LIPID-PROTEIN INTERACTIONS

A. SEMLIKI FOREST VIRUS
B. CTP:PHOSPHOCHOLINE CYTIDYLTRANSFERASE FROM RAT LIVER

by

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ABSTRACT

A. Semliki Forest virus: Lipid Headgroup-Protein Interactions

Semliki Forest virus is an alphavirus enveloped by a lipid bilayer that contains approximately 200 copies each of three glycoproteins: $E_1$, molecular weight 49,000, $E_2$, molecular weight of 52,000, and $E_3$, molecular weight of 10,000. Proton magnetic resonance measurements were done on intact Semliki Forest virus and virus that had been digested with thermolysin. The magnetic resonance line derived from a portion of the N-methyl groups of the choline containing phospholipids narrowed considerably after proteolytic digestion. This reduction in the N-methyl resonance is not inconsistent with increased motion of the headgroups of the phospholipids as a result of thermolysin removal of the viral glycoprotein spikes. To further investigate this phenomenon, experiments were planned using deuterium NMR. Tri-trideuteromethylcholine was chemically synthesized and added to the medium of BHK-21 cells with the hope of labelling a large majority of the choline containing lipids. However, no incorporation of this labelled species could be observed. Two other deuterated cholines, mono-trideuteromethylcholine and di-trideuteromethylcholine, were synthesized and easily incorporated into the BHK-21 cell choline containing lipids. Due to the low yields of Semliki Forest virus, deuterium NMR could not be performed, however, the lack of incorporation of tri-trideuteromethylcholine was of interest. From labelling studies it was concluded that this deuterated choline was not transported across the cell plasma membrane.
B. CTP:phosphocholine Cytidylyltransferase: Lipid-Protein Interactions

Early studies on the rat liver CTP:phosphocholine cytidylyltransferase (E.C. 2.7.7.15) (CT) reported that the enzyme isolated from the cytosolic fraction of 0.9% NaCl solution homogenates increased in activity 4- to 5-fold upon aging several days at 0°C or incubation at 37°C for 3 hours. It was subsequently shown that the activating agent was lysophosphatidylethanolamine (LPE). We have demonstrated that the purified rat liver CT is dependent upon lipid for activity and is activated by oleoyl-LPE and inhibited by oleoyl-lysophosphatidylcholine (LPC). The plot of CT activity at various concentrations of LPE yields a hyperbolic curve with a $K_a$ of 0.3 mM. The activation of CT by LPE results in a decrease of the $K_m$ for CTP from 2 mM at 0.1 mM LPE, to 0.5 mM at 0.4 mM LPE. LPE had no effect on the $K_m$ for phosphocholine. Hence, the activation of CT by LPE is due to an influence on the $K_m$ for CTP. When CT is assayed in the presence of LPE, LPC inhibits the activity with a concentration for half-maximal inhibition of 0.16 mM. Inhibition by LPC was not competitive with phosphocholine.
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LIST OF ABBREVIATIONS

Å  Angstrom unit - $10^{-8}$ cm

ABS absorbance

ACS aqueous counting scintillant

ATP adenosine triphosphate

BHK-21 Baby Hamster Kidney-21 cells

Buffer A $20 \text{ mM Tris-HCl}, 100 \text{ mM NaCl}, (\text{pH } 7.0)$

Ci Curie

CK choline kinase

CDP-choline cytidine diphosphocholine

CHP cytidine monophosphate

CHO carbohydrate

cpm counts per minute

CPT choline phosphotransferase

CT CTP:phosphocholine cytidylyltransferase

CTP cytidine triphosphate

dpm disintegrations per minute

EDTA ethylenediaminetetraacetic acid

FAD flavin adenine dinucleotide

FCS fetal calf serum

Fig. figure

g gram
g  gravity
h  hour
Hz  hertz
K_a  association constant of an enzyme-activator complex
K_m  Michaelis-Menten constant
l  liter
LPC  lysophosphatidylcholine
LPE  lysophosphatidylethanolamine
m  meter
M  molar
min  minute
N  normal
nmol  nanomole
NMR  nuclear magnetic resonance
NAD  nicotinamide adenine dinucleotide
PBS  phosphate buffered saline, pH 7.4 (0.137M NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, and 4.29 mM Na_2HPO_4)
PC  phosphatidylcholine
PE  phosphatidylethanolamine
P.F.U.  plaque forming unit
PG  phosphatidylglycerol
PI  phosphatidylinositol
POPOP  1,4 Bis (2-(5-phenyloxazolyl)) Benzene
PPO  2,5-diphenyloxazole
PS  phosphatidylserine
P_i  inorganic phosphate
ppm  parts per million
ref. reference

$R_f$ ratio of distance moved by a solute to that moved by the solvent front

RNA ribonucleic acid

SDS sodium dodecylsulphate

SFV Semliki Forest virus

t time

TEMED N,N,N',N'-tetramethylethlenediamine

TLC thin layer chromatography

Tris tris (hydroxymethyl) aminoethane

TNE 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4, buffer

$T_1$ spin-lattice relaxation time

TPP thiamine pyrophosphate

UV ultra violet

V volume

$V_0$ void volume

$V_{\text{max}}$ maximal velocity (of an enzyme reaction)

NOTES

Standard prefixes are: c (cent.) - $10^{-2}$; m (milli) - $10^{-3}$; μ (micro) - $10^{-6}$; n (nano) - $10^{-9}$
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DEDICATION

To My Parents

&

The Mountains of British Columbia
INTRODUCTION

A. The Biological Membrane

During the last few years membrane biology has become one of the most exciting and rapidly advancing fields of biochemistry. Many books (1-7), reviews (8-11), and papers have been published in this area dealing with such topics as membrane proteins (12-17), membrane lipids (18-27), bacterial membranes (28,29), membrane transport (30), membrane and cell motility (31), etc.

For some time now, the membranes of eukaryotic cells have been known to be functionally significant. The membranes of the various cellular organelles act as barriers, separating the vital functions of the cell. Compartmentation of these individual cellular functions is necessary for the growth of the individual cell and the organism as a whole. However these barriers are not totally impermeable. This would defeat the purpose of the complex compartmentation required for the cell to function. A number of proteins, in particular receptor and transport proteins have evolved over the ages to aid the various organelles and indeed whole cells to communicate and nourish each other. As well as having proteins involved in transport and communication there are other vital operations required
for cell function, for example, the electron-transport chain of the mito-
chondria and chloroplasts. The proteins of the endoplasmic reticulum are
responsible for a multitude of activities including the synthesis of
phospholipids, as structural components of the cell as well as steroid
biosynthesis, protein synthesis, etc. Membranes play a vital role in the
existence of the living cell, be it from a complex multicellular organism
to the simple yet complex unicellular organism.

For the last 50 years the evidence has suggested that the membrane
consists of lipid which is orientated in a bimolecular leaf structure with
the hydrophobic tails of the phospholipids forming a nonpolar region and
the hydrophilic head groups open to the aqueous environment (32). In the
early 1930's this idea was updated to include a protein layer on the
surface of the bilayer. Since that time the basic model of the biological
membrane has not changed, but the details of the interactions between the
lipids and proteins have. The structure of the biological membrane is in
a constant state of flux. Under physiological conditions most membrane
lipids exhibit rather free lateral diffusion (33-42) with their hydro-
carbon chains in a disordered state (14,36,43). A number of membrane pro-
zeins also diffuse rather freely in the lateral plane of the membrane
(16,17,44,45), a finding which led to the Singer-Nicholson "fluid mosaic
model" of the membrane in 1972 (8-11). A considerable amount of supporting
evidence has come from work on the redistribution of membrane receptors on
lymphocyte surfaces (45-52), and other cells (53-55), the influence of
lipid fluidity and phase changes on membrane transport (56-61), the effect
of lipid on enzyme activities (62-64), and work on the insertion of
integral proteins into membranes (65,66).
Although membrane fluidity and lateral diffusion of membrane components appear to be the general rule, there is clear evidence that this motion is greatly restricted in certain membranes or at least in certain regions of membranes under particular conditions. In the membranes of synapses (67) and gap junctions (68,69) there are regions of specialized and ordered structure. In the prokaryotic cell membrane of *Halobacterium*, similar large regions of ordered structure, known as plaques, are observed in an otherwise random membrane matrix (70). Recent evidence suggests that these regions consist of a single protein or a few protein species which form a regular two-dimensional array by virtue of specific protein-protein interactions.

A much clearer picture of the biological membrane is beginning to emerge, a very tridimensional structure being intricately involved with the existence of the cell. Work in this area is by no means complete. Many areas still remain a mystery, including the exact interactions of lipids and proteins and indeed interactions within the various lipid classes themselves. Questions such as, why are there so many different phospholipid headgroups, some being neutral while others are basic or acidic?, what is the role of cholesterol in the biological membrane? remain unanswered, as do many others.

B. Probes of Membrane Structure

In the previous section a membrane model is described which is compatible with current knowledge. To this point however, the picture has been more qualitative than quantitative. This model has not been assembled
from a few simple experiments or only a few techniques. Rather this picture has been synthesized from a myriad of experiments and from a great number of techniques, with more sophisticated methods being developed. For many of the techniques employed today the intact biological membrane still appears to be too complex to extract information, hence model membranes have been employed which range from simple lipid bilayers, consisting of pure individual lipids or complex mixtures isolated from biological membranes, to the reconstituted systems involving the lipid bilayer with proteins being reinserted into the bilayer. The development of such model membrane systems, which are structurally related to the intact membrane, has greatly aided the search for information on the interaction between lipids themselves as well as lipids and proteins.

What types of physical techniques have been useful in the elucidation of membrane organization? The most successful methods include the scattering techniques (i.e. X-ray, neutron, and raman scattering), fluorescence measurements, magnetic resonance techniques, calorimetry, and freeze-fracture electron microscopy. As was mentioned before, it has not been a single technique or experiment that has yielded a working model of the biological membrane but rather a number of experiments and techniques, each having its advantages and limitations. Each provides an incomplete picture, a part of the jigsaw puzzle, however when assembled together they provide a detailed view of the biological membrane.

Before we deal with the various techniques involved, it is of interest to describe the different types of model systems that have been used. Basically three types of model membrane preparations, with the lipid involved in a bilayer configuration, have been employed.
(1) multilamellar dispersions, in which the lipid is resuspended in an aqueous medium by shaking rapidly. The structures formed are referred to as onions skins and consist of closed concentric lipid bilayers separated by an aqueous region (71),

(2) vesicles, which are prepared by prolonged sonication of the multilamellar dispersions. These consist of spherical single walled lipid bilayers (72), and

(3) orientated multilayers, in which the bilayers are flat and parallel to one another rather than curved. These are prepared in a number of ways as described in the following papers (73,74).

These preparations can be made with a variety of lipid mixtures. As well as preparing these systems with pure lipids or lipid mixtures it is also possible to incorporate other biologically important molecules, including proteins or other lipids which have been labelled in some way (75).

a) **Scattering Methods**

(i) **X-ray Diffraction**

X-ray diffraction has been a technique long involved in structural determination of small molecules and lately in the determination of the three dimensional structure of rather large molecules such as proteins. The high resolution structural determinations of these molecules is due to the ability to form a uniform crystalline array of the molecule under investigation. A membrane on the other hand is incapable of being crystallized or formed into a strictly uniform repeating unit. However structural information can still be obtained although it will not be nearly as detailed.
The technique has found success in determining such structural features as bilayer thickness, distances between the headgroup regions of the membrane, as well as hydrocarbon chain packing. More detailed information about the technique itself and information derived by it may be found in the following reviews and papers (76-78).

(ii) Neutron Diffraction

Another scattering technique which has been rather silent until recently is neutron diffraction. The basis of this technique is very similar to that of X-ray scattering, however instead of electrons scattering an incident beam of X-rays, nuclei scatter a monochromatic beam of neutrons. An elegant experiment of Zaccai et al. (79) involved the study of dipalmitoyl-phosphatidylcholine bilayers dispersed in relatively small quantities of normal and then deuterated water. Analysis of the two sets of data yielded a profile of the neutron scattering density as a function of position across the bilayer. From the profile the polarheadgroup layers are evident as well as the position of the hydrocarbon chains. The least density was found at the junction of the two leaves of the bilayer. Subtracting the two sets of data leaves a profile of the location of the water within the bilayer.

Other elegant experiments involve the use of selectively deuterated molecules. In one study (80,81), selectively deuterated cholesterol (hydrocarbon chain deuterated) was incorporated into dipalmitoyl-phosphatidylcholine bilayers. By using protonated cholesterol in a similar experiment and comparing the two sets of data, it was possible to determine
the position of the tail of the hydrocarbon chain in the bilayer. Schoenborn (80) has recently reviewed the use of neutron scattering in investigating membrane structure.

(iii) Raman Scattering

Raman scattering has not been one of the more important techniques involved with the determination of membrane structure. This is mainly due to the many different physical effects within the membrane that can alter the measurable features of the technique. The assignment of the various absorption lines is normally straightforward since they are usually very similar in all molecules. The frequency of particular types of vibrations such as C-C and C-H stretching vibrations do not vary significantly from molecule to molecule. However the intensities and shapes of the bands are influenced by a myriad of factors including, molecular conformation, molecule size and shape, intermolecular and intramolecular interactions. The Raman spectrum contains a vast amount of knowledge as to the molecular organization of the membrane but a major obstacle to be overcome is to determine which are the important structural properties on a molecular level which influence the line intensities and shapes. As a consequence of such difficulties, the conclusions drawn from this technique are usually regarded as supporting evidence. The following papers deal with more detailed aspects of Raman spectroscopy and membrane structure (82-86).
(iv) **Fluorescence Probes**

The use of fluorescence scattering experiments in membrane research has markedly increased in the past few years. A common property of all fluorescence probes is the existence of one or more aromatic hydrocarbon rings and/or conjugated double bonds. Some of the probes have no resemblance to biologically relevant molecules, such as 1-anilinonaphthalene-8-sulphonate, but others, such as dansyl-phosphatidylethanolamine and parinaroyl-phosphatidylcholine have fluorescent functional groups attached to biologically important molecules (87,88). The latter probe is of interest since it almost completely resembles a normal phospholipid with the exception that it has a non-biological fatty acid - parinaric acid (9,11,13,15-octadecatetraenoic acid).

As with most membrane techniques, fluorescence probe experiments have their advantages and disadvantages. A serious drawback of this technique is the determination of the location of the probe in the membrane. Many of these probes are simply mixed with the sample and with most of these probes being hydrophobic in nature, it is assumed that they enter the hydrophobic environment of the membrane. Since the concentration of the probe in the membrane must be kept to a minimum so as not to disturb the actual membrane structure, it is difficult to be completely sure that the environment the probe is experiencing is typical of the whole membrane. Finally, the measured properties of the probe are usually affected by a variety of physical interactions as in the Raman experiment. Therefore the data obtained must be treated carefully. The following publications will give more detailed information on this technique (89-91).
Magnetic Resonance Techniques

Some of the most useful techniques in membrane research are the magnetic resonance methods including nuclear magnetic resonance and electron spin resonance.

Nuclear Magnetic Resonance

Most of the methods previously mentioned have mainly given a time-independent view of the biological membrane. The magnetic resonance techniques however, answer questions concerned with the dynamic properties of the membrane. For some time people have thought that the membrane was not a stable spatially orientated entity. In the 1930's lipids were being described as possessing properties between solid and liquid, thus the term "liquid-crystalline" state. In the years of 1933-39 Rinne, Zermal, and Schmitt were discussing the 'fluid' properties of the biological membrane. At that point all observations were qualitative. Today we are still talking about the liquid-crystalline state of membrane lipids but it is now time to transform these qualitative formulations into quantitative ones. The magnetic resonance methods are ideally suited for such a task.

