DYNAMIC STRUCTURE OF CHOLESTERYL ESTERS IN MEMBRANES

by

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"Dynamic Structure of Cholesteryl Esters in Membranes"

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ABSTRACT

The dynamic behaviour of cholesteryl esters in several model membrane systems has been investigated by means of $^2$H- and $^{31}$P-NMR.

Cholesteryl palmitate-$d_{31}$ and cholesteryl palmitate-16,16,16-$d_3$ in egg-phosphatidylcholine (egg-PC) multilamellar liposomes give rise to $^2$H-NMR spectra composed of several features. A quantitative comparison of these spectra with the ones for the solid esters yields a solubility of $\sim 0.2$ mol% for the deuterated esters in the bilayer. The quadrupolar splitting for the CD$_2$ groups of incorporated cholesteryl palmitate is $<1/2$ that of the phospholipid acyl chains, indicating a much decreased orientational order of these segments of the ester chain, while the orientational order of the terminal CD$_3$ groups of the ester and the phospholipid is the same.

Experiments performed on aqueous dispersions of sphingomyelin (SPM) containing deuterated cholesteryl palmitate show that the solubility of the ester in the phospholipid is $\sim 1.5$ mol% between 30 and 60 °C, i.e. above and below the gel to liquid-crystalline phase transition ($T_m$) of sphingomyelin. Addition of an equimolar amount (referred to the phospholipid) of cholesterol decreases the solubility $\sim 10$-fold. The orientational order of the ester acyl chains in
the cholesteryl ester/SPM/water system is quite different from that of the phospholipid acyl chains. In addition, \( ^{2}\)H-NMR and \( ^{31}\)P-NMR indicate a substantial (up to \( \sim 30 \) \%) proportion of an isotropic signal above \( T_m \) of the phospholipid, which increases with increasing temperature, and which is interpreted as arising from ester and phospholipid associated in small vesicle-like structures.

Selectively deuterated cholesteryl palmitate and cholesteryl stearate in egg-PC unilamellar vesicles and selectively deuterated cholesteryl palmitate in dipalmitoylphosphatidylcholine (DPPC) vesicles possess very similar behaviour. The solubility of ester in both systems is \( \sim 5 \) mol\%, and ester does not appreciably affect vesicle size. Small (\(<160 \text{ Hz})^{2}\)H-NMR linewidths are obtained for these systems, indicating that the ester acyl chains are highly disordered. Addition of 20 mol\% cholesterol to the vesicles induces an increase in linewidth at all positions, but especially for deuterons attached to C11 and C12 of the ester chain. The \( ^{2}\)H spin-lattice relaxation data are thought to reflect a rate of motion of the ester chain segments which is significantly slower than that of the phospholipid chain segments. Further, the \( ^{2}\)H-NMR linewidths of deuterated cholesteryl palmitate in egg-PC and DPPC vesicles are independent of the vesicle tumbling rate, implying either
very fast lateral diffusion of the ester, or the presence of an important slow (correlation time $< 10^{-8}$ s) motion of the ester chains.

Studies were also performed on selectively deuterated cholesteryl palmitate and stearate in DPPC liposomes at 50 °C. The solubility of ester in this system is $\sim 0.5$ mol%. A characteristic profile of $^2$H-NMR spectral width versus chain position is obtained which is believed to reflect a horseshoe conformation of the cholesteryl ester, i.e. with the carbonyl group near the lipid/water interface, and the cholesteryl and fatty acyl moieties extending into the hydrophobic region of the bilayer. The quadrupolar echo decay times $T_{2e}$ for the cholesteryl ester chain segment are much shorter than those for the phospholipid chains, indicating either a slower rate of motion or the presence of an additional slow motion of the ester chain, as compared to the phospholipid chains.

Preliminary statistical mechanical calculations based on the mean field approximation have also been performed, and these show that a horseshoe conformation for cholesteryl palmitate incorporated into DPPC liposomes is energetically favourable and consistent with the experimental data.
DEDICATION

to Maggie
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Part of this work, viz. the $^2$H-NMR and differential scanning calorimetry studies reported in Sections D. I., D. II., D. III, and D. VI., was performed in collaboration with Prof. Myer Bloom and his research group at the Department of Physics, University of British Columbia. I wish to express my most sincere thanks to Prof. Bloom, as well as to Dr. A.L. MacKay and Dr. M.I. Valic for allowing me the use of their data and for many helpful discussions. I also thank Dr. A.P. Tulloch, Prairie Regional Laboratory, National Science and Engineering Research Council, for collaborating with us and kindly supplying us with some of the deuterated fatty acids.

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A. INTRODUCTION

I. Overview

Biological membranes are structures found in all living organisms. Cell membranes serve as barriers that prevent mixing of the cell contents with its surroundings and thus preserve their structural identity. At the same time, they must allow diverse metabolites to penetrate the cell, and hence they must be selectively permeable to certain substances. Intracellular membranes serve as means for subdividing a cell into organelles having specific structures and functions. Furthermore, membranes possess enzymatic activity (Coleman, 1973). Thus, they serve not only as barriers but also as structural components actively taking part in metabolism. Active transport, photosynthetic and oxidative phosphorylation, and muscle contraction are some of the processes associated with biological membranes.

According to the generally accepted model (Singer and Nicholson, 1972), biological membranes can be represented by a "fluid mosaic" model in which lipid molecules are arranged in a bilayer structure of ~4-6 nm thickness, forming a semifluid medium in which various membrane-bound proteins are
embedded (Fig. 1). The fluidlike state of the lipid bilayer allows lateral diffusion in the plane of the membrane to take place, and as a result the membrane constituents are continuously shifting in their relative positions. Collisions between components which occur as a consequence of this fact may allow processes, for which these interactions are essential, to take place.

Lipids make up ~20-80 % of the membrane, and the composition varies widely and is source dependent (Kotyk and Janacek, 1977). A ubiquitous feature of membranes is their high content of phospholipids (Fig. 2). These substances generally comprise the largest part of the lipids of biological membranes (Kotyk and Janacek, 1977), and this is probably due to their tendency to form bilayers in the presence of water (Tanford, 1973). Thus, phospholipids are believed to serve as the basic structural elements of membranes (Wilkins et al., 1971). There is a striking similarity in the structure and behaviour of bilayers formed when phospholipids are dispersed in an aqueous phase, and the lipid component of biological membranes. This similarity includes properties such as bilayer thickness (Wilkins et al., 1971), rate of lateral diffusion (Scandella et al., 1972; Sackmann et al., 1973), calorimetric behaviour (Steim et al., 1969; Melchior et al., 1970), and orientational order
Fig. 1: The fluid mosaic model of biological membranes (adapted from Singer & Nicholson, 1972).

♀ = lipid molecules
Fig. 2: Structure of a typical phospholipid: dipalmitoylphosphatidylcholine (DPPC)
of the phospholipid acyl chains (Tourtellotte et al., 1970; Rottem et al., 1970; Stockton et al., 1977; Gally et al., 1979). These observations have led to the widespread use of phospholipid/water systems as model systems, in which specific features of membrane behaviour can be studied in a straightforward fashion without interference from the proteins and/or other lipids normally present in biological membranes. In addition, phospholipid model membranes are easily prepared and do not require special care such as that needed when culturing cells as sources of membranes.

Magnetic Resonance Methods as Probes of Membrane Behaviour.

Many different instrumental techniques have been used to investigate membrane structure and behaviour, and among these, magnetic resonance is especially suited to study the dynamic aspects of membrane behaviour. Techniques such as X-ray diffraction and electron microscopy essentially provide a static, time-independent description, while NMR and ESR (Lee et al., 1974) as well as fluorescence spectroscopy (Kinosita et al., 1977) can yield information about those molecular motions that are generally regarded as being the most relevant in terms of biological significance. On the other hand, Raman (Gaber & Peticolas, 1977; Bansil et al., 1980; Bicknell-Brown et al., 1980) and infrared spectroscopy
(Cameron et al., 1980) are sensitive to very fast molecular motions and can complement some of the results obtained by magnetic resonance techniques.

ESR has been widely used to study model and biological membrane systems (Griffith & Jost, 1976; Schreier et al., 1978). In this technique, spin-labelled probe molecules are introduced into the membrane of interest, and, due to the high sensitivity of ESR, amounts of spin-label as small as \( \sim 10^{-9} \) moles may be detected. However, it is now known that the spin label introduces a noticeable perturbation into the system (Seelig & Niederberger, 1974; Taylor & Smith, 1980), and hence, ESR may not report the true bilayer properties. The same may be said of fluorescence spectroscopy, which relies on the incorporation of perturbing fluorophores into the membrane (Badley et al., 1973; Dale et al., 1977). Thus, the results obtained via ESR and fluorescence spectroscopy should be interpreted cautiously and, if possible, complemented by a non-perturbing technique such as NMR.

NMR lacks the high sensitivity characteristic of the ESR technique, but can be regarded as non-perturbing, since it does not have to rely on bulky probe molecules that are rather different from the native molecules in the system. Instead, either the original lipids present in the membrane will give rise to resonance signals (as in \( ^1H-, ^31P- \), and
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high-field

magnets.

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t h e d y n a m i c b e h a v i o u r o f membrane l i p i d s ( L e e e t a l . ,

1974).

However, t h e i n t e r p r e t a t i o n o f t h e s e s p e c t r a i n t e r m s o f t h e
o r i e n t a t i o n a l o r d e r o f t h e membrane c o n s t i t u e n t s i s
c o m p l i c a t e d by t h e o c u r r e n c e o f p a r t i a l l y o v e r l a p p i n g
r e s o n a n c e s a n d by t h e s i g n i f i c a n t d i p o l e - d i p o l e
with neighbouring nuclei.
i n f o r m a t i o n f r o m 'H-

interactions

T h u s , t o e x t r a c t unambiguous

and 1 3 c - N M R

of b i l a y e r l i p i d s is

s o m e t i m e s d i f f i c u l t , w h i c h h a s l e d t o c o n t r o v e r s i e s (Lee e t
al.,

1974; Bloom e t a l . ,

1978; L i c h t e n b e r g

&

Zilberman,

1979). I n s p i t e o f t h e above, I 3 c - N ~ R h a s g i v e n v a l u a b l e
information, e s p e c i a l l y concerning the r a t e of molecular
motions ocurring in l i p i d b i l a y e r s .
D e u t e r i u m N M R is a r e l a t i v e l y new s p e c t r o s c o p i c
technique (Oldfield e t al.,

1 9 7 1 ) , w h i c h h a s b e e n shown t o b e

e x t r e m e l y v a l u a b l e f o r s t u d y i n g t h e d y n a m i c b e h a v i o u r and


orientational order in model and biological membranes
(Seelig, 1977; Mantsch et al., 1977; Smith, 1979). Due to the
spin \( I=1 \) of the deuterium nucleus, \(^2\text{H}-\text{NMR}\) is dominated by
quadrupolar effects, which are modulated by the orientational
order and mobility of the C-D bond in question. Dipole-dipole
interactions with surrounding nuclei are much smaller than
the quadrupolar effects, so that these interactions are
generally unimportant, leading to a relatively
straightforward interpretation of the spectra. In addition,
the assignment of \(^2\text{H}-\text{NMR}\) spectra of membranes is normally
trivial since, due to the low natural abundance of deuterium
(0.016 \%), selectively deuterated compounds are normally used
to obtain spectra in a reasonable length of time, giving rise
to only one (or a few) resonances. The replacement of
hydrogen atoms by \(^2\text{H}\) can be regarded as not significantly
altering membrane behaviour, making \(^2\text{H}-\text{NMR}\) a non-perturbing
technique.

Deuterium NMR has been extensively applied to the study
of model and biological membrane systems (Seelig, 1977;
Stockton et al., 1976; Stockton et al., 1977; Gally et al.,
1979; Kang et al., 1979; Smith, 1979; Nichol et al., 1980),
and it has yielded extremely valuable information.

Phosphorus-31 NMR can be used to study the headgroup
behaviour of membrane phospholipids (Seelig, 1978). Due to
the high natural abundance of $^{31}$P (100%), these spectra can be obtained quite easily. In addition, $^{31}$P-NMR spectra are dominated by the chemical shift anisotropy of the phosphate group in phospholipids, while dipolar interactions may be removed by proton noise-decoupling. Thus, $^{31}$P-NMR is a valuable technique to study headgroup orientation and motion in phospholipid bilayers, and it can also be used to study the phase behaviour of phospholipid membrane systems (Seelig, 1978; Cullis & de Kruijff, 1979).

**Cholesteryl Esters.**

A biologically important class of lipids, encountered principally in mammalian organisms, consists of cholesteryl esters (Fig. 3). These compounds are found in large proportions in the lesions of atherosclerosis (Insull, 1972). Fatty streaks, which are formed during the initial stage of the disease, are known to contain up to 95% cholesteryl ester (Lang and Insull, 1970). Also in the advanced atherosclerotic lesions, large proportions of intracellular and extracellular cholesteryl ester occur (Wissler, 1974) in combination with other compounds. Furthermore, those areas of the aortic intima that seem to be predisposed to the development of atherosclerosis have been shown to possess enhanced permeability, which may be due to a higher cholesteryl ester
Fig. 3: Structure of cholesteryl palmitate (CP)
The cholesteryl esters encountered in atherosclerotic lesions appear to originate primarily from plasma lipoproteins, notably low density and very low density lipoprotein (Wissler, 1974). These lipid-protein aggregates are believed to act as carriers of lipids between different parts of the organism (Getz & Hay, 1979), and they contain up to 40% cholesteryl ester (Scanu, 1979). Although the detailed structure of these lipoproteins has not yet been elucidated, it is believed that the cholesteryl esters are primarily located in their core, while phospholipids, together with small proportions of cholesterol, surround this core in a monolayer arrangement (Morrisett et al., 1977).

Cholesteryl esters are also known to be present in considerable amounts (~5% of the wet weight) in the cytoplasm of adrenal gland cells, where they are found in the form of lipid droplets (Moses et al., 1969), together with phospholipids and small amounts of free cholesterol and proteins (Boyd & Trzeciak, 1973). The cholesterol esters stored in these droplets are believed to be the primary source of cholesterol required for the synthesis of steroid hormones by the adrenal glands (Goodman, 1965; Vahouny et al., 1978).

Further, cholesteryl esters have recently been shown to
occur in small proportions in biological membranes such as rat liver and kidney plasma and intracellular membranes (Zambrano et al., 1975), as well as in some plant microsomal membranes (Musgrave et al., 1976). It is reasonable to assume that membrane cholesteryl ester may be partly responsible for the modulation of membrane properties and function. Thus, it has been recently reported that the increased cholesteryl ester content of rat liver membranes caused by the synthetic estrogen ethinyl estradiol leads to a decrease in Na-K ATPase activity in those membranes (Davis et al., 1978).

II. Purpose of the Present Work.

From what was said in the previous paragraphs, it should be clear that, in order to determine the role of cholesteryl esters in the organism, an understanding of the interactions between this class of compounds and other lipids, notably phospholipids, is required. Little is known about the physical behaviour of cholesteryl esters in phospholipid/water systems, and a deeper knowledge is necessary.

The present thesis reports the results of investigations of cholesteryl esters in various phospholipid model membrane systems, using $^2$H-NMR as the main spectroscopic tool for the determination of solubility, orientational order, and
mobility of the cholesteryl esters. In addition, $^3$P-NMR was utilized to determine the effects of these cholesteryl esters on the headgroup of the phospholipid in these systems. Specifically, we have studied the dynamic behaviour of the saturated cholesteryl esters, cholesteryl palmitate (CP) and cholesteryl stearate (CS), in aqueous dispersions (liposomes) of egg-phosphatidylcholine (egg-PC), bovine brain sphingomyelin (SPM) and dipalmitoylphosphatidylcholine (DPPC), as well as in unilamellar vesicles of egg-PC and DPPC. These results are presented in Sections D. II - VI. In addition, $^2$H-NMR spectra of some pure, polycrystalline esters were determined. These data are important for the interpretation of the $^2$H-NMR spectra of deuterated CP and CS in liposomes; hence they are presented in the first part of the "Results and Discussion" chapter (Section D. I.).

Further, we have performed preliminary statistical mechanical calculations on the CP/DPPC liposome system, and those results are presented in Section D. VII.

In the following paragraphs, a brief account will be given of some of the properties of phospholipid model membrane systems, insofar as they are relevant to the present work. In addition, we will present a brief review of the results presently available regarding cholesteryl ester/phospholipid mixed model membrane systems.
Phospholipid Model Membrane Systems.

Phospholipids are amphiphilic molecules possessing a polar, hydrophilic headgroup, and a non-polar part, generally formed by two fatty acyl chains, and the interaction of phospholipids with water is determined by this amphiphilic nature. Since the rupture of hydrogen bonds of water in order to accommodate the hydrophobic phospholipid chains is energetically unfavourable, these chains will tend to avoid the contact with the aqueous medium by associating among themselves. The polar headgroups of the phospholipid, on the other hand, can interact effectively with the water dipoles by forming hydrogen bonds (Tanford, 1973). Thus, the structures formed by phospholipids in water will have the lipid headgroups exposed to the aqueous phase, while the hydrophobic chains will form a separate, non-polar region. Depending on the kind of phospholipid, water content, temperature, etc., diverse structures consistent with the amphiphilic nature of the lipid may be formed by phospholipid/water systems (Reiss-Husson, 1967; Luzatti et al., 1968).

The most common structure encountered when dispersing a phospholipid in water is the lamellar phase (Fig. 4A), characterized by the occurrence of domains of phospholipid
Fig. 4: Schematic representation of the lamellar (A) and hexagonal (B) liquid crystalline phases
bilayers separated by water (Wilkins et al., 1971), the headgroups facing the aqueous phase, while the acyl chains constitute the inner part of the bilayer. The physical state of the chains depends markedly on the temperature and corresponds to fluidlike, disordered chains at high temperatures (Levine & Wilkins, 1971), while at lower temperatures the chains are believed to exist mainly in the all-trans state, parallel to each other, and having relatively little motion (Tardieu et al., 1973). At a specific temperature called the gel to liquid-crystalline phase transition ($T_m$) the behaviour of the chains changes from relatively ordered to disordered. The value of $T_m$ is dependent upon the type of headgroup, the length and degree of unsaturation of the chains, and the degree of hydration of the phospholipid (Chapman et al., 1967). When a lipid forming a lamellar phase (such as phosphatidylcholine) is dispersed in excess water, structures of concentric bilayers are formed (Bangham and Horne, 1964), which, typically, have diameters of 100-1000 nm and which are called multilamellar liposomes. Ultrasonic disruption of these liposomes gives rise to small spherical structures formed by a single closed bilayer, which separates an inner aqueous compartment from the bulk water (Huang, 1969). These unilamellar vesicles prepared by sonication are generally <50 nm in diameter (Huang, 1969;
Sheetz & Chan; 1972; Gent & Prestegard, 1974; Forge et al., 1978) and, hence, they tumble rapidly in the aqueous medium. This phenomenon, plus lateral diffusion of the phospholipid molecules in the plane of the bilayer, is very important in determining the properties of the NMR spectra arising from these structures (see Chapter B.). There is still a great deal of controversy regarding whether properties such as fatty acyl chain order and mobility are different in these highly curved vesicles as compared to multilamellar liposomes. Different research groups have put forward contradicting reports, based mainly on NMR experiments, claiming that molecular order in small unilamellar vesicles is essentially the same as that in liposomes (Lee et al., 1974; Stockton et al., 1976; Bloom et al., 1978) or different (Sheetz & Chan, 1972; Petersen & Chan, 1977; Lichtenberg & Zilberman, 1979).

Some phospholipids such as phosphatidylethanolamine can form so-called hexagonal phases which are characterized by phospholipid molecules being arranged in long cylindrical rods (Reiss-Husson, 1967; Luzzati et al., 1968). In the normal hexagonal phase, the lipid headgroups face the outside of the cylinders while the hydrocarbon chains lie toward the interior, and water surrounds the cylinders (Fig. 4B). These structures have diameters of about 4-5 nm, and hence they
rotate rapidly in the aqueous medium. In addition, lateral diffusion of the molecules forming the cylinders occurs. The inverted hexagonal phase is characterized by a cylindrical core of water of about 2 nm diameter which is surrounded by lipid molecules such that the phospholipid headgroups face the aqueous cylinder. Fast lateral diffusion of the lipid around the inner channel takes place.

In addition to the lamellar and hexagonal phases, other structures are formed by some phospholipids under certain conditions, such as the cubic and rhombic mesophases. These structures are not very commonly encountered, and so they will not be discussed here. The interested reader is referred to Luzzati et al. (1968) for a description of these phases.

The phase diagram of a typical phospholipid/water system, i.e. that of dipalmitoylphosphatidylcholine (DPPC) aqueous dispersions, is shown in Fig. 5 (Chapman et al., 1967). A lamellar phase is formed at all conditions of temperature and water concentration. Maximum hydration of the phospholipid corresponds to 30% water in the gel phase and ~40% in the liquid-crystalline phase. The temperature delimiting both phases is $T_m = 41 \degree C$.

The phase diagram of egg-phosphatidylcholine (egg-PC) is very similar to that of Fig. 5; the main difference being that $T_m$ is only ~5 \degree C (Chapman et al., 1967) due to the high
Fig. 5: Phase diagram of the DPPC/H$_2$O system (adapted from Chapman et al., 1967)
proportion of unsaturated fatty acyl chains.

The phase behaviour of sphingomyelin is essentially the same as that of DPPC (Shipley et al., 1974). The phase transition of sphingomyelin has been shown to depend to a large extent on the source of the lipid and can be correlated with its fatty acid composition (Calhoun & Shipley, 1979a; Barenholz & Thompson, 1980).

Considerable information is now available on the dynamic behaviour of the acyl chains in the lamellar phase of phospholipids. Studies involving ESR (McConnell & McFarland, 1972; Griffith & Jost, 1976), as well as $^1$H- and $^{13}$C-NMR (Lee et al., 1972; Lee et al., 1974; Stoffel et al., 1974; Gent & Prestegard, 1977), have been performed by a number of researchers. However, due to the difficulties inherent in these methods, as described earlier in this chapter, the most reliable information to date has been obtained via $^2$H-NMR and has been complemented by neutron diffraction studies. These studies have shown that the average alignment of the phospholipid acyl chains above the gel to liquid-crystalline phase transition is perpendicular to the bilayer surface (Seelig & Seelig, 1974a), with the exception of the C2 segment of the sn-2 chain, which appears to be aligned, on the average, parallel to the bilayer surface (Fig. 2) (Seelig & Seelig, 1975). Thus, there is inequivalence of the two
fatty acyl chains, and this has been confirmed by Raman spectroscopy (Gaber et al., 1978) and neutron diffraction studies (Buldt et al., 1978; Zaccai et al., 1979).

The orientational order of the phospholipid fatty acyl chains above the gel to liquid-crystalline phase transition exhibits a characteristic profile versus chain position (Seelig & Seelig, 1974b; Stockton et al., 1976; Davis, 1979). This profile is characterized by a region encompassing the C2 - C9 segments which possesses almost constant orientational order and which is called the "ordering plateau". From C10 onward to the end of the chain the orientational order decreases continuously, presumably due to a progressively lower probability of encountering trans chain segments parallel to the bilayer normal (Schindler & Seelig, 1975), reaching a minimum at the terminal methyl group. This typical profile of chain order has been confirmed for a variety of phospholipids, and there is evidence that at equivalent temperatures above the gel to liquid crystalline phase transition the chain orientational order of many phospholipids is almost identical (Seelig and Browning, 1978).

Lately, $^2$H-NMR has confirmed that the phospholipid in biological membranes shows essentially the same ordering profile as in model systems (Stockton et al., 1977; Gally et
providing convincing evidence of the usefulness of model membranes.

The rate of the reorientational motions of the phospholipid acyl chain segments in model membranes has recently been studied by means of $^2$H-NMR spin-lattice relaxation measurements (Brown et al., 1979). The motions giving rise to spin-lattice relaxation are believed to be mainly trans-gauche chain isomerizations (Schindler & Seelig, 1975), and it appears that the profile of the rate of motion versus chain position closely parallels that of the chain order, i.e. it possesses a "plateau" region of almost constant rate of motion, followed by an almost linear rate increase toward the terminal methyl groups of the chains.

Little is known about the dynamic behaviour of the phospholipid acyl chains below their phase transition. However, it appears that, contrary to the classical concept of the gel phase being characterized by an all-trans chain configuration (Chapman et al., 1967; Tardieu et al., 1973), considerable segmental motion still occurs in the gel phase, which disappears only gradually when the temperature is lowered (Davis, 1979; Marsh, 1980). Thus, in the case of DPPC, most of the chain motion disappears only at temperatures below 0 °C (Davis, 1979; Cameron et al., 1980).

The polar headgroup of the phospholipid in
phospholipid/water dispersions has been studied by means of $^{31}$P-NMR (Yeagle et al., 1976; Griffin et al., 1978) and $^2$H-NMR (Gally et al., 1975; Seelig & Gally, 1976; Seelig et al., 1977) as well as neutron diffraction (Worcester & Franks, 1976; Buldt et al., 1978; Buldt et al., 1979). These studies have indicated that in phosphatidylcholines and phosphatidylethanolamines the polar headgroup is, on the average, aligned parallel to the bilayer surface (Buldt et al., 1978; Seelig & Gally, 1976; Seelig et al., 1977). Furthermore, the experimental data are consistent with rapid reorientational motions of the headgroup about an axis normal to the bilayer. The whole headgroup appears to be rather flexible (Gally et al., 1975), with the choline moiety (in phosphatidylcholine) possessing the greatest motional freedom. When going through the gel to liquid-crystalline phase transition, which is believed to be associated mainly with the fatty acyl chains of the phospholipid (Chapman et al., 1967), headgroup mobility is also affected (Gally et al., 1975), being lower at low temperatures.

Lateral diffusion of the molecules in phospholipid bilayers has been investigated by a variety of methods (Kornberg & McConnell, 1971; Devaux et al., 1972; Cullis, 1976; MacKay et al., 1978; Kuo & Wade, 1979). It has been shown that, above the phospholipid phase transition, lateral
diffusion is relatively fast, with a diffusion coefficient of
\( \sim 5 \times 10^{-8} \text{ cm}^2/\text{s} \) (Devaux et al., 1972; Cullis, 1976; MacKay et al., 1978). On the other hand, below \( T_m \), the lipid molecules diffuse several orders of magnitude more slowly (\( D \sim 10^{-10} \) to \( 10^{-11} \text{ cm}^2/\text{s} \)) (Cullis, 1976; Silva Crawford et al., 1980).

IV. Cholesteryl Esters in Phospholipid Model Membranes.

Comparatively few studies have appeared concerning the behaviour of phospholipid model membrane systems containing cholesteryl esters. The first of these investigations dealt with the unsaturated ester, cholesteryl linolenate, in egg-PC bilayers (Janiak et al., 1974), as studied by means of polarizing light microscopy, X-ray diffraction, and differential scanning calorimetry. These workers found that only small (\( < 4.5 \text{ mol\%} \)) proportions of ester could be incorporated into the phospholipid/water lamellar phase. Ester incorporation was seen to depend on the degree of hydration of the phospholipid, being highest at \( \sim 15-30\% \text{ H}_2\text{O} \) and decreasing at higher and lower water contents. At high water concentrations (\( > 20\% \text{ H}_2\text{O} \)), a conformation for the ester in the bilayer resembling a horseshoe was proposed, in which the carbonyl group of the ester resides near the phospholipid/water interface, while the cholesteryl and fatty acyl moieties are parallel to the phospholipid chains and
extend toward the center of the bilayer. For low-water concentrations, it was proposed that cholesteryl linolenate adopts an extended conformation and is situated in the center of the bilayer.

The interaction of cholesteryl myristate with dimyristoylphosphatidylcholine aqueous dispersions was studied by Janiak et al. (1979). It was reported that the ester can be incorporated into the bilayer only above the phospholipid phase transition, and even then only in small proportions (<5%). High temperatures were concluded to be favourable to higher cholesteryl ester incorporation. As in the case of cholesteryl linolenate, the incorporation of cholesteryl myristate into the phospholipid was reported to depend on the degree of hydration, the maximum incorporation occurring at ~20% H_2O. At maximum hydration (>40% H_2O) essentially no cholesteryl myristate seemed to be taken up by the phospholipid. Janiak et al. could not arrive at definite conclusions regarding the alignment of cholesteryl myristate in bilayers of dimyristoylphosphatidylcholine, but the data were consistent with either a horseshoe configuration or an extended configuration, with the ester molecules spanning the bilayer.

Forrest and Cushley (1977) have investigated the behaviour of cholesteryl esters in egg-PC unilamellar

25
vesicles by means of $^{31}\text{P}-\text{NMR}$ and concluded that CP drastically increases membrane permeability to $\text{Pr}^{+3}$ and EDTA, whereas cholesteryl linoleate does not.

Using $^{13}\text{C}-\text{NMR}$, Cushley and Forrest (1979) also studied the effect of 25 mol% of added CP on the fatty acyl chains of egg-PC in multilamellar liposomes and found no appreciable difference in the spin-lattice relaxation times in the absence and presence of the ester, indicating that phospholipid chain mobility is not affected by the ester. On the other hand, 25 mol% of added cholesteryl linoleate in DPPC liposomes resulted in spectra that were interpreted in terms of ester mobility. These workers concluded that the spin-lattice relaxation times for the cholesteryl linoleate acyl chain are ~2-fold reduced compared to the ones for the DPPC chains, indicating a slower rate of motion of the ester.

ESR studies using spin-labelled cholesteryl esters in planar egg-PC multilayers have indicated that the solubility of spin labelled cholesteryl stearate in the bilayer is low (<1 mol%) (Grover and Cushley, 1979a). In a further study, it was concluded that the C5 segment of the acyl chain of spin labelled cholesteryl palmitate is, on the average, aligned at an angle of $\sim47^\circ$ with respect to the bilayer normal and undergoes slow reorientational motions (Grover et al., 1979). In addition, with the spin-label attached at the $\omega-1$ position...
of the cholesteryl ester acyl chain, the ESR spectra were consistent with a non-tilted chain segment, which undergoes rapid motional fluctuations. These data were interpreted as supporting a horseshoe conformation for spin-labelled cholesteryl ester in egg-PC multilayers.

Recently, physical studies have also been performed on cholesteryl esters in monolayers composed of diverse lipids (Smaby et al., 1979; Smaby & Brockman, 1981a; Smaby & Brockman, 1981b). Those workers concluded that cis-unsaturated and trans-unsaturated esters can incorporate into monolayers, while saturated esters cannot. It was also observed that the solubility of cis-unsaturated cholesteryl esters in the monolayers depends upon the surface pressure. Although proportions of ester as high as ~45 mol% are incorporated at low surface pressures, ester incorporation decreases markedly when the surface pressure is increased (Smaby & Brockman, 1981a; Smaby & Brockman, 1981b), reaching values close to zero at surface pressures of ~30 mN/m, i.e. at conditions where lipid behaviour in monolayers is believed to be very similar to that in bilayers (Blume, 1979). Hence, the ester solubilities determined in monolayers are consistent with those obtained in bilayers. In addition, the molecular areas measured for the cis-unsaturated esters in lipid monolayers support a
horseshoe conformation for the cholesteryl ester, in agreement with the conclusions reached by Janiak et al. (1974) and Grover et al. (1979).
B. THEORY

I. Deuterium Nuclear Magnetic Resonance

Deuterium nuclei possess a spin \( I = 1 \), and, hence, their charge distribution is not spherically symmetrical (Eisenberg & Greiner, 1972). Instead, a deuterium nucleus can be visualized as an ellipsoid, and thus it possesses a quadrupole moment, \( Q \). If a deuterium nucleus is located at a site of symmetry lower than cubic, then the electrons in its vicinity will give rise to an electric field gradient which can interact with the quadrupole, producing \( 2I + 1 \) distinct energy levels which may be detected in a \(^2\text{H}-\text{NMR} \) experiment (Slichter, 1963).

In the following paragraphs, only a simplified account of \(^2\text{H}-\text{NMR} \) theory will be presented. The reader interested in a more rigorous formulation of the theory is referred to the books by Slichter (1963) and Abragam (1961), as well as the excellent review article by Seelig (1977).

Consider a deuterium nucleus in a static magnetic field \( H_0 \) aligned parallel to the laboratory \( z \)-axis. Due to the interaction of the field \( H_0 \) with the nuclear magnetic moment, three Zeeman energy levels are obtained corresponding
to nuclear spin quantum numbers \( m = -1, 0, \) and \(+1\). The energies of these levels are given by (Slichter, 1963):

\[
E_m^{\text{Zeeman}} = -\gamma \hbar H_0 m
\]

(1)

where \( \hbar \) is the modified Planck constant \((= h/2\pi)\), and \( \gamma \) is the magnetogyric ratio of the deuteron. For \(^2\text{H}\) nuclei in the presence of a magnetic field of a magnitude like the ones typically used for NMR \((-2-7 \text{ Tesla})\), the Zeeman energy is of the order of 15-50 MHz.

The interaction of the nuclear quadrupole moment \( Q \) with an electric field gradient at the position of the nucleus gives rise to a modification of the Zeeman energy levels. Quadrupolar effects in \(^2\text{H}\)-NMR are much smaller in magnitude than the Zeeman splitting, and are of the order of 100-200 kHz (Burnett & Muller, 1971), hence the problem of a nucleus in the presence of both interactions can be solved by standard perturbation theory. To simplify matters even further, it will be assumed that the electric field gradient at the position of the deuteron is axially symmetric. This is a valid approximation, which has been shown to hold in the case of C-D bonds (Derbyshire et al., 1969; Barnes & Bloom, 1973). With the above simplifications, the nuclear energy levels are given by (Slichter, 1963):
\[ E_m = -\gamma H \hat{n} m + \frac{e^2 qQ}{4I(2I-1)} \left( \frac{3 \cos^2 \theta - 1}{2} \right) [3m^2 - I(I+1)] \]  

where \( \theta \) is the angle between the axis of symmetry of the electric field gradient and the applied magnetic field, and \( qQ \) is the value of the field gradient along the C-D bond axis.

The selection rule for the allowed spectral transition is \( \Delta m = \pm 1 \), and, since \( I = 1 \), as well as \( \Delta E = \hbar \nu \), we obtain for the frequencies of the allowed transitions

\[ \nu_1 = \frac{\gamma H_0}{2\pi} + \frac{3}{4} \left( \frac{e^2 qQ}{\hbar} \right) \left( \frac{3 \cos^2 \theta - 1}{2} \right) \]

\[ \nu_2 = \frac{\gamma H_0}{2\pi} - \frac{3}{4} \left( \frac{e^2 qQ}{\hbar} \right) \left( \frac{3 \cos^2 \theta - 1}{2} \right) \]  

These values are symmetrical with respect to the nuclear Larmor frequency \( \omega_0 = \frac{\gamma H_0}{2\pi} \), and hence one observes a spectral doublet centered around \( \omega_0 \) and with a separation \( 3/2(e^2qQ/\hbar)(3\cos^2 \theta - 1)/2 \). This doublet will not be infinitely sharp, as would be predicted from the above formulas, due to the fact that minor interactions such as
magnetic dipole-dipole interactions between $^2$H and $^1$H as well as between $^2$H and $^2$H are present (Seelig, 1977), which cause a slight spread of the energy levels among the different deuterium nuclei in the sample.

For a polycrystalline sample containing deuterium nuclei, the quantity $(3\cos^2 \theta - 1)/2$ in eqn. 3 is not fixed, but instead all possible angles $\theta$ are encountered. The probability of finding an angle between $\theta$ and $\theta + d\theta$ is given by (Seelig, 1977):

$$p(\theta) = \frac{1}{2} \sin \theta \ d\theta$$

(4)

This probability is maximum for $\theta = 90^\circ$ and vanishes for $\theta = 0^\circ$ and $\theta = 180^\circ$. Hence, the $^2$H-NMR spectrum for a polycrystalline sample is a "powder pattern" (Fig. 6) (Seelig, 1977) whose characteristic peaks correspond to $\theta = 90^\circ$. The separation between these maxima, which is called the "quadrupolar splitting", has a value of

$$\Delta \nu_Q = \frac{3}{4}(e^2 qQ/h),$$

where $e^2 qQ/h$ is the static quadrupolar coupling constant and has a value of $\approx 170$ kHz for deuterons on methylene or methyl groups (Burnett and Muller, 1971).
Fig. 6: $^2$H-NMR powder pattern
Liquid Crystalline Systems

In a lyotropic liquid crystalline sample, the constituent molecules are not immobile but undergo rapid fluctuations about their average orientation which can be defined by a director $\hat{n}$. Molecular motion is assumed to be cylindrically symmetric about the director axis. It has been shown that, in the case of phospholipid bilayers above their gel to liquid-crystalline phase transition, the director coincides with the normal to the bilayer surface (Seelig & Seelig, 1974a).

For a planar oriented liquid crystal in which a hydrogen atom has been replaced by deuterium, the rapid molecular motion about the director partly averages out the electric field gradient at the position of the deuterium nucleus, and the deuteron will experience an effective field gradient which is axially symmetric about $\hat{n}$. By performing the appropriate coordinate transformations it can be shown (Seelig, 1977) that the spectral shape in this case is the same as that of a single crystal (i.e. a doublet symmetrical about $\omega_0$), and the quadrupolar splitting will be given by

$$\Delta \nu_Q = \frac{3}{2} \left( \frac{e^2 q Q}{h} \right) \left( \frac{3 \cos^2 \theta - 1}{2} \right) \left( \frac{3 \cos^2 \varphi - 1}{2} \right)$$

where $\theta$ is the angle between the C-D bond in question and
the director axis, $\beta$ is the angle between the director and the applied magnetic field, and the angular brackets indicate an average over time. The quantity $<3\cos^2 \theta - 1>/2$ is a convenient measure of the angular fluctuations of the C-D bonds in the system, and it is called the C-D order parameter, $S$; its value equals zero for an isotropically reorienting system, and $S=1$ for a completely immobilized system.

For a random distribution of planar bilayers or for multilamellar phospholipid liposomes, the lineshape is, in close analogy to the case of a polycrystalline sample, the result of the superposition of spectral doublets corresponding to all possible orientations. This gives rise to a powder pattern (Fig. 6), whose splitting is given by

$$\Delta \nu_Q = \frac{3}{4} \left( \frac{e^2 qQ}{h} \right) |S| \quad (6)$$

Hexagonal liquid-crystalline phases are characterized by long cylindrical rods composed of lipid molecules, and the long axes of these molecules are perpendicular to the cylinder surfaces (Fig. 4B) (Luzzati et al., 1968; Brown & Wolken, 1979). Rotation of these cylinders about their axis and lateral diffusion of the lipid molecules around the surface of the cylinders constitutes an additional mechanism
for averaging the quadrupolar interaction. Hence, the $^{2}H$-NMR quadrupolar splittings observed for the hexagonal phases are reduced 2-fold with respect to those of the lamellar phase (Seelig, 1977). Thus, for a random distribution of cylinders, the observed splitting will be given by

$$
\Delta \nu_Q = \frac{3}{8} \left( \frac{e^2 q Q}{h} \right) |s|
$$

(7)

Recently, Petersen & Chan (1977) have discussed the case in which the lipid molecules in a bilayer simultaneously perform two types of motion. According to these authors, the hydrocarbon chain of the lipid molecules may undergo, in addition to trans-gauche chain isomerizations ("kink motions") which are commonly assumed to be the most important molecular motion (Seelig & Niederberger, 1974; Schindler & Seelig, 1975), reorientational motions as a whole ("rigid rod motions") about their average positions. Assuming that these types of motion are independent of each other, then the molecular fluctuations can be thought of as rigid rod motions, in which the whole lipid chain performs cylindrically symmetric reorientations about the director axis, plus fast kink motions which are symmetric about an axis described by the instantaneous position of the molecular
long axis. The C-D order parameter, \( S \), in eqn. 6, may then be decomposed into contributions from both types of fluctuations, i.e.

\[
S = S_\alpha S_\gamma = \left\langle \frac{3 \cos^2 \alpha - 1}{2} \right\rangle \left\langle \frac{3 \cos^2 \gamma - 1}{2} \right\rangle
\]  

(8)

where \( \alpha \) = angle between the C-D bond and the molecular long axis, and \( \gamma \) = angle between the molecular long axis and the director.

The above result shows that the order parameters, \( S \), determined from a \(^2\)H-NMR experiment, will be influenced not only by the angular fluctuations of the C-D bonds as a result of trans-gauche chain isomerizations, but also by the orientation of the molecular long axis or molecular segment with respect to the director. This fact will be important for the interpretation of some of our \(^2\)H-NMR data of deuterated cholesteryl esters in phospholipid bilayers.

In unilamellar lipid vesicles, the residual quadrupolar interaction present in the lamellar phase is further averaged by vesicle tumbling and lateral diffusion of the lipid
molecules in the bilayer plane (Stockton et al., 1976). Instead of the \(^2\text{H}-\text{NMR}\) powder pattern encountered for multilamellar liposomes a single Lorentzian line is observed for a specific deuteron site. Assuming that the molecular motions occurring in the system can be divided into two classes, one whose correlation time is much shorter than the inverse of the nuclear Larmor frequency plus a second much slower class, the linewidth of this absorption may be written as the sum of two terms:

\[
\Delta \nu_2 = \frac{1}{\pi T_2^*} = \frac{1}{\pi T_1} + \frac{1}{\pi T_{2\text{slow}}} \tag{9}
\]

If we identify the slow motions producing line-narrowing with vesicle tumbling combined with lateral diffusion, then \(1/T_{2\text{slow}}\) may be replaced by (Stockton et al., 1976):

\[
\frac{1}{T_{2\text{slow}}} = \frac{4}{5} \frac{\pi^2}{\Delta v_Q^2} \tau_e = \frac{9}{20} \left( \frac{e^2 qQ}{\hbar} \right)^2 s^2 \tau_e \tag{10}
\]

where \(\Delta v_Q\) is the quadrupolar splitting in the lamellar phase, and \(\tau_e\) is the effective correlation time for vesicle reorientation given by

\[
\frac{1}{\tau_e} = \frac{1}{\tau_v} + \frac{1}{\tau_d} = \frac{3}{4} \frac{kT}{\pi \eta r^3} + \frac{6D}{R^2} \tag{11}
\]
In the above expression, $\tau_v$ is the correlation time for vesicle tumbling, $\tau_d$ is the correlation time for lateral diffusion, $\eta$ is the viscosity of the aqueous medium, $r$ is the hydrodynamic vesicle radius, $k$ is the Boltzmann constant, $T$ is the temperature, $R$ is the effective distance from the center of gravity of the vesicle to the midpoint of the bilayer, and $D$ is the coefficient for lateral diffusion.

**Spin-Lattice Relaxation**

The spin-lattice relaxation time $T_1$ measures the decay of the macroscopic magnetization of the sample, after having been perturbed from its equilibrium value. If we define the axis of the applied magnetic field as the $z$ axis, then the dependence of the magnetization of the sample along this axis ($M_z$) is given by (Abragam, 1961)

$$\frac{d M_z}{dt} = -\frac{M_z - M_0}{T_1}$$

(12)

where $M_0$ is the equilibrium magnetization of the system. This equation implies that decay of $M_z$ to its equilibrium value $M_0$ is described by a single exponential with a time constant $T_1$, and this simple definition of $T_1$ has been shown to hold in the case of lipid bilayers (Lee et al., 1974; Brown et al., 1979).
Spin-lattice relaxation is caused by time-dependent fluctuations acting on the spins in the system (Carrington & McLachlan, 1967) such as alternating magnetic fields or quadrupolar interactions. These fluctuations are produced mainly by reorientations of the molecules constituting the system. The energy of the fluctuations available at a specific angular frequency $\omega$ is measured by the spectral density $J(\omega)$ (Carrington & McLachlan, 1967). In the case of isotropic motion, the spin-lattice relaxation rate $1/T_1$ is related to the spectral densities at the Larmor frequency $\omega_0$ and at $2\omega_0$ by (Abragam, 1961)

$$\frac{1}{T_1} = \frac{3}{80} \left( \frac{e^2 qQ}{\hbar} \right)^2 \left[ J(\omega_0) + 4 J(2\omega_0) \right] \quad (13)$$

which, in the case of extreme narrowing ($\omega_0^2 \tau_c^2 \ll 1$, where $\tau_c$ is the correlation time of the relevant motion(s)), reduces to

$$\frac{1}{T_1} = \frac{3}{8} \left( \frac{e^2 qQ}{\hbar} \right)^2 \tau_c \quad (14)$$

The theory of $^2$H-NMR spin-lattice relaxation in lipid bilayers is not yet well established. The molecular motions inside a bilayer are likely to be complex, and any appropriate theory will have to take into account this
complexity. On the other hand, in many lipid bilayers the motional anisotropy does not seem to be very large (Seelig & Seelig, 1974b; Davis, 1979), hence the formulas for isotropic reorientation (eqs. 13 and 14) are thought to provide a reasonable first approximation to the real situation. On the other hand, several workers have derived expressions for $T_1$ in bilayers based on specific assumptions. Brown et al. (1979), assuming that one molecular motion is responsible for spin-lattice relaxation and that this motion is in the extreme narrowing limit, obtained

$$\frac{1}{T_1} = \frac{3}{8} \left( \frac{e^2 q Q}{\hbar} \right)^2 \left( 1 + \frac{1}{2} S - \frac{3}{2} S^2 \right) \tau_c$$

(15)

for liposomes, and

$$\frac{1}{T_1} = \frac{3}{8} \left( \frac{e^2 q Q}{\hbar} \right)^2 \left( 1 - S^2 \right) \tau_c$$

(16)

for unilamellar vesicles. Since generally in lipid bilayers $S < 0.3$, the terms involving $S$ in eqs. 15 and 16 are likely to be rather small, and the results arrived at by use of these formulas and the simple isotropic approximation (eqn. 14) are expected to be very close.
Davis (1979) assumed a model involving two motions, one being very fast ($\omega_0^2 \tau_C^2 \ll 1$) and the other being much slower than the first one, and derived the expression

$$\frac{1}{T_1} = \frac{3}{8} \left( \frac{e^2 qQ}{h} \right)^2 f (1 - S^2) \tau_c$$

(17)

where $f(1 - S^2)$ is the fraction of the total spectral density corresponding to the fast motion alone.

**Spin-Spin Relaxation**

The spin-spin relaxation time $T_2$ is commonly defined by means of the Bloch equations (Abragam, 1961) as

$$\frac{d M_{xy}}{dt} = - \frac{M_{xy}}{T_2}$$

(18)

As in the case of $T_1$ (see previous section), eqn. 18 assumes that the magnetization in the $xy$ plane, $M_{xy}$, decays exponentially with a single time constant $T_2$. Since the free induction decay (FID) measures $M_{xy}$, the above implies an exponential shape for the FID. However, due to the residual static quadrupolar interactions occurring in deuterated liquid crystalline systems, the $^2$H-NMR FID's do not decay exponentially, i.e. the absorption lineshape is not
Lorentzian, and this fact makes a definition of $T_2$ in terms of the Bloch equations difficult.

The $^2$H-NMR FID for a lipid bilayer is dependent on the residual static quadrupolar interactions and on the motional processes taking place in the system. The contribution to the FID of the residual static interactions can be removed by the quadrupolar echo pulse sequence

$$
\begin{pmatrix}
\frac{\pi}{2} & 0 \\
0 & \pi - \frac{\pi}{2}
\end{pmatrix}
\begin{pmatrix}
\tau_1 - \frac{\pi}{2} & 90^\circ
\end{pmatrix}
$$

(Davis et al., 1976), where $\tau_1$ is the time delay between the two pulses of the sequence, and one can then define a relaxation time $T_{2e}$, called the echo decay time, which is related only to the motional fluctuations occurring in the bilayer. The height of the quadrupolar echo is related to $2\tau_1$ by a simple exponential expression, and $T_{2e}$ is the time constant of that exponential decay (Davis et al., 1976; Davis, 1979). In the isotropic approximation, $T_{2e}$ is given by (Abragam, 1961; Davis, 1979)

$$
\frac{1}{T_{2e}} = \frac{1}{160} \left( \frac{e^2 qQ}{\pi} \right)^2 \left[ 9 J(0) + 15 J(\omega_o) + 6 J(2 \omega_o) \right]
$$

(20)

Comparison with eqn. 13 shows that $T_{2e}$ depends not only on $J(\omega_o)$ and $J(2\omega_o)$, but also on $J(0)$, and, hence, will
reflect slow \((\omega_0^2 \tau_c^2 > 1)\) motions in the system (Davis et al., 1976; Davis, 1979; Rice & Oldfield, 1979). Since the molecular motions in lipid bilayers are anisotropic, the above formula is, rigorously speaking, inappropriate, but it provides a reasonable first approximation to the real case.

For unilamellar lipid vesicles, the spin-spin relaxation time \(T_2\) is more easily defined since, due to the rapid vesicle reorientation and lateral diffusion in the plane of the bilayer, the \(^2H\)-NMR powder pattern is averaged out, and a Lorentzian absorption line is obtained (see above). The FID decays in an exponential fashion, and, ignoring magnetic field inhomogeneity, its time constant is \(T_2 = 1/\pi \Delta \nu_{1/2}\), where \(\Delta \nu_{1/2}\) is the width at half height of the Lorentzian absorption line.

II. Phosphorus 31 NMR

Phosphorus-31 NMR is a non-perturbing probe of the headgroup behaviour in phospholipid systems. In the present work, this technique was used to determine the phase behaviour of phospholipid membrane systems, as well as to determine the average size of unilamellar phospholipid vesicles. Hence, only the theory relevant to these applications will be presented in this section. For a more complete discussion of \(^{31}\text{P}-\text{NMR}\) in membranes, the reader is
referred to the review by Seelig (1978).

The $^{31}$P-NMR spectra of phospholipid liquid crystalline samples are dominated by the chemical shift anisotropy of the phosphate moiety and by dipolar interactions between the $^{31}$P atoms and neighbouring protons (Seelig, 1978). The effect of the chemical shift anisotropy is particularly useful for extracting information about the headgroup motion and the phase behaviour of the system. Therefore, dipolar interactions with the protons in the sample have to be removed by the application of proton-noise decoupling.

For phospholipid molecules in a lamellar phase, the long chain-like molecules undergo reorientational motions about the director axis which are assumed to be rapid and axially symmetric. For a sample composed of randomly distributed planar bilayers, the rapid axial motion gives rise to a typical $^{31}$P-NMR spectrum characterized by a low-field shoulder which corresponds to the magnetic field being parallel to the director axis and which has a chemical shift $\sigma_\parallel$, and by a high field peak, corresponding to the magnetic field being perpendicular to the director and with a chemical shift $\sigma_\perp$ (Fig. 7A). The quantity $\sigma_\parallel - \sigma_\perp$ is called the residual chemical shift anisotropy ($\Delta \sigma$ ) of the phosphate group, and for phospholipid bilayers above the gel to liquid-crystalline phase transition, is typically -35 to
Fig. 7: Proton noise-decoupled $^{31}\text{P}-\text{NMR}$ spectra of the lamellar (A) and hexagonal (B) liquid crystalline phases.
-45 ppm (Cullis et al., 1976; Seelig, 1978).

In the normal and inverted hexagonal phases, the rotation of the long cylinders and/or lateral diffusion of the phospholipid molecules provide an averaging mechanism for the chemical shift anisotropy of the phosphate moiety. In the case of a random distribution of cylinders, a proton-noise decoupled $^{31}$P-NMR spectrum (Fig. 7B) is obtained, whose residual chemical shift anisotropy is $-1/2$ that of the corresponding lamellar phase spectrum (Seelig, 1978).

In small, unilamellar phospholipid vesicles, fast isotropic tumbling of the approximately spherical structures and lateral diffusion average out the $^{31}$P lamellar phase powder pattern in a fashion similar to that of $^2$H-NMR (see above). A single Lorentzian absorption is observed having a width at half height $\Delta \nu_{1/2}$ given by

$$\Delta \nu_{1/2} = M_2 \tau_e + C$$

In this expression, $M_2$ is the residual second moment of the $^{31}$P powder pattern ($= 4/45 \Delta \sigma^2$) (McLaughlin et al., 1975), $\tau_e$ is the effective correlation time for vesicle reorientation given by eqn. 11, and C is a constant which reflects linewidth contributions which are independent of the vesicle tumbling rate, such as very fast molecular motions and magnetic field inhomogeneities.
C. EXPERIMENTAL

I. Materials

Crude egg-phosphatidylcholine (egg-PC) was extracted from fresh hen egg yolks according to Singleton et al. (1965) and then purified by column chromatography on silica gel as described by Richter et al. (1977). The pure egg-PC was dissolved at known concentration in chloroform and stored at -20 °C until used.

Bovine brain sphingomyelin was either purchased from Sigma Chemical Co., or extracted as described in Section C. II.

Cholesterol was purchased from Fisher Scientific Co. and recrystallized from benzene before being used. Other materials and their suppliers are as follows:

Dipalmitoylphosphatidylcholine and cholesteryl palmitate: Sigma Chemical Co.; deuterium depleted water (containing ~1% of the natural abundance of deuterium): Aldrich Chemical Co. and Sigma Chemical Co.; deuterium oxide (99.7%): Merck, Sharp & Dohme, Canada, Ltd.; hexadecanoic acid and glycerol: Fisher Scientific Co.; hexadecanoic-5,5,6,6-d₄ acid and hexadecanoic-11,11,12,12-d₄ acid: Merck, Sharp & Dohme,

Hexadecanoic-3,3-d₂ acid and hexadecanoic-4,4-d₂ acid, synthesized as described by Gorrissen et al. (1980), were a generous gift of Dr. A.P. Tulloch, Prairie Regional Laboratory, National Science and Engineering Research Council, Saskatoon, Saskatchewan, Canada. Octadecanoic-8,8-d₂ and octadecanoic-13,13-d₂ acids, prepared as described (Tulloch, 1977), were also gifts of Dr. A.P. Tulloch.

Hexadecanoic-2,2-d₂ and octadecanoic-2,2-d₂ acids were prepared by reaction with NaOD (Atkinson et al., 1968) in a stainless steel bomb. Only two instead of five exchanges were used. Temperatures were 220 °C (exchange 1) and 245 °C (exchange 2). Completion of the reaction was judged by ¹H-NMR and mass spectrometry, and >95% of the protons at C2 were exchanged by deuterons.

5-doxy1 palmitic acid (2-(3-carboxypropyl)-2-undecyl-4,4,4,4-dimethyl-3-oxazolidinylloxy1), prepared as described by Hubbell & McConnell (1971), was a gift from Dr. A.K. Grover, while 16-doxy1 stearic acid (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinylloxy1) was purchased from Syva, Palo Alto, CA.
II. Methods

Preparation of Cholesteryl Esters

Cholesteryl palmitate and stearate, deuterated in the fatty acyl chain, were prepared from cholesterol and the corresponding fatty acid, essentially as described by Grover & Cushley (1979b), except that purification was achieved by column chromatography on silica gel, eluting with benzene.

For spin labelled cholesteryl palmitate and stearate the procedure described by Grover & Cushley (1979b) was used unchanged.

Preparation of Bovine Brain Sphingomyelin

In a typical extraction, 500 g of bovine brain, obtained from a local slaughterhouse, were used, and crude sphingolipids were obtained essentially as described by Carter et al. (1947). The dry sphingolipids were then dissolved in 100 mL of warm glacial acetic acid, cooled to room temperature to precipitate cerebrosides (Marinetti & Stotz, 1954), and filtered. The filtrate was brought to dryness on a rotary evaporator. The dry residue was subjected to alkaline hydrolysis, as described by Sweeley (1963) and then purified on a silica gel column (50 cm x 2.5 cm).
Impurities were eluted with chloroform/methanol (1:1 and 1:4 v/v) and pure sphingomyelin with chloroform/methanol (1:9 v/v). The yield was typically 1.5 g.

Preparation of Lipid Dispersions and Unilamellar Vesicles

Lipids were mixed in sample tubes and co-dissolved in ~2-3 mL of chloroform (in the case of samples containing egg-PC) or chloroform/methanol (3:1 v/v) (in the case of samples containing DPPC or sphingomyelin). The solvent was removed by evaporation under a stream of nitrogen and subsequent overnight pumping under high vaccum.

Lipid dispersions were prepared by adding an equal weight of water or deuterium depleted water and vigorously shaking with a vortex mixer at a temperature above the gel to liquid-crystalline phase transition ($T_m$) of the respective phospholipid, until the sample appeared homogeneous.

Unilamellar vesicles were prepared by adding water or deuterium depleted water and sonicating the resultant mixture for ~15 min. on a Biosonik III probe-type sonicator at a temperature above $T_m$ of the phospholipid. The vesicles were centrifuged for ~15 min. on a small laboratory centrifuge, in order to eliminate titanium fragments and undispersed lipid. The vesicles were used immediately for the experiments.

Samples for which a quantitative determination of the
phospholipid and/or cholesteryl ester was required or which were to be used for analytical ultracentrifugation (see below), were subjected to an additional preparative ultracentrifugation for 3 h at 160,000 g on a Spinco Model L ultracentrifuge, in order to remove any traces of residual undispersed lipid and multilamellar liposomes (Barenholz et al., 1977).