Due to the complexity of the intact biological membrane, the majority of useful information using the magnetic resonance techniques has been obtained from the study of model systems. The amount of research done on these systems is overwhelming and it would be near to impossible to cover this area in great detail. However there have been significant contributions made to membrane research using these techniques that should be mentioned.
The NMR technique can monitor the lipid component of the membrane, even when protein is present in the bilayer. By studying what happens to the lipid upon the addition of membrane-associated polypeptides it is possible to deduce the types of interactions that take place between the lipid and protein.

(1.1) Headgroup Studies

Recently three groups have been investigating the headgroup conformation of phospholipids in membranes. Using $^{31}$P and $^2$H NMR, Seelig and Gally (92) studied bilayers of dipalmitoylphosphatidylethanolamine, above and below the phase transition. They selectively deuterated the ethanolamine carbon hydrogens for the deuterium NMR study. The data suggested a model in which the headgroup rotates flat on the surface of the bilayer and makes rapid transitions between two conformations. Studies on dipalmitoylphosphatidylcholine headgroup orientation by Seelig, Gally, and Wohlgemuth (93) suggest a model in which the choline headgroup is aligned parallel to the bilayer plane. Kohler and Klein (94) have measured $^{31}$P NMR spectra of dipalmitoylphosphatidylethanolamine, dipalmitoylphosphatidylcholine, egg phosphatidylcholine, and brain phosphatidylcholine. Cullis et al. studying phosphatidylcholine liposomes, using $^{31}$P NMR, discuss factors affecting the motion of the polar headgroup (237). The spectra of unsaturated and saturated phosphatidylcholines in the liquid-crystalline state are very similar indicating that the motion of the polar headgroup is not sensitive to fatty acid composition. They also noted that there was a reduction in the motion of the phosphate when the phospholipids
were taken below their hydrocarbon phase transition temperature. The addition of equimolar concentrations of cholesterol eliminated this effect. A recent review by Seelig (213) deals with the use of $^{31}$P NMR and head-group structure of phospholipids in membranes.

(1.2) Order Parameter Studies

Deuterium NMR has several advantages over the other nuclei used in membrane research.

Since the deuterium resonance is sensitive to restricted motion, this attribute can be used to yield information on the local order experienced by the nucleus. The parameter that can be easily measured is known as the order parameter. Seelig and Seelig using deuterated DPL (95) and deuterated 1-palmitoyl-2-oleoyl PC (96) studied the order parameter as a function of position along the chain. In a similar experiment Stockton et al. studied egg PC and egg PC-cholesterol bilayers containing deuterated stearic acid (97). This group also biosynthetically incorporated deuterated fatty acids, deuterated at selective positions along the chain, to study the mobility gradient along the hydrocarbon chains in A. laidlawii (134,135).

The results from each of these experiments are qualitatively the same. They noted that the order parameters are nearly constant at the top and middle of the fatty acid chain but then decrease toward the methyl ends. The values of the order parameters are subject to temperature, degree of unsaturation, and mole fraction of cholesterol. Even though the measurement of such order parameters are relatively easy to obtain, the actual interpretation has not been simple (95, 99). Most of these
analyses involve many assumptions and oversimplifications, making it
difficult sometimes to believe the conclusions. Peterson and Chan (98)
have dealt with this topic indicating several other possible explanations
to account for the change in order parameters, including the importance
of reorientation of the chains as well as rotational isomerization.

Many membrane studies have been performed using sonicated
vesicles. The use of such systems for membrane research has been ques­tionable for some time. It is not clear that the local orientational
order of the lipid chains is the same in the sonicated systems and in the
multilamellar system. If the order were considerably different, then the
sonicated vesicle system would not be a good model for the
structure of the biological membrane. Several reports by Lichtenburg
(100) and Finer (101) have attempted to answer this question. Recently
Bloom et al. (129) using proton NMR and Stockton et al. (97) have concluded
that the lipid packing in vesicles is not substantially more disordered
than in the multilamellar dispersions.

(1.3) $^{13}$C NMR Studies

The use of $^{13}$C in membrane studies has been hampered by strong
proton-$^{13}$C dipolar broadening. Urbina and Waugh (104) have applied a
double resonance technique to study DPL dispersions. This technique not
only eliminates dipolar coupling, but also yields greater sensitivity.
Opella, Yesinowski, and Waugh (105) applied the technique to the study
of cholesterol in DPL dispersons, using cholesterol specifically enriched
with carbon-13 in two positions.
Several other groups have used shift reagents in conjunction with $^{13}\text{C}$ in model systems, to distinguish signals from the outside and inside leaflets of the bilayer (106,107).

(1.4) **Reconstituted Systems**

When proteins were finally added to the lipid dispersions, many questions began to arise as to the nature of the interactions between the polypeptide and the lipid. Using reconstituted systems it was possible to control the number of components of a system under study. One of the most popular systems was the cytochrome oxidase system initially studied by Jost *et al.* (108) using electron spin resonance, and then by Longmuir *et al.* (109) using both deuterium and $^{19}\text{F}$ NMR. Early reports indicated the presence of several different classes or types of lipid in the sample:

1. **nonexchanging lipid**, which was always present even on the purified cytochrome oxidase complex and could not be removed by a variety of techniques;

2. **boundary lipid** (annular lipid), which is that segment of the lipid population that is in direct contact with the protein surface;

3. **motionally perturbed lipid**, which is associated with the 'solvation' layers of lipid extending several layers away from the boundary lipid. It is this class along with the boundary lipid which gives rise to a class of slowly exchanging, restricted lipid, as observed by deuterium NMR;

4. **finally there is the free lipid**, those regions of lipid many 'solvation' layers away (109,110).

It appears however the Dahlquist *et al.* have been the only group to observe this 'boundary lipid' phenomenon using NMR. Oldfield *et al.* (111) have studied a
number of deuterated lipids and various proteins including gramicidin A, cytochrome oxidase, cytochrome b5, myelin proteolipid apoprotein, and bacteriophage fl coat protein. Studying the above listed proteins, above and below the phase transition using DMPC deuterated in the terminal methyl groups and along with results on the interaction of cytochrome oxidase with DPL deuterated in the terminal methyl groups of chain number 1, no examples of such 'boundary lipid' could be observed above the transition temperature. Instead they conclude that proteins and polypeptides disorder the phospholipid hydrocarbon chains as judged from the deuterium NMR quadropole splittings. Below the transitions temperature they prevent crystallization and as a result cause bilayer disorder (111).

Work recently completed by Devaux et al. (112) studying rhodopsin boundary lipids in spin-labelled rhodopsin-lecithin complexes point to a very interesting criticism of work done on reconstituted systems. It was their experience that if the lipid/protein ratio of the complex being studied was very low (lipid/protein - 10/1) then a two component system is observed. Such a ratio is however very much lower than the physiological ratio of about 80/1. The system they were studying had rhodospin spin labelled with spin label I.

\[
\text{CH}_3\text{CH}_2\text{C}(\text{CH}_2)_{14}\text{COO(CH}_2\text{)}_{22}\text{N}^\text{O}
\]

SPIN LABEL I
With a lipid/protein ratio in the physiological range no trace of two components was observed. It is their opinion that a low lipid-protein ratio would correspond to a smaller distance between rhodopsin molecules which may result in an increase in the boundary effects imposed by the protein. However an alternative explanation may be postulated (112, 113). Immobilization of the probe (spin label I) at low lipid/protein ratios may reflect protein aggregation which is quite possible when the ratio is decreased.

Whatever the basis of this immobilization the problem remains that at physiological temperature no immobilization appears to take place. This fact must be reconciled with the previous experiments on the cytochrome oxidase system (108, 114-16), the Ca$^{++}$ ATPase (117) and rhodopsin systems (118).

(1.5) **Intact Systems**

Although most membrane research using NMR tends to involve model systems there are a few reports of studies done on intact systems. The data obtained from such systems tends to be rather complex, but by using specific labelling techniques and the knowledge obtained from model systems it is now possible to extract information on the intact biological membrane.

*Adholeplasma laidlawii* has been a system of considerable interest for the last few years. DeKruijff et al. (119) using $^{31}$P NMR studied *A. laidlawii* cell membranes and derived liposomes. It was noted that the phosphorous spectrum of the intact membrane is very similar to the spectrum of the liposomes. When the membranes were treated with pronase, a non-specific protease, the spectrum appeared to be insensitive with 40-60%
of the membrane protein being removed. This indicated that either no long-
lived lipid headgroup-protein interactions occur or that the lipid-protein
complexes in the membrane have a fast rotation time ($t_c < 10^{-6}s$) along an
axis perpendicular to the plane of the membrane.

DeKruijff et al. (120) have shown evidence for isotropic motion
of phospholipids in liver microsomal membranes using $^{31}P$ NMR. This isotropic
motion was not due to rapid tumbling of the microsomal vesicles nor to
rapid lateral diffusion of the phospholipids. They discuss the possible
formation of a transitory non-bilayer lipid configuration with the bulk
lipid in rapid exchange. The existence of anything but a bilayer phase
in biological membranes has not been considered until recently. However,
there is a strong possibility that other lipid phases can exist in membranes
(Fig. 1) and play functional roles. Membrane fusion, for example, must
require that some of the lipids adopt, at least on the short term, a non-
bilayer configuration during the intermediate steps. Studies by Cullis
and Hope, using $^{31}P$ NMR, have suggested the involvement of the hexagonal
H$_{11}$ phase in the fusion event (121). The addition of oleic acid or glycerol
mono-oleate at concentrations found to induce fusion of erythrocytes in vitro
are found to produce a transition of a variable portion of the membrane
phospholipids from the classical bilayer configuration to the hexagonal H$_{11}$
phase. They propose a model for oleic acid induced fusion of the erythrocyte
membrane and also suggest this to be the mechanism of fusion events in vivo.
To face page 17.

Figure 1. Illustration of the various phases lipids may assume along with their corresponding $^{31}$P NMR spectra (reproduced by permission of P.R. Cullis).
Cullis and Verkleij have studied a phosphatidylserine/phosphatidyl-ethanolamine mixture with regards to the effects of Ca$^{++}$ and the local anaesthetic dibucaine (122) on membrane phase behaviour. It was reported that Ca$^{++}$ can induce the bilayer to hexagonal $H_{II}$ phase transition but the addition of dibucaine can reverse this effect. This result is discussed in terms of a model for membrane fusion and the mechanics of anaesthesia. An article by Cullis and McLaughlin (123) discusses the recent progress of $^{31}$P NMR as a probe of membrane structure and motion of phospholipids in membrane systems.

Dratz et al. have centred their attention on the use of proton NMR in studying rod outer segment disk membranes (124). The use of proton NMR presents certain problems in interpreting the data due to the closely overlapping reasonances. However some general conclusions about molecular motion can be made. The study of the linewidths suggest that the rhodopsin does not greatly affect relatively low frequency motions of the phospholipids such as lateral diffusion. However the study of the spin-lattice
relaxation rates indicate that the membrane resonances can be decomposed into two components, one corresponding to phospholipids interacting with the rhodopsin and the other corresponding to the bulk phospholipid.

The theory for the interpretation of proton NMR lineshapes has been investigated by several groups. Chan and coworkers have published several articles in this area (98,125,126) however there appears to be conflicting views on the subject as seen in publications by Ulmius et al. (127) and Bloom et al. (128,129).

Two of the most popular nuclei for use in studying intact membrane systems appear to be deuterium and $^{13}$C. Due to the low natural abundance of these nuclei ($^{13}$C = 1.1%; $^2$H = 0.015%) they are useful labels for membrane study since virtually no background signal is obtained in spectra of isotopically enriched membranes.

London and coworkers (130) reported a $^{13}$C Fourier transform (FT) NMR study on the fractionated membranes of Candida utilis. By growing the organism on a medium enriched with 20 atom % $^{13}$C acetate (doubly labelled), they were able to nonspecifically label the yeast. From spin lattice measurements they found evidence to suggest a mobility gradient along the hydrocarbon chain with increased mobility from the glycerol backbone towards the terminal methyl group as well as towards the choline methyls. Metcalfe et al. (131) reported $^{13}$C NMR spectra of Acholeplasma laidlawii membranes containing $^{13}$C labelled phospholipids. The organism was grown on a medium enriched with [1-$^{13}$C]-palmitic acid and by doing so the spectrum of the membrane was reduced to a single well defined resonance. A major disadvantage of this labelling procedure is that it is expensive, especially for placing $^{13}$C labels along the chain. Smith
et al. (132) labelled another organism, *Aureobasidium pullulans*, using 1- and 2-[13C]-acetate as previously described by London et al. (130). The use of 1-[13C]-acetate and 2-[13C]-acetate allows the study of the odd and even carbon atoms, respectively, of the fatty acyl chains. $T_1$ analysis of the data obtained indicated qualitatively at least, an increase in mobility from the glycerol backbone towards the terminal methyl group. Similar work is also underway on two other organisms, *Micrococcus freudenreichii* and *Halobacterium cutirubrum*.

*A. Laidlawii* was the first organism to be studied by deuterium NMR (133). Oldfield et al. employed the use of perdeuterated fatty acid in the growth medium to specifically label the phospholipids. However, due to a lack of resolution of the individual resonances no conclusions could be made. Smith et al. (134,135) have recently completed work on the *A. Laidlawii* system in which they incorporated individual specifically deuterated fatty acids. This work is the result of an incredible amount of time and expense in preparing the individually labelled fatty acids. The profiles of the order parameters and quadropole splittings versus position along the acyl chain are very similar to those obtained with the synthetic systems for egg PC (136,137) and DPL (138). These results are very encouraging because they confirm the relevance of model system studies.

Stoffel et al. (139,140) have used $^{13}$C NMR to study the lipid organization of the enveloped virion, Vesicular Stomatitus virus. Moore et al., using phosphorous-31 NMR, have also been studying this virus (141). These papers will be discussed in a separate section.
(2) Electron Spin Resonance

ESR has been a very popular and useful magnetic resonance technique in membrane study. It has the advantage of being able to use small samples and yet obtain spectra in a very short time.

The most frequently used 'spin labels' are nitroxides which contain a N-O group. This group can be incorporated into a number of molecules which are soluble in both aqueous and non-aqueous phases. An example of this type of probe is 2,2,6,6-tetramethylpiperadone-1-oxyl (TEMPO). This type of probe is believed to partition itself between the aqueous and hydrophobic regions of the membrane. Other types of probes include derivatives of fatty acids with the nitroxide being attached at various positions along the chain (doxyl derivatives). Phospholipids can also be labelled, either along the fatty acid chain, or in the headgroup region. Finally, sterol derivatives have been prepared including 3-doxyl derivatives of cholestane-3-one and androstane-3-one-17-01 (142).

An important difference between ESR and NMR is in the time scales. ESR is sensitive to motions on the order of 10^{-8}s while NMR is sensitive to motions on the orders of 10^{-5}-10^{-6}s. Therefore, ESR is sensitive to motions that take place on a much shorter time scale compared to NMR. Theories for line shape analysis are discussed elsewhere (142).

A common method of analysing a spectrum involves the extraction of the ESR 'order parameter', which is conceptually similar to the proton and deuterium order parameters. The ESR order parameter is a number between 0 and +1 and has a well defined meaning in terms of the average ability of the nitrogen p orbital to rotate during times on the order of 10^{-8}s. If the order parameter is near unity, then in a time of 10^{-8}s the p orbital
containing the unpaired electron does not appreciably change direction. However a value significantly less than 1 is consistent with a wide variety of possible motions.

ESR experiments have measured a number of membrane properties. The partitioning of TEMPO between the aqueous and non-aqueous membrane regions has been used to investigate lipid phase transitions and lateral diffusion (238). TEMPO is found to be more soluble in the membrane interior above the phase transition temperature. Using this property of the probe the effect of membrane proteins may be studied, looking for changes in the phase transition temperature of model membranes induced by the introduction of the protein (117). In this same way it is possible to estimate the fraction of lipids in a biological membrane which are in a fluid state (143). The partitioning of phospholipid spin labels between the fluid and solid phase is one method of studying the calcium-induced lateral phase separations in phospholipid bilayers (144).

By affixing doxyl spin labels at various positions along the hydrocarbon chains of phospholipids it is possible to obtain information about the mobility of the chain as a function of distance from the glycerol backbone. Studies using this system have found that the electron spin resonance order parameter decreases as the spin label is moved away from the glycerol backbone. This variation, referred to as the 'flexibility gradient' or 'fluidity gradient' has been observed in a variety of systems using deuterium and $^{13}$C NMR as previously mentioned (43,145).

Butterfield et al. (146) have used spin labels to compare order parameters derived from erythrocytes obtained from normal subjects and from patients with myotonic muscular dystrophy, Duchenne muscular dystrophy,
and congenital myotonia. Increased membrane fluidity was demonstrated in both myotonic muscular dystrophy and congenital myotonic ethrocyte membranes by means of ESR. The ethrocyte membranes from patients having Duchenne muscular dystrophy exhibited normal membrane fluidity. Although the specific mechanism responsible for these phenomena is not known, the spin label measurements suggest a correlation of increased ethrocyte membrane fluidity with the presence of myotonia.

A number of papers have been published dealing with spin labelling of intact viral membranes (147-152). These will be discussed in a separate section.

(vi) Calorimetry

Differential scanning calorimetry (DSC) is a useful technique for determining phase transition temperatures. In the case of lipids, it detects the latent heat associated with the transition from the crystalline to the liquid-crystalline state. Most of the work using DSC has been on model systems. The transition temperature and enthalpies for various one component systems have been reported (153-156).

The phase transition temperature can be affected by changing the external environment of the lipids such as changing the pH or the concentration of divalent cations such as Ca$^{++}$ or Mg$^{++}$ (90). Binary mixtures of phosphatidylcholines have been studied as well as the effect of cholesterol on the phase transition temperature of these lipids (156,157). This techniques has also been used to study the phase behaviour of the membranes of *A. laidlawii* (158) and *E. coli* (159).
(vii) Freeze Fracture Electron Microscopy

For years electron microscopy has been a very powerful tool in membrane research. Some of the first evidence for the bilayer structure of the membrane was contributed by the negative staining electron microscopy procedure (160). A more popular technique today is freeze fracture electron microscopy which involves 'photographing' the interior surface of the membrane (161).

From electron micrographs of one or two component lipid mixtures, different textures can sometimes be seen, presumably due to different phases in the bilayer. When two or more such textures appear on the same bilayer, it is clear that there is a lateral phase separation of the phospholipids. Freeze fracture electron microscopy studies of biological membranes usually show particles on relatively smooth surfaces which may be homogeneously distributed or concentrated in patches. These particles are interpreted as reflecting intra-membrane proteins dispersed in smooth lipid surfaces.

Van Dijck et al. (162) and Papahadjopoulos et al. (163,164) have used this technique in conjunction with DSC to study the effect of Ca$^{++}$ and Mg$^{++}$ on the structure of bilayers composed of dimyristoyl phosphatidylglycerol. Chapman et al. (165) studied the effect of monovalent ions on the phase transition of phosphatidylcholine bilayers while Verkleij et al. (166) studied the outer membrane of E. coli mutants.

C. Membrane Studies of Viruses

Studies on biological membranes have been hampered by their complex nature. As seen in the previous section, most work has centred on
model and reconstituted membrane systems. Lipid enveloped viruses on the other hand offer an opportunity to study a biological membrane which in most cases is more simplified compared to whole cell membranes. These viruses offer a number of attractive features including:

(i) they can be obtained with a high degree of purity;

(ii) they have a limited number of polypeptides associated with the membrane which simplifies the number of interactions within the bilayer;

(iii) by growing the virus in different host cells it is possible to alter the lipid composition while keeping the protein composition the same, and

(iv) the reverse case is also possible - by growing different viruses, which vary in protein composition in the same host cell, the lipid composition will be the same while the protein content will be different.

A number of viruses including influenza, parainfluenza, SV-5 (147,149,167), Rauscher Murine Leukemia virus (148), Sindbis virus (150), Venezuelan Equine Encephalomyelitis virus (151), and Vesicular Stomatitis virus (152,139-141) have been studied by such techniques as ESR, $^{13}$C NMR, $^{31}$P NMR, and fluorescence scattering. Landsberger et al. (147-149, 152) have been very active studying influenza, Rauscher leukemia, parainfluenza SV5, and Vesicular Stomatitis viruses using ESR probes. Their studies have indicated that all of these viruses have the classic bilayer structure, and that the host cell membrane is considerably more fluid than the viral lipid. Protease digestion of these viruses leads to the removal of the surface glycoprotein "spikes" usually observed by electron microscopy studies (149). Removal of these surface "spikes" from influenza virus (147) does not appear to affect the organization of
the lipid bilayer, however in a study on Vesicular Stomatitis Virus the removal of the "spikes" increases the fluidity of the lipid bilayer (152).

By growing influenza and parainfluenza SV5 virus on two different cell lines, BHK21-F and MBDK cells (149), Landsberger et al. using ESR were able to show that the rigidity of the viral membrane depends largely on the lipid composition and is not affected by the different protein composition of the two viruses.

Sefton and Gaffney (150) studied the fluidity of the lipids in the membrane of Sindbis Virus using ESR. Again the viral membrane was found to be more rigid than the host cell membrane, however their data suggests that the difference in fluidity is not due simply to differences in lipid composition but rather the result of the interaction of the viral proteins with the membrane lipids. They found that the viral lipids were more fluid after chloroform/methanol extraction than in the intact virus and the viral membrane is made more fluid by proteolytic digestion of the viral glycoproteins.

Using $^{13}$C NMR Stoffel et al. (139,140) studied the lipid organization of Vesicular Stomatitis virus. From $^{13}$C $T_1$ relaxation data it was concluded that the motions of the phospholipid choline headgroups of Vesicular Stomatitis virus are more restricted by the digestion of the viral glycoprotein with trypsin. They were also able to study the mobility of the central part of the fatty acid chains by growing the virus on host cells prelabelled with $[^{13}$C]-oleic acid. Using $[3-^{13}$C]- and $[11-^{13}$C]-oleic acid and $[16-^{13}$C]palmitic acid as labels, the data suggested a high rigidity limited to a depth of about 15 Å which is primarily due to the high cholesterol content of the membrane as well as interactions of the membrane associated G- and possibly M-protein with the lipids. The inner core of the bilayer
was found to be more fluid. Tryptic digestion of the glycoprotein "spikes" of the G-protein caused a reduction in the fluidity of the inner core of the viral membrane, possibly due to the further entry of the remaining hydrophobic peptide of the G-protein into the bilayer.

Moore et al. using $^{31}P$ $T_1$ relaxation measurements to study headgroup motion in the same virus, Vesicular Stomatitis virus, concluded that proteolytic digestion of the G-protein leads to an increase in the motion of the phosphate, and therefore presumably an increased mobility of the phospholipid headgroups (141) which is opposed to the conclusion of Stoffel et al. (139,140). The results obtained using $T_1$ relaxation data is sometimes difficult to interpret when studying such complex heterogeneous systems.

D. Lipid-dependent Proteins

In the previous sections the topic of interest has been the bulk interactions of lipids and proteins. Using such techniques as NMR, ESR, DSC, fluorescence, X-ray, and neutron diffraction, it is the average interactions between lipids and proteins that are being studied. For some time now it has been known that certain proteins, be they membrane bound or soluble, are dependent on certain classes of lipids or lipid mixtures for activity. Investigations in this area have proven to be rather difficult due to the low monomer concentrations of lipids in aqueous solution. Most studies report protein-lipid interactions on self-associated lipids (phospholipid liposomes) in contact with protein solutions. A wide range of protein classes have been studied including hydrophilic proteins, serum apolipoproteins, soluble peripheral membrane proteins, insoluble proteins and
small peptides. Of interest are the group of enzymes which have been purified and found to be dependent on lipid for activity.

Malate-vitamin K reductase isolated from *M. phlei* (purified to near homogeneity) is found to be dependent upon added phospholipid for activity (171). The enzyme activity is found to be dependent upon the degree of aggregation of the protein. In high salt solution the protein exists as a monomer and is most active, while in low salt it aggregates and is less active. Protein in lipid complexes have been isolated and it is suggested that a phospholipid binding site(s) appears to be involved with the aggregation-disaggregation process. While studying the activation process by phospholipids Imai et al. found asolectin (major components are PC and PE) to be most effective. Individual lipids alone or cardiolipin exhibited less ability to activate. Since the isolated protein-lipid complexes can be dissociated using high salt, it would seem that electrostatic interactions may be important.

Differential centrifugation of sonicated cell extracts were used to localize the enzyme. The protein activity was predominantly found in the supernatant fraction with some residual activity in the particulate fraction. However, ghost preparations obtained by the treatment of cells with lysozyme in the presence of glycine, contained >85% of the enzymatic activity which can then be released by sonic oscillation (172). This suggests that the enzyme is loosely associated with the cell membrane and could be classified as a "peripheral" protein using the Singer and Nicholson nomenclature (8).

A phospholipid requiring enzyme, NAD-dependent malate dehydrogenase, isolated from *M. sp.* strain Takeo (173), is found to be different
from the enzyme isolated from \textit{M. phlei} although they have similar molecular weights. Enzyme activity is dependent upon the presence of cardiolipin, without which the enzymatic activity is only about 3% of maximum.

(a) \textbf{L-lactate Dehydrogenase}

L-lactate dehydrogenase from \textit{E. coli} is isolated by detergent treatment of bacterial membranes. The enzyme is activated about 3-fold with the addition of phosphatidylglycerol, cardiolipin, or a lipid mixture (174). When activated by phospholipids the enzyme exhibits a similar $K_m$ value for L-lactate to that of the membrane bound enzyme. Several points of information indicated that the enzyme has a different conformation in the detergent free system. The $\alpha$-helical content of the protein is increased 1.7-fold during preincubation with the lipids and the $\alpha$-helix becomes more stable during heat treatment. This suggests that the enzyme is showing monomolecular dispersion in the lipid bilayer. In the detergent free system the protein is an aggregate or oligomeric complex of 10 or more molecules in aqueous solution. In this form the enzyme is only partially sensitive to a specific antibody suggesting that not all of the antigenic sites in the oligomer are accessible. However upon incubation of the aggregate with phosphatidylglycerol or cardiolipin, the enzyme became completely sensitive to the antibody presumably due to all the protein molecules becoming dispersed in the monomolecular form in the lipid bilayer and thus exposing their antigenic sites.

Kinetic evidence also supports a different conformation of the enzyme in the detergent-free system. It was found that both the $K_m$ and $V_{\text{max}}$ increase for L-lactate when the enzyme is incubated with phospholipids.
From preliminary studies it appears that the specificity of phospholipid fatty acid chains is rather broad since cardiolipin from *E. coli* or bovine heart have similar effects even though they differ considerably in fatty acid composition. Phosphatidylethanolamine or phosphatidylcholine along with cholate have a similar effect as phosphatidylglycerol suggesting that phospholipids in suitable liposomes with an acidic component have similar effects regardless of fatty acid composition.

(b) **Pyruvate Oxidase**

This enzyme from *E. coli*, which is a soluble tetrameric flavoprotein, has been purified to homogeneity and crystallized (175-177). It binds both thiamine pyrophosphate and FAD and is activated 15- to 100-fold by phospholipids and long chain fatty acids. Maximal activation requires preincubation of the enzyme for at least six min with the lipid activator in the presence of substrates and cofactors (pyruvate, TPP, and MgCl$_2$). Minimal activation occurs if any of these components are missing or if the enzyme is preincubated with lipid before the substrates are added.

Initially lysophosphatidylethanolamine was found to be the only activating lipid, however water soluble micellar preparations of other phospholipids were found to exhibit higher stimulation of activity. It appears that the nature of the phosphoryl base is not of primary importance in determining the capacity of the phospholipid to activate pyruvate oxidase, since phosphatidylcholine and the hydrophobic moieties of lecithin fully activate the enzyme, while 1-α-glycerophosphate and 1-α-glycerophosphocholine have no effect on enzyme activity. It was also noted that several fatty
acids can activate the enzyme with a specific activity only slightly lower than that given by the phospholipids. Palmitoleic and oleic acids are particularly effective in enzyme activation. This activation by fatty acids could serve as an important control mechanism in *E. coli* metabolism.

Phosphatides dramatically affect the kinetic parameters of pyruvate oxidase. The $K_m$ for pyruvate and TPP are lowered 13- and 3- to 4-fold respectively in the presence of phospholipids. In addition, the phosphatides bestow co-operativity to TPP binding to the enzyme. In the absence of phospholipid, TPP binds with the usual Michaelis-Menten type saturation kinetics, however in the presence of phospholipids, TPP is found to bind co-operatively and shifts the $K_m$ for TPP to a lower value. Using stopped-flow kinetics it is clearly evident that in measuring the rate of FAD reduction, the presence of phospholipid affects the rate-controlling step leading to the formation of the enzyme-FADH$_2$ complex. The rate of reduction of enzyme bound FAD is increased some 100-fold in the presence of lipid.

It would seem that there is a good case for a regulation mechanism involving phospholipids and long chain fatty acids acting as allosteric modulators of pyruvate oxidase activity. By studying the pathways of *E. coli* for conversion of pyruvate to the acetate level, a small build up of free fatty acid could serve to switch on the pyruvate oxidase to produce acetate and carbon dioxide, which would be a low energy pathway for pyruvate utilization instead of having pyruvate dehydrogenase producing acetyl-CoA and carbon dioxide.
(c) **Malate Oxidase**

In mutant strains of *E. coli*, lacking NAD-dependent malate dehydrogenase activity, malate oxidase is present which utilizes L-malate and FAD as substrates (178). The enzyme is localized on the inner face of the cytoplasmic membrane to which it is loosely bound and easily released by sonication or membrane disruption. As with pruvate oxidase, malate oxidase, considered a peripheral enzyme, can be obtained in a soluble state free of lipids and detergent. This makes it an ideal model system for the study of lipid-protein interactions.

Phosphatidylglycerol and cardiolipin are the two major acidic phospholipids produced by *E. coli* cells (179) and both are potent activators of malate oxidase. The enzyme is also activated by nonionic detergents such as TX-100 and some other lipids, such as asolectin, oleoyl-acetate, and palmitoleoylacetate. The allosteric substrate for malate oxidase is FAD and not malate. The addition of phospholipid to the enzyme does not affect the $K_m$ of malate or ferricyanide (artificial substrate) but has a large effect on the $K_m$ for FAD (9 μM in the absence of phospholipid to 0.2 μM in its presence).

(d) **D-β-hydroxybutyrate Dehydrogenase**

D-β-hydroxybutyrate dehydrogenase is tightly bound to the mitochondrial membrane and can be released from the membrane by digestion with phospholipase A or by detergents such as cholic acid (180). As with most of these systems the lipid-free protein exhibits no activity, however upon reconstitution with mitochondrial lipids of lecithins from a variety of
sources, an active lipid-protein complex is formed (181-183). The enzyme specifically requires unsaturated phosphatidylcholines for maximum activation (184). Most other common lipids have been shown to be ineffective in the formation of an active lipid-protein complex and often inhibit the enzyme (184,185).