Analytical Methods

Purity of phospholipids and cholesteryl esters was checked by means of thin layer chromatography on Silica Gel G plates (Macherey-Nagel & Co., Duren, West Germany), eluting with chloroform/ methanol/water (65:25:4 by volume) for phospholipids, and chloroform for cholesteryl esters.

The amount of phospholipid in unilamellar vesicle samples was quantified by phosphorus determination (Ames, 1966), while cholesteryl esters were determined either by the colorimetric method of Rudel & Morris (1973) or by $^2$H-NMR (see Sections D. IV. and V.).

For the fatty acid analysis of sphingomyelin, fatty acid methyl esters were prepared by dissolving 100 mg of the lipid in 2 mL of 3 M methanolic HCl and 2 mL of 2,3-dimethoxypropane, and then heating the solution in a stoppered tube under nitrogen for 3 h at 65-70 °C. The fatty
Acid methyl esters were extracted with petroleum ether, washed three times with water and dried under vacuum. The methyl esters were analyzed on a Varian Series 2100 gas chromatograph equipped with a flame ionization detector, using a 23 m x 0.27 mm inner diameter capillary column coated with Silar 10C. The instrument was programmed from 150 to 215 °C at a rate of 2 °C/min. In order to assign the peaks on these chromatograms, the mixture of fatty acid methyl esters was also analyzed on a Hewlett Packard HP 5985 GC/MS system possessing a Silar 10C GC column, and each peak was identified by mass spectrometry. The assignments were then obtained by comparison of these peaks with the ones obtained on the Varian 2100 gas chromatograph.

Differential scanning calorimetry experiments were performed on a Perkin Elmer DSC-1B calorimeter using a scanning rate of 5 °C.

**Analytical Ultracentrifugation**

Diffusion studies were carried out on Spinco Model E analytical ultracentrifuge equipped with a Schlieren optical system and a RTIC temperature control unit, at 20 °C and 42,040 rpm, essentially as described by Newman & Huang (1975). After subjecting the vesicles to preparative ultracentrifugation as described under "Preparation of Lipid
Dispersions and Unilamellar Vesicles" (see above), they were diluted to the desired concentration (typically between 0.15 and 0.50%) with distilled water and deuterium oxide in order to bring the composition of the aqueous phase to 86 vol.% H$_2$O :14 vol.% D$_2$O. Under these conditions, the vesicles showed negligible sedimentation during the centrifugation runs, and the spreading of the Schlieren peaks could be recorded over long periods of time.

**Electron Spin Resonance**

ESR spectra of samples containing spin-labelled cholesteryl esters were obtained on a Varian E-4 spectrometer operating at ~9.4 GHz. Temperature was regulated by means of a Varian gas-flow temperature controller. The microwave power used was 50 mW, and the amplitude of the 100 kHz field modulation was typically 0.4 G.

**Phosphorus-31 Nuclear Magnetic Resonance**

Phosphorus-31 NMR spectra were acquired on a Varian XL-100-15 spectrometer operating in the Fourier transform (FT) mode and interfaced to a Nicolet 1080 computer. Temperature was controlled by means of a gas-flow system. The free induction decays (FID's) were recorded at 40.5 MHz, using an external $^{19}$F field-frequency lock, and in the
presence of proton noise-decoupling which usually covered a 2 kHz bandwidth. Chemical shifts were measured relative to an external \( \text{H}_3\text{PO}_4 \) (85%) reference.

For unilamellar vesicle samples, proton decoupling was normally gated on only during acquisition of the FID's, in order to eliminate nuclear Overhauser effects. Spectral parameters used were: Pulse length = 15 \( \mu \)s (90° flip angle); sweep width = 2 kHz; dataset size = 2K zero-filled to 4K; pulse delay = 15 s; line broadening = 2 Hz. In order to determine the ratio of \(^{31}\text{P}\) atoms on the outside and inner monolayer of unilamellar vesicles, enough \( \text{Pr(NO}_3\text{)}_3 \) solution (~25 \( \mu \)L of 0.1 M solution per mL of 10% w/v vesicle dispersion) was added to produce a shift of ~150-200 Hz between the outside and inside signals. Signal intensities were obtained by spectral integration on the Nicolet 1080 computer.

In the case of multilamellar liposomes, the output of the decoupler was applied to the transmitter coils, while the transmitter pulses were applied to the decoupler coils. This was seen to provide an increase in decoupling power at the sample compared to the conventional arrangement, yielding improved spectral shape. Parameters used were: Pulse length = 20 \( \mu \)s (~30° flip angle); sweep width = 10 kHz; dataset size = 4K; number of transients ~2000; pulse delay = 2 s; line
broadening = 5 Hz.

**Deuterium Nuclear Magnetic Resonance**

Deuterium NMR spectra of unilamellar vesicles were obtained on a Varian XL-100-15 NMR spectrometer operating at 15.4 MHz in the FT mode and interfaced to a Nicolet 1080 computer. Sufficient rf power was achieved by use of a 300 W ENI power amplifier. Temperature was controlled by means of a gas-flow system, and the field-frequency lock was an external $^{19}$F signal. Unless otherwise indicated, a pulse length of ~60 μs (90° flip angle) was used. Calculations of spectral intensities as well as spectral simulations in order to obtain the best fit Lorentzian lines were carried out on the Nicolet 1080 computer. Spin-spin relaxation times ($T_2$) were determined from the $^2$H-NMR peak widths at half height ($\Delta \nu_2$). Spin-lattice relaxation times ($T_1$) were determined by the inversion-recovery method (Vold et al., 1968). In all experiments a phase-alternating pulse sequence was used in order to minimize baseline errors. In a few of the $^2$H-NMR spectra recorded for unilamellar vesicles, a DOH resonance appeared in spite of the use of deuterium depleted water in the preparation of the sample. When accurate linewidth measurements were demanded on these spectra, the DOH signal was eliminated by spectral subtraction of a $^2$H signal from
distilled water.

Deuterium NMR experiments on multilamellar liposomes were performed on a Bruker SXP-100 spectrometer with a Nalorac superconducting magnet, at 34.4 or 37.2 MHz. The NMR signals were collected on an Intel 8080 microprocessor-based computer, interfaced to a Nicolet 1090AR Explorer digital oscilloscope. Spectral processing in order to obtain FT spectra was done on a Nicolet BNC-12 computer. The sample temperature was regulated by means of a Bruker BST 100-700 controller. The quadrupolar echo pulse sequence

\( \left( \frac{\pi}{2} \right)_{0^o} - \tau_1 - \frac{\pi}{2} \left| 90^o \right. \), where \( \tau_1 \) is the delay between the two pulses) (Davis et al., 1976) was used in order to obtain spectra without distortions due to the receiver recovery time. The phase of the first pulse of the sequence was changed by 180° for every second sequence, and alternate scans were subtracted to eliminate effects from coherent interference. Echo decay times (\( T_{2e} \)) were measured from the dependence of the signal intensity upon \( \tau_1 \). Spin-lattice relaxation times (\( T_1 \)) were obtained using a modification of the quadrupolar echo sequence in which the 180° phase shift in every second sequence was replaced by a \( \pi \) pulse preceding the sequence by a time \( \tau_2 \) (Valic et al., 1979), and \( T_1 \) was calculated by the expression:

\[
A(\tau_2) = A(0) \exp(-\tau_2/T_1) \quad (22)
\]
where $A(\tau_2) = \text{signal amplitude corresponding to a delay } \tau_2$, and $A(0) = \text{signal amplitude corresponding to } \tau_2 = 0$.

Measurements of the areas of the various features in the $^2\text{H-NMR}$ spectra of liposomes were performed on a Nicolet BNC-12 computer. The uncertainty in these measurements was 10-20%, depending on the signal-to-noise ratio of the spectra.

Data processing required to obtain the spectra corresponding to an aligned sample (Bloom et al., 1981) was carried out on an Amdahl V6 computer.

Statistical mechanical computer calculations based on the mean-field approximation were done on an IBM 4341 computer.
D. RESULTS AND DISCUSSION

I. Deuterium NMR of Solid Cholesteryl Esters

The $2H$-NMR spectrum of a sample of solid, polycrystalline cholesteryl palmitate (CP)-16,16,16-$d_3$ is shown in Fig. 8. It consists of a typical $2H$-NMR powder pattern with a quadrupolar splitting of 38 kHz, plus a narrow central line. The splitting of 38 kHz observed can be explained if fast rotation of the $CD_3$ group about the $CH_2-CD_3$ bond of the the acyl chain is the dominant line-narrowing mechanism. For such a rotation, the angle $\theta$ between the C-D bond in question and the axis of symmetry of reorientational motions equals 109.3°, and hence $S = -1/3$, yielding (from eqn. 6) a quadrupolar splitting of 42 kHz. The reduction from this value to the observed one is due to small amplitude thermal motions (librations) of the ester acyl chain (Craven & DeTitta, 1976), which provide an additional spectral averaging mechanism.

From the dependence of the intensity of the spectra of solid CP-16,16,16-$d_3$ with the time delay $\tau_R$ between successive pulse sequences (also called the "repetition time"), a spin-lattice relaxation time $T_1$ of 0.24 s for the
Fig. 8: 2H-NMR spectrum of polycrystalline cholesteryl palmitate-16,16,16-d$_3$.

$\tau_R$ = time delay between quadrupolar pulse sequences; #SC = number of scans; rf pulse length = 4 $\mu$s (90° pulse); number of datapoints = 4096; line broadening = 200 Hz.
SOLID CHOLESTERYL PALMITATE - 16, 16, 16 - d₃

\[ \tau_R = 2 \text{ sec.} \]

\# SC = 3,600

38 kHz
CD₃ deuterons was calculated.

The narrow central spike in the spectrum of solid CP-16,16,16-d₃ (Fig. 8) must arise from deuterons in an isotropic environment and may be due to ester dissolved in small amounts of residual solvent, water of hydration, etc. Since the proportion of this narrow signal is very small (<1% of the total intensity), we will ignore it in our discussion.

The 2H-NMR spectrum of solid, polycrystalline CP-d₃₁ is presented in Fig. 9. This spectrum was taken using a very short pulse of 1 μs (22.5° flip angle) in order to obtain a faithful representation of the spectral shape across its whole width. This spectrum is interpreted as the superposition of two powder patterns (plus a narrow central line) with quadrupolar splittings of 38 and 118 kHz, respectively, and having a ratio of intensities of about 1:9. On the basis of our observations using CP-16,16,16-d₃, we again assign the 38 kHz splitting to the three CD₃ deuterons, whereas the 118 kHz feature is assigned to the 28' (CD₂)ₙ deuterons of CP-d₃₁. The observed intensity ratio corroborates this interpretation. For deuterons on CD₂ groups undergoing no motions, we expect from eqn. 6 a quadrupolar splitting $\Delta v_Q = 126$ kHz. The reduction to the observed value of 118 kHz can, again, be interpreted as being due to slight librational motions of the CD₂ groups of solid CP-d₃₁ (Craven
Fig. 9: 2H-NMR spectrum of polycrystalline cholesteryl palmitate-d$_{31}$.  

\[ \tau_R = \text{time delay between quadrupolar pulse sequences}; \#SC = \text{number of scans}; \text{rf pulse length} = 1 \mu s (22.5^\circ \text{ pulse}); \text{number of datapoints} = 4096; \text{line broadening} = 1 \text{ Hz}. \]
SOLID

$d^{31}_{31} -$ CHOLESTERYL PALMITATE

$\tau_R = 60$ sec.

$\#\ SC = 240$
& DeTitta, 1976).

The effect of the length of the rf pulse on the 2H-NMR spectrum of solid polycrystalline CP-d$_{31}$ is apparent from Figs. 9 and 10, where the pulse length is 1 µs (22.5° flip angle) and 4 µs, (90° flip angle), respectively. The use of the longer pulses is advantageous because it gives rise to a much improved signal to noise ratio. However, due to the large spectral width of the solid spectra, distortions will result. This is due to the falloff of the power of the rf pulse at frequencies away from the carrier frequency (Farrar & Becker, 1971). It is, however, possible to account for such effects, and this will be done when dealing with the 2H-NMR spectra of CP-d$_{31}$ in egg-PC liposomes.

The average spin-lattice relaxation time T$_1$ of the methylene deuterons of CP-d$_{31}$, determined from the spectral intensities as a function of $\tau_2$ (see eqn. 22) is $T_1 = 11 \pm 1$ s at ~20 °C, and such a high value is consistent with the CD$_2$ segments of solid CP-d$_{31}$ being rather immobile.
Fig. 10: 2H-NMR spectra of polycrystalline cholesteryl palmitate-d$_{31}$.

$\tau_R$ = time delay between quadrupolar pulse sequences; $\# SC$ = number of scans; rf pulse length = 4 $\mu$s (90° pulse); number of datapoints = 4096; line broadening = 75 Hz.
II. Multilamellar Liposomes of Egg-Phosphatidylcholine

Multilamellar liposomes composed of 50 wt% egg-PC (plus 5 mol% CP-16,16,16-d$_3$) + 50 wt% H$_2$O give rise to the $^2$H-NMR spectrum depicted in Fig. 11. This spectrum consists of the spectral feature attributed to the solid ester (with a splitting of 38 kHz) and the narrow central line (see Fig. 8), plus a relatively narrow feature having a quadrupolar splitting of ~3 kHz. Since the solubility of CP in water is negligible (see section D. IV.), the 3 kHz feature must arise from molecules of cholesteryl ester dissolved in the egg-PC bilayers.

The absolute value of the order parameter S for the C-D bonds of CP-16,16,16-d$_3$, incorporated into egg-PC liposomes, was calculated by means of eqn. 6 and using $\Delta \nu_Q = 3$ kHz, and it is $|S| = 0.02$. This value is identical to the results obtained for palmitic-16,16,16-d$_3$ acid probes in egg-PC bilayers (Stockton et al., 1976) and for the CD$_3$ group of bilayers composed of DPPC-d$_{62}$ (Davis, 1979), indicating that the orientational order of the cholesteryl ester and the phospholipid CD$_3$ groups is quite similar.

When comparing the intensities of the features attributed to incorporated and solid cholesteryl ester in Fig. 11, one finds that the solubility of CP in egg-PC...
Fig. 11: $^2$H-NMR spectrum of 50 wt% egg-PC (plus 5 mol% cholesteryl palmitate-16,16,16-\textit{d}_3 ) + 50$ wt% H$_2$O multilamellar liposomes.

$\tau_R =$ time delay between quadrupolar pulse sequences; $\#SC =$ number of scans; rf pulse length = 4 $\mu$s (90 $^\circ$ pulse); number of datapoints = 512 zero-filled to 4096; line broadening = 50 Hz.
$\tau_R = 0.12 \text{ sec.}$

$\# SC = 32,000$
bilayers must be quite low (< 1 mol%). Due to the relatively low signal to noise ratio of the spectra involving CP-16,16,16-d₃, a quantitative calculation of the amount of dissolved ester is difficult. This number can, however, be obtained from the $^2$H-NMR spectra of CP-d₃ in egg-PC liposomes (Fig. 12). These spectra consist of four features labelled A, B, C, and D, with widths of: (A) 3 kHz, (B) 12 kHz, (C) 38 kHz, (D) 118 kHz. Comparison with the spectra presented in the previous section indicates that features C and D are due to the deuterons on the CD₃ and (CD₂)ₙ groups of solid CP-d₃, respectively, while feature A is due to the deuterons on the CD₃ group of ester dissolved in the bilayers. Feature B is thought to arise from the (CD₂)ₙ groups of CP-d₃ molecules dissolved in the phospholipid bilayer. As in the case of the solid esters, there is an isotropic spectral component of negligible intensity compared to the other features.

The spectra in Fig. 12 were taken with a delay between successive pulse pairs $\tau_R$ of 10 s. Therefore, on the basis of our results involving the solid esters (Section D. I.), we know that, due to the long $T_1$ of the solid (CD₂)ₙ groups, feature D will be attenuated with respect to its expected value. This reduction is given by $1 - \exp(-\tau_R/T_1)$. In addition, feature D will be attenuated due to the long rf
Fig. 12: $^2$H-NMR spectra of 50 wt% egg-PC (plus X mol% cholesteryl palmitate-d$_{31}$) + 50 wt% H$_2$O multilamellar liposomes.

Top spectrum: X = 5 mol%  
Bottom spectrum: X = 1 mol%

$\tau_R$ = time delay between quadrupolar pulse sequences; 
#SC = number of scans; rf pulse length = 4 $\mu$s (90° pulse); number of datapoints = 4096; line broadening = 400 Hz.

The features A, B, C, and D are described in the text and the dotted lines represent the separations chosen for the measurement of the areas of each feature.
50 wt. % EYL (+X mol % d_{31} -CP)

+ 50 wt. % H_2 O

\[ \tau_R = 10 \text{ sec} \]
\[ \# SC = 3600 \]

X = 5 %

X = 1 %

-250 kHz

0

+250 kHz
pulse length of 4 \( \mu s \). This attenuation can be accounted for by comparing the areas of the C + D features in the spectrum of CP-d\(_{31}\) taken at a very long (>5\( T_1 \)) repetition time, and using the 4 \( \mu s \) pulses. Analysis of a spectrum acquired using a repetition time \( T_R = 60 \) s yields Area D/Area C = 7, whereas the theoretical ratio is given by the relation between the number of (CD\(_2\))\(_n\) and CD\(_3\) deuterons in the CP-d\(_{31}\) molecules, i.e. 28/3. The finite pulse length of 4 \( \mu s \) will therefore attenuate feature D by an amount "d" given by\(^1\):

\[
d = \frac{\text{measured ratio}}{\text{predicted ratio}} = \frac{7}{(28/3)} = 0.75
\]

(23)

In order to obtain the concentration of dissolved CP-d\(_{31}\) (\( f_{\text{diss}} \)) from Fig. 12, we define the ratio

\[
f/f_{\text{diss}} = 1 + \frac{N_C + N_D}{N_A + N_B}
\]

(24)

where \( f \) = total concentration of CP-d\(_{31}\) in the sample, and \( N_i \) = number of deuterons contributing to feature "i". The area \( E_i \) of the spectral feature "i" is proportional to the number of deuterons \( N_i \), except in the case of feature D which is attenuated. Hence

\[\text{---------}\]

For this analysis we have used the reasonable assumption that the 4 \( \mu s \) pulse is short enough not to attenuate features A, B and C.
\[ E_i = K N_i \quad \text{for} \quad i = A, B, C \]  

and \[ E_D = K N_D d \left( 1 - \exp \left( - \frac{\tau_R}{T_{1(D)}} \right) \right) \]

where \( K \) is a proportionality constant.

The measured ratio of the areas of the signals due to solid and dissolved \( \text{CP-d}_31 \) is

\[ R = \frac{E_C + E_D}{E_A + E_B} = \frac{N_C + N_D d \left( 1 - \exp \left( - \frac{\tau_R}{T_{1(D)}} \right) \right)}{N_A + N_B} \]  

or

\[ R = \frac{N_C + N_D d \left( 1 - \exp \left( - \frac{\tau_R}{T_{1(D)}} \right) \right)}{N_C + N_D} \frac{N_C + N_D}{N_A + N_B} \]  

hence,

\[ \frac{f}{f_{\text{diss}}} = 1 + R \frac{N_C + N_D}{N_C + N_D d \left( 1 - \exp \left( - \frac{\tau_R}{T_{1(D)}} \right) \right)} \]  

The ratio \( R \) can be measured, \( N_C/N_D = 3/28, d = 0.75, T_{1(D)} = 11 \text{ s}, \) and \( \tau_R = 10 \text{ s}, \) therefore, \( \frac{f}{f_{\text{diss}}} \) can be calculated\(^2\).

\(^2\) I am indebted to Prof. Myer Bloom for the original derivation of eqn. 28.
It should be noted that for the above derivation we did not take into account another possible source of discrepancy between the ratio of the number of deuterons and the observed ratio of the corresponding spectral components: If the decay time of the quadrupolar echo ($T_{2e}$) is not much longer than the delay between the two pulses in the pulse sequence ($\tau_1$), then the observed spectral intensity will be reduced by an amount $\exp(-2\frac{\tau_1}{T_{2e}})$. From the dependence of the spectral component "B" from CP-d$_{31}$ in egg-PC liposomes with $\tau_1$, an echo decay time $T_{2e} = 850$ µs has been calculated. This value is much larger than the $\tau_1$ values between 60 and 100 µs used to obtain our spectra, hence we can ignore the above correction.

Based on the spectrum in Fig. 12 (upper trace), an incorporation $f_{\text{diss}} \sim 0.2 \ (\pm 0.1)$ mol% is calculated from eqn.28 for CP-d$_{31}$ in egg-PC liposomes. This low incorporation is somewhat surprising, since the solubility of CP in egg-PC unilamellar vesicles (Forrest & Cushley, 1977; see also section D. IV.) can reach up to 5 mol%, whereas the incorporation of the spin-labelled ester, 5-doxy1 cholesteryl palmitate, in egg-PC multilayers is $\sim 0.8$ mol% (Grover & Cushley, 1979). In addition, Janiak et al. (1974) found that the solubility of cholesteryl linolenate in egg-PC bilayers is $\sim 3$ mol% at similar water concentrations. Thus, our results
may indicate that the solubility of cholesteryl esters in phospholipid membranes is dependent upon the structure of the ester, and it may be that saturated esters are less soluble than unsaturated ones. This is consistent with the conclusion reached by Janiak et al. (1979), who studied the saturated ester, cholesteryl myristate, in dimyristoylphosphatidylcholine bilayers, and found negligible incorporation at maximum hydration of the phospholipid.

A solubility of ~0.2 mol% is also calculated from the spectrum of egg-PC liposomes containing only 1 mol% CP-d_{31} (Fig. 12, lower trace). Comparison of the two spectra in this figure reveals that the shape and intensity of the features attributed to incorporated CP-d_{31} are practically the same, whereas the solid signal is reduced ~5-fold in the 1 mol% spectrum with respect to the one of the sample containing 5 mol% ester. Using the expression derived previously (eqn. 28), we find for this sample f_{diss} ~ 0.2 mol%, in agreement with the previous calculation.

In order to obtain information regarding the dynamic behaviour of the dissolved portion of CP-d_{31} in egg-PC liposomes, we have obtained another spectrum of a sample containing 1 mol% CP-d_{31} (Fig. 13). This spectrum was obtained using a very fast repetition time of 0.1 s, which allowed the acquisition of a large number of scans. The low
Fig. 13: $^2$H-NMR spectrum of 50 wt% egg-PC (plus 1 mol% cholesteryl palmitate-d$_{31}$ ) + 50 wt% H$_2$O multilamellar liposomes.

$\tau_R$ = time delay between quadrupolar pulse sequences; #SC = number of scans; rf pulse length = 4 $\mu$s (90° pulse); number of datapoints = 4096; line broadening = 100 Hz.
50 wt. % EYL (+1 mol % d_31-CP) 
+ 50 wt. % H_2O (deuterium depleted)

\( \tau_R = 0.1 \text{ sec.} \)

\( \# \text{ SC} = 108,000 \)
value of $\tau_R$ caused saturation not only of feature D but also of feature C, so that essentially only the "dissolved" signal is seen. The spectrum has a shape somewhat reminiscent to that obtained from dipalmitoylphosphatidylcholine perdeuterated in both acyl chains (DPPC-$d_{62}$), which is characterized by sharp edges of the powder pattern (Davis, 1979). These sharp spectral edges, which in DPPC-$d_{62}$ occur at a splitting of $\sim 26$ kHz at 45 °C (Davis, 1979), are a manifestation of the well known "ordering plateau" (Seelig & Seelig, 1974b; Stockton et al., 1976; Davis, 1979) and arise as a consequence of the fact that a relatively large number of deuterons give rise to essentially the same quadrupolar splitting. By analogy, we may conclude that, in the case of the cholesteryl ester, a number of deuterons must give rise to quadrupolar splittings near $\sim 12$ kHz, and this value is >2-fold lower than that encountered for the phospholipid.

A possible reason for the low width of the $^2$H-NMR signal from incorporated CP-$d_{31}$ in egg-PC liposomes could be the existence of hexagonal phase of the membrane. In such a phase the observed quadrupolar splittings are reduced by a factor of two with respect to the lamellar phase (Seelig, 1977; Section B. I.). The hexagonal phase can be easily recognized by its characteristic proton noise-decoupled $^{31}$P spectrum, whose chemical shift anisotropy is $-1/2$ that of the lamellar
phase spectrum (Seelig, 1978; Section B. II.). However, the proton noise-decoupled $^{31}\text{P}$-NMR spectrum of egg-PC liposomes containing 5 mol% CP shows the typical shape and chemical shift anisotropy corresponding to the lamellar phase, and this rules out the existence of any appreciable amounts of hexagonal phase.

Since the decreased average quadrupolar splitting observed for CP-d$_{31}$ in egg-PC liposomes, as compared to bilayers of pure phospholipid, is not due to the existence of hexagonal phase, it must stem from a reduced "average" segmental order parameter for the CD$_2$ segments of the ester chain. The average value of S for CP-d$_{31}$ is ~0.1, versus S~0.23 for the plateau region of DPPC (Seelig & Seelig, 1974b; Davis, 1979) and for deuterated fatty acid probes in egg-PC membranes (Stockton et al., 1976). By eqn. 8 (Section B. I.), the order parameter S is related to the degree of motional averaging due to "kink" motions taking place in the molecule, and to the angle $\gamma$ between the axis of the molecular segment in question and the director. This last contribution may be due either to the presence of "rigid stick" motions as proposed by Petersen & Chan (1977) or to a specific molecular alignment such that $\gamma$ in eqn. 8 is constant. Applying these concepts to the case of CP-d$_{31}$ in egg-PC bilayers, the observed order parameter decrease for
the ester may be due to a higher degree of "kink" motions and/or to a changed value of $<3 \cos^2 \gamma - 1>/2$ as compared to the phospholipid. The idea of a higher degree of intramolecular trans-gauche isomerizations may not be discarded on account of the present data. However, on the basis of the proposal of a "horseshoe" conformation for some cholesteryl esters in phospholipid bilayers (Janiak et al., 1974; Grover et al., 1979), it is conceivable that several molecular segments may be aligned at an angle with respect to the bilayer normal (which is assumed to constitute the director axis), thus giving rise to a decrease in the value of $<3 \cos^2 \gamma - 1>/2$ in eqn. 8. The "horseshoe" conformation could then be manifested in a lower average value of $S$. A study using selectively deuterated cholesteryl esters might be useful to obtain more detailed information in this respect. However, due to the very low ester solubility in this system, such a study would require extremely long spectral averaging times, and a better alternative is to look for membrane systems in which ester solubility is higher than in the present case.
III. Aqueous Dispersions of Sphingomyelin

During the progress of atherosclerosis, the phospholipid composition of human aorta shows an increase in sphingomyelin (SPM) content from 35 to 63%, whereas the proportion of phosphatidylcholine decreases substantially from 43 to 23% (Bottcher & Van Gent, 1961). Since the cholesteryl ester content of the aorta is also known to increase during the atherosclerotic disease (Insull, 1972), the interaction of cholesteryl esters with SPM may be important to the understanding of the factors responsible for the disease. In addition, attempts to produce SPM/CP unilamellar vesicles showed that CP is somewhat more soluble in these (~8-10 mol%) than in egg-PC vesicles (~5 mol%). Hence, we decided to study the dynamic behaviour of cholesteryl esters in SPM bilayers, hoping to obtain stronger $^2$H-NMR signals than for deuterated CP in multilamellar egg-PC liposomes.

Fatty Acid Analysis and Differential Scanning Calorimetry.

It has been reported that SPM obtained from different sources can vary greatly in its fatty acid composition and in its calorimetric behaviour (Calhoun and Shipley, 1979a). We have therefore analyzed the two types of SPM used in this
study and found their fatty acid composition as shown in Table I. The results show that the composition of the two SPM's is essentially the same, and they were used interchangeably throughout the study.

A differential scanning calorimetry experiment performed on SPM/CP dispersions showed that the gel to liquid-crystalline phase transition $T_m$ encompasses the range of 30-45 $^\circ$C, with maxima at 34 and 40 $^\circ$C, in agreement with published data (Shipley et al., 1974; Barenholz et al., 1976). The differential scanning calorimetry trace of 47.5 mol% SPM/47.5 mol% cholesterol/5 mol% CP dispersions (not depicted) showed no phase transition in the temperature range of 20 to 60 $^\circ$C, indicating that cholesterol removes the transition of the phospholipid, and this is identical to the behaviour of SPM/cholesterol aqueous dispersions in the absence of cholesteryl ester (Calhoun & Shipley, 1979b; Estep et al., 1979).