From studies on the activation of the enzyme by phosphatidylcholines, the minimal requirement for activation was a hydrophobic chain and a phosphorylcholine group such as stearoyl phosphorylcholine. It is not enough to have the two charges and a hydrophobic chain present, as in a mixture of SDS and N-trimethyl-n-dodecylamine. This suggests that there is a spatial requirement as well. These results indicate that the enzyme has a specific activating site(s) which has a specific requirement for a hydrophobic chain linked to phosphorylcholine. However, a membrane-like liposome structure appears necessary for the stabilization of an enzyme-lipid complex. Gazzotti et al. (186,187) studied the interaction of phospholipids and \( \beta \)-hydroxybutyrate dehydrogenase in terms of restoration of enzymatic activity and the ability of the phospholipids to complex with the enzyme. Optimal reactivation takes place when various lecithins are microdispersed with phosphatidylethanolamine. Since water soluble phosphatidylcholines (PC6:0 and PC8:0) were able to reactivate the enzyme it suggests that a bilayer structure is not necessary for activation however, as shown before (180) a bilayer structure is necessary for enzyme stabilization. Studies on the formation of phospholipid-protein complexes indicates that those phospholipids reactivating the enzyme also form complexes and the amount of reactivation is proportional to the phospholipid binding affinity.
This was the first enzyme in which the role of a specific lipid in a particular step of the reaction mechanism was demonstrated (187).

The enzyme when complexed with phosphatidylcholine or a lipid mixture containing phosphatidylcholine binds NADH with a $K_d = 6-16 \mu$M while, with no phosphatidylcholine present, no NADH is bound. The binding of NADH is dependent on the formation of a protein-lipid complex.

(e) **CTP:phosphocholine Cytidylyltransferase**

The synthesis of CDP-choline from CTP and phosphocholine by CTP:phosphocholine cytidylyltransferase (E.C. 2.7.7.15) was first described by Kennedy and Weiss (188). It was also shown that CDP-choline was an intermediate in the biosynthesis of phosphatidylcholine. Schneider (189) reported that the cytidylyltransferase, isolated from the soluble fraction of rat liver 0.9% NaCl solution homogenates, increased in activity about 4-5 fold upon storage at 0°C for several days or incubation at 38°C for 3 h. Subsequently Fiscus and Schneider (190) found that the enzyme could be stimulated by the addition of boiled, previously aged particulate fractions of rat liver. This stimulation was attributed to the phospholipids present in the particulate fractions. It was interesting to find that lipid extracts from fresh rat liver cytosol had little activating potential unless they were oxidized by being exposed to a stream of air for 15 h (190). Lipid analysis indicated a higher proportion of lysolipids in the oxidized sample. This finding raised the possibility of some type of control mechanism via degraded phospholipids.

To this point all of the work had been done using crude homogenates or at best semi-purified enzyme. Choy et al. described the first successful
purification of the cytidylyltransferase (191). Two forms of the enzyme were identified in the cytosol as judged by gel filtration chromatography, a low molecular weight form (L-form) and a high molecular weight aggregate (H-form). Fresh rat liver cytosol contains predominately the L-form of the cytidylyltransferase, which is found to require lipid for activity. Upon aging the cytosol at 4°C for several days the L-form is converted to the H-form, which has no dependence on lipid for enzyme activity. A study of all the lipids in rat liver cytosol revealed that lysophosphatidylethanolamine (LPE) was the most potent activator of the enzyme although phosphatidylserine and phosphatidylinositol could activate but to a lesser extent (212). Some species of lysophosphatidylcholine (LPC) inhibited the cytidylyltransferase by 80%. All of these effects could be demonstrated on the purified enzyme.

The increase in enzyme activity by storing the cytosol at 4°C for several days was correlated to an increase in LPE concentration as well as a decrease in phosphatidylethanolamine concentration. This result implicated LPE as a possible physiological activator of the rat liver cytidylyltransferase. In a subsequent study Choy et al. investigated the aggregation process that accompanies the activation of the enzyme when stored at 4°C (192). An exhaustive analysis of the lipids of rat liver cytosol revealed that diacylglycerol was the active aggregating factor.

The study of the cytidylyltransferase from rat lung has some interesting differences compared to the rat liver enzyme. As with the rat liver enzyme, the adult lung enzyme is located in both the microsomal and cytosolic fractions when the tissue is homogenized in isotonic saline. However, when fetal rats were studied, the enzyme activity was predominately
located in the cytosolic fraction. Further investigation revealed that two forms of the enzyme were present in the adult lung, a situation similar to the rat liver system. The fetal form has a low molecular weight and requires lipid for activity, while the adult form is an aggregate of the fetal form and has no lipid dependence. A significant difference between these two systems however is that phosphatidylglycerol is both the activating and aggregating factor in the rat lung. It was also noted that the conversion of the fetal form of the cytidylyltransferase to the adult form paralleled an increase in phosphatidylglycerol concentration in the lung, indicating the possibility that phosphatidylcholine biosynthesis may be regulated by the concentration of phosphatidylglycerol.

E. Conclusions

The majority of enzymes which are affected by the presence of lipid are associated in some way with membranous organelles such as mitochondria (193), microsomes (194), or bacterial membranes (195). The association of these proteins with membranes might lead to the conclusion that reactivation by the addition of membrane lipid merely reflects the need of the enzyme to be in a hydrophobic environment. This possibility is further substantiated in that most of these proteins respond in a non-specific manner to the added lipid. However the question that remains is, can phospholipids perform a regulatory function as well as forming the supporting structures of the cell. From the studies on lactate dehydrogenase, pyruvate oxidase, malate oxidase, β-hydroxybutyrate dehydrogenase, and the CTP:phosphocholine cytidylyltransferase it would appear they can and do
act as regulators of enzyme activity although the specificity normally associated with enzyme regulation appears to be much broader. The interactions between lipids and proteins are very complex and with further study of more purified and controlled systems and the improvement of existing techniques, more knowledge about these interactions will be discovered.
F. The Structure of Semliki Forest Virus

The structures of all the Group A Togaviruses are almost if not entirely identical (224, 225). These viruses, of which Semliki Forest virus and Sindbis virus are the most studied, consist of an icosahedral nucleocapsid surrounded by a spherical lipid envelope. Three glycoproteins denoted as $E_1$, $E_2$, and $E_3$ are situated in the envelope and lie in close proximity to the virus nucleocapsid (226). The structure of Semliki Forest virus is shown diagrammatically in Fig. 2. The virus genome consists of a single strand of 42S RNA.

![Diagram of Semliki Forest Virus](image)

**SEMLIKI FOREST VIRUS**

Figure 2. The structure of Semliki Forest virus.
The structural proteins of Semliki Forest virus have been resolved with SDS polyacrylamide gel electrophoresis. Originally studies were performed using the gel system of Weber and Osborne (227). $E_1$ and $E_2$ were not resolved in the studies of Hay, Skehel and Burke (228), Kaariainen et al. (229), and Acheson and Tamm (230). Nucleocapsid protein was clearly evident while no trace of $E_3$ was found on the gels and its existence remained unknown. More recently the discontinuous buffer SDS gel electrophoresis systems of Neville (231) and Laemmli (232) were applied to purified virus preparations by Simons (233) and Pfefferkorn (225). These results show the molecular weights of $E_1$, $E_2$ and nucleocapsid to be 52,000, 49,000 and 34,000 respectively.

The existence of $E_3$ in Semliki Forest virus was not evident until very recently (234). As yet, this protein has not been demonstrated to be present in Sindbis virus. Garoff and Simons showed that although $E_3$ cannot be detected on 7.5% and 10% SDS acrylamide gels by classical staining techniques, the small protein could be detected when $[^{35}\text{S}]\text{Met}$ labelled SFV was applied to 10% SDS gels - the gels were then sliced and assayed for radioactivity. More conclusive evidence for the existence of $E_3$ was presented when delipidated membrane protein was eluted from an SDS hydroxylapatite column (234). $E_1$, $E_2$, $E_3$, and nucleocapsid proteins appear in equimolar amounts in the mature virion and constitute 35.7%, 35.7%, 4.9%, and 23.7% of the total protein respectively.

All three SFV membrane proteins are glycosylated. Residues of N-acetylgalcosamine, mannose, galactose, fucose, and sialic acid appear in all three proteins (234). The carbohydrate sequences for some of the glycoproteins of Semliki Forest virus have recently been proposed (235).
Table 1 (12)
Moles CHO Residue per Mole Protein

<table>
<thead>
<tr>
<th>Protein</th>
<th>N-acetyl glucosamine</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Fucose</th>
<th>Sialic Acid</th>
<th>Total CHO % by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>7.5%</td>
</tr>
<tr>
<td>E₂</td>
<td>8</td>
<td>12</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>11.5%</td>
</tr>
<tr>
<td>E₃</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>45.1%</td>
</tr>
</tbody>
</table>

The lipids of the viral membrane consist of 32% neutral lipids, 61% phospholipids and 7% glycolipids (205). The neutral lipid fraction of SFV consists almost exclusively of free cholesterol while the main components of the phospholipids are sphingomyelin, phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine. The glycolipid fraction contains almost exclusively sialic-lactosyl ceramides. The distribution of the various lipid types is shown as mole ratios in Table 2 (205).

Table 2
Lipid Class Composition of SFV shown as Mole Ratio Relative to Phospholipids

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Mole Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.99</td>
</tr>
<tr>
<td>Glycolipids</td>
<td>0.08</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>1.00</td>
</tr>
<tr>
<td>PE</td>
<td>0.23</td>
</tr>
<tr>
<td>PC</td>
<td>0.33</td>
</tr>
<tr>
<td>PS</td>
<td>0.13</td>
</tr>
<tr>
<td>PI</td>
<td>0.02</td>
</tr>
<tr>
<td>Spingomyelin</td>
<td>0.20</td>
</tr>
</tbody>
</table>
It is believed that the lipid class composition resembles that of the host plasma membrane. Such a relationship is also reflected in the fatty acid composition of the phospholipids in the virus and plasma membranes of infected BHK-21 cells (205). Recent studies by Richardson and Vance (236) have shown that Semliki Forest virus obtains its lipid envelope by "budding" from the plasma membrane of the host cell. In summary, a single particle of Semliki Forest virus contains the molecular composition listed in Table 3.

Table 3 (234,235)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Number of Molecules Per Virion</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>1</td>
</tr>
<tr>
<td>Nucleocapsid</td>
<td>200</td>
</tr>
<tr>
<td>Membrane proteins</td>
<td>550</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>15,000</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>16,000</td>
</tr>
<tr>
<td>PE</td>
<td>3,500</td>
</tr>
<tr>
<td>PC</td>
<td>6,400</td>
</tr>
<tr>
<td>PS</td>
<td>2,000</td>
</tr>
<tr>
<td>PI</td>
<td>200</td>
</tr>
<tr>
<td>Sphingomyelins</td>
<td>2,400</td>
</tr>
<tr>
<td>Gangliosides</td>
<td>1,000</td>
</tr>
</tbody>
</table>

G. The Thesis Investigations

a) Semliki Forest Virus: Lipid Headgroup-Protein Interactions

As has been mentioned several times in the introduction, up to the present time most studies of lipid-protein interactions have been done on model and reconstituted systems. Although these systems have been
extremely helpful in elucidating the various interactions between lipids and proteins, they do have their limitations. To overcome the problems associated with the preparation of such reconstituted systems but more importantly, to be confident that the proteins within the membrane are experiencing natural lipid-protein interactions, the only solution is to study an intact biological membrane. But as has been mentioned, these intact systems tend to be extremely complex and as a result, not much useful information has been obtained. Lipid enveloped viruses, on the other hand, are an attractive alternative to the complex membranes of most eukaryotic and prokaryotic cells. Some of the advantages of these viruses have been outlined previously (Introduction, sec. C).

We chose to study the lipid-protein interactions within the polarheadgroup of Semliki Forest virus using high resolution proton NMR. The use of proton NMR to study membrane systems has generally not been very fruitful, due to the complexity of the spectrum and the close overlapping resonances. However in studying changes in the motion of the polarheadgroups of the choline containing lipids, the analysis of the spectrum is simplified, since the $\text{N(CH}_3\text{)}_3$ resonance is substantially separated from the remaining portion of the spectrum.

Initially experiments were planned to investigate the effect of protease digestion of the viral glycoprotein 'spikes' on the motion of the polarheadgroup region of the membrane. If there are interactions between the glycoprotein 'spikes' and the lipid polarheadgroups, then protease digestion should remove the 'spikes' and therefore the interactions. A decrease in any interactions should be reflected by changes in the linewidth of the choline resonance. Further experiments were
planned involving the selective deuteration of the choline methyl groups. By growing the host cell on medium supplemented with deuterated choline we should be able to enrich the deuterium content of the choline containing lipids. Since the virus obtains its envelope by 'budding' from the host cell plasma membrane (236) the virus would also become enriched with deuterium labeled choline containing lipids. By using deuterium NMR we would then be able to quantitatively evaluate changes in the motion of the lipid polarheadgroups. However, due to the lack of incorporation of tri-trideuteromethyl choline into the BHK-21 cell lipids and the overall low yields of Semliki Forest virus, this aspect of the project was discarded. Instead, the lack of uptake of tri-trideuteromethyl choline was studied.

b) CTP:phosphocholine Cytidylyltransferase: Lipid-Protein Interactions

The second portion of this thesis deals with the interactions between the two lysolipids, oleoyl-LPE and oleoyl-LPC, and the CTP:phosphocholine cytidylyltransferase. Schneider (189) initially reported that this enzyme was activated by storage at 4°C for several days and subsequently Fiscus and Schneider (190) showed that this activation was due to phospholipid. Choy et al. (212) found that LPE was the activating lipid in rat liver cytosol stored at 4°C for several days. They also noted that phosphatidylserine and phosphatidylinositol could also activate the enzyme but not to the same extent. Lysosphatidylcholine was found to strongly inhibit the cytidylyltransferase. The finding that LPE activated and LPC inhibited the enzyme was interesting with regards to a system for studying lipid-protein interactions and possibly as a mechanism for phospholipid regulation.
Initially experiments were planned using physical techniques such as electron spin resonance and equilibrium binding studies, however the amounts of protein needed for such studies could not be obtained. Therefore a kinetic approach was adopted by studying the effect of these lysolipids on the kinetic parameters of the cytidylyltransferase.

The last part of the studies on the cytidylyltransferase deal with the mechanism of the aggregation process of this enzyme when stored at 4°C for several days.
MATERIALS AND METHODS

A. Chemicals and Isotopes

Chemicals and isotopes were obtained from the following suppliers.

Sigma Chemical Company, P.O. Box 14508, Saint Louis, Missouri, 63178 U.S.A.
Choline chloride, choline iodide, phosphocholine, Tris, yeast choline kinase, thermolysin, trypsin, chymotrypsin, bovine serum albumin, and phosphatidyl-DL-glycerol.

Oleoyl-lysophosphatidylethanolamine and oleoyl-lysophosphatidylcholine.

Fisher Chemical Company, 196 West Third Avenue, Vancouver, Canada, V5Y 1E9
Phenol reagent (Folins, 2N), Reneicke salt, and POPOP

P-L Biochemicals, 1037 West McKinley Avenue, Milwaukee, Wisconsin, 53205, U.S.A.
Cytidine triphosphate and cytidine diphosphocholine.

Swartz-Mann, 2646 South Sheridan Way, Mississauga, Ontario, L5J 2M8
Enzyme grade sucrose.
J.T. Baker Chemical Company, c/o Canadian Laboratory Supplies, 237-7080 River Road, Richmond, B.C., V6X 1X5

Potassium tartrate.

Matheson, Coleman, and Bell, c/o North American Scientific Chemical Ltd., 268 East Second Avenue, Vancouver, B.C., V5T 1B7

Acrylamide.

Bio-Rad Laboratories, 2580 Wharton Glen Avenue, Mississauga, Ontario, L4X 2A9

N-N'-Methylene bis-acrylamide, cation exchange resin AG50W-X8, and anion exchange resin AG1-X10.

British Drug House Chemicals, 15 West 6th Avenue, Vancouver, B.C., V5Y 1K2

Sodium dodecyl sulphate.

Grand Island Biological Company, 4534 Manilla Road S.E., Calgary, Alberta.

Dulbecco's Modified Eagles Medium and Medium 199.

Flow Laboratories, 1625 Sismet Road, Unit 10, Mississauga, Ontario, L4W 1V6

Fetal calf serum and BHK-21 clone 13 cells.

Pharmacia, 2044 St. Regis Blvd., Dorval, Quebec, H9P 1H6

Sepharose 6B.

New England Nuclear, 2453 46th Avenue, Lachine, Quebec, H8T 3C9

[Methyl-\(^3\)H]-choline and [\(^3\)H]-toluene.

Amersham/Searle, 505 Iroquois Road, Oakville, Ontario, L6H 2R3

[Methyl-\(^3\)H]-choline, n-[1,2-\(^3\)H]-hexadecane, ACS, and [1-\(^3\)H]-ethanolamine.
Brinkman Instruments, 50 Galaxy Blvd., Rexdale, Ontario, M9W 4Y5

Prespread silica gel (G-25) thin layer chromatography plates, 20cm x 20cm x 0.25mm.

Mallinckrodt Company, c/o North American Scientific Company (see above).

Prespread silica gel (Chromar 7GF) thin layer chromatography plates, 20cm x 20cm x 0.25mm, bulk silica gel (chromar 7GF), and PPO.

Merck, Sharpe, and Dohme, Montreal, Canada.

Trideuteromethyl iodide, Deuterium oxide.


Ethanolamine, monomethylethanolamine, and dimethylethanolamine.

All other chemicals were of reagent grade.

B. General Methods

(i) Protein Determination

Protein was determined by the method of Lowry et al. (196), with bovine serum albumin as a standard. The assay of protein in membrane fractions required a minor modification of the general procedure. In this instance the samples, in 0.66N NaOH, were immersed in a boiling water bath for 5 min, cooled, and assayed in the normal way.

Reagent A: 100 µl 4% NaK Tartrate

100 µl 2% CuSO₄

70 ml 2% K₂CO₃

Reagent B: 300 µl IN Folins reagent
General procedure:

(i) Samples are made up to 200 μl with water

(ii) Three hundred μl 1N NaOH is added and the samples are immersed in a boiling water bath for 5 min and then cooled.

(iii) Add 3 ml Reagent A.

(iv) Wait 15 min.

(v) Add 300 μl Reagent B.

(vi) Wait 45 min and read absorbance at 550 nm.

A standard curve was prepared simultaneously and shown to be linear from 0-100 μg bovine serum albumin.

(ii) Thin-Layer Chromatography

TLC was performed on 20cm x 20cm x 0.25mm prespread (or homemade) silica gel plates. Routinely used solvent systems were:

A. CHCl₃ - CH₃OH - H₂O (70/30/4 ; v/v/v)

This system is used for the separation of phospholipids.

B. CH₃OH - 0.6% NaCl - NH₄OH (50/50/5 ; v/v/v) (197).

This system is useful for separating choline (Rₐ 0-0.13), phosphocholine (Rₐ 0.25-0.38), and CDP-choline (Rₐ 0.53-0.63).

(iii) Gas Chromatography

Fatty acid analysis of phospholipids.

Phospholipid samples (1-5mg) in chloroform were dried down under
a stream of $\text{N}_2$. Three ml of 1N $\text{HCl}$ in anhydrous methanol was added to each sample and $\text{N}_2$ bubbled through the solution before tightly capping and incubation at 80°C overnight. The samples were then dried under a stream of $\text{N}_2$ and redissolved in 200 µl of hexane for direct analysis (198).

The analyses were performed on a Hewlett-Packard high efficiency gas chromatograph (Model 7610A) operated at 170°C with a carrier gas ($\text{N}_2$) flow rate of 50 ml/min. The column (180 cm) contained 12% (w/v) polyethylene glycol succinate supported on Gaschrom P (80-100 mesh) (Applied Science Laboratories).

Fatty acid standards were prepared from pure fatty acid species and were esterified as described above.

(iv) **Phospholipid Phosphorous Analysis**

Total organic phospholipid phosphorous was measured by the method of Raheja et al. (199). The lipid sample (1-10 µg lipid P) in chloroform was added to a glass tube and dried under a stream of $\text{N}_2$. Chloroform (0.4 ml) and chromogenic solution (0.1 ml) were added and the tubes placed in a boiling water bath for 90 s. After cooling to room temperature, an additional 5 ml of chloroform was added and the tubes gently shaken. The absorbance at 710 nm was then measured. A standard curve (0-10 µg lipid P) was prepared using dipalmitoylphosphatidylcholine as standard. The assay was found to be linear over the range of 1-10 µg lipid P (about 25-250 µg phospholipid).
(v) **Liquid Scintillation Counting**

Radioactive lipid samples were counted in a toluene based scintillant containing PPO (4 g/l) and POPOP (50 mg/l). Radioactive aqueous samples were counted in ACS.

Liquid scintillation counting was done in an ISOCAP/300 counter (Nuclear Chicago). Counting efficiency was determined by the external standards ratio of chloroform-quenched standards containing either $[^3H]$-toluene or $[^3H]$-hexadecane. Standards in the appropriate scintillation fluid were counted with each set of samples.

(vi) **SDS-Polyacrylamide Gel Electrophoresis**

One dimensional SDS-polyacrylamide slab gel electrophoresis was performed by the method of Laemmli and Favre (200). The separation gel was 8.5cm high, 14cm wide, and 1.5mm thick. A 1cm high stacking gel which contained ten 8mm sample slots was cast on top of the separation gel. The separation gel consisted of a specified percentage of acrylamide, 0.375M Tris-HCl (pH 8.8), and 0.1% SDS. The stacking gel consisted of 4% (w/v) acrylamide, 0.08% (w/v) N-N'-methylene bis-acrylamide, 0.125M Tris-HCl (pH 6.8), and 0.1% SDS.

Buffers and solutions:

1. Lower gel buffer (prepared 4X final concentration)
   
   1.5M Tris-HCl pH 8.8 (36.4 g)
   
   0.4% SDS (0.8 g)

   Make up to 200 ml with water.
2. Upper gel buffer (prepared 4X final concentration)
   0.5M Tris-HCl pH 6.8  (6.06 g)
   0.4% SDS  (0.4 g)
   Make up to 100 ml with water.

3. SDS sample buffer
   10% glycerol
   2% β-mercaptoethanol
   1% SDS
   0.0625M Tris-HCl pH 6.8

4. Running buffer (prepare 5X final concentration)
   0.125M Tris  (15.15 g)
   0.96M glycine  (72.0 g)
   0.5% SDS  (5.0 g)
   Make up to 1% with water
   NOTE: pH 8.3 DO NOT ADJUST.

5. Gel stock solution
   30.0 g acrylamide
   0.8 g N,N'-methylene bis-acrylamide
   Make up to 100 ml with water.

Preparation of gels

Lower gel (separating gel)
   (1) Lower gel buffer  7.5 ml
   (2) water  22.5 ml - X ml
   (3) gel stock  X ml
(4) TEMED  30 µl
(5) Ammonium persulphate  150 µl

(10%; w/v) (FRESHLY PREPARED)

NOTE: X corresponds to the percentage gel desired.

Upper gel (stacking gel)

(1) Upper gel buffer  2.5 ml
(2) water  6.5 ml
(3) gel stock  1.0 ml
(4) TEMED  20 µl
(5) Ammonium persulphate  30 µl

(10%; w/v)

The separating gel was allowed to polymerize for 1-2 h while the stacking gel required 0.5 h to polymerize. The reservoir buffer consisted of 0.25M Tris, 0.192M glycine and 0.1% SDS (pH 8.3). The gel was electrophoresised at a constant current of 30 mA for 2.5 h. The gels were stained for protein using a solution of 0.1% (w/v) Coomassie Blue and 50% (w/v) trichloroacetic acid for a period of 1 h and destained overnight with 7.5% (v/v) acetic acid.

(vii) **Non-denaturing Polyacrylamide Gel Electrophoresis**

Non-denaturing polyacrylamide gel electrophoresis was performed as described by Nelson et al. (201). Preparation of the polyacrylamide gels involved mixing 11.1 ml of 0.1M Tris-glycine (pH 8.7), 5 ml of water (for 5% polyacrylamide gels), 5 ml of stock acrylamide solution (22.2 g acrylamide
and 0.6 g N-N'-methylene bis-acrylamide in a final volume of 100 ml of water), 1.1 ml of freshly prepared ammonium persulphate (15 mg/ml) and 30 µl of TEMED. The mixture was poured into glass tubes (0.5 cm x 7 cm), covered with a few millimeters of water and allowed to polymerize for 2 h. The upper and lower reservoir buffer consisted of 50 mM Tris-glycine (pH 8.7). The gels were electrophoresed at a constant current of 2 mA/gel. The samples, up to 75 µl, were added in 10% (w/v) glycerol plus 0.001% bromophenol blue as a dye marker. Protein was detected as described above.

(viii) Preparation of [³H]-phosphocholine

[³H]-phosphocholine was prepared according to the method of Paddon and Vance (202).

\[
\text{yeast choline kinase} \quad [³H]\text{-choline + ATP} \xrightarrow{\text{Mg}^{++}} [³H]\text{-phosphocholine + ADP}
\]

Briefly, 1 mCi of [³H]-choline was incubated with 20 µl of 100 mM ATP, 20 µl 1M Tris-HCL (pH 7), 20 µl 0.1M MgCl₂, and 150 µl dialysed yeast choline kinase (0.2 units). The reaction was terminated after 2 h at 37°C by immersing the tube in a boiling water bath for 2 min. The whole reaction mixture was spotted on a silica gel (G-25) TLC plate over a length of 2 cm. The chromatogram was developed in solvent B.

A marker lane, containing a small amount of the reaction mixture, was also spotted to enable location of the radioactive product. This lane was scraped at 1 cm intervals and the silica gel placed into scintillation vials containing 2 ml 0.1N NaOH plus 10 ml ACS. Before liquid scintillation
counting 100 μl of glacial acetic acid was added. Upon locating the radioactive phosphocholine in the marker lane, the corresponding area in the major lane was scraped and the silica gel washed with 8 ml of water to elute the [3H]-phosphocholine. This sample was evaporated in vacuo and the residue normally dissolved in 5 mM phosphocholine at a radioactive concentration of 100-150 μCi/ml.

C. Cell Culture

The cells used throughout this work were Baby Hamster kidney-21 cells, clone 13 (BHK-21). The cells were grown as monolayer cultures at 37°C in a controlled atmosphere of 5% CO₂/95% air and 100% humidity. They were grown on large (150mm x 15mm, Lux Scientific) and medium (100mm x 15mm, Falcon) petri dishes or roller bottles (725 cm²). The cells were maintained on Dulbecco's Modified Eagles Medium with 5% fetal calf serum. By visual inspection, cells were used when they were nearly confluent unless otherwise stated.

D. Propagation of Semliki Forest Virus

Semliki Forest virus originated as described by Vance and Burke (203). For propagation, the virus was added to nearly confluent roller bottle cultures of cells at a multiplicity of infection of about 0.05 plaque forming units/cell (p.f.u.) in 10 ml of Medium 199 plus 2% fetal calf serum. After adsorption for 1 h, an additional 40 ml of the same medium was added. The cultures were incubated at 37°C for 18-24 h (rolling rate about 1-2 r.p.m.)
at which time the medium was removed and stored in 3 ml aliquots at -70°C until needed. Infectivity of each preparation was determined by the monolayer plaque assay (204).

E. Preparation of Large Amounts of Pure Semliki Forest Virus

The preparation of large amounts of Semliki Forest virus required up to 200 roller bottles of BHK-21 cell cultures. The regular growth medium was removed and the cells infected with virus at a multiplicity of infection of 0.01-0.05 p.f.u./cell in 10 ml of Medium 199 plus .2% fetal calf serum. After 1 h of adsorption at 37°C an additional 30 ml of Medium 199 plus 2% fetal calf serum was added and the cultures incubated overnight (18-24 h). The medium was removed and centrifuged at 10,000 x g for 10 min at 4°C to remove dead cells and debris. Solid ammonium sulphate was added to the supernatant fraction over a period of 20 min to yield 65% saturation at 0°C (430g ammonium sulphate/l of supernatant fraction). The solution was stirred during the addition and the pH maintained at 7 by dropwise addition of 1N NaOH. The solution was allowed to stir for an additional 1 h at 0°C and was then centrifuged at 10,000 x g for 20 min. The precipitate was resuspended in ice-cold PBS and centrifuged at 10,000 x g for 10 min to remove any insoluble debris. The supernatant fraction was then layered onto either 15-50% linear sucrose gradients or 15-50% linear potassium tartrate gradients and centrifuged at 65,000 x g (25,000 r.p.m. in a SW27 rotor) for 3 h. The virus band was removed either by dripping the tube from the bottom or using a pipette to selectively remove the band from the gradient.

To prepare the virus for proton NMR investigations the sample was centrifuged at 100,000 x g for 1 h to sediment the virus. The pellet was
resuspended in 8 ml D₂O/0.9% NaCl and recentrifuged at 100,000 x g for 1 h. The pellet was again resuspended in 8 ml D₂O/0.9% NaCl and recentrifuged before finally resuspended in 0.25 ml D₂O/0.9% NaCl for the actual NMR experiment. All NMR measurements were done at 30°C.

F. Thermolysin Digestion of Semliki Forest Virus

The virus sample (45 mg protein) was resuspended in 250 μl 0.1M Tris-HCl (pH 7.5), 250 μl water, and 500 μl thermolysin (2.4 mg in 0.05M Tris-HCl, pH 7.5) and incubated at 37°C for 1 h. At the end of the incubation the treated virus was cooled to 4°C and repurified on a 15-50% linear potassium tartrate gradient. The sample was then prepared for the NMR experiment as described above.

In another experiment the total virus sample (28 mg protein) was resuspended in 6 ml PBS. One-half was treated with 2.4 mg thermolysin plus 35 μmol CaCl₂ for 1 h at 37°C. The other half of the sample was treated similarly except without the addition of the thermolysin. At the end of the incubation both tubes were cooled in an ice bath and subsequently centrifuged at 100,000 x g for 1 h. The samples were not repurified on potassium tartrate gradients due to the low yield of virus. The samples were prepared for the NMR experiment as described above. This experiment required about 200 roller bottle cultures to obtain 28 mg of viral protein.

G. Preparation of a Mock Semliki Forest Virus Lipid Sample

(i) Preparation of the Sample

A lipid sample consisting of the same lipid class composition as Semliki Forest virus was prepared according to the proportions indicated in Table 4 (205).
Table 4
Lipid Class Composition of Semliki Forest Virus

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Mole Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.99</td>
</tr>
<tr>
<td>Glycolipids</td>
<td>0.08</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>1.00</td>
</tr>
<tr>
<td>PE</td>
<td>0.23</td>
</tr>
<tr>
<td>PC</td>
<td>0.33</td>
</tr>
<tr>
<td>PS</td>
<td>0.13</td>
</tr>
<tr>
<td>PI</td>
<td>0.02</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>0.20</td>
</tr>
</tbody>
</table>

All lipids except PI were shown to be chromatographically pure. The appropriate amount of each lipid (except PI and glycolipid), in chloroform, was placed in a flask and the solvent was removed in vacuo.

(ii) Preparation of Liposomes
A sample of Semliki Forest virus (28 mg protein) was extracted by the procedure of Folch et al. (207). The lipid extract was transferred to a 5mm NMR tube and the solvent was removed under a stream of N₂. Deuterium oxide/0.9% NaCl (0.25 ml) was added and the sample was vigorously agitated.