**Nuclear Magnetic Resonance**

Deuterium spectra of dispersions containing 50 wt% (SPM + 5 mol% CP-d$_{31}$) + 50 wt% deuterium depleted water at different temperatures are presented in Fig. 14. These spectra are rather similar to the ones obtained for CP-d$_{31}$ in egg-PC liposomes, and we interpret them in a like manner.
### TABLE I: Fatty acid composition of sphingomyelin.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Bovine brain sphingomyelin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sigma</td>
<td>Sigma</td>
</tr>
<tr>
<td>16:0</td>
<td>6.0</td>
<td>2.9</td>
</tr>
<tr>
<td>18:0</td>
<td>38.2</td>
<td>46.6</td>
</tr>
<tr>
<td>20:0</td>
<td>1.9</td>
<td>1.3</td>
</tr>
<tr>
<td>22:0</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>23:0</td>
<td>2.5</td>
<td>3.4</td>
</tr>
<tr>
<td>24:0</td>
<td>6.9</td>
<td>10.5</td>
</tr>
<tr>
<td>24:1</td>
<td>32.8</td>
<td>27.3</td>
</tr>
<tr>
<td>other unsaturated fatty acids</td>
<td>7.7</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Fig. 14: $^2$H-NMR spectra of 50 wt% sphingomyelin (plus 5 mol% cholesteryl palmitate-$d_{31}$) + 50 wt% H$_2$O dispersions. The features A - E are described in the text. The arrows indicate the position at which the width of feature C was measured. Time delay between quadrupolar pulse sequences ($\tau_R$) = 1.5 s; number of scans = 8000; rf pulse length = 5 $\mu$s (90° pulse); number of datapoints = 4096.
Features D and E, with quadrupolar splittings of 38 and 118 kHz, respectively, are ascribed to solid ester in the sample. Due to the short repetition time $T_R$ of 1.5 s, feature E is greatly attenuated ($T_1$ for $E = 11$ s; see Section D. I.). Feature C is attributed, as in the case of CP-d$_{31}$ in egg-PC liposomes, to deuterons on ester molecules intercalated in SPM bilayers. Spectral feature B is only observed above ~40°C and appears as a relatively narrow signal having a width of <1 kHz which becomes more intense at higher temperatures. Thus it cannot arise from residual DOH in the sample, and we attribute it to ester molecules undergoing isotropic motion. We will elaborate on this point later. Finally, a small and narrow central signal (A) is observed, whose intensity is independent of the temperature and thus probably arises from residual DOH.

The width of the spectral feature attributed to ester in SPM bilayers (feature C) varies dramatically with temperature, and this is represented graphically in Fig. 15. For comparison, we have also included the data for N-palmitoyl-10,10-d$_2$ SPM (Neuringer et al., 1979). It is clear that the width of the signal from incorporated CP-d$_{31}$ changes much more with temperature than that of deuterated SPM, which implies that the structural organization of the CP chains is quite different from that of the phospholipid chains. At
Fig. 15: Plot of the width of the $^2$H-NMR signal from cholesteryl palmitate-d$_{31}$ incorporated in sphingomyelin aqueous dispersions, versus temperature. The continuous line represents the width of the $^2$H-NMR spectra of N-palmitoyl-10,10-d$_2$ sphingomyelin (Neuringer et al., 1979).
temperatures below $T_m$ of the phospholipid, the splitting of feature C is so broad that it cannot be separated from the solid ester signal.

Since the $^2$H-NMR spectra of CP-d$_{31}$ in SPM, especially below the phase transition of the phospholipid, contain several overlapping features, and since $T_1$ of the solid (CD$_2$)$_n$ signal is very long, we did not calculate the incorporation of CP-d$_{31}$ in the bilayer from these spectra. Instead, we have obtained $^2$H-NMR spectra of CP-16,16,16-d$_3$ in SPM dispersions, which are simpler than those of CP-d$_{31}$, and $T_1$ of the solid signal is only 0.25 s. Spectra obtained at four temperatures are depicted in Fig. 16, and the features A - D are assigned as also shown in this figure. The numbers of deuterons $N_i$ giving rise to B, C, and D are related to the solubility of the ester in the bilayer by:

$$f_{\text{diss}}/f = \frac{N_B + N_C}{N_B + N_C + N_D}$$

(29)

Since the broadest quadrupolar splitting in these spectra is 38 kHz and the pulse length is 5 $\mu$s, we can ignore the spectral distortions caused by the finite pulse length. (Farrar & Becker, 1971; see also Section D. I.) The delay between pulse sequences $\tau_R$ is 1.5 s, which is much longer than the $T_1$ of 0.25 s for the solid ester. Hence we do not have to resort to the relatively complicated treatment.
Fig. 16: $^2$H-NMR spectra of 50 wt% sphingomyelin (plus 5 mol% cholesteryl palmitate-16,16,16-d$_3$) + 50 wt% H$_2$O dispersions. The features A-D are assigned as in Fig. 14 and are described in the text. The dotted lines indicate the separation between features C and D.

Time delay between quadrupolar pulse sequences ($\tau_R$) = 1.5 s; number of scans = 8000; rf pulse length = 5 $\mu$s (90° pulse); number of datapoints = 4096.
outlined in Section D. I. to calculate the solubility of CP in the bilayer. However, in the present case we have found that there is an effect of the echo decay time $T_{2e}$ on the observed spectral intensity. $T_{2e}$ for CP-16,16,16-d₃ in SPM bilayers varies between ~200 μs at 25 °C and ~1000 μs at 50 °C, whereas $T_{2e}$ for the solid ester is 400 μs. On the other hand, the delay $\tau_1$ between the two pulses in the quadrupolar echo sequence was 80 μs. Thus, the relation $T_{2e} \gg \tau_1$ is not valid at all temperatures, and we have to extrapolate the measured peak areas $E_i(2\tau_1)$ to $\tau_1 = 0$ by use of the relation:

$$E_i(2\tau_1) = E_i(0) \exp(-2\tau_1/T_{2e})$$  \hspace{1cm} (30)$$

$E_i(0)$ is directly proportional to the number of deuterons $N_i$. The percentage of dissolved CP, calculated using eqs. 29 and 30, was found to be constant at 1.5 (±0.3) mol% at all temperatures between 25 and 60 °C (Fig. 17). Hence, the solubility of CP in SPM bilayers is the same both above and below the gel to liquid-crystalline phase transition of the phospholipid. In addition, it is about 8 times higher than that of CP in egg-PC liposomes. These results are in contrast with those obtained by Janiak et al. (1979) for the related system cholesteryl myristate/ dimyristoylphosphatidylcholine/
Fig. 17: Graph of the percentage incorporation of cholesteryl palmitate-16,16,16-\textsuperscript{d}_3 in sphingomyelin aqueous dispersions, versus temperature. The error bar on the first point indicates the typical uncertainty of the values.
water by use of polarizing light microscopy, X-ray diffraction, and differential scanning calorimetry. Those workers concluded in their study that ester incorporation into the phospholipid was negligible below the phase transition temperature.

We have also obtained $^2$H-NMR spectra of a dispersion of 47.5 mol% SPM/47.5 mol% cholesterol/5 mol% CP-d$_{31}$ in deuterium depleted water at four temperatures between 25 and 55 °C and found that the incorporation of cholesteryl ester is decreased >10-fold with respect to its value in SPM dispersions without cholesterol. This effect of cholesterol is similar to that observed in egg-PC vesicles, where addition of 20 mol% of the sterol decreases ester solubility several-fold (see Section D.IV.).

The quadrupolar splitting of spectral feature C in Fig. 16 can be used to calculate the segmental order parameter S for the CD$_3$ group of CP-16,16,16-d$_3$ using eqn. 6. Fig. 18 represents a plot of S versus temperature for this chain position. For comparison, we have included in Fig. 18 the values obtained for the CD$_3$ groups of DPPC-d$_{62}$ (Davis, 1979). Above the phase transition of SPM, S for the ester is very close to those of DPPC-d$_{62}$ as well as CP-16,16,16-d$_3$ (Section D. II.) and palmitic-16,16,16-d$_3$ acid (Stockton et al., 1976) in egg-PC liposomes. This similarity is not
Fig. 18: Graph of the C-D order parameter for the CD₃ group of cholesteryl palmitate-16,16,16-d₃ in sphingomyelin aqueous dispersions, versus temperature. The continuous line represents the order parameter for the CD₃ groups of DPPC-d₆₂ (Davis, 1979).
surprising, since the terminal methyl groups of hydrocarbon chains are known to have a high degree of motional freedom. At temperatures within and below the region of the SPM phase transition, the $S$ values of CP-16,16,16-$d_3$ increase significantly, indicating that the ester acyl chain packing is affected by the increased orientational order of the SPM chains.

Spectral feature B, with a narrow width of $\sim 0.4$ kHz, is attributed by us to cholesterol ester in SPM membranes undergoing rapid isotropic motion. The percentage of this isotropic portion of ester is related to the areas $E_B$ and $E_C$ of the spectral features B and C in Fig. 17 by:

$$\% \text{ isotropic} = \frac{E_B}{E_B + E_C} \cdot 100 \quad (31)$$

and this number is plotted in Fig. 19 as a function of temperature. The proportion of isotropic ester is very small below the SPM phase transition and increases in a nearly linear fashion above $T_m$, reaching $\sim 30\%$ of the total incorporated ester at $\sim 60^\circ C$.

We have also obtained proton noise-decoupled $^{31}P$ spectra of SPM/5 mol$\%$ CP aqueous dispersions at different temperatures, and these are shown in Fig. 20. Whereas the spectra below $T_m$ of SPM essentially consist of a lamellar
Fig. 19: Graph of the percentage of isotropic component of cholesteryl palmitate-16,16,16-d₃ in sphingomyelin aqueous dispersions, as determined by $^2$H-NMR ($\bullet$), and of the percentage of sphingomyelin giving rise to an isotropic signal, as determined by $^{31}$P-NMR ($\Delta$), versus temperature.
Fig. 20: Proton noise-decoupled $^{31}$P-NMR spectra of 50 wt% sphingomyelin (plus 5 mol% cholesteryl palmitate) + 50 wt% H$_2$O dispersions.
$33^\circ$C

$37^\circ$C

$55^\circ$C

ppm relative to external H$_3$PO$_4$
phase powder pattern (Seelig, 1978), above $T_m$ we obtain a lamellar phase powder pattern plus a superimposed narrow line at a position identical to that expected for an isotropic phospholipid signal. This narrow signal must, therefore, arise from SPM undergoing isotropic reorientation, and its proportion increases at higher temperatures. The fraction of isotropic component in the $^{31}$P-NMR spectra versus temperature is also plotted in Fig. 19. The striking similarity between the fractions of isotropic ester and isotropic SPM is readily apparent and suggests that the two are associated. These isotropic signals may arise from small bilayer structures or vesicles for which tumbling and lateral diffusion produce motional narrowing, and this is consistent with the fact that the isotropic part of the $^{31}$P-NMR signal observed in SPM dispersions has been observed to be absent at low water concentrations (Cullis & Hope, 1980) and to become more intense at high water concentrations (Cushley et al., 1980).

We can estimate an average size of the small structures giving rise to the isotropic $^{31}$P line, since the $^{31}$P-NMR linewidth $\Delta \nu_{1/2}$ is related to the effective correlation time $\tau_e$ of the isotropic motion by (McLaughlin et al., 1975):

$$\pi \Delta \nu_{1/2} \approx \frac{4}{45} \Delta \sigma^2 \tau_e$$  \hspace{1cm} (32)
where $\Delta \sigma$ is the residual chemical shift anisotropy of the $^{31}$P lamellar powder pattern in rad s$^{-1}$. Using our experimental value of $\Delta \nu_{z} = 200$ Hz and $\Delta \sigma = 40$ ppm, we calculate $\tau_{e} = 5 \times 10^{-5}$ s, which is related to the average radius of the hypothetical structures by eqn. 11. Using $D = 5 \times 10^{-8}$ cm$^2$/s (Cullis, 1976; Bloom et al., 1978) and $T = 320$ K we obtain $r \sim 50.0$ nm.

On the other hand, the deuterium linewidth of feature B can be related to $\tau_{e}$ by (Section B. I.):

$$\pi \Delta \nu_{z} = \frac{4 \pi}{5} \Delta \nu_{Q} \tau_{e}$$

Using $\Delta \nu_{z} = 400$ Hz and $\Delta \nu_{Q} = 3$ kHz we find $\tau_{e} = 2 \times 10^{-5}$ s.

Considering that these calculations are only crude approximations, the $\tau_{e}$ values obtained from the $^{31}$P and $^2$H spectra are consistent with each other. These data plus the similarity in the temperature behaviour of the isotropic fraction leads us to our proposal that the cholesteryl ester giving rise to the narrow $^2$H-NMR signal is dissolved in SPM undergoing isotropic reorientation.

The $^{31}$P-NMR spectra of aqueous dispersions of 47.5 mol% SPM/ 47.5 mol% cholesterol/ 5 mol% CP indicate a complete absence of the isotropic line. Only a lamellar powder pattern is apparent at all temperatures, and this behaviour is
identical to that observed previously in SPM/cholesterol dispersions (Cullis & Hope, 1980). We are not certain whether the isotropic $^2$H-NMR signal from deutero-CP/SPM/cholesterol dispersions is also absent, since the extremely low ester solubility in this system causes the signal to noise ratio of these $^2$H spectra to be very poor.
IV. Unilamellar Vesicles of Egg-Phosphatidylcholine

It has been reported (Forrest & Cushley, 1977) that up to 5 mol% CP can be incorporated into unilamellar phospholipid vesicles. This value is much higher than the value of ~0.2 mol% found for CP-d_{31} in egg-PC liposomes. Additionally, the $^2$H-NMR absorptions obtained from vesicles are much narrower and, hence, much more intense than the ones from liposomes. This fact results from the small size of the unilamellar vesicles (diameter < 30 nm), which gives rise to motional narrowing of the spectra due to the rapid vesicle tumbling plus lateral diffusion (see Chapter B.). Thus, unilamellar vesicles appeared to be a promising system to undertake a $^2$H-NMR study using selectively deuterated cholesteryl esters, and we have investigated the behaviour of selectively deuterated CP and CS in egg-PC vesicles, both in the absence and presence of 20 mol% cholesterol.

Cholesteryl Ester Incorporation

We have found that CP can be incorporated into egg-PC unilamellar vesicles at concentrations up to ~5 mol%. However, we observed that, in order to prepare vesicles containing a given concentration of CP, excess ester has to be added to the chloroform solution containing phospholipid
(see Section C. II.) prior to sonication, and a proportion of ester remains undispersed after the sonication step. This CP can be easily removed by preparative ultracentrifugation at 160,000 g for 3 h (see Section C. II.). That all the undispersed ester is indeed removed by this step will be shown below. In addition, in the absence of phospholipid, CP cannot be dispersed in water in measurable amounts, even after prolonged sonication. The relation of CP actually incorporated into the vesicles after the sonication step to that added initially is represented in Fig. 21. It should be pointed out that this graph is very approximate (as indicated by the large error bars), and the exact amount of ester incorporated in each sample can vary greatly. We do not understand the reason for this, but speculate that the amount of CP incorporated may depend on the exact power and length of the sonication, temperature, etc. In spite of our efforts to keep these factors as constant as possible from sample to sample, variations in the proportion of CP incorporated still occurred.

The 5 mol% solubility of CP in egg-PC vesicles determined in the present work is in agreement with the results of Forrest & Cushley (1977), but in contrast with those of Kellaway & Saunders (1967) who reported an ester incorporation of ~30 mol%. Kellaway & Saunders only used
Fig. 21: Plot of the proportion of cholesteryl palmitate incorporated into egg-PC unilamellar vesicles, as a function of the proportion of cholesteryl palmitate added prior to sonication. The curve indicates a smooth line drawn through the datapoints.
centrifugation of the vesicles at 10,000 g for 1 h to eliminate excess lipid, whereas we have used ultracentrifugation at 160,000 g for 3 h (Barenholz et al., 1977; see Section C. II.). As pointed out by Janiak (1977, p. 46), "cosonication of cholesteryl ester and phosphatidylcholine may result in the formation of microemulsion particles consisting of a stabilizing surface monolayer of phosphatidylcholine and a cholesteryl ester core." Hence, these hypothetical particles, having a structure similar to that of blood serum lipoproteins (Morrisett et al., 1977) and difficult to remove by centrifugation at moderate accelerations, may account for the discrepancy between our results and those of Kellaway & Saunders.

We found that the solubility of CP in egg-PC/20 mol% cholesterol vesicles is somewhat less than in egg-PC vesicles containing no cholesterol, and only ~2 mol% CP can be incorporated. It was also observed that egg-PC vesicles containing CP as well as CP/20 mol% cholesterol seem to be stable up to about 2 weeks at 4°C, as judged by their appearance and by our NMR results.
Vesicle Characterization

The results obtained in an analytical centrifugation study of egg-PC, egg-PC/2 mol% CP, and egg-PC/20 mol% cholesterol/1 mol% CP vesicles are summarized in Fig. 22. From the spreading of the Schlieren peaks with time, apparent diffusion coefficients in water at 20 °C ($D_{20,w}$) have been calculated at different vesicle concentrations, as described by Newman & Huang (1975). All the Schlieren peaks were highly symmetrical, which indicates a narrow size distribution of vesicles in all three cases. The value of $D_{20,w}^0$, extrapolated to zero concentration ($D_{20,w}^0$) is related to the average radius of the structures by the Stokes-Einstein relationship:

$$r = \frac{kT}{6 \pi \eta D_{20,w}^0} \quad (34)$$

where $k$ is the Boltzmann constant, $T$ the absolute temperature and $\eta$ the viscosity of the solvent. From the data of Fig. 22, we calculate values of $D_{20,w}^0$ of $1.85 \pm 0.10 \times 10^{-7}$, $1.98 \pm 0.10 \times 10^{-7}$, and $1.90 \pm 0.10 \times 10^{-7}$ cm$^2$/s for egg-PC, egg-PC/CP, and egg-PC/cholesterol/CP, respectively. These values are identical within experimental error and in excellent agreement with the value of $1.87 \times 10^{-7}$ cm$^2$/s reported by Huang (1969) for egg-PC vesicles. Hence, the
Fig. 22: Plot of the apparent diffusion coefficient in water at 20 °C of egg-PC (●), egg-PC/cholesteryl palmitate (▲), and egg-PC/20 mol% cholesterol/cholesteryl palmitate (□) unilamellar vesicles, versus phospholipid concentration.
phospholipid concentration (g/L)

diffusion coefficient ($cm^2 s^{-1} \cdot 10^7$)
average vesicle radius in the three cases is the same within experimental uncertainty, and we calculate from the Stokes-Einstein equation, \( r = 11.0 \pm 0.7 \) nm. Vesicles containing 20 mol\% cholesterol have the same size as the ones without cholesterol, which agrees with the work of Newman & Huang (1975) who observed a change in size of less than 10% when adding up to 25 mol\% of the sterol to egg-PC vesicles.

To make certain that the ultracentrifugation used in the preparation of the vesicles indeed removes all of the undispersed cholesteryl ester from the sample, a series of experiments was performed using CP-d_{31} in egg-PC vesicles. \(^{2}H\)-NMR spectra were determined for vesicles containing various amounts of the ester. These consist of a NMR absorption (Fig. 23) with a width at half height \( \Delta \nu_{1/2} = 26 \) Hz and with broad "skirts" extending up to ~300 Hz from the center frequency. The lineshape of this signal is not Lorentzian; instead it is the result of the superposition of NMR lines of different widths and corresponding to the various chain positions (see below). The lineshape and linewidth for the signal from CP-d_{31} in egg-PC vesicles was found to be independent of the amount of ester in the sample. Further, the intensity of the absorption, as determined by the integration of spectra taken under identical conditions, is proportional to the amount of ester in the preparation.
Fig. 23: $^2$H-NMR spectrum of cholesteryl palmitate-$d_{31}$ in egg-PC vesicles at ~30 °C.
Sweep width = 1000 Hz; pulse delay = 1.02 s; number of datapoints = 2048; number of scans = 5000.
(determined by chemical analysis), at all concentrations used (0.3–10 mol% CP-d$_{31}$ added).

An additional experiment was performed in which egg-PC/CP-d$_{31}$ vesicles were prepared using a carefully measured amount of distilled water, and the $^2$H-NMR spectrum was obtained under conditions such that the delay between successive pulses was $>5T_1$, in order to obtain correct spectral intensities (for CP-d$_{31}$, $T_1<0.25$ s; while for DOH, $T_1=0.5$ s). The integrated signal intensities of the CP-d$_{31}$ and the DOH absorptions were obtained and compared with their expected values as determined from chemical analysis of the vesicle sample. This experiment showed that the observed intensity of the CP-d$_{31}$ resonance was $>95\%$ of that expected. Thus, all the ester present in the sample gives rise to a narrow NMR line. If crystalline patches of undispersed ester such as proposed by Grover and Cushley (1979) for spin-labelled CP in egg-PC multilayers, or particles in which a core of solid ester is surrounded by a phospholipid monolayer (Janiak, 1977, p.46), were present, they would give rise to broad $^2$H resonances, which would be undetected on our spectrometer setup. This is clearly not the case and we conclude

a.) that the ultracentrifugation step removes all of the undispersed cholesteryl ester, and

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b.) that no microcrystalline regions of ester are present in the system.

The discrepancy of CP solubility in the two egg-PC membrane systems described in this work (0.2 mol% for liposomes versus 5 mol% for vesicles) is surprising, and it is not clear why such a discrepancy should exist. However, it has been reported that the incorporation of cholesterol (Lundberg, 1977; McCabe & Green, 1977) and several steroid hormones (Lundberg, 1979) in unilamellar vesicles is higher than in multilamellar liposomes. It may be that the sonication step used in the preparation of the vesicles simply forces an excess amount of cholesteryl ester into the bilayer, so that the final sample represents a supersaturated system. This is consistent with the fact that the amount of ester added to the mixture prior to sonication must be larger than the amount actually incorporated into the bilayer. If this situation were the case, the vesicles would try to achieve equilibrium by releasing cholesteryl ester over a period of time. However, such a process may be slow and, since the vesicles themselves have only a limited stability, may be very difficult to prove.

On the other hand, it is well known that certain properties of vesicles such as the phase transition temperature and enthalpy (Kantor et al., 1977; Van Dijck et
al., 1978) and possibly the lipid packing (Sheetz & Chan, 1972; Huang & Mason, 1978) are different than in liposomes. Thus the enhanced solubility of cholesteryl ester in the case of vesicles may be a real phenomenon, possibly related to the high radius of curvature of these vesicles. It might be interesting to carry out a study of the dependence of cholesteryl ester solubility and packing on the curvature of the bilayer by using vesicles of different sizes (Olson et al., 1979).

The apparent decrease of CP solubility in egg-PC vesicles containing 20 mol% cholesterol versus vesicles without cholesterol may be related to the fact that the saturated esters CP and cholesteryl myristate have been reported to disrupt the phospholipid bilayer (Forrest & Cushley, 1977; Janiak et al., 1979). Since cholesterol increases the orientational order of the phospholipid acyl chains (Ladbrooke et al., 1968; Stockton & Smith, 1976), the tight packing it causes may not allow for an effective incorporation of the ester and could result in a decreased solubility.
Deuterium NMR of Selectively Deuterated Esters

The 2H-NMR spectrum of CP-d$_{31}$ in egg-PC vesicles has been discussed in the previous section. The shape and width of this absorption signal, hence the orientational order of the ester, are independent of the concentration of the ester in the bilayer. This is important insofar as it allows us to obtain 2H-NMR spectra at any ester concentration. For all the 2H-NMR spectra of selectively deuterated esters in vesicles we have worked with samples to which 5 mol% ester had been added. Control experiments also showed that the 2H-NMR absorptions are not affected by the total lipid concentration of the vesicle samples. Hence, generally, preparations containing about 20 wt% lipid were used. In addition we observed that the 2H-NMR lineshapes, hence $\Delta v_{1/2}$, were identical whether the vesicles were subjected to preparative ultracentrifugation or not. Therefore, for the 2H-NMR experiments involving vesicles containing selectively deuterated esters, the ultracentrifugation step was not required.

Deuterium-NMR spectra for a series of selectively deuterated cholesteryl palmitates and cholesteryl stearates in egg-PC vesicles have been obtained at $\sim$30 °C. The measured linewidths and spin-spin relaxation times are given in Table II, part A, and representative spectra for some
TABLE II: $^2$H-NMR linewidths and spin-spin relaxation times for deuterated cholesteryl palmitate and cholesteryl stearate in egg-PC and egg-PC/20 mol% cholesterol vesicles, at ~30°C.

<table>
<thead>
<tr>
<th>ester</th>
<th>linewidth (Hz)</th>
<th>$T_2 \times 10^3$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Without cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP-2,2-d$_2$</td>
<td>145 ± 15</td>
<td>2.2</td>
</tr>
<tr>
<td>CS-2,2-d$_2$</td>
<td>160 ± 15</td>
<td>2.0</td>
</tr>
<tr>
<td>CP-3,3-d$_2$</td>
<td>135 ± 15</td>
<td>2.4</td>
</tr>
<tr>
<td>CP-4,4-d$_2$</td>
<td>85 ± 10</td>
<td>3.7</td>
</tr>
<tr>
<td>CP-5,5,6,6-$d_4$</td>
<td>63 ± 15</td>
<td>5.1</td>
</tr>
<tr>
<td>CS-8,8-d$_2$</td>
<td>72 ± 10</td>
<td>4.4</td>
</tr>
<tr>
<td>CP-11,11,12,12-$d_4$</td>
<td>59 ± 15</td>
<td>5.4</td>
</tr>
<tr>
<td>CS-13,13-$d_2$</td>
<td>46 ± 10</td>
<td>6.9</td>
</tr>
<tr>
<td>CP-16,16,16-$d_3$</td>
<td>10 ± 2</td>
<td>31.8</td>
</tr>
<tr>
<td>(B) With 20 mol% cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP-2,2-d$_2$</td>
<td>160 ± 15</td>
<td>2.0</td>
</tr>
<tr>
<td>CS-2,2-d$_2$</td>
<td>145 ± 20</td>
<td>2.2</td>
</tr>
<tr>
<td>CP-3,3-d$_2$</td>
<td>205 ± 30</td>
<td>1.6</td>
</tr>
<tr>
<td>CP-4,4-d$_2$</td>
<td>160 ± 20</td>
<td>2.0</td>
</tr>
<tr>
<td>CP-5,5,6,6-$d_4$</td>
<td>115 ± 10</td>
<td>2.8</td>
</tr>
<tr>
<td>CS-8,8-$d_2$</td>
<td>125 ± 20</td>
<td>2.5</td>
</tr>
<tr>
<td>CP-11,11,12,12-$d_4$</td>
<td>270 ± 50</td>
<td>1.2</td>
</tr>
<tr>
<td>CS-13,13-$d_2$</td>
<td>145 ± 25</td>
<td>2.2</td>
</tr>
<tr>
<td>CP-16,16,16-$d_3$</td>
<td>23 ± 3</td>
<td>13.8</td>
</tr>
</tbody>
</table>

1: The linewidth was measured at half-height, and it is related to the spin-spin relaxation time $T_2^*$ by $T_2^* = 1/(\pi \Delta v_1)$.

2: Values recorded are averages of all runs.
positions are shown in Fig. 24. Superimposed on these are the Lorentzian lineshapes fitting the experimental spectra best, and it is apparent that a single Lorentzian gives a good fit to all the spectra.

The effect on the spectra of adding 20 mol% cholesterol to the egg-PC/deutero-CP(or CS) vesicles can be seen in Fig. 25 which represents spectra for the same chain positions as Fig. 24, as well as in Table II, part B. It is apparent that a general linewidth increase is induced by cholesterol. Again, a single Lorentzian gives a satisfactory fit to the experimental spectra.

The complete picture of the measured linewidths for the system studied here is shown in Fig. 26. Comparison with the results for deuterated fatty acid probes in egg-PC vesicles (Stockton et al., 1976) shows that the behaviour of cholesteryl esters and fatty acids is very different. In the case of the fatty acid probes one sees an essentially constant linewidth (~1250 Hz at 30°C) for deuterons on C2 - C10, which is a manifestation of the well known ordering "plateau" exhibited by the phospholipid acyl chains (Seelig & Seelig, 1974b; Davis, 1979). Further down the chain the orientational order decreases, and this shows up as a progressive decrease of $\Delta v_{1/2}$ between positions 11 and 16.

In the case of egg-PC vesicles containing deuterated CP
Fig. 24: $^{2}$H-NMR spectra (——) and best-fit single Lorentzian lineshapes (---) of selectively deuterated cholesteryl palmitate in egg-PC vesicles at ~30°C. (A): 3,3-d$_2$; (B): 4,4-d$_2$; (C): 11,11,12,12-d$_4$; (D): 16,16,16-d$_3$. Parameters: Sweep width = 2500 Hz; pulse delay = 0.4 s (A-C) and 1.3 s (D); number of datapoints = 2048; number of scans = 3000-100,000.
Fig. 25: 2H-NMR spectra (---) and best-fit single Lorentzian lineshapes (---) of selectively deuterated cholesteryl palmitate in egg-PC/20 mol% cholesterol vesicles at ~30°C. (A): 3,3-d₂; (B): 4,4-d₂; (C): 11,11,12,12-d₄; (D): 16,16,16-d₃. Parameters: Sweep width = 2500 Hz; pulse delay = 0.4 s (A-C) and 1.3 s (D); number of datapoints = 2048; number of scans = 5000-300,000.
Fig. 26: 2H-NMR linewidths of selectively deuterated cholesteryl esters in egg-PC or egg-PC/20 mol% cholesterol vesicles at ~30 °C, versus chain position. (△): cholesteryl palmitate in egg-PC vesicles; (○): cholesteryl stearate in egg-PC vesicles; (▲): cholesteryl palmitate in egg-PC/cholesterol vesicles; (●): cholesteryl stearate in egg-PC/cholesterol vesicles. The curved lines join the points in the graph.
\[ \Delta U (\text{Hz}) \]

chain position

111b
or CS, the $^2$H-NMR linewidths of the CD$_2$ segments are much lower than for the corresponding fatty acids. The linewidths are largest for deuterons near the ester linkage and decrease gradually toward the terminal methyl group, but even the largest value of $\sim 160$ Hz found for CS-2,2-d$_2$ is much lower than the value of $\sim 1250$ Hz observed for the respective fatty acid. On the other hand, the linewidth observed for CP-16,16,16-d$_3$ (10 $\pm$ 2 Hz) is identical to that for palmitic-16,16,16-d$_3$ acid. Hence, deuterated CP shows an $\sim 16$-fold decrease in linewidth between positions 2 and 16, whereas for fatty acid probes an $\sim 125$-fold decrease is observed. Concerning the possible reason for the difference in the behaviour of cholesteryl ester and fatty acid, it may be hypothesized that the hydrophilic carboxyl group of an acid can interact more effectively with the aqueous phase than can the ester group, in which the fatty acyl moiety is bound to the bulky cholesteryl moiety. This could give rise to a much different motional behaviour and/or orientational order of the chain.

The profile of the $^2$H-NMR linewidth versus chain position for deuterated CP and CS is also shown in Fig. 26 for egg-PC vesicles containing 20 mol% cholesterol. Due to the lower solubility of the esters and the greater linewidths, the signals in this case are weaker, giving rise
to a larger uncertainty in the values of $\Delta v_{1/2}$. However, we see a substantial increase in linewidth at every position except C2, and an especially large increase for CP-11,11,12,12-d$_4$. Please note that, both in the case of egg-PC and egg-PC/20 mol% cholesterol vesicles, the linewidths observed for deuterated CS either agree with or fall on the lines joining the $\Delta v_{1/2}$ values for deuterated CP, indicating that the acyl chains of both esters behave identically within experimental error.

We have also obtained $^2$H spin-lattice relaxation times $T_1$ for selectively deuterated esters in egg-PC, and these are summarized in Table III. Short (<25 ms) values of $T_1$ were measured for all deuterated segments except for the terminal CD$_3$ group, which has a distinctly longer $T_1$ of 250 ms. The latter value is identical to that of palmitic 16,16,16-d$_3$ acid in egg-PC vesicles (Stockton et al., 1976). On the other hand, the $T_1$ values for the CD$_2$ segments of deuterated CP are shorter by a factor of ~2-3 compared to the corresponding values for DPPC at the equivalent temperature (Brown et al., 1979). Assuming that eqn. 14 (Section B. I.) holds in the present case, we may conclude that the correlation time of the fast molecular motions of CP (and CS) in egg-PC vesicles is larger than for the phospholipid molecules by a factor of ~2-3. The fast molecular motions giving rise to $T_1$
TABLE III: Spin-lattice relaxation times of deuterated cholesteryl palmitate and cholesteryl stearate in egg-PC and egg-PC/20 mol% cholesterol vesicles, at ~30 °C.

<table>
<thead>
<tr>
<th>ester</th>
<th>spin-lattice relaxation time (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>(A)</td>
<td>Without cholesterol</td>
</tr>
<tr>
<td>CP-2,2-d₂</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>CS-2,2-d₂</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>CP-3,3-d₂</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>CP-4,4-d₂</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>CP-5,5,6,6-d₄</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>CS-8,8-d₂</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>CP-11,11,12,12-d₄</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>CS-13,13-d₂</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>CP-16,16,16-d₃</td>
<td>250 ± 30</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>(B)</td>
<td>With 20 mol% cholesterol</td>
</tr>
<tr>
<td>CP-5,5,6,6-d₄</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>CP-11,11,12,12-d₄</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>CP-16,16,16-d₃</td>
<td>190 ± 30</td>
</tr>
</tbody>
</table>
are generally interpreted as trans-gauche chain isomerizations ("kink motions") (Schindler & Seelig, 1975; Petersen & Chan, 1977), and our results imply that the ester chains may have some motional restriction that decreases the rate of these isomerizations, at least at the chain positions near the carbonyl linkage.

Spin-lattice relaxation times $T_1$ were also obtained for several selective deuterated cholesteryl esters in egg-PC vesicles containing 20 mol% cholesterol, and these have also been included in Table III. The lower ester solubility and greater linewidths for these vesicles decreased the spectral intensities to such a degree that $T_1$ values for CP (and CS) containing only two deuterons could not be obtained in a reasonable length of time; hence, only CP-5,5,6,6-d$_4$, CP-11,11,12,12-d$_4$, and CP-16,16,16-d$_3$ have been used. The addition of 20 mol% cholesterol seems to lower the $T_1$ values of the ester chain segments, as shown in Table III. Although this decrease is not dramatic, it is consistent with cholesterol inducing a tighter packing of the chains together with a restriction of their motions (Ladbrooke et al., 1968; Stockton & Smith, 1976).
Viscosity Dependence of NMR Spectra

In order to determine a possible reason for the unexpectedly narrow $^2\text{H}-\text{NMR}$ linewidths of CP (and CS), compared to fatty acids, in egg-PC vesicles, we have performed a study using CP-$d_{31}$ and palmitic-$d_{31}$ acid probes in egg-PC unilamellar vesicles. The $^2\text{H}$-linewidths were measured as a function of the viscosity of the suspending medium, which can be altered significantly by adding aliquots of glycerol to the sample. This technique has been shown to be useful for determining the lateral diffusion coefficient of the phospholipid in unilamellar vesicles (Cullis, 1976). Representative $^2\text{H}-\text{NMR}$ spectra of egg-PC vesicles containing CP-$d_{31}$ or palmitic-$d_{31}$ acid and varying proportions of glycerol are shown in Fig. 27, and a plot of the linewidths $\Delta \nu_{1/2}$ as a function of the viscosity of the aqueous medium is depicted in Fig. 28. It is readily apparent that the linewidth of palmitic-$d_{31}$ acid shows a clear increase with viscosity, as expected from eqs. 9, 10, and 11 (Section B. I.). In contrast, the width of the $^2\text{H}-\text{NMR}$ resonance of CP-$d_{31}$ is constant over the whole viscosity range studied, to within experimental error, and this was also observed when using the selectively deuterated ester, CP-$16,16,16-d_3$, instead of CP-$d_{31}$. From the integrated intensities of the spectra of Fig. 27 we also conclude that, within the

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Fig. 27: 2H-NMR spectra of cholesteryl palmitate-d\textsubscript{31} and palmitic-d\textsubscript{31} acid in egg-PC vesicles containing various proportions of glycerol, at \textasciitilde28 °C. Parameters: Sweep width = 1000 Hz; pulse delay = 1.02 s; number of datapoints = 2048; number of scans = 1100-8700.
CP-d$_{31}$

DOH

0% glycerol

12 Hz

50% glycerol

12 Hz

65% glycerol

12 Hz

palmitic-d$_{31}$ acid

0% glycerol

12 Hz

40% glycerol

15 Hz

75% glycerol

21 Hz
Fig. 28: Plot of the $^2$H-NMR linewidths versus viscosity, for cholesteryl palmitate-$d_{31}$ (△) and palmitic-$d_{31}$ acid (●) in egg-PC vesicles containing various amounts of glycerol, at ~28°C. The upper curved line represents the best fit of the data for palmitic-$d_{31}$ acid to eqn. 9 (Section B. I.), while the lower line represents the best fit straight line through the data for cholesteryl palmitate-$d_{31}$. 
experimental error, the intensity of the CP-d$_{31}$ signal is proportional to the amount of ester in the sample.

We have also obtained $^{31}$P spectra of egg-PC/CP-d$_{31}$ vesicles in mixtures of H$_2$O and glycerol, which were acquired immediately prior to performing the $^2$H-NMR runs discussed in the previous paragraph. The results are presented in Fig. 29 and clearly show the linewidth increase expected on the basis of eqn. 21. These results rule out the possibility of an artifact, e.g. poor mixing or other faults of the experimental technique, being responsible for the unexpected $^2$H-NMR results.

The lateral diffusion coefficient of the phospholipid in the CP-d$_{31}$/egg-PC vesicles, calculated from the $^{31}$P spectra by means of eqn. 20, and using the experimental vesicle radius $r = 11.0$ nm as well as $T = 300$ K, is $D = 3 \times 10^{-8}$ cm$^2$/s, in agreement with published values (Cullis, 1976; Bloom et al., 1978).

The measured $^2$H linewidths for palmitic-d$_{31}$ acid as a function of viscosity were used to calculate the coefficient of lateral diffusion for the fatty acid in the bilayer. The least squares fit in this case yields $D = 7 \times 10^{-8}$ cm$^2$/s, which is in agreement with the values reported for fatty acids in the lamellar phase of egg-PC (Rigaud et al., 1977).
Fig. 29: Plot of the $^{31}$P-NMR linewidths versus viscosity, for egg-PC/cholesteryl palmitate-d$_{31}$ vesicles containing various amounts of glycerol, at $\sim 30^\circ$C. The curved line represents the best fit of the data to eqn. 21 (Section B. II.).
The graph shows the relationship between linewidth (Hz) and viscosity (cP). The data points are plotted along with a fitted curve, indicating an increasing linewidth with increasing viscosity.
Petersen & Chan (1977) have proposed that the orientational order of the lipids in unilamellar vesicles may be different to that in liposomes, due to a higher degree of "rigid rod" motion (see Section B. I.) in vesicles. According to their model, this fact would be manifested by the independence of the vesicle NMR linewidths with the viscosity of the aqueous medium. Our results can be used to obtain information about the possible validity of the Petersen-Chan model. Since deuterated fatty acid probes have been shown to reproduce satisfactorily the orientational order of the phospholipid acyl chains in model membranes (Stockton et al., 1976), the presence of considerable rigid rod motion in vesicles should show up in the viscosity behaviour of the $^2H$-NMR linewidths of deuterated fatty acid probes incorporated in the vesicle bilayers. This is clearly not borne out by our observations of palmitic-d$_{31}$ acid in egg-PC vesicles. Indeed, if considerable rigid rod motion existed in vesicles, then the $^2H$-NMR linewidth of the fatty acid resonance would be predicted to change little or not at all upon the addition of glycerol to the aqueous medium, resulting in the calculation of value of D higher than in the case of the lamellar phase. However, the fact that the value of D calculated for vesicles is essentially identical to the
values reported for the lamellar phospholipid phase (Sackmann et al., 1973; Rigaud et al., 1977), must mean that the contribution from the rigid-stick motion, if it exists, must be very similar in both systems. We point out that this conclusion is not necessarily true in the case of cholesteryl esters, since with them we do observe a viscosity independence, and this point will be further discussed below.

Stockton et al. (1976) have interpreted their $^2$H-NMR data for deuterated fatty acid probes in egg-PC vesicles in terms of segmental order parameters $S$ as calculated by eqs. 9-11. These equations predict that the $^2$H-NMR linewidth observed for vesicles will be a function of the correlation time for vesicle tumbling and, hence, will be related to the viscosity of the suspending medium. Since we have obtained evidence that the ester in egg-PC/deutero-CP vesicles shows no such viscosity dependence, we prefer not to use these expressions in order to calculate the order parameter $S$ of the esters in vesicles. Neither do we wish to employ the more detailed theory put forward by Petersen and Chan (1977) in which the effects of motional averaging due to reorientation of the lipid chains as a whole ("rigid rod motions") are included. Instead, we will calculate the more general quantity $1/T_{2\text{slow}}$ defined by eqn. 9 and say that it reflects changes in the orientational
order and/or rate of slow molecular motions along the acyl chain.

Fig. 30 shows the values of $1/T_{2\text{slow}}$ versus chain position calculated from our data of $\Delta v_2^*$ and $T_1$, for egg-PC vesicles containing deuterated CP or CS, in the absence and presence of 20 mol% cholesterol. For the cholesterol containing vesicles, we have used the $T_1$ values determined for CP-5,5,6,6-d$_4$, CP-11,11,12,12-d$_4'$ and CP-16,16,16-d$_3$ to estimate, by interpolation, the values for the other chain positions, assuming that a similar behaviour of $T_1$ versus chain position exists as in the case of vesicles without cholesterol. Thus, we have used $T_1 = 0.005$ s for CP (and CS)-2,2-d$_2$, CP-3,3-d$_2$ and CP-4,4-d$_2'$, 0.008 s for CS-8,8-d$_2$, and 0.020 s for CS-13,13-d$_2$. It is clear that the shapes of the plots are rather similar to the ones of $\Delta v_2^*$ versus chain position (Fig. 26). The values of $1/T_{2\text{slow}}$ for cholesteryl esters are, with the exception of CP-16,16,16-d$_3'$, much lower than the ones for fatty acids. One possible reason for this could be a smaller size of egg-PC/CP (or CS) vesicles as compared to egg-PC vesicles containing no ester, giving a lower effective correlation time for vesicle tumbling (eqn. 11), hence lower $\Delta v_2^*$. This is definitely not the case, since we have previously shown that egg-PC, egg-PC/CP and egg-PC/CP/20 mol% cholesterol vesicles have the
Fig. 30: Plot of $1/T_2^* - 1/T_1$ versus chain position for selectively deuterated cholesteryl palmitate and cholesteryl stearate in egg-PC and egg-PC/20 mol% cholesterol vesicles at $-30$ °C. (△): cholesteryl palmitate in egg-PC vesicles; (○): cholesteryl stearate in egg-PC vesicles; (▲): cholesteryl palmitate in egg-PC/cholesterol vesicles; (●): cholesteryl stearate in egg-PC/cholesterol vesicles. The curved lines join the datapoints. The error bars are indicative of the typical uncertainty of the data.
same average radius.

The low $1/T_{2\text{slow}}$ values of cholesteryl esters in egg-PC vesicles and the viscosity-independence of the $^2H$-NMR linewidths might in principle be caused by rapid lateral diffusion of the esters in the vesicle bilayer. The minimum value of $D$ to produce such an effect is $\sim 10^{-6}$ cm$^2$/s. On the other hand, the lateral diffusion coefficient of phospholipid molecules has been reported to be $\sim 5 \times 10^{-8}$ cm$^2$/s (Cullis, 1976; Bloom et al., 1978). It seems difficult to imagine molecules such as CP having a rate of lateral diffusion >20 times faster than that of the host (phospholipid) molecules, when they are of nearly equal size. One would rather imagine that, since these ester molecules are dispersed in the bilayer, they would diffuse at a rate dictated by that of the molecules forming the membrane matrix. It could be argued that the hydrophobic nature of cholesteryl esters may influence their lateral diffusion coefficient in the membrane, since, in contrast to the phospholipid molecules, they will not experience strong polar interactions with the aqueous phase. However, while small non-polar molecules like benzene are known to diffuse quite rapidly in phospholipid bilayers ($D \sim 3 \times 10^{-6}$ cm$^2$/s) (Rigaud et al., 1972), somewhat bigger solutes such as pyrene (Mol. wt. = 194) diffuse much more slowly ($D \sim 10^{-7}$ cm$^2$/s) (Galla & Sackmann, 1974). Hence,
we expect the far bulkier cholesteryl esters (Mol. wt. > 625) to diffuse at a rate still slower than pyrene.

In spite of what was said in the previous paragraph, we have attempted to rule out fast lateral diffusion of the ester as the reason for the unusual behaviour of CP in egg-PC vesicles, by measuring the coefficient of lateral diffusion for spin-labelled CP in this system, as described by Trauble & Sackmann (1972) and Scandella et al. (1972). In this method, the exchange broadening of the ESR absorption lines observed at high (>1 mol%) concentrations of spin-label is related to the lateral diffusion coefficient of the spin-labelled molecules in the bilayer. For this method to be useful, the observed ESR spectra must reflect rapid motion of the spin-label. Such a situation was seen not to be the case for 5-doxy1 cholesteryl palmitate in egg-PC vesicles. This ester gives rise to ESR spectra that are indicative of slow motion (Grover et al., 1979) and for which the published analysis cannot be applied. On the other hand, 16-doxy1 cholesteryl stearate was seen to give rise to spectra corresponding to very fast motion, which would be well suited for the experiment. However, the solubility of this ester in egg-PC vesicles is very low, and at no time could we incorporate more than ~2 mol% into the system. Thus the exchange broadening in this case is too low to be analyzed.
meaningfully.

Due to the failure of the ESR spin-label method to determine the lateral diffusion coefficient of CP in phospholipid vesicles, it would be desirable to determine this value by some means other than ESR or NMR, e.g. by fluorescence spectroscopy. It is our expectation, however, that very fast diffusion of the ester molecules is unlikely to be the reason for the unusual behaviour of cholesteryl esters in unilamellar vesicles.

It is interesting to compare the $^2$H-NMR results obtained for deuterated CP (or CS) in egg-PC vesicles and in multilamellar liposomes with respect to the orientational order of the ester acyl chain. For CP-$d_{31}$ in multilamellar liposomes (Section D. II.) we obtained an average quadrupolar splitting for the $CD_2$ segments of 12 kHz, corresponding to a segmental order parameter $S_d = 0.1$. Assuming that the only difference between the liposome and vesicle systems is the smaller size of the vesicles and that lateral diffusion of the ester has a rate similar to that of the phospholipid, we can calculate an average linewidth for the $^2$H-NMR spectra of the $CD_2$ segments of deuterated CP in egg-PC vesicles from eqs. 9-11. Using our experimental vesicle radius $r = 11.0$ nm, $\eta = 0.8$ cP, $T = 303$ K, $D = 5 \times 10^{-8}$ cm$^2$/s, and assuming a bilayer thickness of 4 nm, we obtain from eqn. 11 an
effective correlation time for vesicle tumbling of 7 x 10^{-7} s. This compares favourably with the value of 5.8 x 10^{-7} s obtained for egg-PC vesicles containing deuterated fatty acids at the same temperature (Stockton et al., 1976). Introducing \( \tau_e = 7 \times 10^{-7} \) s, plus \( S = 0.1 \) and \( T_1 = 0.01 \) s, into eqs. 9 and 10, we obtain \( \Delta \nu_{1/2} = 340 \) Hz, whereas the observed value is \( \sim 60 \) Hz. On the other hand, for the terminal CD\(_3\) group of CP-16,16,16-d\(_3\), using \( S = 0.02 \) and \( T_1 = 0.25 \) s, we obtain \( \Delta \nu_{1/2} = 13 \) Hz versus the observed value of 10 Hz. Whereas the difference of the predicted versus observed values of \( \Delta \nu_{1/2} \) for CP-16,16,16-d\(_3\) may simply be due to experimental uncertainties, this is clearly not the case for the average linewidth of the CD\(_2\) segments, which is \( >5 \) times lower than the predicted value. This behaviour, together with the different solubility of CP, might be interpreted as evidence for a different packing of the cholesteryl ester in vesicles compared to liposomes. However, in light of the difficulties encountered in interpreting our results, due to the viscosity-independence of the \(^2\)H-NMR linewidths, we prefer not to speculate further on this subject.

In the case of egg-PC vesicles containing 20 mol% cholesterol and deuterated CP(or CS), an increase in the value of \( 1/T_{2s1ow} \) is found compared to vesicles without
cholesterol, consistent with the fact that cholesterol increases the orientational order of the phospholipid chains (Stockton and Smith, 1976). Since egg-PC/CP and egg-PC/20 mol% cholesterol/ CP vesicles possess the same average radius, the observed increase in linewidth cannot be attributed to a decreased rate of vesicle tumbling, hence, probably stems from an enhancement of the orientational order of the ester acyl chains. The origin of the especially large linewidth increase at positions 11 and 12 is unclear, but we may speculate that it arises from some selective interaction between these positions and the cholesterol ring system. Space-filling models show that, if the cholesteryl ester intercalated in the bilayer in an arrangement approximating an "inverted horseshoe" (Janiak et al., 1974; Grover et al., 1979), then ester chain segments close to the carbonyl group would not be greatly affected by the increased order induced by cholesterol, whereas the central part of the ester chain, i.e. segments 8-13 would be affected most, thus possibly giving rise to the observed behaviour.
V. Unilamellar Vesicles of Dipalmitoylphosphatidylcholine

Due to its fixed chemical composition, dipalmitoylphosphatidylcholine (DPPC) is a convenient phospholipid to use for model membrane studies, whereas, e.g. egg-PC and beef-brain SPM may have different fatty acid compositions depending upon their source and method of extraction. In addition, the gel to liquid-crystalline phase transition temperature of DPPC ($T_m = 41 \, ^0C$) is such that it is straightforward to study both the region above and below $T_m$. Therefore we have investigated the behaviour of deuterated CP in DPPC unilamellar vesicles by $^2H$- and $^{31}P$-NMR.

Ester Incorporation and Vesicle Characterization

DPPC vesicles have been prepared containing up to $\sim 5$ mol% CP. In order to prepare vesicles with more than $\sim 1$ mol% ester, excess CP must be added to the lipid mixture prior to sonication. This is identical to the results for egg-PC vesicles (Section D. IV.). We have also determined, in the same manner described previously (Section D. IV.), that the narrow $^2H$-NMR line from CP-d$_{31}$ in DPPC vesicles above $T_m$ accounts for $>90\%$ of the expected intensity, confirming that no appreciable amount of undispersed ester and/or
microcrystalline CP patches (Grover & Cushley, 1979) remain in the sample after ultracentrifugation at 160,000 g for 3 h. Also, the lineshape and linewidth of the CP-\textsuperscript{d\textsubscript{31}} signal are identical whether the vesicles are sized by preparative ultracentrifugation or not, and they are independent of the amount of ester in the sample.

When 20 mol\% cholesterol is added to DPPC/CP-\textsuperscript{d\textsubscript{31}} vesicles, no reduction in the solubility of the ester is observed, and this is in contrast to our previous results using egg-PC vesicles and SPM liposomes.

As in the case of egg-PC/CP vesicles (Section D. IV.), in the present study of CP in DPPC vesicles we have used, for all the NMR runs, preparations to which 5 mol\% ester had been added before sonication, i.e. which contained ~2 mol\% ester after sonication.

Attempts to determine the average radius of the vesicles by analytical ultracentrifugation, as in the case of egg-PC vesicles, were unsuccessful. The Schlieren peaks of the DPPC vesicles, obtained at 20 °C, were not symmetrical, even after extended periods of preparative ultracentrifugation after sonication, and we attribute this to fusion of the small vesicles that takes place below the phase transition of DPPC (Schullery et al., 1980).

We also attempted to determine vesicle size by electron
microscopy using the negative staining technique (Sheetz & Chan, 1972), but could not obtain reproducible results. Furthermore, determination of the vesicle size by means of Sepharose gel filtration was not done, since it is known that fusion of DPPC vesicles may occur on a Sepharose column (Sheetz & Chan, 1972).

We were able, however, to determine an average size of the DPPC/CP and DPPC/20 mol% cholesterol/CP vesicles from $^{31}$P-NMR spectra using the lanthanide shift technique (Bystrov et al., 1972). Spectra for these samples at 50 °C, in the presence of Pr(NO$_3$)$_3$, are shown in Fig. 31. The ratios of the signals corresponding to phosphorus atoms on the outside (downfield signal) and inside (upfield signal) monolayers of the vesicles, are 1.9 (+0.2) and 1.7 (+0.2) for DPPC/CP and DPPC/ cholesterol/ CP vesicles, respectively. These values agree, within the experimental uncertainty, with the results obtained for vesicles of pure DPPC (De Kruijff et al., 1976). On the other hand, if we estimate the outside/inside ratio by comparing the intensity of the inside signal in Fig. 31 with the total line intensity before the addition of the shift reagent, we obtain outside/inside ratios of 2.1 (+0.2) and 1.9 (+0.2) for DPPC/CP and DPPC/cholesterol/CP, respectively. These results indicate that the average radius of the DPPC vesicles is not appreciably altered by the addition of CP or
Fig. 31: Proton noise-decoupled $^{31}\text{P}-\text{NMR}$ spectra at 50 °C of vesicles after the addition of Pr$^{3+}$. Upper spectrum: DPPC/cholesteryl palmitate vesicles; lower spectrum: DPPC/20 mol% cholesterol/cholesteryl palmitate vesicles.
CP/20 mol% cholesterol, and this conclusion is identical to that reached for egg-PC vesicles. Assuming an identical packing density of phospholipid in the two monolayers of the vesicles and a bilayer thickness of 3.5 nm (Wilkins et al., 1971), we calculate an average vesicle radius of 13.5 nm from the observed outside/inside ratios. This is in agreement with the sizes reported for pure DPPC vesicles (Sheetz & Chan, 1972; Bloom et al., 1978; Schullery et al., 1980). Another indication for an unchanged size of DPPC/CP and DPPC/cholesterol/CP vesicles as compared to DPPC vesicles is that the linewidths of the $^{31}$P signals in the absence of Pr$^{43}$ are practically identical in the three cases, with values of 15, 16, and 14 (+1) Hz, respectively. An unexpected result from Fig. 31 was that the linewidth of the downfield (outside) $^{31}$P-NMR line in the presence of Pr$^{43}$ was 31 Hz in the absence of cholesterol and 17 Hz in the presence of 20 mol% cholesterol. Although this fact is not essential to our discussion, we believe it has not been reported previously.

In order to determine the incorporation of CP in DPPC vesicles below the phospholipid phase transition, we have obtained the $^2$H-NMR spectrum of CP-16,16,16-d$_3$ in DPPC vesicles at 30 °C. This spectrum was taken after an initial $^2$H-NMR run at 45 °C and consisted of a signal having
Δν½ ~ 200 Hz, with an integrated intensity at equivalent experimental conditions of ~90% of that obtained at 45 °C. Due to the low signal intensity as a result of the large linewidth, the 30 °C run required 25 h in order to obtain sufficient signal-to-noise. After that time, the sample was warmed to 45 °C and another spectrum taken at the higher temperature. The signal intensity in the final 45 °C spectrum was ~80% of the intensity found in the original 45 °C spectrum, whereas its linewidth was 10 Hz versus 8 Hz for the initial 45 °C run. These results are interpreted as signifying that the solubility of CP is not significantly reduced below the DPPC phase transition. Indeed, the 10% loss in signal intensity at 30 °C is attributed to vesicle aggregation. It is known that DPPC vesicles are unstable below Tm and tend to fuse forming larger structures (Schullery et al., 1980). This phenomenon would be enhanced by trace amounts of free fatty acids that might be present in the bilayer (Kantor & Prestegard, 1978). The additional 10% loss of signal intensity between the run at 30 °C and the final 45 °C experiment, as well as the increase in linewidth from 8 to 10 Hz between the first and the second 45 °C run, support the conclusion that irreversible aggregation occurred below Tm.

The fact that CP incorporation is not greatly decreased
below the phase transition of the phospholipid is in agreement with our results for the SPM/CP system (Section D. III.) and in contrast with the conclusion reached by Janiak et al. (1979). Those authors studied the related system dimyristoylphosphatidylcholine/cholesteryl myristate/H₂O using polarizing light microscopy, X-ray diffraction and differential scanning calorimetry and reported that practically no ester remains in the bilayer below the phospholipid phase transition.

**Deuterium NMR of Selectively Deuterated Esters**

We have obtained ²H-NMR spectra, at 45 and 55 °C, of CP, selectively deuterated at several positions along the acyl chain, incorporated into DPPC vesicles. Representative spectra are shown in Fig. 32, and superimposed on these are the Lorentzian lineshapes giving the best fit. It is apparent that a single Lorentzian gives a satisfactory representation of the experimental spectra.