(iii) Preparation of Vesicles
(a) Mock Virus Lipid Sample
A lipid mixture (30 mg) composed of the same lipid class composition as Semliki Forest virus (Table 4) except without PI and glycolipid was sonicated 3 x 30s (Fisher SONIC Dismembrator, setting 8) in 2 ml 100mM NaCl, 10mM Tris-acetate, pH 7.4 in 100% D₂O. The sample was immediately transferred to a 5 mm NMR tube for the experiment.

(b) Egg Phosphatidylcholine Lipid Sample
Vesicles of egg phosphatidylcholine (50 mg) were prepared as described above.
H. Preparation of Deuterated Cholines

Three differently labelled cholines were prepared by deutero-nmethylation of ethanolamine or methylated derivatives of ethanolamine with trideuteromethyl iodide.

(i) Synthesis of Tri-trideuteromethylcholine

Ethanolamine (18 mmol) was dissolved in 5 ml ice-cold ethanol. CD$_3$I (55 mmol) was added slowly to the reaction flask with constant stirring. The flask was kept cold in an ice-ethanol bath and in the dark by covering with tin foil. The reaction was allowed to proceed over 3 h while slowly warming up to room temperature. Some product was found to precipitate at this time. The reaction mixture was then kept at 4°C for several days. Afterwards the solvent was evaporated in vacuo to yield a yellow oil. Some product was able to be crystallized at this point (725 mg).

The remaining portion of the mixture was dissolved in water and passed through a Dowex AG50-X8 cation exchange resin (3cm x 25cm column) and eluted with a 800 ml 0-4N HCl gradient. The choline containing fractions were detected by assaying with reniecke salt (Ammonium tetrathio-cyanodiammonochromate), a reagent specific for detecting tertiary amino compounds. The fractions were pooled and the solvent removed in vacuo. Total yield of tri-trideuteromethylcholine chloride was 1.5 g (10.1 mmol).

(ii) Synthesis of Di-trideuteromethylcholine

Monomethylethanolamine (33 mmol) was dissolved in 5 ml ice-cold ethanol. Trideuteromethyl iodide (69 mmol) was added slowly to the reaction
flask with constant stirring. The vessel was kept in an ice-ethanol bath and in the dark. The reaction was allowed to warm up to room temperature over 3 h and was then cooled to -20°C for overnight. The product crystallized from solution and was filtered. Total yield of di-trideuteromethylcholine iodide was 2.6 g (11 mmol).

(iii) **Synthesis of Mono-trideuteromethylcholine**

Dimethylethanolamine (63 mmol) was dissolved in 5 ml of ice-cold ethanol. Trideuteromethyl iodide (69 mmol) was added slowly to the flask with constant stirring. The reaction was again kept cold and in the dark. The product precipitated almost immediately. The flask was kept at -20°C overnight at which time the product was filtered. Total yield of mono-trideuteromethylcholine iodide was 13.6 g (58 mmol).

(iv) **Incorporation of Labelled Cholines into BHK-21 Cells**

Baby Hamster Kidney-21 cells have been shown to incorporate exogenous choline which has been added to the culture medium (203). Therefore by supplementing the culture medium with these labelled (deuterated) species of choline, one would expect to observe incorporation into the choline containing lipids. The ability of each of the deuterated cholines to be incorporated into the cellular choline containing lipids was determined by a long term labelling experiment. The cells were grown on Dulbecco's Modified Eagles Medium supplemented with the deuterated cholines plus [3H]-choline. By knowing the specific radioactivity of the choline in the medium and by determining the specific radioactivity of the choline
containing lipids it should be possible to determine the ability of the deuterated cholines to be incorporated.

In detail, three large dishes of BHK-21 cells were grown on Dulbecco's Modified Eagles Medium supplemented with 80 \( \mu g/ml \) of tri-trideuteromethylcholine chloride plus 0.167 \( \mu \text{Ci}/ml \) \( ^3\text{H} \)-choline. Another three plates were grown on medium supplemented with 130 \( \mu g/ml \) of protonated choline iodide plus 0.167 \( \mu \text{Ci}/ml \) \( ^3\text{H} \)-choline. Finally three dishes of cells were grown on medium supplemented with only 0.167 \( \mu \text{Ci}/ml \) \( ^3\text{H} \)-choline. (Note: Dulbecco's Modified Eagles Medium has 4 \( \mu g/ml \) of protonated choline chloride).

After the cells reached confluence the cells were harvested and the lipids were extracted (207) and purified by thin layer chromatography using solvent A. The various lipid classes were visualized using \( I_2 \) vapours. The phosphatidylcholine band was identified and scraped from the plate. The lipid was eluted with 10 ml \( \text{CHCl}_3 - \text{CH}_3\text{OH} - \text{NH}_4\text{OH} \) (1/1/0.1 ; v/v/v). Total lipid phosphorous was determined by the method of Rajeha et al. as described earlier, and the radioactive content measured by liquid scintillation counting. The specific radioactivity of the phosphatidylcholine was then calculated.

The same experiment was performed when studying the incorporation of mono-trideuteromethylcholine and di-trideuteromethylcholine.

(v) Preparation of tri-trideuteromethyl-[1,2-\(^{3}\text{H}\)]-choline iodide

\([1,2-^{3}\text{H}]\)-ethan-1-ol-amine (2 mCi) was dissolved in 0.5 ml ethanol at 0°C. Trideuteromethyl iodide (6.9 mmol) was added to the reaction
vessel with constant agitation. The reaction mixture was kept at room
temperature for 3 h at which time the solvent was removed by evaporation
under a stream of $N_2$. The mixture was dissolved in a small volume of ethanol
and spotted on a silica gel G-25 thin layer chromatography plate and
developed using solvent B. A marker lane was also spotted (2 µl of the
reaction mixture) to locate the product of the reaction. The total yield
of tri-trideuteromethyl-[1,2-$^3$H]-choline iodide was 10 µCi.

(vi) **Choline Transport**

Large dishes of cells were used for the transport studies. The cells
were washed 3 times with Medium 199. Tri-trideuteromethyl-[1,2-$^3$H]-choline
(2.86 µCi) was added in 6 ml Medium 199. The cells were incubated at
37°C and at 37, 60, and 126 min, 2 x 50 µl aliquots of medium were removed
and the radioactivity determined by liquid scintillation counting. At
these same time points, cells were harvested to determine the amount of
transport into BHK-21 cells. The medium was removed and the cells were
washed with 5 x 10 ml ice-cold PBS. The cells were scraped off the dish
with a rubber policeman and extracted according to the procedure of Folch
*et al.* (207). The total aqueous and organic soluble radioactivity was
determined by liquid scintillation counting.

The same experiment was repeated using [$^3$H]-choline as a control.

I. **Enzyme Assays**

(i) **Choline Kinase**

Choline kinase was assayed essentially by the method of Weinhold
and Rethy (208). Cytosol was prepared from BHK-21 cells and added to a
reaction mixture containing 100 μmol Tris-HCl (pH 8), 7.5 μmol MgCl₂, 7.5 μmol [γ-³²P]-ATP (0.03 Ci/mol), and 0.25 μmol choline, using either protonated choline or the deuterated species (tri-trideuteromethylcholine or mono-trideuteromethylcholine) in a final volume of 1 ml. After incubation at 37°C for 20 min, the reaction was terminated by immersing the tubes in boiling water for 2 min. Each reaction mixture was drained into a 0.5cm x 2cm column of activated charcoal/celite (1/1). The assay tubes were rinsed with 2 x 1 ml water and this rinse was also drained into the column. Water (5 ml) was forced through the column to elute the product of the reaction. Radioactivity in the product was determined by liquid scintillation spectrometry.

(ii) CTP:phosphocholine Cytidylyltransferase

The cytidylyltransferase was assayed by a procedure similar to that of Ansell and Chojnacki (209) except that thin layer chromatography on silica gel was used to separate CDP-choline from phosphocholine, instead of charcoal adsorption. Rat liver cytosol or purified cytidylyltransferase was added to a reaction mixture containing 10 μl 1M Tris-succinate (pH 7) and 0.1M MgCl₂, 20 μl 10mM CTP, and 20 μl 5mM [³H]-phosphocholine (100-150 μCi/ml) in a final volume of 100 μl. The mixture was incubated at 37°C for 15 min and the reaction stopped by immersing the tubes into a boiling water bath for 2 min. Usually 60 μl of the reaction mixture was spotted onto a silica gel thin layer plate along with 0.5 mg CDP-choline as carrier. The TLC plate was developed in solvent B. The product of the reaction was visualized under UV light and the band scraped from the plate into a scintillation vial containing 2 ml 0.1N NaOH and 10 ml ACS. Glacial
Acetic acid (100 µl) was added to reduce chemiluminescence before liquid scintillation counting. The assay was found to be linear up to 15 min at 37°C and up to 3.5 mg cytosolic protein (Fig. 3).

J. Purification of the CTP:phosphocholine Cytidylyltransferase

The purification of the cytidylyltransferase is described by Choy et al. (191). Briefly, fresh rat liver obtained from 150-200 g male or female Wistar rats, was homogenized in isotonic saline (20% w/v) and centrifuged at 100,000 x g for 1 h. The supernatant fraction was aged at 4°C for 5 days and was then centrifuged at 10,000 x g to remove any debris. The supernatant fraction was adjusted to 25% saturation with ammonium sulphate (pH 7) and left at 4°C for 1 h. The solution was centrifuged at 10,000 x g for 10 min and the pellet was resuspended in Buffer A (20 mM Tris-HCl, pH 7 and 0.1 M NaCl) at 1/10 the initial volume.

A 4 ml aliquot was applied to a Sepharose 6B column (2.5 cm x 80 cm) equilibrated with Buffer A. The majority of the cytidylyltransferase activity was located in the void volume of the column (Fig. 21). Fractions containing cytidylyltransferase activity were pooled and SDS was added to a final concentration of 0.05%. The sample was kept at 5°C for 1 h and then concentrated by ultrafiltration, using an XM100A Amicon filter, to a volume of 4 ml. This sample was then applied to a second Sepharose 6B column (2.5 cm x 80 cm) equilibrated with Buffer A plus 0.001% SDS. Upon assaying the column fractions for cytidylyltransferase activity, the enzyme activity was found to split into two peaks, some remaining in the void volume of the column and a new fraction located in the included volume of the column (Fig. 22). This second peak of activity, which
Figure 3. CT activity vs. time and protein.

The enzyme was assayed in 10 μl 1M Tris-succinate (pH 7) and 0.1M MgCl₂, 20 μl 10 mM CTP, and 20 μl 5mM [³H]-phosphocholine (100-150 μCi/ml) in a final volume of 100 μl. The upper graph shows the effect of incubation time at 37°C on enzyme activity. The assay was linear to approximately 15 min. The lower graph shows the effect of cytosolic protein concentration on CT activity. The assays were performed at 37°C for 15 min with varying amounts of protein. The assay was linear up to ~ 3.5 mg cytosolic protein.
Figure 3.
requires lipid for activity, was pooled and concentrated by ultrafiltration to a volume of about 1 ml for use in subsequent experiments.

K. Assay of the Cytidylyltransferase from Non-Denaturing Polyacrylamide Gels

Non-denaturing 5% polyacrylamide gels were prepared as described in section B (vii). The cytidylyltransferase was initially too large to enter the 5% polyacrylamide gel. Therefore SDS (final concentration 0.05%) was added and the sample was electrophoresised for 2.5 h at 2 mA/gel. Duplicate gels were electrophoresised with one being stained for protein, and the other being assayed for cytidylyltransferase activity. Only one band was observed in the gel although some material stained for protein at the top of the gel. Three sections were cut from the gel corresponding to the areas indicated in Fig. 4. Each section was crushed and assayed for cytidylyltransferase activity. The assay contained the following components:

(i) 20 μl Tris-succinate (pH 7)
(ii) 40 μl 10mM CTP
(iii) 40 μl 5mM [3H]-phosphocholine (150 μCi/ml)
and (iv) 100 μl water

The reaction mixture was incubated at 37°C for 1 h at which time the reaction was terminated by immersing the tubes in a boiling water bath for 2 min. A series of aliquots (5 x 20 μl) were spotted on a silica gel G-25 TLC plate along with 0.5 mg CDP-choline as carrier. The plate was developed in solvent B and the radioactivity in CDP-choline determined as described above.
Figure 4. Schematic drawing of a non-denaturing polyacrylamide gel of the CT.
RESULTS

A. Lipid-Protein Interactions in the Polar Headgroup Region of Semliki Forest Virus

The study of lipid-protein interactions has mainly used model membrane preparations. However several groups have attempted to investigate the effect of protein on the motion of the phospholipid polarheadgroup in intact biological membrane systems (119, 139-141). Several studies on Vesicular Stomatitis virus by two groups are of particular interest since they are studying a relatively simple and uniform membrane preparation (139, 140, 141). As mentioned previously in the introduction, lipid enveloped viruses offer a number of attractive features over whole cell membranes. Stoffel et al. (139, 140) and Moore et al. (141) in studying the effect of trypsin digestion of the virus on the motion of the polarheadgroups of the lipids of Vesicular Stomatitis virus, arrived at opposing conclusions as to the effect of protease digestion. This may be related to problems associated with the interpretation of spin-lattice relaxation data. We have chosen to examine the effect of thermolysin removal of the viral glycoproteins on the headgroup motion of the membrane lipids of Semliki Forest virus using high resolution proton magnetic resonance. The information obtained about the motion of the headgroups of choline containing lipids with this technique is less ambiguous than that obtained from spin-lattice relaxation studies.
(a) Preparation of Semliki Forest Virus

Semliki Forest virus was prepared by infecting monolayer cultures of BHK-21 cells and harvested as described previously (Materials and Methods, see E). Briefly, the virus was precipitated from Medium 199 plus 2% fetal calf serum by addition of ammonium sulphate and subsequently purified on either 15-50% linear potassium tartrate gradients or 15-50% linear sucrose gradients (Fig. 5). The purity of the preparation was determined by SDS-polyacrylamide slab gel electrophoresis in 9% polyacrylamide (Fig. 6). In all cases the virus was shown to be pure with the gel pattern exhibiting only two bands, a higher molecular weight band corresponding to the E₁E₂ combination, and a lower molecular weight band corresponding to the nucleocapsid protein (NC). As mentioned before, the third membrane protein of Semliki Forest virus, E₃, is not detected in this system due to its low molecular weight and high carbohydrate composition.

The integrity of the virus particles was determined by negative staining transmission electron microscopy using phosphotungstic acid. The virus preparations consisted of intact, uniformly shaped particles of about 650 Å diameter (Fig. 7).

(b) Proteolytic Digestion of Semliki Forest Virus

Initially trypsin and chymotrypsin were chosen to remove the glycoprotein "spikes" of Semliki Forest virus. It was felt that since these enzymes were readily available in pure form, the use of other proteases such as pronase and thermolysin would be avoided since they are relatively impure and non-specific. However the use of trypsin or chymotrypsin
Figure 5. Profile of a 15-50% linear sucrose gradient containing Semliki Forest virus.

Semliki Forest virus, grown in BHK-21 cells, was precipitated from Medium 199 plus 2% fetal calf serum using ammonium sulphate (65% saturation at 4°C) as described in Materials and Methods, sec.E. The resuspended precipitate, in PBS, was layered onto a 15-50% linear sucrose gradient and centrifuged at 65,000 x g for 3 h. The gradient was dripped from the bottom of the tube and 0.5 ml fractions were collected. The purified virus was detected by its absorbance at 260 nm.
Figure 5.
Figure 6. SDS-polyacrylamide gel electrophoresis of purified Semliki Forest virus.