Figure 33 summarizes the linewidths obtained for deuterated CP in DPPC vesicles at 45 and 55 °C. It is apparent that the linewidths are largest for the C2 segment, tend to decrease toward the terminal methyl group, and decrease when raising the temperature from 45 to 55 °C. Most of the variation of Δν₁/₂ occurs near both ends of the acyl
Fig. 32: $^2$H-NMR spectra (---) and best-fit Lorentzian lineshapes (-----) of selectively deuterated cholesteryl palmitate in DPPC vesicles at 45 °C. Parameters: Sweep width = 5000 Hz (2,2-d$_2$), 2500 Hz (3,3-d$_2$), 2000 Hz (5,5,6,6-d$_4$ and 11,11,12,12-d$_4$), 1000 Hz (16,16,16-d$_3$); pulse delay = 0.2 s (2,2-d$_2$ and 3,3-d$_2$), 0.25 s (5,5,6,6-d$_4$), 0.5 s (11,11,12,12-d$_4$), 2.4 s (16,16,16-d$_3$); number of datapoints = 2048 (2,2-d$_2$ and 16,16,16-d$_3$), 1024 (3,3-d$_2$, 5,5,6,6-d$_4$ 11,11,12,12-d$_4$); number of scans = 3800-114,000.
Fig. 33: Plot of the $^2$H-NMR linewidth versus chain position of selectively deuterated cholesteryl palmitate in DPPC or DPPC/20 mol% cholesterol vesicles. ($\triangle$): cholesteryl palmitate in DPPC at 55°C; ($\blacksquare$): cholesteryl palmitate in DPPC at 45°C; (○): cholesteryl palmitate in DPPC/cholesterol at 55°C; (●): cholesteryl palmitate in DPPC/cholesterol at 45°C. The curved lines join the experimental data.
chain, with less change apparent in the central positions. This behaviour is very similar to that observed in egg-PC vesicles. Also, the $^2H$-linewidths are quite close for both systems, signifying that the packing of the ester chain in both phospholipids is essentially the same and quite different from the behaviour of deuterated fatty acid probes in egg-PC vesicles (Stockton et al., 1976). Additionally, experiments with DPPC/CP-d$_{31}$ vesicles revealed that the $^2H$-NMR lineshape and linewidth of the ester signal above $T_m$ is not affected by the viscosity of the suspending medium. Addition of up to 75% glycerol to the aqueous phase did not change the width of the $^2H$-NMR ester signal, and this is the same result as observed for egg-PC vesicles.

Addition of 20 mol% cholesterol to the DPPC/deutero-CP vesicles results in an increase of the $^2H$-NMR linewidths. Representative spectra for these vesicles are shown in Fig. 34, whereas the summary of the measured $\Delta v_L$ values is presented in Fig. 33. A single Lorentzian line was also found to give a satisfactory fit to the spectra in this case. The general trend is that cholesterol increases the linewidths with an especially large increase at positions 11 and 12. This behaviour is very similar to that observed in egg-PC vesicles. The fact that the very large linewidth increase at the C11 and C12 segments also occurs in DPPC rules out the
Fig. 34: $^2$H-NMR spectra (—) and best-fit Lorentzian lineshapes (---) of selectively deuterated cholesteryl palmitate in DPPC/20 mol% cholesterol vesicles at 45°C. Parameters: Sweep width = 5000 Hz (2,2-d$_2$, 3,3-d$_2$ and 11,11,12,12-d$_4$), 2000 Hz (5,5,6,6-d$_4$), 1000 Hz (16,16,16-d$_3$); pulse delay = 0.1 s (2,2-d$_2$, 3,3-d$_2$ and 11,11,12,12-d$_4$), 0.25 s (5,5,6,6-d$_4$), 1 s (16,16,16-d$_3$); number of datapoints = 1024 (2,2-d$_2$, 3,3-d$_2$ and 11,11,12,12-d$_4$), 2048 (5,5,6,6-d$_4$ and 16,16,16-d$_3$); number of scans = 40,000-500,000.
possibility that it may be caused by some steric interaction of these segments of the ester chain with the rigid double bonds found in the oleate, linoleate and linolenate chains of egg-PC. Furthermore, the linewidth increase upon cholesterol addition, also seen previously with the egg-PC system, cannot be related to an increase in the vesicle tumbling correlation time, since we have shown that DPPC/CP vesicles have the same size with or without cholesterol.

We have also obtained spin-lattice relaxation times $T_1$ for selectively deuterated CP in DPPC unilamellar vesicles at 45 and 55 °C, and the results are summarized in Fig. 35. In the same figure we have plotted, for comparison, the $T_1$ values of CD$_2$ and CD$_3$ segments of deuterated DPPC (Brown et al., 1979; Davis, 1979). The $T_1$ values of the CD$_2$ and CD$_3$ segments of deuterated CP in DPPC vesicles at 55 °C are longer than at 45 °C. In addition, they are ~2-3 times shorter than the corresponding values of deuterated DPPC at the same temperatures. The discrepancy is largest (~3-fold) near the carbonyl end of the chain, decreases to ~2-fold at positions 11 and 12, and to 1.2-fold at the terminal methyl group. Essentially, this behaviour is very similar to that observed for CP (or CS) in egg-PC vesicles.

The positive temperature dependence of the spin-lattice relaxation times of deuterated CP in DPPC implies that the
Fig. 35: $^2$H-NMR spin-lattice relaxation times of selectively deuterated cholesteryl palmitate in DPPC vesicles, versus chain position. For comparison, the data for deuterated DPPC at 51°C (Brown et al., 1979) are also plotted. The curved lines join the experimental data.
\[ T_1 (\text{ms}) \]

- \( \square = \text{DPPC, 51}^{\circ} \text{C} \)
- \( \triangle = \text{CP in DPPC, 55}^{\circ} \text{C} \)
- \( \Delta = \text{CP in DPPC, 45}^{\circ} \text{C} \)

chain position

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relevant molecular motions are in the fast correlation time regime \( \omega_0^2 \tau_C^2 \ll 1 \). Thus, we can estimate an effective correlation time \( \tau_C \) by means of eqn. 14. These values of \( \tau_C \) are \(~2-3\) times larger than the ones of deuterated DPPC (Brown et al., 1979), hence, the fast molecular motions are slower in CP than in DPPC. This behaviour was also observed for deuterated CP (and CS) in egg-PC vesicles (Section D. IV.). The longer correlation times of CP versus the phospholipid may be due to a decrease of the rate of fast segmental motions of the ester chain as a result of steric interactions with the bulky cholesterol moiety. This effect would not be surprising if deuterated CP and CS intercalated into the phospholipid bilayer in the average shape of a "horseshoe" (Janiak et al., 1974; Grover et al., 1979).

We can calculate the quantity \( 1/T_{2\text{slow}} \) defined by eqn. 9, which reflects the orientational order and/or rate of slow molecular motions of the ester chain. The values of \( 1/T_{2\text{slow}} \) versus chain position are presented in Fig. 36, as are the equivalent values estimated for vesicles in the presence of 20 mol\% cholesterol. The \( T_1 \)'s obtained for egg-PC/ 20 mol\% cholesterol/deutero-CP(or CS) vesicles were used in the calculation of \( T_{2\text{slow}} \) for deuterated CP in DPPC/cholesterol vesicles, and we think this is a valid assumption given the close agreement of the \( T_1 \) values of
deuterated CP in DPPC vesicles and egg-PC vesicles in the absence of cholesterol (Section D. IV.). The behaviour of $1/T_{2\text{slow}}$ versus chain position is rather similar to that for CP (and CS) in egg-PC vesicles (Section D. IV.). However, in the present case, the maximum at positions 11 and 12, observed in the presence of cholesterol, is also present in the absence of the sterol, although it is less pronounced. This maximum is also higher at 45 than at 55 °C, corresponding to increased order and/or slower rate of motion, and this is consistent with the behaviour expected on the basis of the known results for pure phospholipids.

The origin of the maximum of $1/T_{2\text{slow}}$ in Fig. 36 is not understood. It is likely a reflection of the spatial arrangement of the ester chain in the bilayer giving rise to some interaction with neighboring molecules. On the other hand, there is a clear minimum in $1/T_{2\text{slow}}$ at positions 5 and 6, which might be related to a very low orientational order of the ester chain in that region. This is reminiscent of the behaviour reported for spin-labelled CP in oriented multilayers (Grover et al., 1979) where the order parameter was found to be very close to zero at C5 of the ester chain, due to a tilt of this chain at an angle close to the "magic angle" (54.7 °) with respect to the bilayer normal.
Fig. 36: Plot of $\frac{1}{T_2} - \frac{1}{T_1}$ versus chain position for selectively deuterated cholesteryl palmitate in DPPC or DPPC/20 mol% cholesterol vesicles. (Δ): cholesteryl palmitate in DPPC at 55 °C; (▲): cholesteryl palmitate in DPPC at 45 °C; (〇): cholesteryl palmitate in DPPC/cholesterol at 55 °C, (●): cholesteryl palmitate in DPPC/cholesterol at 45 °C. The curved lines join the datapoints. The error bars are representative for positions 5, 6 and 11, 12; the error is higher at positions 2 and 3 and lower at position 16. Errors are lower at 55 °C than at 45 °C.
At all positions of the CP acyl chain except C16 the values of $1/T_{2\text{slow}}$ are much lower (~4-16 times) than for fatty acid probes in egg-PC vesicles, indicating a much different ordering and/or motional behaviour. The observed viscosity independence of the $^2$H-NMR linewidth of deutero-CP supports this conclusion.
VI. Multilamellar Liposomes of Dipalmitoylphosphatidylcholine

The $^2$H-NMR spectra from multilamellar liposomes are more easily interpreted than those from unilamellar vesicles. This is due to the fact that line narrowing due to reorientation and lateral diffusion is not encountered in liposomes, because of their large size compared to vesicles. Hence, we have studied deuterated CP and CS in DPPC multilamellar liposomes. In the case of egg-PC liposomes (Section D. II.) it had been concluded that, due to the very low incorporation of cholesteryl ester, spectra from esters selectively labelled on the acyl chain would be very difficult to obtain. Ester solubility in DPPC was found to be higher, and this fact made possible a study of selectively deuterated CP and CS using DPPC as the membrane matrix.

Cholesteryl Ester Incorporation

The $^2$H-NMR spectrum of a dispersion of 50 wt% (DPPC + 5 mol% CP-16,16,16-d$_3$) + 50 wt% deuterium depleted water at $50^\circ$C is shown in Fig. 37h. It consists of three features, labelled A, B, and C, having spectral widths of <1, 3, and 38 kHz, respectively, which are interpreted in the same manner as for egg-PC and SPM liposomes: Feature C is attributed to regions of solid ester in the sample, feature B
Fig. 37: $^2$H-NMR spectra of 50 wt% DPPC (plus 5 mol% selectively deuterated cholesteryl palmitate) + 50 wt% H$_2$O dispersions. Temperature = 50 °C, except for (d), where it was 52 °C. Parameters: Pulse length = 5-6 µs (90° pulse); spectral width = 100 kHz; number of datapoints = 1024 zero-filled to 4096; delay between the two pulses of the quadrupolar echo sequence ($\tau_1$) = 70 µs (b,c,d,e,f), 80 µs (a), 200 µs (h); line broadening = 40 Hz (d), 100 Hz (b,e,f,g), 150 Hz (h); delay between quadrupolar pulse sequences ($\tau_R$) = 0.1-0.3 s (a-g), 1 s (h). The features A-C are described in the text, and the dashed lines separate the features.
to ester dissolved (incorporated) in DPPC bilayers, and
feature A is due to either residual DOH and/or a small
fraction of "isotropic" ester similar to that found in SPM/CP
dispersions (Section D. III.).

For the calculation of the incorporation of cholesteryl
into the DPPC bilayers we can essentially ignore spin-lattice
relaxation effects, since the spectrum of Fig. 37h was
acquired using a delay between pulse sequences $\tau_R = 1$ s, and
$T_1$ for solid CP-16,16,16-d$_3$ is $\sim 0.25$ s (Section D. I.), while
$T_1 < 0.4$ s for the incorporated ester (Section D. V.).
However, spectra obtained at different values of $\tau_1$, the
delay between the two pulses of the quadrupolar echo
sequence, showed that the ratio of the areas of features B
and C depended on $\tau_1$, signifying that the echo decay times
$T_2e$ (Section B. I.) for these two features are not the same.
From a plot of the relative spectral intensity of features B
and C (see Fig. 37h), versus $2\tau_1$, we calculate $T_{2e} = 340$ $\mu$s
for feature C, and $T_{2e} = 850$ $\mu$s for feature B. In order to
calculate the fraction of ester incorporated into the
bilayer, we have to obtain the ratio of spectral intensities
$E_B/(E_B + E_C)$ and extrapolate this value to $\tau_1 = 0$. This
calculation is very similar to that performed for CP in SPM
bilayers (Section D. IV.). Our experimental data yield an
incorporation of CP-16,16,16-d$_3$ in DPPC of 0.5 ($\pm$ 0.3) mol%.

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Dynamic Behaviour of the Cholesteryl Ester Chains.

Deuterium NMR spectra of selectively deuterated cholesteryl esters in DPPC liposomes, obtained at ~50 °C, are shown in Fig. 37. As expected from the low solubility of CP in this system, these spectra suffer from very limited signal-to-noise. The spectral widths as a function of chain position are also indicated on the spectra represented in Fig. 37. For CP-5,5,6,6-d₄ two spectral components are apparent which are of ~1:1 relative intensity. On the basis of the spectrum from CP-6,6-d₂, we assign the narrower of the two components to deuterons on C5 and the broader one to deuterons on C6. In contrast, for CP-11,11,12,12-d₄ only one splitting is apparent which indicates that the deuterons on C11 and C12 have a similar behaviour.

A graph of ²H-NMR splittings versus position for deuterated CP and CS incorporated into DPPC liposomes at 50 °C is presented in Fig. 38, and the values are accurate to within 1 kHz. At those positions where the peaks of a powder pattern cannot be discerned, the width of the component, as shown in Fig. 37, was taken as a measure of the splitting. This point will be further discussed below. The profile for bilayers of deuterated DPPC (Seelig & Seelig, 1974b; Davis, 1979) at 50 °C is also shown in Fig. 38 for comparative
Fig. 38: Plot of the $^2$H quadrupolar splitting versus chain position for selectively deuterated cholesteryl palmitate in DPPC liposomes at $\sim 50 \, ^\circ\text{C}$ (▲). The data for liposomes of deuterated DPPC at $\sim 50 \, ^\circ\text{C}$ (●) (Seelig & Seelig, 1974b; Davis, 1979) are plotted for comparison. The lines represent smooth curves through the experimental data.
purposes. It is readily seen that the spectral widths for deuterated CP and CS are quite different from those for the equivalent positions of deuterated DPPC. In DPPC, a "plateau" region exists that comprises C2 - C9 and for which the \(^2\)H linewidths are essentially the same, whereas the region comprising C10 - C16 is characterized by a continuous decrease in the spectral width going toward the terminal methyl group. On the other hand, in the case of CP and CS we observe a decrease of the spectral width from 4.5 kHz at C2 to only \(~1.5\) kHz at positions 4 and 5, followed by an increase to a maximum at C11 and C12. Between C2 and C10, the splittings observed for the ester are at least a factor of two lower than those of DPPC. Only approaching the terminal methyl group, i.e. from C12 onward, does the behaviour of both types of lipid tend to be similar. The shape of the profile of splitting versus chain position for the esters is reminiscent to the linewidth profile observed for deuterated CP and CS in egg-PC (Section D. IV.) and DPPC (Section D. V.) vesicles, which may signify that the conformation adopted by the ester in these systems is similar.

Inspection of the \(^2\)H-NMR spectra of selectively deuterated CP and CS in DPPC liposomes in Fig. 37 reveals that, for deuterons at positions 2, 4, and 5, where the widths are small, a characteristic powder pattern (Seelig,
1977) is not observed. Instead, these spectra are rather "bell-shaped" with no clear indication of the normally expected $90^\circ$ peaks. This behaviour could arise from the superposition of powder patterns of slightly different splittings, caused by the presence of more than one ester conformation in the bilayer. However, we believe that it is due to line broadening being comparable to the quadrupolar splitting, which leads to an unresolved powder pattern, especially in the presence of a central isotropic component (Seelig, 1977). The amount of broadening can be estimated by measuring the echo decay time $T_{2e}$. For CP-2,2-d$_2$ we determined $T_{2e} = 250 \mu$s, whereas for CP-16,16,16-d$_3$, $T_{2e} = 850 \mu$s. In addition, from the $\tau_1$ dependence of the $^2$H-NMR spectra of CP-d$_3$ in DPPC (Fig. 39a), an average $T_{2e}$ of ~250 $\mu$s was obtained for the CD$_2$ segments of the ester. These values correspond to a line broadening (= $1/\pi T_{2e}$) of ~1.3 kHz for CD$_2$ and 0.4 kHz for CD$_3$. Thus, for positions 2, 4, and 5, the line broadening is comparable to the quadrupolar splitting, leading to the observed spectral shape. On the other hand, for CP-16,16,16-d$_3$ (Fig. 37h), the powder pattern peaks are obscured by the presence of the central isotropic component, in spite of the smaller line broadening of ~0.4 kHz.

The incorporation of 0.5 mol% calculated for CP in DPPC
liposomes is approximately on the borderline of the amount above which $^2$H-NMR spectra of selectively deuterated samples could be acquired in a reasonable length of time. Hence, when using selectively deuterated esters we obtained spectra having a low signal to noise ratio. However, a new data analysis technique has recently been developed in order to extract the NMR spectrum corresponding to an oriented sample from a powder pattern (Bloom et al., 1981). This technique is applicable to cases in which the spectra are indicative of local axial symmetry, e.g. $^2$H-NMR spectra corresponding to an asymmetry parameter $\eta = 0$. From an experimentally determined powder pattern, a spectrum can be obtained by means of an iterative computer algorithm, which corresponds to a planar sample having the applied magnetic field parallel to the symmetry axis for reorientational motions. This technique is also applicable to a superposition of powder patterns, and allows the identification of the individual contributions to a complex powder pattern without the need for spectral simulations. Hence, in order to corroborate the information gained from the spectra of selectively deuterated CP (and CS) in DPPC liposomes, we have applied this data analysis procedure to the spectra of CP-d$_{31}$ in DPPC. The "aligned" spectrum of CP-d$_{31}$ in DPPC is shown in Fig. 39b. For comparison, we have also obtained the corresponding "aligned"
Fig. 39: High-field half of the $^2$H-NMR powder patterns for
(a): cholesteryl palmitate-$d_{31}$ in DPPC liposomes at ~50 °C
(in this spectrum the Larmor frequency is slightly offset and corresponds to the top of the highest peak); (b): the "aligned" spectrum (Bloom et al., 1981) corresponding to (a);
(c): DPPC-$d_{62}$ liposomes at 50 °C (Davis, 1979); (d): the "aligned" spectrum corresponding to (c).
spectrum of DPPC-d\textsubscript{62} (Davis, 1979) (Fig. 39d). It it apparent that the two are quite different. For the phospholipid, one observes a clearly resolved peak from the terminal CD\textsubscript{3} group close to the Larmor frequency (\(\Delta v_Q = 3\) kHz) and a series of nearly equally spaced resonances from deuterons on C15 to C10, whereas the plateau region is manifested as a very intense resonance at a splitting of 26 kHz. On the other hand, in the "aligned" spectrum from CP-d\textsubscript{31} in DPPC liposomes only five resolved peaks are visible. The most intense peak occurs at a splitting of \(\sim 14\) kHz, while the other four peaks have smaller splittings. Comparison of Fig. 39b with the spectra from the selectively deuterated esters (Fig. 37), together with an analysis of the integrated intensities, yields the peak assignments shown on Fig. 39b.

The "aligned" spectra obtained by data processing of the signals from CP-d\textsubscript{31} in DPPC liposomes at 50 °C, acquired using different values of \(\tau_1\), show that the relative intensities of the five peaks of Fig. 39b are essentially independent of \(\tau_1\), and this fact suggests that \(T_{2e}\) for the ester CD\textsubscript{2} groups does not vary significantly along the chain. Thus, all \(T_{2e}\)'s of the CD\textsubscript{2} segments must be \(\sim 250\) \(\mu s\), and this is more than 2-times lower than the equivalent values for DPPC-d\textsubscript{62} (Davis, 1979). This peculiar behaviour may reflect either some slow motion of the ester chain that is absent in
the phospholipid chains, or a rate of ester chain motions that is significantly slower than for the phospholipid. This may be the result of the particular molecular arrangement of CP (and CS) in the bilayer and may be caused by the proximity of the chain to the bulky cholesterol moiety. In this context we should remember that the $^2$H-NMR spin-lattice relaxation times of deuterated CP in DPPC unilamellar vesicles are shorter than those of the phospholipid (Section D. V.), which may indicate a slower rate of the motions giving rise to $T_1$ in that case.

A conformation for CP and CS in DPPC liposomes which is thought to be consistent with the observations presented in this work is the horseshoe conformation (Janiak et al., 1974; Grover et al., 1979). In this arrangement, the long axes of both the cholesterol fused ring system and most of the fatty acyl chain are thought to be aligned parallel to the bilayer normal, and with the ester linkage close to the aqueous interface. Such a structure would result in the initial segments of the fatty acyl chain to be aligned at an angle with respect to the bilayer normal. On the basis of eqn. 8, and assuming that the bilayer normal is identical to the axis of symmetry for reorientational motions of the acyl chain, such a situation will result in reduced order parameters $S$ for these positions. Hence, rather than interpreting the low
spectral widths observed for deuterons on C2, C4, and C5 of the ester chain in terms of an improbably high degree of local motion, we believe that these segments are aligned in such a manner as to make the average angles to these segments with the bilayer normal close to the magic angle (54°44'). On the other hand, the similarity in the splittings of incorporated CP and CS with those of DPPC, near the terminal methyl ends of the chains, is taken as evidence that the ester chain in that region lies, on average, parallel to the bilayer normal and undergoes motional fluctuations similar to those of the phospholipid.

Further support for a horseshoe conformation adopted by CP incorporated into DPPC bilayers comes from statistical mechanical calculations based on the mean-field approximation. Such calculations have been performed in this laboratory and will be discussed in the next section.
VII. Statistical Mechanical Calculations: Cholesteryl Palmitate in Dipalmitoylphosphatidylcholine Bilayers.

In order to obtain a deeper understanding of the structural organization and motional behaviour of cholesteryl esters in phospholipid bilayers, we have performed preliminary statistical mechanical calculations based on the mean-field approximation, using the model proposed by Marcelja (1974a, 1974b). This model is particularly useful since it considers the possible conformations of the acyl chains under investigation in an explicit manner and, thus, is able to predict segmental order parameters which may be directly compared to data obtained by means of $^2$H-NMR.

Schindler & Seelig (1975) have applied the Marcelja model to DPPC bilayers in the liquid-crystalline phase and were able to reproduce, quite accurately, the experimental $^2$H-NMR order parameter profile. In addition, properties such as the bilayer thickness and the probabilities of trans and gauche conformations could be calculated. Thus, this type of statistical mechanical computation allows the interpretation of experimental data from a molecular point of view and may permit us to understand the microscopic basis of some macroscopic observations.
We have attempted to use the Marcelja model in order to gain insight into the conformation of CP in DPPC bilayers. This system was chosen because DPPC is the only phospholipid for which mean-field calculations have been performed, hence, the corresponding program parameters are readily available. In addition, the experimental $^2$H-NMR data for selectively deuterated cholesteryl ester in DPPC bilayers (Section D. VI.) are present.

In order to study the conformation of cholesteryl ester in a phospholipid bilayer by means of the Marcelja model, we have written a computer program which, in its first stage, was designed to reproduce the data obtained by Schindler & Seelig (1975) for DPPC bilayers at 41 $^\circ$C. The algorithm used in our program to generate the possible conformations of the ester chain was taken from the program BURLESK (courtesy of C.D. Barry, L.O. Ford, & A.C.T. North; Laboratory of Molecular Biophysics, Department of Zoology, University of Oxford), while the different energy contributions for each conformation as well as the statistical weights, partition function, C-D order parameters, and average chain coordinates were calculated exactly as described previously (Schindler & Seelig, 1975). ¹

¹I wish to thank Prof. J. Seelig and Dr. J.P. Meraldi for their advice when setting up the present program.
The order parameter profile for DPPC bilayers at 41 °C (Fig. 40), calculated by our program, shows excellent agreement with the data of Schindler & Seelig (1975). The minor differences between the two sets of data are probably due to slight variations in the algorithms used. In addition, our program provides a fit to the experimental order parameter profile (Seelig & Seelig, 1974b) which is even better than that of Schindler & Seelig's calculation.

In order to perform calculations involving CP in DPPC bilayers, the original computer program had to be modified, and the final program adopted for our calculations is listed in the Appendix. For simplicity, it has been assumed that the presence of a small amount of cholesteryl ester in the membrane will not noticeably alter the bilayer properties, i.e. that the ester molecules will experience the same molecular field as DPPC. The rotational isomeric states as well as the energies of these states were taken to be the same as those of the phospholipid chains, where each segment is thought to exist either in a trans or one of two possible gauche conformations, and the energy of a gauche state is 500 cal/mol higher than that of the trans state. The rigid cholesteryl moiety was taken into account only in an extremely crude fashion as defining a "forbidden" region in which no segment of the ester chain can be found, hence,
Fig. 40: C-D order parameters for liposomes of deuterated DPPC at 41 °C, versus chain position. (●): experimental data (Seelig & Seelig, 1974b); (▲): calculated by statistical mechanical program (Schindler & Seelig, 1975); (□): calculated by statistical mechanical program (our program).
chain conformations in which any of the segments enter the "forbidden" region are discarded. For simplicity, the cholesteryl moiety was assumed to be a cylinder, with a length of 0.9 nm and a diameter of 0.72 nm, approximately corresponding to the dimensions of the cholesteryl fused ring system in cholesteryl esters (Janiak et al., 1979). Control runs showed that altering these values within reasonable limits did not drastically change the results of the computations. The position of the "forbidden" region in the bilayer is determined by the assumed geometry of the ester in the bilayer. In the present case, we have performed calculations only for the horseshoe conformation of CP, and the excluded region consisted of the cylinder long axis lying parallel to the bilayer normal.

The position of the cylindrical region corresponding to the cholesteryl fused ring system with respect to the C2 segment of the acyl chain was obtained by assuming that the geometry of the ester carbonyl region is essentially identical to that in cholesteryl myristate molecules (Fig. 41) (Craven & DeTitta, 1976). Initial calculations showed that the results were not significantly affected when changing the geometry of the carbonyl region within reasonable limits.

In order to obtain a reasonable fit of the order
Fig. 41: Geometry of the carbonyl region of cholesteryl palmitate in DPPC bilayers assumed for the statistical mechanical calculations. The angles $\alpha$ and $\beta$ are defined in the text.
"forbidden" region
(cholesteryl)
parameters calculated by means of the Marcelja model to the experimental data, Schindler & Seelig (1975) assumed that the C2 segment of the phospholipid acyl chain undergoes rapid transitions between three discrete conformational states, each of them having a different probability. These conformations were obtained as a result of varying the initial segment orientation and performing calculations for each of these orientations. The three conformational states adopted for the C2 segment were found to correspond to minima in the average chain energy. Such an approach might also give satisfactory results in the case of the CP acyl chain. However, this procedure is extremely time consuming since it involves performing computations for many different initial orientations. In addition, since the acyl chain of CP is attached to the rigid cholesteryl moiety, the motional freedom of the C2 chain segment may be relatively restricted, especially in the horseshoe conformation, in which there is bound to be considerable steric hindrance of this segment from the nearby cholesteryl fused ring system. We have therefore attempted to treat the C2 segment of the CP acyl chain as possessing only one orientation, i.e. fixed values of the angles $\alpha$ and $\beta$, where $\alpha$ is the angle between the bilayer normal and the CO-CH$_2$ bond vector, and $\beta$ is the angle between the bilayer normal and the normal on the plane.
defined by the two C-H vectors of the C2 segment (also called the segment direction) (Schindler & Seelig, 1975). As pointed out by Schindler & Seelig, the choice of the orientation of the C2 segment should only affect the numerical results of the first few chain segments, i.e. up to C4 or C5. In spite of the drastic simplification adopted for the C2 ester chain segment, there is still a large number of angles $\alpha$ and $\beta$ to be considered. Specifically, under the assumption of the geometry of the carbonyl region given in Fig. 41, the angle $\alpha$ can have values between $-9$ and $131^\circ$. We have performed calculations for a considerable number of initial segment orientations, without being able to exhaust all the possibilities.

Results.