Semliki Forest virus was purified as outlined in Materials and Methods and solubilized in sample buffer which contained 0.5% β-mercaptoethanol and 0.5% SDS, final concentration. The virus proteins (20 μg protein) were separated on a 9% polyacrylamide slab gel. Proteins were stained with Coomassie Blue. Envelope proteins are designated E$_1$E$_2$ since they resolve poorly in this system, and nucleocapsid protein is denoted NC. E$_3$ was not detected on these gels.
Figure 6.
Figure 7. Negative staining transmission electron micrograph of purified Semliki Forest virus. (89,367 x magnification)
Figure 7.
resulted in no digestion of the viral glycoprotein 'spikes' as judged by SDS-polyacrylamide slab gel electrophoresis (Fig. 8) or negative staining transmission electron microscopy (Fig. 9). This result necessitated the use of thermolysin which has already been shown to digest the viral glycoproteins of Semliki Forest virus (210, 211).

After incubation of the virus with thermolysin and particle repurification (Materials and Methods, see F), a small aliquot (50 μg protein) was removed and subjected to SDS-polyacrylamide slab gel electrophoresis. From the gel pattern it was demonstrated that the protein band corresponding to the E₁E₂ combination had been completely removed (Fig. 10).

(c) **High Resolution Proton NMR of Intact and Thermolysin Treated Semliki Forest Virus**

The electron micrographs of Semliki Forest virus show protruding material from the envelope encapsulating the core of the virus. We were interested to see what effect, if any, the removal of these 'spikes' would have on the motion of the headgroup of the choline containing lipids. Although the proton NMR spectrum of the virus is rather complex, the resonance of the N-(CH₃)₃ group of the choline containing phospholipids exhibits a distinct line. If there were an alteration of the membrane so that interactions between membrane protein and lipid were disturbed (if any interactions exist at all), we might detect these changes by observing variations in the linewidth of the choline methyl resonance.

The proton NMR spectrum of intact Semliki Forest virus is shown in Fig. 11. The choline methyl resonance is found to be distinct from
Figure 8. SDS-polyacrylamide slab gel electrophoresis of trypsin-treated Semliki Forest Virus.

Semliki Forest virus (70 μg protein) was incubated with 5 μg trypsin in 55 μl TNE buffer at 37°C for 20 min. Trypsin inhibitor (20 μg) was added to terminate the reaction. The sample was prepared for SDS-polyacrylamide gel electrophoresis in a 7.5% polyacrylamide slab gel as described in the Materials and Methods.
Figure 8.
Figure 9. Negative staining transmission electron microscopy of trypsin-treated Semliki Forest virus. (89,367 x magnification). The virus was treated as noted in Fig. 8.
Figure 10. SDS-polyacrylamide slab gel electrophoresis of thermolysin-treated Semliki Forest virus.

Semliki Forest virus (45 mg protein) was resuspended in 250 μl water, 250 μl 0.1M Tris-HCL (pH 7.5), and 500 μl thermolysin (2.4 mg in 0.05M Tris-HCL, pH 7.5) and incubated at 37°C for 1 h. At the end of the incubation the sample was cooled to 0°C and layered onto a 15-50% linear potassium tartrate gradient. The gradient was centrifuged at 65,000 x g for 3 h at 4°C and the thermolysin-treated virus band isolated. An aliquot was removed (50 μg protein) and solubilized in sample buffer containing 0.5% β-mercaptoethanol and 0.5% SDS, final concentration. The sample was then applied to the top of a 9% SDS-polyacrylamide slab gel and electrophoresised at 30 mA for 2.5 h. The proteins were detected by staining with Coomassie Blue and destaining overnight with 7.5% acetic acid.
Figure 10.
Figure 11. High resolution proton NMR spectrum of intact Semliki Forest virus.

Semliki Forest virus (45 mg) was prepared for the NMR experiment as described in Materials and Methods, sec. E. The virus was resuspended in 0.25 ml D_2O/0.9% NaCl. The spectrum was recorded on a Nicolet TT.23 100 MHz NMR spectrometer using a 2 KHz spectral width and a 2 s acquisition time. The spectrum required 1000 scans.
the rather broad resonances of the fatty acyl terminal methyl groups and
the methylene protons. After thermolysin digestion of the virus and repurifi-
cation of the particles, the NMR spectrum exhibited a narrow resonance
superimposed on a broader resonance, both corresponding to the absorption
frequency of the choline \( \text{N-}(\text{CH}_3)_3 \) group (Fig. 12). The linewidth of a
particular resonance is directly related to the motion experienced by the
nucleus as described in equation 1.

\[
\frac{1}{\tau_2} \propto t_c \quad (1)
\]

where \( t_c \) is the rotational correlation time which can be obtained as a
solution of the rotational diffusion equation (239) as

\[
\frac{1}{t_c} = \frac{6}{a^2} D_{\text{diff}} \quad (2)
\]

where \( a \) is the radius of the vesicle and \( D_{\text{diff}} \) is the diffusion rate.

\( D_{\text{diff}} \) can be divided into two components, rotational diffusion due to
Brownian tumbling (\( D_r \)) which is given by the Stokes-Einstein relation
as \( D_r = kT/8\pi n a \) (where \( n \) is the viscosity of the medium) and lateral
diffusion \( D_t \) (240). Therefore equation 2 becomes,

\[
\frac{1}{t_c} = \frac{6}{a^2} \left[ \frac{kT}{8\pi n} + D_t \right] \quad (3)
\]

It can be seen that the rotational diffusion component (\( D_r \)) varies as the
cube of the radius, whereas the lateral diffusion component (\( D_t \)) varies
as the square of the radius. The linewidth of a resonance from a nucleus which is unable to reorient rapidly will be much broader than that from a nucleus which is experiencing rapid motion. Since the thermolysin only digests protein on the outside of the membrane (210, 211), the narrow resonance (4 Hz, Fig. 12) superimposed on the broad resonance is assigned to the choline containing lipids on the inside of the membrane, which are now experiencing more rapid motion. Consequently, the broad underlying resonance is assigned to the choline containing lipids on the inside of the membrane.

A second experiment (Figs. 13 and 14) was performed, however, this time two samples of virus were prepared and both treated identically except one portion of virus was not incubated in the presence of thermolysin. The thermolysin treated virus (Fig. 14) again indicated a narrowing of the choline resonance (6 Hz), but not to the same extent as shown in Fig. 12. There are several possible mechanisms for producing an N-methyl resonance as narrow as illustrated in Figs. 12 and 14. During the digestion with thermolysin, it may have been possible to have degraded the total structure of a small portion of the virus, causing the formation of small vesicles which may have a narrower line width than the intact virus. However, since the treated virus (in experiment #1) was repurified on a potassium tartrate gradient, such small structures would have been removed, ruling out such a mechanism. Another mechanism would involve the use of thermolysin itself. Since the enzyme preparation is not pure, a possible phospholipase A contamination could lead to the production of lysophospholipids, in particular lysophosphatidylcholine. The formation of such a product could lead to disruption of the virus membrane with the formation of micelles which would exhibit a very narrow N-methyl resonance. Although this mechanism cannot be completely ruled out, the fact that the purified treated virus particle had the same
Figure 12. High resolution proton NMR spectrum of thermolysin-treated Semliki Forest virus.

Semliki Forest virus was treated with thermolysin as described in Fig.10. After treatment and repurification the sample was prepared for the NMR experiment as described in the Materials and Methods, sec. E. The spectrum was recorded under the same conditions as in Fig.11.
Figure 13. High resolution proton NMR spectrum of intact Semliki Forest virus.

The virus sample (14 mg protein) was incubated with 35 μmol CaCl₂ in 3 ml PBS for 1 h at 37°C and subsequently centrifuged 100,000 x g for 1 h. The virus was then prepared for the NMR experiment as described in Materials and Methods, sec. E. The spectrum was recorded on a Varian XL-100 100 MHz spectrometer using a 2 KHz spectral width and a 2 s acquisition time. The spectrum required 30,000 scans.
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Figure 14. High resolution proton NMR spectrum of thermolysin treated Semliki Forest virus.

The virus sample (14 mg protein), in 3 ml PBS, was treated with 2.4 mg thermolysin plus 35 μmol CaCl₂ for 1 h at 37°C. At the end of the incubation the sample was cooled and centrifuged at 100,000 x g for 1 h to pellet the particles. The sample was prepared for the NMR experiment as described previously (Materials and Methods, sec. E). The spectral conditions were the same as described in Fig. 13.
A sample of Semliki Forest virus (28 mg viral protein) was extracted by the procedure of Folch et al. (207). The lipid phase was isolated and transferred to a 5 mm NMR tube and the chloroform evaporated under a stream of N₂. D₂O/0.9% NaCl (0.25 ml) was added and the sample rapidly agitated. The spectrum was recorded on a Varian XL-100 100 MHz spectrometer using a 2 KHz spectral width and a 2 s acquisition time. The spectrum required 1000 scans.
density as non-treated virus leads one to believe that the treated particles were intact. During the repurification the particles are centrifuged several times at 100,000 x g and resuspended in 0.9% NaCl/D$_2$O before carrying out the NMR measurements. Such treatment may cause destruction of the viral membrane leading to the formation of smaller lipid structures. However, this same procedure was employed when preparing the intact virus particles for the NMR experiment and clearly no small structures were formed.

To see if a vesicle sample would exhibit a choline resonance linewidth of comparable size to that obtained in Fig. 12, a lipid sample was prepared that was comprised of the lipid class composition of Semliki Forest virus less phosphatidylinositol and glycolipid (Table 2) (these lipids represent less than 10% of the total lipid) (205). This sample was sonicated (Materials and Methods, sec. G(iii)) and the proton NMR spectrum taken. From Fig. 16 the choline resonance was measured as 16 Hz, substantially broader than the resonance in Fig. 12. This might suggest the the linewidth of the choline resonance observed in Fig. 12 is anomalously narrow. However, the result of this experiment may not mean much since the sample does not contain the exact lipid composition of the intact virus. A vesicle preparation of egg phosphatidylcholine was also studied (Fig. 16). From the spectrum a choline resonance linewidth of 7 Hz was observed, comparable to that published in the literature (242). A liposomal preparation of the extracted viral lipid was prepared. From Fig. 15 the linewidth of the choline resonance was 10 Hz. Due to the large structures in a liposomal lipid sample some broadening of the resonances will occur.

Considering the choline linewidths obtained from the viral liposomal sample and the egg phosphatidylcholine vesicles, it is not unreasonable to observe such a narrow resonance after thermolysin digestion of the virus.
Figure 16. High resolution proton NMR spectra of mock Semliki Forest virus lipid vesicles and egg phosphatidylcholine vesicles.

A. A lipid mixture (30 mg) composed of the same lipid class composition as Semliki Forest virus (205) was prepared as described in Materials and Methods, sec. G(i). The sample was sonicated 3 x 30 s (Fisher Sonic Dismembrator, setting 8) in 2 ml 100 mM NaCl, 100 mM Tris-acetate, pH 7.4 in 100% D₂O.

B. Vesicles of egg phosphatidylcholine (50 mg) were prepared in a similar manner as described above. The spectra were recorded on a Bruker WP200 200 MHz spectrometer using a 5 KHz spectral width. The spectrum of the mock virus lipid sample required 32 scans while the egg phosphatidylcholine vesicle spectrum required 4 scans.
Figure 16.
B. Incorporation of Deuterated Species of Choline into BHK-21 Cell Lipids

The magnetic resonance techniques are useful for studying the dynamic aspects of the biological membrane. The use of proton NMR is however, somewhat limited in its ability to quantify changes in the motion of a particular nucleus. Therefore it would be advantageous to find a nucleus which is sensitive to restrictive motion and at the same time yield a quantitative evaluation of changes in motion. Deuterium is such a nucleus, however, it suffers from the problem of low sensitivity, requiring the production of large samples.

We wanted to obtain a more quantitative evaluation of the increased motion of the headgroups of the choline containing lipids as a result of protease digestion of the virus. A series of isotopically enriched cholines were prepared for incorporation into the choline containing lipids of BHK-21 cells.

(a) Characterization of the Deuterated Cholines

A series of three isotopically labelled cholines were prepared (Materials and Methods, sec. H(i,ii,iii)) with either 1,2, or 3 deuterated N-methyl groups (Fig. 17). The synthesis of all three deuterated cholines followed the same general scheme, that is, the methylation of an amine with trideuteromethyl iodide.

All three labelled products were purified by crystallization and shown to react strongly with reinecke salt (ammonium tetrathiocyanodiammonochromate) forming a red insoluble complex in aqueous solution.
Figure 17.

Choline

 mono-trideuteromethyl Choline

 di-trideuteromethyl Choline

 tri-trideuteromethyl Choline
\[
\text{NH}_2\text{CH}_2\text{CH}_2\text{OH} + 3 \text{CD}_3\text{I} \rightarrow \\
\text{Ethanolamine} \quad \text{trideuteromethyl Iodide}
\]

\[
\begin{array}{c}
\text{I} \\
\text{CD}_3
\end{array}
\quad
\begin{array}{c}
\text{D}_3\text{C}-\text{N}-\text{CH}_2\text{-CH}_2\text{OH} + 2\text{HI} \\
\text{Hydrogen Iodide}
\end{array}
\]

\[
\begin{array}{c}
\text{CD}_3
\end{array}
\quad
\text{tri-trideuteromethyl Choline Iodide}
\]

This reagent is specific for detecting tertiary amino compounds. NMR spectra were taken of each of the three labelled species (Figs. 18 A, B, C, D). The integrated areas of the peaks are given in Table 5.

Table 5

Integrated Areas of the Peaks from Spectra Obtained from Choline, Mono-trideuteromethyl Choline, Di-trideuteromethyl Choline, and Tri-trideuteromethyl Choline

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ratio of areas under the peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>regular choline</td>
<td>2 : 2 : 9</td>
</tr>
<tr>
<td>mono-trideuteromethyl choline</td>
<td>2 : 2 : 6</td>
</tr>
<tr>
<td>di-trideuteromethyl choline</td>
<td>2 : 2 : 3</td>
</tr>
<tr>
<td>tri-trideuteromethyl choline</td>
<td>2 : 2 : 0</td>
</tr>
</tbody>
</table>
Choline and the three deuterated species of choline (100 mg each) were dissolved separately in 0.3 ml D$_2$O. The spectra of the deuterated cholines were recorded on a Bruker WP200 200 MHz NMR spectrometer using a 2 KHz spectral width. The spectra required 5 scans. The spectrum of choline was recorded on a Varian T-60 CW NMR spectrometer using a 500 Hz spectral width.

A. choline, B. mono-trideuteromethyl choline, 
C. di-trideuteromethyl choline, and
D. tri-trideuteromethyl choline.
The successive deuteration of the choline methyl groups will result in the loss of 3 protons per deuterated methyl group.

(b) Incorporation of the Deuterated Cholines in BHK-21 Cell Choline Containing Lipids

Regular choline and the three deuterated species plus \(^{3}\text{H}\)-choline were added to the medium above the BHK-21 cells as described previously (Materials and Methods, sec. H(iv)). The cells were grown to confluence, the cell lipids were isolated and the phosphatidylcholine purified. By calculating the specific radioactivity of the added choline and measuring the specific radioactivity of the phosphatidylcholine, it was possible to determine the ability of each species of choline to be incorporated.

Theoretically, the specific radioactivity of the phosphatidylcholine should be the same as the specific radioactivity of the added choline, that is if there are no problems in incorporating the various choline species. From Table 6, the specific radioactivity of the phosphatidylcholine labelled with regular choline, mono-trideuteromethyl choline, and di-trideuteromethylcholine is very similar to the specific radioactivity of the added choline suggesting that the deuterated and tritiated species of choline are finding no difficulty in being incorporated into the phosphatidylcholine. However, the specific radioactivity of the phosphatidylcholine isolated from cells grown on medium supplemented with tri-trideuteromethylcholine is much higher than the phosphatidylcholine isolated from cells grown on regular choline. The specific radioactivity of the
phosphatidylcholine from cells incubated with tri-trideuteromethylcholine plus $[^3]$H-choline is very similar to the specific radioactivity of the phosphatidylcholine from cells incubated with only $[^3]$H-choline (specific radioactivity of phosphatidylcholine - 4.66, 3.43, and 5.0 μCi/μmol in three different experiments). This suggests that this deuterated species of choline is not being incorporated into the lipid.