Typical plots of the C-D order parameter versus chain position for CP incorporated in DPPC bilayers at $41^\circ$ C are shown in Fig. 42. Please note, that in this figure, the absolute value of the order parameter $|S|$ is depicted, in order to compare it more easily with the experimental profile of quadrupolar splittings (Fig. 38). The profiles of Fig. 42 all show a striking minimum in the orientational order of the CP acyl chain between C3 and C5, its exact position depending upon the orientation of the C2 segment, and this minimum was
Fig. 42: C-D order parameter profiles for the acyl chain of cholesteryl palmitate in DPPC bilayers at 41°C, calculated by our statistical mechanical program. The values of the angles $\alpha$ and $\beta$ (see Fig. 41 and text) were: (A): $\alpha = 110^\circ$, $\beta = 101^\circ$; (B): $\alpha = 90^\circ$, $\beta = 110^\circ$; (C): $\alpha = 70^\circ$, $\beta = 120^\circ$; (D): $\alpha = 50^\circ$, $\beta = 100^\circ$; Please note that • corresponds to C-D order parameters $S>0$, while ○ corresponds to $S<0$. 
observed for a large variety of C2 segment orientations.

The order parameter profile shown in Fig. 42A is quite similar to the experimental profile (Fig. 38), although the theoretical order parameters are somewhat higher than those calculated from the experimental data by means of eqn. 6 (Section B. I.). This discrepancy can be partly accounted for by the fact that the experimental data were recorded at a temperature of 50 °C, while our computation corresponds to 41 °C (see above). In addition, it is probable that the long axis of the cholesteryl moiety is not, as was assumed in our program, always aligned parallel to the bilayer normal. Instead, the cholesteryl moiety will likely undergo some "wobbling" motion, and this would reduce the computed order parameters of the CP chain, giving a better fit to the experimental values.

As is evident from Fig. 42 and from our other computer calculations, the minimum in the order parameter of the CP chain at C3 - C5 generally corresponds to a sign change, with the segments close to the carbonyl group having positive C-D order parameters, while those segments closer to the methyl end of the chain have negative C-D order parameters. Based on these observations we propose that, in the profile of quadrupolar splitting versus position for CP in DPPC (Fig. 38), positions 2 and 3 correspond to \( S>0 \), while
positions 6 - 16 have $S < 0$.

The average calculated positions of the atoms constituting the acyl chain of CP in DPPC bilayers, were found to be in agreement with the "horseshoe" conformation (Janiak et al., 1974; Grover et al., 1979). In Table IV we have listed the average depths in the bilayer of the carbon atoms of the CP acyl chain, corresponding to the profile of Fig. 42A, calculated by our computer program. Here, we have assumed that the depth of the C2 atom of the ester chain is 0.2 nm, but a different value does not alter the relative average positions of the chain atoms appreciably. Clearly, the first few segments of the acyl chain point toward the aqueous interface, while between C4 and C16 the chain bends back into the hydrophobic bilayer interior. This is precisely what we would expect on the basis of the horseshoe conformation.

The horseshoe conformation for CP in DPPC bilayers can be further rationalized by considering the different energy contributions to the total acyl chain energy. If we assume the cholesteryl moiety to be parallel to the bilayer normal and the carbonyl region to be positioned near the aqueous interface, then inspection of the bond angles of the CP carbonyl region (Fig. 41) shows that the acyl chain would, in its all-trans form, be tilted with respect to the bilayer
TABLE IV: Average depths in DPPC bilayers, for the carbon atoms of the cholesteryl palmitate acyl chain, calculated by our statistical mechanical program.

<table>
<thead>
<tr>
<th>Carbon atom</th>
<th>average depth in bilayer (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.200</td>
</tr>
<tr>
<td>3</td>
<td>0.099</td>
</tr>
<tr>
<td>4</td>
<td>0.143</td>
</tr>
<tr>
<td>5</td>
<td>0.214</td>
</tr>
<tr>
<td>6</td>
<td>0.252</td>
</tr>
<tr>
<td>7</td>
<td>0.356</td>
</tr>
<tr>
<td>8</td>
<td>0.408</td>
</tr>
<tr>
<td>9</td>
<td>0.519</td>
</tr>
<tr>
<td>10</td>
<td>0.580</td>
</tr>
<tr>
<td>11</td>
<td>0.693</td>
</tr>
<tr>
<td>12</td>
<td>0.757</td>
</tr>
<tr>
<td>13</td>
<td>0.864</td>
</tr>
<tr>
<td>14</td>
<td>0.925</td>
</tr>
<tr>
<td>15</td>
<td>1.015</td>
</tr>
</tbody>
</table>
normal. Such an orientation of the ester acyl chain was shown by our calculations to be energetically highly unfavourable. In fact, although in an orientation with the ester chain in an all-trans conformation the internal chain energy reaches a minimum, the steric repulsion with the neighboring DPPC chains is very high, and the Van der Waals attraction is very low. Hence, the ester chain will tend to adopt a bend in the upper chain region in order to align the maximum number of chain segments parallel to the bilayer normal. When considering the different energy contributions, it was seen that the increase in internal energy caused by the introduction of a bend in the upper chain region is more than offset by the decrease in the energies corresponding to steric repulsion and Van der Waals attraction.

It may be hypothesized that an alignment of the long axis of the rigid cholesteryl moiety parallel to the bilayer normal would also minimize steric repulsions, tending to support our a priori assumption concerning the alignment of this part of the ester molecule.

From what has been said in the previous paragraphs we can conclude that, while we did not obtain a quantitative agreement of the computer calculations of CP in DPPC bilayers with the experimental $^2$H-NMR data, qualitatively our computations are certainly consistent with a horseshoe
conformation for the ester. However, we are aware of the many approximations that our model entails, and it is clear that a more rigorous calculation of the system is desirable. The present model involves many drastic assumptions, and a more refined method would be needed. Such an investigation could be a worthwhile topic for future research.
E. CONCLUSIONS.

In the present investigation we have studied the incorporation and dynamic behaviour of saturated cholesteryl esters, specifically cholesteryl palmitate and cholesteryl stearate, in several phospholipid model membrane systems, and we can now draw the following general conclusions from our work:

1.) The incorporation of cholesteryl palmitate and stearate in phospholipid liposomes has been observed to be very low. Specifically, it is 1.5 mol% in bovine brain sphingomyelin (Section D. III.), 0.5 mol% in dipalmitoylphosphatidylcholine (Section D. VI.), and 0.2 mol% in egg-phosphatidylcholine (Section D. II.). Thus, while other researchers (Janiak et al., 1979; Smaby et al., 1979) have concluded that saturated esters are insoluble in phospholipids at maximum hydration, we have been able to show, by use of $^2$H-NMR, that small proportions of saturated cholesteryl esters can be incorporated into phospholipid bilayers. We believe that this is the first application of $^2$H-NMR to determine the solubility of compounds in membranes. Thus, this technique is extremely sensitive and especially useful for studying bilayer components which are present in
small amounts and which might remain undetected by other methods. Since, in spite of its low incorporation, cholesteryl palmitate is known to greatly enhance bilayer permeability (Forrest & Cushley, 1977), the effect of the minor membrane constituents such as cholesteryl esters may be very important for understanding the behaviour of biological membranes.

The low solubility of cholesteryl palmitate and cholesteryl stearate in phospholipid membranes, under conditions of maximum hydration, observed by our $^2$H-NMR experiments supports the hypothesis (Small & Shipley, 1974) that the formation of lipid droplets in the aorta ("fatty streaks") which consist mainly of cholesteryl esters (Lang & Insull, 1970), may be interpreted in terms of the phase behaviour of the lipids involved, and that these droplets represent excessively metabolized cholesteryl ester which cannot be solubilized by the organism.

On the other hand, we have observed that the solubility of cholesteryl palmitate in sphingomyelin is several-fold higher than in phosphatidylcholines. Since the sphingomyelin content of the aortic wall is known to increase during progressive stages of atherosclerosis, while that of phosphatidylcholine decreases, (Bottcher & Van Gent, 1961), such a change in lipid composition may represent an attempt
by the organism to limit the formation of fatty streaks by incorporating a larger amount of cholesteryl ester into the aortic cell membranes.

Cholesteryl esters in plasma lipoproteins are generally believed to exist in a nonpolar core surrounded by a phospholipid/cholesterol monolayer and proteins (Morrisett et al., 1977). In addition, based on the similar chemical composition of plasma lipoproteins and the lipid droplets encountered in the cytoplasm of adrenal gland cells (Boyd & Trzeciak, 1973), we may hypothesize that these lipid droplets may have a structural organization quite similar to that of plasma lipoproteins. Since the present study has shown that phospholipid membranes may incorporate small amounts of saturated cholesteryl esters, it is probable that cholesteryl ester will be found in the lipoprotein surface monolayer as well as in the hypothetical surface monolayer of adrenal gland lipid droplets, and this finding may be important in order to understand the behaviour and metabolism of these structures.

2.) The incorporation of cholesteryl esters in phospholipid vesicles is several-fold higher than in multilamellar liposomes, and up to 5 mol% cholesteryl
palmitate (or stearate) may be incorporated into egg-phosphatidylcholine and dipalmitoylphosphatidylcholine sonicated bilayers. It is not clear whether phospholipid vesicles containing higher proportions (\textgreater{}1 mol\%) of cholesteryl ester merely constitute a supersaturated system, or whether the increased ester incorporation may be related to the high curvature of the vesicles. If indeed ester solubility were dependent upon the curvature of the bilayer, it could have physiological importance, since cholesteryl ester in the organism would tend to be more abundant in highly curved regions of membranes.

Saturated cholesteryl esters such as cholesteryl palmitate and cholesteryl stearate cannot be dispersed in water. On the other hand, in order to prepare phospholipid/ester mixed vesicles containing higher proportions (\textgreater{}1 mol\%) of cholesteryl ester, excess ester has to be added prior to sonication. Hence, that part of the cholesteryl ester which is not incorporated into the vesicles must form other structures together with some of the phospholipid. It would be interesting to determine if these other entities are indeed composed of a cholesteryl ester core surrounded by phospholipid (Janiak, 1977), since such a system would possess a structure very similar to that of plasma lipoproteins and, possibly, to that of the lipid
droplets found in adrenal gland cells. Thus, the cholesteryl ester/phospholipid aggregates might represent a simple model system for many biological structures.

3.) For selectively deuterated cholesteryl palmitate in dipalmitoylphosphatidylcholine, our data are consistent with the ester adopting a horseshoe conformation in the bilayer, with the carbonyl linkage close to the aqueous interface and the cholesteryl and fatty acyl moieties extending into the hydrophobic interior. Thus, \(^2\)H-NMR using selectively deuterated compounds has been shown to be a useful technique for determining the orientational order of bilayer components that are present in low proportions (in this case, \(\sim 0.5 \text{ mol}\%\)). In addition, our data for selectively deuterated cholesteryl palmitate and stearate have been corroborated and extended by use of the data analysis technique of Bloom et al. (1981), which we have applied to the spectra of cholesteryl palmitate-\(d_{31}\) in dipalmitoylphosphatidylcholine bilayers. We believe that the present application of this data analysis technique is the first one to a membrane component other than a phospholipid, and we have shown it to be very useful.

Preliminary statistical mechanical calculations, based on the Marcelja model, of cholesteryl palmitate in
dipalmitoylphosphatidylincholine bilayers, reported in Section D. VII. of the present thesis, give additional support to our contention that the cholesteryl ester adopts a horseshoe conformation in the bilayer. This conformation had been suggested for cholesteryl linolenate in egg-phosphatidylcholine bilayers (Janiak et al., 1974), cholesteryl myristate in dimyristoylphosphatidylcholine bilayers (Janiak et al., 1979), and, lately, for several unsaturated cholesteryl esters in lipid monolayers (Smaby et al., 1979), whereas such a conformation has been shown to be adopted by spin labelled cholesteryl palmitate in egg-phosphatidylcholine bilayers (Grover et al., 1979). In addition, the similarity of the $^2$H-NMR spectra of cholesteryl palmitate-$d_{31}$ in egg-phosphatidylcholine and dipalmitoylphosphatidylincholine liposomes (Sections D. II. and VI.) suggests that the conformation of the ester in egg-phosphatidylcholine is very similar to that in dipalmitoylphosphatidylincholine, i.e. a horseshoe conformation of cholesteryl palmitate may also be present in egg-phosphatidylcholine bilayers. Thus, a horseshoe conformation has been found to be consistent with a wide range of experimental observations, involving diverse esters and phospholipids, and we believe that such a conformation is a general feature of cholesteryl esters incorporated into
phospholipid membranes.

It is probable that a horseshoe conformation of cholesteryl ester is also encountered in biological membranes as well as in structures such as the surface monolayer of plasma lipoproteins and, perhaps, in the lipid droplets of adrenal gland cells. In such a conformation the ester carbonyl group will be placed near the aqueous interface, thus being readily accessible to ester hydrolases. Since the enzyme-mediated hydrolysis of cholesteryl ester is an important step in the metabolism of plasma lipoproteins (Kane, 1977) and of adrenal gland lipid droplets (Vahouny et al., 1978), the horseshoe conformation of the ester is probably significant for an understanding of the in vivo behaviour of these structures.

4.) The profiles of $1/T_{25\text{low}}$ for deuterated cholesteryl palmitate and cholesteryl stearate in egg-phosphatidylcholine and dipalmitoylphosphatidylcholine unilamellar vesicles (Sections D. IV. and V.), and the quadrupolar splitting profile of deuterated cholesteryl palmitate in dipalmitoylphosphatidylcholine liposomes (Section D. VI.) are similar, suggesting that the conformation of cholesteryl ester in vesicles is related to that in liposomes, i.e. some type of horseshoe conformation may also be involved in the
sonicated systems. The spin-lattice relaxation times measured for deuterated ester in vesicles are consistent with this proposal, as is the $^2H$-NMR linewidth change upon addition of 20 mol% cholesterol. However, our data do not allow us to answer this question with certainty. In addition, the low $^2H$-NMR linewidths of the signals from deuterated cholesteryl esters in vesicles, and the viscosity-independence of these linewidths, indicate a unique motional behaviour of the ester chain in these systems, which is not understood at present. It may be that cholesteryl ester in highly curved bilayers has a dynamic behaviour different to that in membranes of less curvature, and this might have important physiological implications. Specifically, the ester molecules in vesicles are believed by us to either possess a very high lateral diffusion coefficient, or, more probably, to undergo some unique slow motion(s). It may be hypothesized that such a slow ester motion may create transient pores in the bilayer through which ions can penetrate. Hence, it may be responsible for the drastically enhanced membrane permeability observed in cholesteryl ester/phospholipid vesicles (Forrest & Cushley, 1977), and such an effect may also exist in highly curved regions of biological membranes containing cholesteryl ester.

Clearly, the cholesteryl ester/phospholipid mixed
vesicle systems require further study, in order to determine the rate of lateral diffusion and/or the nature of the additional slow motion(s). The same may be said regarding the cholesteryl palmitate/dipalmitoylphosphatidylcholine liposome system, where we have measured unusually short values of the echo decay time $T_{2e}$. Hence, slow ester chain motions may also be important in liposomes, and it is desirable to know more about the nature of these motions.

5.) The dynamic behaviour of cholesteryl esters in model membranes, as evidenced from the $^2$H-NMR results presented in Sections D. II. - VI., is quite different from that of the phospholipids constituting the membrane matrix. The ester chains appear more disordered and seem to undergo slower motions than the phospholipid acyl chains. In the light of these data, it is conceivable that cholesteryl esters, in spite of being soluble only in small proportions in phospholipid bilayers, may serve to modulate membrane behaviour in vivo, either directly, by altering the properties of the lipid component, and/or indirectly, by having an effect on the function of membrane-bound proteins. The increased permeability of phospholipid bilayers observed by Forrest & Cushley (1977) and the observations of Davis et al. (1978), who saw a change in the activity of the hepatic...
Na-K ATPase in cholesteryl ester containing membranes, certainly point toward such roles. However, more data will be needed in order to be able to correlate the dynamic behaviour of cholesteryl esters with their metabolic functions. It is hoped that the work described in this thesis will help to stimulate further research in that direction.
F. APPENDIX.

This appendix comprises the Fortran IV computer programs used for performing the statistical mechanical calculations described in Section D. VII. The first program, INPUT, calculates the atomic coordinates of the acyl chain to be considered, in its all-trans form. The second program, ESTERS, performs the actual statistical mechanical calculations, using the initial chain coordinates generated by INPUT.
PROGRAM INPUT


DIMENSION XNAME(20)
COMMON/ONE/XCARB(25),YCARB(25),XHYD1(25),YHYD1(25),ZHYD1(25),XHYD2(25),YHYD2(25),ZHYD2(25),XTERM,YTERM
COMMON/TWO/NCHAIN
DATA PI/3.141592654/

READ INPUT TITLE
READ(5,1) (XNAME(I),I=1,20) 1 FORMAT(20A4)

READ NUMBER OF C ATOMS IN CHAIN, REQUIRED VALUES OF ALPHA AND BETA, DEPTH (Z COORDINATE) OF CARBON ATOM OF FIRST CH2 GROUP IN CHAIN.
READ(5,2) NCHAIN,ALPHA,BETA,ZZERO 2 FORMAT(I5,3F10.3)
WRITE(6,5) (XNAME(I),I=1,8) 5 FORMAT(/,8A4,/) WRITE(6,4) NCHAIN,ALPHA,BETA,ZZERO 4 FORMAT(' NUMBER OF CH2 UNITS IN CHAIN=',I5,/, ' ALPHA=',F10.3,' DEG.',5X,' BETA=',F10.3,' DEG.',6X, ' DEPTH OF C' 2,' ATOM IN FIRST CH2 GROUP=',F10.3,' ANGSTROMS',/) TRANSFORM TETRAHEDRAL ANGLE/2 (54.65 DEG.) INTO RADIANS
ANGLE= (54.65/360.0) *2.0*PI
SENO = SIN(ANGLE)
COSENO = COS(ANGLE)
IFLAG=0

SET UP INITIAL COORDINATES
DO 10 I=1,NCHAIN
ZNUM = I - 1

CARBON ATOMS
C-C DISTANCE = 1.541 ANGSTROMS
DISTINGUISH BETWEEN EVEN AND ODD NUMBERED ATOMS.
ASSUME THAT FOR ODD I, H ATOMS LOOK TO THE LEFT (NEGATIVE Y VALUES), WHILE FOR EVEN I, H ATOMS LOOK TO THE RIGHT (POSITIVE Y VALUES)
NUMBERING OF H ATOMS IS SUCH THAT THE CROSS PRODUCT OF THE C-HYD1 AND C-HYD2 VECTORS COINCIDES WITH THE POSITIVE Z AXIS;
I.E. (C-HYD1 X C-HYD2) IS A VECTOR POINTING IN THE
POSITIVE Z DIRECTION

CHECK IF C ATOM IS EVEN OR ODD
NNUM = I/2
FNUM = FLOAT(I)/2.0
IF(FNUM - FLOAT(NNUM))6,7,6

EVEN NUMBERED ATOMS
7 XCARB(I) = 0.0
YCARB(I) = (1.541/2.0) * COSENO
ZCARB(I) = ZZERO + ZNUM*1.541*SENO
XHYD1(I) = -1.073 * SENO
YHYD1(I) = 1.073*COSENO + YCARB(I)
ZHYD1(I) = ZCARB(I)
XHYD2(I) = - XHYD1(I)
YHYD2(I) = YHYD1(I)
ZHYD2(I) = ZCARB(I)
GO TO 10

ODD NUMBERED ATOMS
6 XCARB(I) = 0.0
YCARB(I) = -(1.541/2.0)*COSENO
ZCARB(I) = ZZERO + ZNUM*1.541*SENO
XHYD1(I) = 1.073*SENO
YHYD1(I) = - 1.073*COSENO + YCARB(I)
ZHYD1(I) = ZCARB(I)
XHYD2(I) = - XHYD1(I)
YHYD2(I) = YHYD1(I)
ZHYD2(I) = ZCARB(I)
10 CONTINUE

THIRD H-ATOM OF TERMINAL CH3 GROUP:
XTERM = 0.0
ZTERM = ZCARB(I) + 1.073*SENO
IF(FNUM - FLOAT(NNUM))8,9,8
8 YTERM = YCARB(I) + 1.073*COSENO
GO TO 11
9 YTERM = YCARB(I) - 1.073*COSENO

CALCULATE HOW MUCH MOLECULE HAS TO BE TILTED ABOUT
X AXIS, IN ORDER TO GET REQUIRED
VALUE OF ALPHA. IN INITIAL ORIENTATION, ALPHA=35.35
DEGREES. ALPHA IS MEASURED CLOCKWISE FROM NEGATIVE
Z AXIS.
11 TILTAL = ALPHA - 35.35

TRANSFORM TO RADIANS
RADAL = (TILTAL/360.0) * 2.0 * PI
CALCULATE DIRECTION COSINES OF ROTATION AXIS
(COINCIDES WITH X AXIS)
\[ \cos 11 = 1.0 \]
\[ \cos 12 = 0.0 \]
\[ \cos 13 = 0.0 \]

PERFORM ROTATION ABOUT X AXIS
CALL COOR(RADAL, \( \cos 11 \), \( \cos 12 \), \( \cos 13 \))

ROTATION ABOUT CO-CH\(_2\) BOND:

CALCULATE DIRECTION COSINES OF CO-CH\(_2\) BOND
AFTER FIRST ROTATION:
\[ \cos 21 = 0.0 \]
\[ \text{RAD} = (\text{ALPHA}/360.0) \times 2.0 \times \pi \]
\[ \text{C WITH Y AXIS:} \]
\[ \cos 22 = -\sin(\text{RAD}) \]
\[ \text{C WITH Z AXIS:} \]
\[ \cos 23 = \cos(\text{RAD}) \]

ROTATION ABOUT CO-CH\(_2\) BOND HAS TO BE PERFORMED IN SUCH A WAY AS TO OBTAIN THE CORRECT VALUE OF BETA.
EASIEST WAY: PERFORM SMALL ROTATIONS (E.g. 0.05 DEG. AT A TIME) AND CALCULATE BETA AFTER EACH ONE.
DO THIS UNTIL THE DESIRED VALUE OF BETA IS REACHED.
NOTE THAT BETA MUST BE ALWAYS BETWEEN 0 AND 180 DEGREES.
BETA IS ALSO MEASURED FROM THE NEGATIVE Z AXIS.
IT IS THE ANGLE BETWEEN THE NORMAL TO BOTH C-H BONDS IN THE FIRST CH\(_2\) SEGMENT AND THE NEGATIVE Z AXIS.

PERFORM SUCCESIVE ROTATIONS ABOUT CO-CH\(_2\) BOND

TRANSFORM 0.05 TO RADIANS:
\[ \text{AINC} = (0.05/360.0) \times 2.0 \times \pi \]
DO 20 I=1,7200
\[ \text{ANGLE} = \text{AINC} \times \text{FLOAT}(I-1) \]
CALL ROTA(ANGLE,RESULT,COS21,COS22,COS23)
IF(ABS(RESULT-BETA).GT.0.025) GO TO 20

IF REACH THIS POINT, CORRECT ROTATION ANGLE HAS BEEN FOUND. PERFORM ROTATION AND GET NEW COORDINATES:
CALL COOR(ANGLE,COS21,COS22,COS23)

WRITE FINAL COORDINATES:

WRITE(6,30)
30 FORMAT(/,' CONVERGENCE. FINAL COORDINATES ARE:'/'
1,4X,'X',7X,'Y',7X,'Z',3X,'TYPE')
DO 40 K=1,NCHAIN
40 WRITE(6,41) XCARB(K),YCARB(K),ZCARB(K),XHYD1(K),
1YHYD1(K),ZHYD1(K),XHYD2(K),YHYD2(K),ZHYD2(K)
41 FORMAT(3F9.4,' C',/,'C1,/,3F9.4,' H',/,'F9.4,' H')
WRITE(6,42) XTERM,YTERM,ZTERM
42 FORMAT(3F9.4,' H')
IFLAG=1
IF(IFLAG.EQ.1) STOP
20 CONTINUE
WRITE(6,777)
777 FORMAT(/,' NO CONVERGENCE ACHIEVED. CHECK DATA.')
STOP
END
SUBROUTINE COOR(RADAL,COS1,COS2,COS3)
COMMON/ONE/XCARB(25),YCARB(25),ZCARB(25),XHYD1(25),
1YHYD1(25),XHYD2(25),YHYD2(25),ZHYD2(25),XTERM,YTERM
2,ZTERM,ZCARB(25)
COMMON/TWO/NCHAIN
C RADAL=ROTATION ANGLE IN RADIANS
C COS1,COS2,COS3= DIRECTION COSINES OF ROTATION AXIS
C WITH RESPECT TO X, Y, Z AXIS
C THIS SUBROUTINE PERFORMS THE ROTATION AROUND THE C ATOM
C OF THE FIRST CH2 UNIT
C
COSE = COS(RADAL)
SINE = SIN(RADAL)
ONCOS= 1.0 - COSE
C
C SET UP ROTATION MATRIX
C
T1=COS1*COS1*ONCOS + COSE
T2=COS1*COS2*ONCOS - COS3*SINE
T3=COS1*COS3*ONCOS + COS2*SINE
T4=COS1*COS2*ONCOS + COS3*SINE
T5=COS2*COS2*ONCOS + COSE
T6=COS2*COS3*ONCOS - COS1*SINE
T7=COS1*COS3*ONCOS - COS2*SINE
T8=COS2*COS3*ONCOS + COS1*SINE
T9=COS3*COS3*ONCOS + COSE
C
C PERFORM TRANFORMATION, ALL GROUPS EXCEPT
C EXCEPT LAST H ATOM IN CH3 GROUP:
C
XCARBO=XCARB(1)
YCARBO=YCARB(1)
ZCARBO=ZCARB(1)
DO 10 I=1,NCHAIN
DEXCAR = XCARB(I) - XCARB0
DEYCAR = YCARB(I) - YCARB0
DEZCAR = ZCARB(I) - ZCARB0
DEXH1 = XHYD1(I) - XCARB0
DEYH1 = YHYD1(I) - YCARB0
DEZH1 = ZHYD1(I) - ZCARB0
XXCAR = DEXCAR*T1 + DEYCAR*T2 + DEZCAR*T3
YYCAR = DEXCAR*T4 + DEYCAR*T5 + DEZCAR*T6
ZZCAR = DEXCAR*T7 + DEYCAR*T8 + DEZCAR*T9
XCARB(I) = XXCAR + XCARB0
YCARB(I) = YYCAR + YCARB0
ZCARB(I) = ZZCAR + ZCARB0

XXHYD1 = DEXH1*T1 + DEYH1*T2 + DEZH1*T3
YYHYD1 = DEXH1*T4 + DEYH1*T5 + DEZH1*T6
ZZHYD1 = DEXH1*T7 + DEYH1*T8 + DEZH1*T9
XHYD1(I) = XXHYD1 + XCARB0
YHYD1(I) = YYHYD1 + YCARB0
ZHYD1(I) = ZZHYD1 + ZCARB0

XXHYD2 = DEXH2*T1 + DEYH2*T2 + DEZH2*T3
YYHYD2 = DEXH2*T4 + DEYH2*T5 + DEZH2*T6
ZZHYD2 = DEXH2*T7 + DEYH2*T8 + DEZH2*T9
XHYD2(I) = XXHYD2 + XCARB0
YHYD2(I) = YYHYD2 + YCARB0
ZHYD2(I) = ZZHYD2 + ZCARB0

10 CONTINUE

C CH3 GROUP, FINAL H ATOM ONLY
DEXMET = XTERM - XCARB0
DEYMET = YTERM - YCARB0
DEZMET = ZTERM - ZCARB0
XXTERM = DEXMET*T1 + DEYMET*T2 + DEZMET*T3
YYTERM = DEXMET*T4 + DEYMET*T5 + DEZMET*T6
ZZTERM = DEXMET*T7 + DEYMET*T8 + DEZMET*T9
XTERM = XXTERM + XCARB0
YTERM = YYTERM + YCARB0
ZTERM = ZZTERM + ZCARB0
RETURN

END

SUBROUTINE ROTA(ANGLE, RESULT, COS21, COS22, COS23)
COMMON/ONE/XCARB(25), YCARB(25), XHYD1(25), YHYD1(25),
XHYD2(25), YHYD2(25), ZHYD2(25), XTERM, YTERM,
ZTERM, ZCARB(25)
C
C THIS SUBROUTINE PERFORMS THE ROTATION ABOUT THE
C CO-CH2 BOND, AT
AN ANGLE "ANGLE" (IN RADIANS), AND IT CALCULATES THE
NEW VALUE OF BETA (CALLED "RESULT").
COS21, COS22, COS23 = DIRECTION COSINES OF
THE CO-CH2 BOND.
COSINE = COS(ANGLE)
SINE = SIN(ANGLE)
COCOS = 1.0 - COSINE

ELEMENTS OF ROTATION MATRIX

T1 = COS21*COS21*COCOS + COSINE
T2 = COS21*COS22*COCOS - COS23*SINE
T3 = COS21*COS23*COCOS + COS22*SINE
T4 = COS21*COS22*COCOS + COS23*SINE
T5 = COS22*COS22*COCOS + COSINE
T6 = COS22*COS23*COCOS - COS21*SINE
T7 = COS21*COS23*COCOS - COS22*SINE
T8 = COS22*COS23*COCOS + COS21*SINE
T9 = COS23*COS23*COCOS + COSINE

DO TRANSFORMATION FOR FIRST CH2 GROUP ONLY

XCAR0 = XCARB(1)
YCAR0 = YCARB(1)
ZCAR0 = ZCARB(1)
XHYD01 = XHYD1(1) - XCAR0
YHYD01 = YHYD1(1) - YCAR0
ZHYD01 = ZHYD1(1) - ZCAR0
XHYD02 = XHYD2(1) - XCAR0
YHYD02 = YHYD2(1) - YCAR0
ZHYD02 = ZHYD2(1) - ZCAR0
X1 = XHYD01*T1 + YHYD01*T2 + ZHYD01*T3
Y1 = XHYD01*T4 + YHYD01*T5 + ZHYD01*T6
Z1 = XHYD01*T7 + YHYD01*T8 + ZHYD01*T9
XX1 = X1 + XCAR0
YY1 = Y1 + YCAR0
ZZ1 = Z1 + ZCAR0
X2 = XHYD02*T1 + YHYD02*T2 + ZHYD02*T3
Y2 = XHYD02*T4 + YHYD02*T5 + ZHYD02*T6
Z2 = XHYD02*T7 + YHYD02*T8 + ZHYD02*T9
XX2 = X2 + XCAR0
YY2 = Y2 + YCAR0
ZZ2 = Z2 + ZCAR0
A1 = XX1 - XCAR0
B1 = YY1 - YCAR0
A2 = XX2 - XCAR0
B2 = YY2 - YCAR0
CALCULATE NEW VALUE OF BETA

CORES = -(A1*B2 - A2*B1)/1.08662414551
RES = ARCOS(CORES)

TRANSFORM TO DEGREES:

RESULT = (RES*360.0)/(2.0*3.141592654)
RETURN
END
C PROGRAM ESTERS
COMMON/ONE/ETOTAL,RT,RIGID(25,2),CX(100,26)
1,CY(100,26),CZ(100,26),SCD(25),NORIGD
COMMON/TWO/IWRITE,PARFC,FIELD,ICHOL,ALPHA
1,OMEGA,CONT(25)
INTEGER RIGID
READ(5,810) IWRITE
810 FORMAT(2X,I3)
C
C IWRITE=FLAG WHICH INDICATES IF ALL ALLOWED
C CONFORMATIONS ARE TO BE PRINTED OUT (0=NO,1=YES).
C USE IWRITE=1 ONLY FOR SMALL MOLECULES.
C
WRITE(6,811) IWRITE
811 FORMAT(/,2X,'IWRITE=',I3,/)  
C
C FIELD=MOLECULAR FIELD IN CAL/MOL.
C ICHOL=FLAG WHICH INDICATES IF CHOLESTEROL ESTER OR NOT
C (0 = NO, OTHER VALUES = YES).
C
READ(5,813) FIELD,ICHOL
813 FORMAT(F11.4,I5)
WRITE(6,812) FIELD,ICHOL
812 FORMAT(/,' MOLECULAR FIELD =',F11.4,' CAL/MOL',
15X,' ICHOL =',I3,/)  
READ(5,814) ALPHA,BETA,OMEGA
814 FORMAT(3F11.4)
C
C READ ANGLES ALPHA AND BETA WHICH GIVE
C INITIAL SEGMENT ORIENTATION
C SEE SCHINDLER AND SEELIG (1975).
C OMEGA=ANGLE BETWEEN CHOLESTEROL LONG
C AXIS AND BILAYER NORMAL.
C OMEGA=0 FOR HORSESHOE CONF. OF ESTER.
C NOTE THAT ALPHA IS MEASURED FROM THE NEGATIVE Z AXIS,
C WHILE OMEGA AND BETA ARE MEASURED FROM
C THE POSITIVE Z AXIS
C
WRITE(6,815) ALPHA,BETA,OMEGA
815 FORMAT(' ALPHA=',F11.4,' DEG.',5X,  
1'BETA=',F11.4,' DEG.'
2,5X,' OMEGA=',F11.4,' DEG.',/)  
CALL CONFOR
STOP
END
SUBROUTINE CONFOR
THIS SUBROUTINE CALCULATES THE CONFORMATIONS FOR THE MOLECULE
MAXIMUM NO OF UNITS 10, MAXIMUM NO OF COORDS 100
MAXIMUM NUMBER OF CHAIN SEGMENTS=25

COMMON/ONE/ETOTAL,RT,RIGID(25,2),CX(100,26)
1,CY(100,26),CZ(100,26),SCD(25),NORIGD
COMMON/TWO/IWRITE,PARFC,FIELD,ICHOL,ALPHA
1,OMEGA,CONT(25)
INTEGER RIGID,STANG,FINANG
DIMENSION XNAME(20),AVZ(25),COB(25),CODISP(25)
DIMENSION NTRANS(25)
1,SINE(361),COSINE(361)
2,CXX(100),CYY(100),CZZ(100)
INTEGER TYPE(100),HMU(25)
1,PHI(25),BA(25),EA(25)
DATA ITOTAL/0/
C CONST=MODULE OF THE CROSS-PRODUCT OF TWO C-H VECTORS IN A METHYLENE CHAIN SEGMENT. ASSUMED: H-C-H ANGLE=109.3 DEG., C-H DISTANCE=1.073 ANGSTROM.
C TEMP=TEMPERATURE
C
DATA CONST/ 1.08662414551/
DATA TEMP/314.0 /
DATA AVZ/25*0.0/
C SUM = ESTIMATED DISTANCE FROM FIRST CARBON ATOM OF CHAIN TO NEAREST C-ATOM OF CHOLESTEROL RING SYSTEM, IN THE CASE OF CHOLESTERYL ESTERS.
C TLENG = ESTIMATED LENGTH OF CHOLESTEROL RING SYSTEM
C XCHOL = ESTIMATED RADIUS OF CHOLESTEROL RING SYSTEM
DATA SUM,TLENG,XCHOL/2.0,9.0,3.6/
C EQUIVALENCE (CX,CXX),(CY,CYY),(CZ,CZZ)
C INPUT NAME OF COMPOUND AND NUMBER OF COORDINATES
READ(5,200)(XNAME(I),I=1,20)
WRITE(6,200)(XNAME(I),I=1,20)
200 FORMAT (20A4)
C GAMMA= SURFACE PRESSURE IN BILAYER, IN DYNES/CM
C EZERO = ENERGY OF THE FIRST CH2 SEGMENT (CAL/MOL)
C COUPLG = COUPLING CONSTANT (CAL/MOL)
C
READ(5,931) GAMMA,EZERO,COUPLG
931 FORMAT(3F10.3)
C READ NUMBER OF ATOMIC COORDINATES TO BE READ
READ(5,201)NOCOOR
201 FORMAT (I3)
C SET PARTITION FUNCTION EQUAL TO 0.
PARFC=0.0
C READ COORDINATES OF CHAIN ATOMS
C IN THE ALL TRANS
C CONFORMATION, AS WELL AS TYPE
C OF ATOM FOR EACH COORDINATE.
779 READ(5,202) (CX(I,1),CY(I,1),CZ(I,1),TYPE(I),
   1I=1,NOCOOR)
202 FORMAT (3F9.4,A4)
C INPUT NO OF RIGID UNITS AND 1ST ATOM AND LAST ATOM OF EACH
READ(5,201)NORIGD
READ(5,203) (RIGID(I,1),RIGID(I,2),I=1,NORIGD)
203 FORMAT (2I4)
C READ ROTATABLE BONDS (ONE LESS THAN
C THE NUMBER OF RIGID UNITS),
C HOW MANY UNITS AFFECTED, STARTING ANGLE,
C FINISHING ANGLE, INCREMENT
NOBOND = NORIGD-1
READ(5,204) (BA(I),EA(I),HMU(I),
   1,I=1,NOBOND)
   STANG=0
   FINANG=240
   INC=120
204 FORMAT (315)
C WRITE (6,944) GAMMA,COUPLG,EZERO
944 FORMAT(' GAMMA =',F8.3,'CAL/MOL',5X,
   1' COUPLING CONSTANT ='
   2,F10.3,'CAL/MOL',5X,' EZERO = ',F10.3,'CAL/MOL',/)
WRITE(6,208)NOCOOR
208 FORMAT (' NUMBER OF COORDINATES ',/)
WRITE(6,209) (CX(I,1),CY(I,1),CZ(I,1),TYPE(I),
   1I=1,NOCOOR)
209 FORMAT (6X,3F11.4,3X,A4)
WRITE(6,210)NORIGD
210 FORMAT ('// NUMBER OF RIGID UNITS ',/) WRITE(6,211)
211 FORMAT (16X,' FIRST ATOM NO OF UNIT'
   1,' END ATOM OF UNIT',/)
WRITE(6,212) (I,RIGID(I,1),RIGID(I,2),I=1,NORIGD)
212 FORMAT (6X,I4,16X,I4,16X,I4)
WRITE(6,213)
213 FORMAT ('// PHI ANGLE',10X,' BOND ATOM NOS',
   1' HOW MANY UNITS ',
   2' STARTING ANGLE FINISHING ANGLE INCREMENT'/)
WRITE(6,214) (I,BA(I),EA(I),HMU(I),STANG,FINANG,INC
   1,I=1,NOBOND)
214 FORMAT (5X,I4,16X,I4,3X,I4,10X,I5,12X,I5,16X,I5,15,I2X,I5)
C CALCULATE VALUE OF R*TEMP AND LENGTH OF HYDROCARBON
CHAIN IN ALL TRANS CONFORMATION:
ONLY C-C BONDS WILL BE CONSIDERED (SEE SEELIG & SEELIG, BIOCHEMISTRY 13, 4839 (1974))

RT = 1.987 * TEMP
CLENG0 = FLOAT(NORIGD) * 1.25689
VN = COUPLG/(FLOAT(NOBDON))
COFILD = 0.0
AVENCO = 0.0
OMEG = (OMEGA/360.0) * 6.283186
AVLENG = 0.0

COALPH = PROJECTED LENGTH (ON Z AXIS) OF CO-CH2 BOND.
COALPH = COS((ALPHA/360.0) * 6.283186) * 1.541

IF(ICHOL.EQ.0) GO TO 12
CALCULATE ANGLE BETWEEN LONG AXIS OF CHOLESTEROL AND POSITIVE Z AXIS.

CALCULATE COORDINATES OF RESTRICTED ZONE DUE TO CHOLESTEROL FUSED RING SYSTEM

COSO = COS(OMEG)
SINO = SIN(OMEG)
ZZMIN = CZZ(1) + SUM
ZZMAX = ZZMIN + TLENG

ASSUME THAT ANGLE BETWEEN CHOLESTEROL LONG AXIS AND BOND BETWEEN STEROL AND O ATOM IS ZERO.
CALCULATE HORIZONTAL (Y DIRECTION) DISTANCE FROM O ATOM BOUND TO STEROL AND C ATOM OF FIRST CH2 GROUP

VALENCE ANGLE OF O ATOM = 119 DEG.
AG = ((119.0 - OMEGA)/360.0) * 6.283186
YOC = 1.34 * SIN(AG) - 1.49 * SIN((ALPHA/360.0) * 6.283186)

VERTICAL (Z DIRECTION) DISTANCE BETWEEN O ATOM BOUND TO STEROL AND C ATOM OF FIRST CH2 GROUP

ZOC = 1.34 * COS(AG) + COALPH

SET UP SINE AND COSINE TABLES

12 DO 101 I = 1, 361
   ANG = (I-1) * 0.0174532925

194
SINE(I) = SIN(ANG)

101  COSINE(I) = COS(ANG)
C
CALCULATE PARAMETERS FOR FIRST CH2 GROUP
C
VMIN = CZZ(1) - COALPH
VMAX = VMIN
INUM = 1
NONE = RIGID(INUM, 1)
NTWO = NONE + 1
NTHREE = NONE + 2
C
CALCULATE COSINE OF ANGLE BETWEEN NORMAL TO BOTH C-H BONDS AND Z AXIS (COB) FOR THIS SEGMENT
C
A1 = CXX(NTWO) - CXX(NONE)
B1 = CXX(NTHREE) - CXX(NONE)
A2 = CYY(NTWO) - CYY(NONE)
B2 = CYY(NTHREE) - CYY(NONE)
COB(1) = ((A1*B2 - A2*B1)/CONST)
C
CALCULATE CONTRIBUTION TO SEGMENTAL ORDER PARAMETERS FOR THIS SEGMENT. C1, C2 = DIRECTION COSINES FOR C-H BONDS IN SEGMENT.
C
C1 = (CZZ(NTWO) - CZZ(NONE))/1.073
C2 = (CZZ(NTHREE) - CZZ(NONE))/1.073
CONT(1) = ((3.0*C1*C1 - 1.0) + (3.0*C2*C2 - 1.0))/4.0
C
CALCULATE VALUE OF 3COS2(\beta) - 1 REQUIRED FOR CALCULATION OF DISPERSION ENERGY
CODISP(1) = (3.0*COB(1)*COB(1) - 1.0)
C
INITIALIZE VALUES FOR LENGTH OF CHAIN PROJECTION ON BILAYER NORMAL
VMAX = CZZ(1)
VMIN = VMAX
C
NTRANS(1) = 0
C
AT THIS POINT ALGORITHM FOR CALCULATING CHAIN CONFORMATIONS BEGINS.
C
COPY COORDS OF UI+1 TO UN INTO COORDS LEVEL 2
I = 1
   NRGD = RIGID(I+1, 1)
DO 13 J = NRGD, NOCOORD
   CX(J, I+1) = CX(J, I)
   CY(J, I+1) = CY(J, I)
   CZ(J, I+1) = CZ(J, I)
13  CONTINUE
14  PHI(I)=STANG
15  II=1
C  CHECK IF THERE IS A GAUCHE-/GAUCHE+ CONFORMATION
C  FIRST SEE IF BOND I IS THE FIRST ONE
C  IF(I-1)300,301,300
C  FOR A FORBIDDEN GAUCHE-/GAUCHE+ SEQUENCE, THE SUM OF
C  PHI(I-1) AND PHI(I) IS 360
300  IF(PHI(I-1)+PHI(I).EQ.360) GO TO 47
301      LINK=1
      IB=BA(I)
      IE=EA(I)
      DX=CX(IE,I+1)-CXX(IB)
      DY=CY(IE,I+1)-CYY(IB)
      DZ=CZ(IE,I+1)-CZZ(IB)
      DIST=SQRT((DX*DX)+(DY*DY)+(DZ*DZ))
      A=DX/DIST
      B=DY/DIST
      C=DZ/DIST
      IST=RIGID(I+1,1)
      IEND=RIGID(I+1,2)
      XZERO=CXX(IB)
      YZERO=CYY(IB)
      ZZERO=CZZ(IB)
      GO TO 16
C  TESTS
17  CONTINUE
C  DELETE CONFORMATIONS WITH SEGMENTS HAVING
C  NEGATIVE Z COORDINATES, I.E. WHICH PROTRUDE
C  INTO THE AQUEOUS REGION.
      IF(CZZ(IST).LT.0.0) GO TO 47
C  CHECK FOR BUMPS WITH CHOLESTEROL
C  IF(ICHOL.EQ.0) GO TO 302
      IF(ABS(CXX(IST)).GT.XCHOL) GO TO 302
      XN = CXX(IST)
      YN = (CYY(IST)+YOC)*COSO + (CZZ(IST)+ZOC)*SINO
      ZN =-(CYY(IST)+YOC)*SINO + (CZZ(IST)+ZOC)*COSO
      IF(ZN.LT.ZZMIN.OR.ZN.GT.ZZMAX) GO TO 302
      IF(XN*XN+YN*YN.GT.XCHOL*XCHOL) GO TO 302
      GO TO 47
302  INUM = I + 1
      NONE=RIGID(INUM,1)
      NTWO=NONE+1
NTHREE=NONE+2

CALCULATE COSINE OF ANGLE BETWEEN NORMAL TO BOTH C-H BONDS AND Z-AXIS FOR THIS CONFORMATION (COB)

\[
\begin{align*}
A1 &= \text{CXX}(\text{NTWO}) - \text{CXX}(\text{NONE}) \\
B1 &= \text{CXX}(\text{NTHREE}) - \text{CXX}(\text{NONE}) \\
A2 &= \text{CYY}(\text{NTWO}) - \text{CYY}(\text{NONE}) \\
B2 &= \text{CYY}(\text{NTHREE}) - \text{CYY}(\text{NONE}) \\
\text{COB(INUM)} &= ((A1*B2 - A2*B1)/\text{CONST})
\end{align*}
\]

CALCULATE CONTRIBUTIONS TO ORDER PARAMETERS C1, C2 = DIRECTION COSINES OF C-H BONDS OF SEGMENT

\[
\begin{align*}
C1 &= \frac{(\text{CZZ}(\text{NTWO}) - \text{CZZ}(\text{NONE}))}{1.073} \\
C2 &= \frac{(\text{CZZ}(\text{NTHREE}) - \text{CZZ}(\text{NONE}))}{1.073} \\
\text{CONT(INUM)} &= \frac{(3.0*\text{C1}^2 \cdot 1.0) + (3.0*\text{C2}^2 \cdot 1.0)}{4.0}
\end{align*}
\]

CALCULATE VALUE OF \(3\cos^2(\beta) - 1\) REQUIRED FOR CALCULATION OF DISPERSION ENERGY

\[
\text{CODISP(INUM)} = \text{CODISP(I)} + (3.0*\text{COB(INUM)}*\text{COB(INUM)} - 1.0)
\]

CALCULATE MAXIMUM AND MINIMUM Z COORDINATES OF THE C ATOMS OF THE CHAIN FOR THIS CONFORMATION

IF(CZZ(NONE).GT.VMAX) VMAX=CZZ(NONE)
IF(CZZ(NONE).LT.VMIN) VMIN=CZZ(NONE)

CALCULATE NUMBER OF TRANS SEGMENTS

IF(\(\phi(I).EQ.0.\) OR \(\phi(I).EQ.360\)) \(\text{INTRANS(INUM)} = \text{NTRANS(I)} + 1\)

IF(\(\phi(I).EQ.120.\) OR \(\phi(I).EQ.240\)) \(\text{INTRANS(INUM)} = \text{NTRANS(I)}\)

TEST IF COMPLETE CONFORMATION GENERATED
IF(I-NOBOND)303,34,34

APPLY \(\phi(I)\) TO ALL UNITS AFFECTED AND ADVANCE I BY ONE

TEST IF ANY MORE ROTATIONS NEED TO BE APPLIED BEFORE TRANSFERING C
303 IF(HMU(I)-1)304,35,304
304 IST=RIGID(I+2,1)
ICOL=I+HMU(I)
IEND=RIGID(ICOL,2)
II=I+2
LINK=2
GO TO 36
C TEST IF ANY MORE TO BE COPIED
37 IF(ICOL-NORIGD)305,38,38
305 ISTC=RIGID(ICOL+1,1)
GO TO 39
C
C COPY REST OF COORDS
35 ISTC=RIGID(I+2,1)
39 I2=I+2
I1=I+1
NNRGD = RIGID(NORIGD,2)
DO 40 NK=ISTC,NNRGD
  CX(NK,I2)=CX(NK,I1)
  CY(NK,I2)=CY(NK,I1)
  CZ(NK,I2)=CZ(NK,I1)
40 CONTINUE
38 I=I+1
GO TO 14
34 CONTINUE
C
C CHECK IF TERMINAL METHYL CARBON HAS NEGATIVE Z COORDINATE:
C
NTERM=RIGID(NORIGD,1) + 3
IF(CZZ(NTERM).LT.0.0) GO TO 47
C
C IF PASS THIS POINT THEN ALL TESTS SATISFACTORY
C FOR A CERTAIN CONFORMATION
C INCREMENT COUNTER OF NUMBER OF CONFORMATIONS (ITOTAL)
C
ITOTAL=ITOTAL + 1
C
WRITE COORDINATES OF ALLOWED CONFORMATION IF IWRITE.NE.0
IF(IWRITE.EQ.0) GO TO 901
WRITE(6,807)
807 FORMAT(/,' ATOM CONF. COORDINATES',/)
DO 900 KK=1,NOCOR
  WRITE(6,808) KK,ITOTAL,CXX(KK),CYY(KK),CZZ(KK)
808 FORMAT(2(I3,3X),3(F11.4,3X))
 WRITE(6,806)(PHI(I),I=1,NOBOND)
806 FORMAT(6(3X,I3))
901 KKP= NONE + 3
 IF(CZZ(KKP).GT.VMAX) VMAX=CZZ(KKP)
  IF(CZZ(KKP).LT.VMIN) VMIN=CZZ(KKP)
C
C CALCULATE LENGTH OF PROJECTION ON Z AXIS
C OF C-C BONDS FOR THIS CONFORMATION.
CLENG = VMAX - VMIN
C CHECK IF CLENG IS ZERO, AND IF SO, PRINT OUT AND
C ADOPT VALUE OF 0.1 FOR CLENG IN ORDER TO ELIMINATE
C DIVISION BY ZERO IN CALCULATION OF ESTRIC
IF(CLENG) 47,819,818
819 WRITE(6,817)
817 FORMAT(/,' CONFORMATION WITH CLENG=0 ENCOUNTERED!'/
1,' FOR THIS CONFORMATION, CLENG = 0.1 WILL BE TAKEN',/)
CLENG = 0.1
C INTERNAL BOND ENERGY (MARCELJA, J. CHEM. PHYS. 60, 3599 (1974)
C ASSUME ENERGY FOR GAUCHE BOND = 500 CAL/MOL.
C
818 EINT=EZERO + 500.0*FLOAT(NOBOND-NTRANS(INUM))
C CALCULATE DISPERSION ENERGY OF CHAIN FOR THIS CONFORMATION
C    FRAC = FRACTION OF BONDS IN TRANS CONFORMATION
C
    FRAC = FLOAT(NTRANS(INUM))/(FLOAT(NOBOND))
    EDISP = -FIELD*FRAC*0.5*CODISP(INUM)

C CALCULATE ENERGY FOR STERIC REPULSION
C
C THE FACTOR 1.4395 CONVERTS FROM DYN*ANGSTROM2/CM TO CAL/MOL
ESTRIC = GAMMA * 20.4 *CLENG0/CLENG *1.4395
C
C CALCULATE TOTAL ENERGY FOR THIS CONFORMATION
C
ETOTAL = EINT + EDISP + ESTRIC
C
C CALCULATE STATISTICAL WEIGHT OF THIS CONFORMATION
WEIGHT = EXP(-ETOTAL/RT)
C
C CALCULATE PARTITION FUNCTION
C
PARFC = PARFC + WEIGHT
C
C CALCULATE CONTRIBUTION TO NEW MOLECULAR FIELD
C
COFILD = COFILD + FRAC*CODISP(INUM)*0.5*WEIGHT
CALL ORDER(WEIGHT)
C
C CALCULATE CONTRIBUTION TO AVERAGE LENGTH OF
C C-C BONDS ALONG Z AXIS
AVLENG = AVLENG + WEIGHT*CLENG
C
C CALCULATE CONTRIBUTION TO AVERAGE COORDINATES OF C ATOMS
DO 51 JP=1,NORIGD
   JJ=RIGID(JP,1)
   AVZ(JP) = WEIGHT*CZZ(JJ) + AVZ(JP)
51    CONTINUE
C
C CALCULATE CONTRIBUTION TO AVERAGE ENERGY
C AVENCO = AVENCO + WEIGHT*ETOTAL
C
C BEFORE CALCULATING NEXT CONFORMATION, REINITIALIZE
C REQUIRED VALUES
C
47   VMIN = CZZ(1)
     VMAX = VMIN
C TESTS IF LAST VALUE OF PHI(I) USED
IF(PHI(I)-FINANG)305,48,48
306  PHI(I)=PHI(I)+INC
     GO TO 15
48   I=I-1
     IF(I)49,49,47
49   WRITE(6,237)ITOTAL
237  FORMAT ('TOTAL NUMBER OF SOLUTIONS ',I7)
     WRITE(6,991)TEMP,PARFC
991  FORMAT('CALCULATED PARTITION FUNCTION AT ',
     1F6.1,' DEG. KELVIN = ',E11.4)
     WRITE(6,665)
665  FORMAT('CALCULATED SEGMENTAL ORDER PARAMETERS:
     1, CARBON ATOM',4X,'SCD',/)
C
C CALCULATE FINAL ORDER PARAMETERS AND PRINT THEM OUT
C
DO 987 I=1,NORIGD
   NONE = RIGID(I,1)
   SEGM = SCD(I)/PARFC
987  WRITE(6,986) NONE,SEGM
986  FORMAT(3X,I2,5X,F11.4)
C
C CALCULATE MOLECULAR FIELD
FIELD2 = VN*COFILD/PARFC
     WRITE(6,908)FIELD2
908  FORMAT('CALCULATED MOLECULAR FIELD = ',
     1E11.4,' CAL/MOL')
C
C CALCULATE AVERAGE ENERGY AND PRINT OUT
C
AVEN = AVENCO/PARFC
     WRITE(6,60) AVEN
60   FORMAT('AVERAGE ENERGY = ',E11.4,' CAL/MOL')
     WRITE (6,53)
53 FORMAT(/,' AVERAGE Z COORDINATES OF C ATOMS:',/)

C CALCULATE AVERAGE COORDINATES OF C ATOMS
C
DO 52 JP=1,NORIGD
   AVZ(JP) = AVZ(JP)/PARFC
   WRITE(6,209) AVZ(JP)
52 CONTINUE
C
C CALCULATE AVERAGE CHAIN LENGTH
   AVLENG = AVLENG/PARFC
   WRITE(6,54) AVLENG
54 FORMAT(/,' AVERAGE SUM OF PROJECTIONS OF',
     1 ' C-C BONDS IN Z DIRECTION='
     2,F10.3,' ANGSTROMS',/)
C
C RETURN
C
C SUBSECTION FOR T MATRIX
16 CONTINUE
C ASSUME ANGLE AND DIRECTION COSINES PASSED
C WITH IST,IEND,X,Y,ZZERO
   ICOL=PHI(I)+1
   SINR=SINE(ICOL)
   COSR=COSINE(ICOL)
   X=1.-COSR
   T1=(A*A)+((1.-(A*A))*COSR)
   T2=(A*B*X)-(C*SINR)
   T3=(A*C*X)+(B*SINR)
   T4=(A*B*X)+(C*SINR)
   T5=(B*B)+((1.-(B*B))*COSR)
   T6=(B*C*X)-(A*SINR)
   T7=(A*C*X)-(B*SINR)
   T8=(B*C*X)+(A*SINR)
   T9=(C*C)+((1.-(C*C))*COSR)
36   I1=I+1
   DO 50 K=IST,IEND
      X=CX(K,I1)-XZERO
      Y=CY(K,I1)-YZERO
      Z=CZ(K,I1)-ZZERO
      ZZ=(X*T7)+(Y*T8)+(Z*T9)
      CZ(K,I1)=ZZ+ZZERO
50 CONTINUE

   XX=(X*T1)+(Y*T2)+(Z*T3)
   YY=(X*T4)+(Y*T5)+(Z*T6)
   CX(K,I1)=XX+XZERO
   CY(K,I1)=YY+YZERO
50 CONTINUE
SUBROUTINE ORDER(WEIGHT)

C
C THIS SUBROUTINE CALCULATES THE ORDER PARAMETERS OF
C THE CHAIN SEGMENTS
C
COMMON/ONE/ETOTAL,RT,RIGID(25,2),CX(100,26)
1,CY(100,26),CZ(100,26),SCD(25),NORIGD
COMMON/TWO/IWRITE,BARFC,FIELD,ICHOL,ALPHA
1,OMEGA,CONT(25)
INTEGER RIGID
C CALCULATE CONTRIBUTIONS TO ORDER PARAMETERS FOR
C THIS CONFORMATION.
C
DO 989 KKK=1,NORIGD
SCD(KKK) = SCD(KKK) + CONT(KKK)*WEIGHT
989 CONTINUE
RETURN
END

BLOCK DATA
COMMON/ONE/ETOTAL,RT,RIGID(25,2),CX(100,26)
1,CY(100,26),CZ(100,26),SCD(25),NORIGD
COMMON/TWO/IWRITE,BARFC,FIELD,ICHOL,ALPHA
1,OMEGA,CONT(25)
INTEGER RIGID
DATA SCD/25*0.0/
END
REFERENCES


Insull, W. (1972) in "Atherosclerosis and Coronary Heart Disease" (Likoff, W., Segal, B.L., Insull, W., Jr., eds.) Grune & Stratton, New York, pp. 20-27.