Several possible explanations for this lack of incorporation into phosphatidylcholine may be: (1) The inability of the cell to transport this heavily deuterated species of choline across the plasma membrane of the cell, or (2) the enzymes involved in the biosynthesis of phosphatidylcholine cannot utilize such a heavily deuterated molecule. Since choline is the substrate for the first enzyme in the biosynthesis of phosphatidylcholine, we were interested to see if these deuterated cholines would be effective as a substrate for the enzyme choline kinase.

Choline kinase from BHK-21 cell cytosol was found to be effective in phosphorylating both mono-trideuteromethyl choline and tri-trideuteromethyl choline (Table 7). Unless the other two enzymes in the biosynthetic pathway, the CTP:phosphocholine cytidylyltransferase or the choline phosphotransferase, are unable to utilize the deuterated phosphocholine, the only alternative for the lack of incorporation of tri-trideuteromethyl choline into phosphatidylcholine is due to problems in transport.

To investigate this possibility, tri-trideuteromethyl-$[1,2-^3]$H-choline was prepared by deuteromethylation of $[1,2-^3]$H-ethanolamine with CD$_3$I (Materials and Methods, Sec. H(v)), for use in transport studies. If the lack of incorporation of tri-trideuteromethyl choline were due to failure to transport the molecule across the plasma membrane of the cell,
Table 6
Incorporation of Deuterated Cholines into BHK-21 Phosphatidylcholine

<table>
<thead>
<tr>
<th>Specific Radioactivity of choline in the medium (μCi/μmol)</th>
<th>Specific radioactivity of phosphatidylcholine in cells grown on the following isotopically labelled cholines (μCi/μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>choline</td>
</tr>
<tr>
<td>1.4</td>
<td>1.44</td>
</tr>
<tr>
<td>1.4</td>
<td>1.44</td>
</tr>
<tr>
<td>0.86</td>
<td>0.42</td>
</tr>
<tr>
<td>0.87</td>
<td>0.46</td>
</tr>
<tr>
<td>0.8</td>
<td>--</td>
</tr>
<tr>
<td>0.8</td>
<td>0.62</td>
</tr>
</tbody>
</table>

1. mono = mono-trideuteromethylcholine
2. di = di-trideuteromethylcholine
3. tri = tri-trideuteromethylcholine

BHK-21 cells (3 large dishes) were grown on Dulbecco's Modified Eagles Medium supplemented with 80 μg/ml of tri-trideuteromethylcholine chloride plus 0.167 μCi/ml [\(^3\)H]-choline. Another three dishes were grown on medium supplemented with 130 μg/ml of choline iodide plus 0.167 μCi/ml [\(^3\)H]-choline. Finally three dishes of cells were grown on medium supplemented with only 0.167 μCi/ml [\(^3\)H]-choline. (Note: Dulbecco's Modified Eagles Medium has 4 μg/ml choline chloride.) After the cells reached confluence they were harvested and the lipids extracted (207) and purified by TLC (solvent A). The phosphatidylcholine was isolated and extracted from the silica gel with 10 ml CHCl\(_3\)/CH\(_3\)OH/NH\(_4\)OH (1/1/0.1 : v/v/v). Total lipid phosphorous was determined by the method of Rajeha et al. (199), and the radioactive content measured by liquid scintillation counting. The specific radioactivity of the phosphatidylcholine was then calculated. The same experiment was performed when studying the incorporation of mono-trideuteromethylcholine and di-trideuteromethylcholine.
Choline kinase activity using choline, mono-trideuteromethyl choline, and tri-trideuteromethyl choline as substrate was assayed using choline, mono-trideuteromethyl choline, and tri-trideuteromethyl choline as substrate. The assay was performed as described in Materials and Methods using $^{32}\text{P}$-ATP as a labelled substrate. Enzyme activities are expressed as counts above background.

Then no uptake of the tri-trideuteromethyl-[1,2-$^3$H]-choline should take place. Unfortunately there was some contamination of the tri-trideuteromethyl-[1,2-$^3$H]-choline with [1,2-$^3$H]-ethanolamine, therefore, some uptake of radioactivity into the cell was expected. However all of the radioactivity absorbed by the cell should be associated with phosphatidylethanolamine and its precursors. As indicated in Fig. 19, there was an initial loss of radioactivity from the medium above the cells which were incubated with tri-trideuteromethyl-[1,2-$^3$H]-choline. However after 37 min the uptake of radioactivity plateaued indicating that no further transport was taking place. Analysis of the organic soluble radioactivity in these cells showed that all of the radioactivity was associated with phosphatidylethanolamine (Table 8). There was no incorporation into BHK-21 cell phosphatidylcholine (Fig. 20).

In comparison, cells incubated with [methyl-$^3$H]-choline showed no plateau in uptake (Fig. 19), with the label being rapidly incorporated.
Table 8
Time Course of Incorporation of Radioactivity into the Organic and Aqueous Soluble Fractions of BHK-21 Cells

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Aqueous (dpm)</th>
<th>Organic&lt;sup&gt;a&lt;/sup&gt; (dpm)</th>
<th>Aqueous (dpm)</th>
<th>Organic (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>37</td>
<td>0.26 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.7 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.42 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.8 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>0.12 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.25 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.8 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>126</td>
<td>0.28 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.9 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.23 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.3 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

BHK-21 cells (large dishes) were incubated at 37°C with 6 ml Medium 199 containing either tri-trideuteromethyl-[1,2-<sup>3</sup>H]-choline (2.86 μCi/dish) or [<sup>3</sup>H]-choline (3 μCi/dish). At 37, 60, and 126 min the cells were harvested to determine the amount of transport of radioactivity into the cells. The medium was removed and the cells were washed with 5 x 10 ml ice-cold PBS. The cells were scraped off the dishes with a rubber policeman and extracted according to the procedure of Folch et al. (207). The total aqueous and organic soluble radioactivity was determined by liquid scintillation counting. (a). All organic soluble radioactivity associated with cells incubated with the tri-trideuteromethyl-[1,2-<sup>3</sup>H]-choline was associated with phosphatidylethanolamine.
Figure 19. Decrease of radioactivity from the cell medium.

BHK-21 cells were incubated over a 1.5 h period with 6 ml Medium 199 containing either tri-trideuteromethyl-[1,2-3H]-choline (2.86 μCi/dish) (●) or [methyl-3H]-choline (3 μCi/dish (▲) as control. At 37, 60, and 126 min, 2 x 50 μl aliquots of medium were removed and the radioactivity determined by liquid scintillation counting. [3H, 2H]-choline = tri-trideuteromethyl-[1,2-3H]-choline.
Figure 19.

- $[^3\text{H},^2\text{H}]$-Choline

- $[^3\text{H}]$-Choline

Loss of Radioactivity (dpm x $10^{-6}$) vs. Time (min)
BHK-21 cells were treated as described in Fig. 19. At 37, 60, and 126 min, cells were harvested and the total lipids extracted (207). The phosphatidylcholine was purified by TLC (solvent A) and the radioactivity determined by liquid scintillation counting. $[^{3}\text{H},^{2}\text{H}]$-choline = tri-tri-deuteromethyl-$[^{1}\text{H},^{3}\text{H}]$-choline.
Figure 20.

Radioactive Incorporation (dpm x 10^-9)

[Diagram showing radioactive incorporation over time (min)]
into phosphatidylcholine (Fig. 20). Since the uptake of radioactivity in cells incubated with the tri-trideuteromethyl-[1,2-\(^3\)H]-choline (contaminated with [1,2-\(^3\)H]-ethanolamine) plateaued at about 37 min, this suggests that there was a rapid uptake of most of the contaminating ethanolamine leaving the tri-trideuteromethyl-[1,2-\(^3\)H]-choline in the medium.

The data suggests that tri-trideuteromethyl choline is unable to be incorporated into BHK-21 cell choline containing lipids because it is unable to be transported across the plasma membrane of the cell.
C. Studies on the CTP:phosphocholine Cytidylyltransferase

It has been known for some time that the activity of the rat liver CTP:phosphocholine cytidylyltransferase is modulated by lipid (190). Investigations by Choy et al. (212) have shown that the activation of the enzyme, which is observed when the rat liver cytosol is aged at 4° C for several days, is due to an increase in the concentrations of LPE in the cytosol. Two other lipids, phosphatidylserine and phosphatidylinositol, were also able to activate the enzyme but not to the same level as LPE. Another lysolipid, LPC, was found to strongly inhibit the cytidylyltransferase. The finding that two different lysolipids are capable of regulating the cytidylyltransferase activity led to the present investigation, which was to study the effects of these two lysolipids on the cytidylyltransferase.

(a) Preparation of the CTP:phosphocholine Cytidylyltransferase

The cytidylyltransferase was purified according to the method of Choy et al. (191) (Materials and Methods, sec. J). The purification of the enzyme is dependant on the aggregation of the light form (L-form) of the enzyme to the heavy form (H-form) of the enzyme.

The cytidylyltransferase was precipitated from aged cytosol using ammonium sulphate (25% at 4°C). This fraction containing the heavy form of the enzyme was applied to the first of two Sepharose 6B columns (Fig. 21). The majority of the cytidylyltransferase, located in the void volume of the column, was pooled and treated with SDS (final
Rat liver cytosol (50 ml of a 20% homogenate in isotonic saline) was aged for 5 days at 4°C. The cytosol was centrifuged at 10,000 x g for 10 min to remove any debris. The supernatant fraction was adjusted to 25% saturation with ammonium sulphate (pH 7) and left at 4°C for 1 h. The solution was centrifuged at 10,000 x g for 10 min and the pellet resuspended in Buffer A at 1/10 the initial volume. A 4 ml sample was then applied to a Sepharose 6B column (2.5 cm x 80 cm) equilibrated with Buffer A. Fractions (6 ml) were collected and assayed for enzyme activity in the presence of exogenous rat liver phospholipid (1 mg/assay). Activity is expressed per ml of column eluant.
Figure 21.

Figure 22.
concentration 0.06%) for 1 h at 4°C. This sample was then ultrafiltrated and applied to the second Sepharose 6B column which had been equilibrated with Buffer A plus 0.01% SDS (Fig. 22). The column profile showed that the cytidylyltransferase activity split into two fractions. The activity located within the included volume of the column was pooled and concentrated by ultrafiltration for use in subsequent experiments.

(b) Purity of the Cytidylyltransferase

Although SDS was able to dissociate the H-form of the enzyme to the L-form, which was located within the included volume of the second Sepharose 6B column, the size of the protein (or aggregate) did not allow it to enter a 5% nondenaturing polyacrylamide gel. To overcome this problem, SDS (final concentration of 0.05%) was added to the sample of L-form. After electrophoresis and staining the gel for protein, only one band of protein was observed inside the gel although some material still resided on top of the gel (Fig. 23). By running duplicate gels, staining one for protein and assaying the other for cytidylyltransferase activity (Materials and Methods, sec. K) the single band in the gel was found to contain enzyme activity. The material on top of the gel also contained cytidylyltransferase activity suggesting that not all of the enzyme was completely dissociated to the L-form, or it had reaggregated after the column chromatography.
Figure 23. Non-denaturing polyacrylamide gel electrophoresis of purified CTP:phosphocholine cytidylyltransferase.

The purified cytidylyltransferase was electrophoresed on 5% polyacrylamide tube gels, prepared as described in Materials and Methods, sec. B(vii). The dissociation of the H-form to the L-form was not totally complete, therefore, SDS was added to a final concentration of 0.05%. Duplicate gels were electrophoresed (2 mA/gel for 2 h at 4°C) with one gel being stained for protein using Coomassie Blue, and the other gel being assayed for cytidylyltransferase activity. Three sections were cut out of the gel corresponding to the three regions, TOP, MID, and BAND. Each section was crushed and assayed for cytidylyltransferase activity as described in Materials and Methods, Sec. K. The enzyme activity associated with each region is expressed as a percentage of the total recovered activity.
Figure 23.
(c) **Effects of Lysolipids on CTP:phosphocholine cytidylyltransferase Activity**

It was the initial observation by Schneider (189) that storage of rat liver cytosol at 4°C for several days led to an increase in cytidylyltransferase activity (Fig. 24). The enzyme can also be activated over a shorter time period by incubation of the fresh cytosol at 37°C for a few hours (Fig. 25). Choy et al. (212) studied this activation phenomenon in more detail and reported that lysophosphatidylethanolamine (LPE) was responsible for the activation of the cytidylyltransferase. It was also noted that lysophosphatidylcholine (LPC) strongly inhibited cytidylyltransferase activity. (NOTE: Throughout the study only the oleoyl-derivatives of both lysolipids were used due to their solubility in aqueous solution.)

The purity of oleoyl-lysophosphatidylcholine and oleoyl-lysophosphatidylethanolamine was determined by silica gel TLC (Solvent A) and gas chromatography (Materials and Methods, Sec. B(iii)). Both lysolipids were shown to be 100% pure by lipid class (Fig. 26A). Oleoyl-LPC contained >99% of the C\textsubscript{18:1}-species of fatty acid while oleoyl-LPE contained >90% of the C\textsubscript{18:1}-species with minor contamination with C\textsubscript{16:0} and C\textsubscript{16:1}-species (3.5% each) (Fig. 26B).

(d) **Effect of LPE on the Activity the Purified CTP-phosphocholine Cytidylyltransferase**

The purified enzyme was assayed at optimal substrate concentrations
Figure 24. Activation of CTP:phosphocholine cytidylyltransferase in rat liver cytosol at 4°C.

Rat liver cytosol (20% homogenate in isotonic saline) was stored at 4°C. The activity of the enzyme was determined as previously described (Materials and Methods, sec. I(ii)), after 1, 2, 3, 4, and 5 days. Enzyme activity was determined in the absence of exogenous rat liver lipid.

Figure 25. Activation of CTP:phosphocholine cytidylyltransferase in rat liver cytosol at 37°C.

Rat liver cytosol (20% homogenate in isotonic saline) was incubated at 37°C for 4 h. At hourly intervals an aliquot (20 µl) of cytosol was removed and assayed for enzyme activity in the absence of exogenous rat liver lipid (Materials and Methods, sec. I(ii)).
Figure 24.

CT ACTIVITY (nmol·min⁻¹·mg⁻¹ × 10²)

TIME (hours)

Figure 25.
Figure 26. A. Thin layer chromatography of oleoyl-lysophosphatidylethanolamine and oleoyl-lysophosphatidylcholine on silica gel G-25. Lipids were visualized using I$_2$ vapor.

B. Gas chromatography of the fatty acid methyl esters of oleoyl-lysophosphatidylethanolamine and oleoyl-lysophosphatidylcholine (Materials and Methods, sec. B(iii)).
Figure 26.
in the presence of increasing amounts of LPE. The cytidylyltransferase was found to be stimulated about 10-fold (Fig. 27 and Fig. 28) with a $K_a$ for LPE of about 0.3 mM.

(e) Effect of LPC on the Activity of Purified CTP:phosphocholine Cytidylyltransferase

In contrast to the activating ability of LPE, the oleoyl-derivative of LPC was found to strongly inhibit the cytidylyltransferase (Fig. 29). The purified enzyme was assayed at optimal substrate concentrations in the presence of an increasing LPC/LPE ratio. The enzyme activity is very low in the presence of LPC alone. Therefore, to obtain more reliable results, LPE (20 μg/assay, 0.402 mM) was added to raise the basal level of activity.

The initial approach to the study of the interactions between these two lysolipids and the cytidylyltransferase was to be of a physical biochemical nature, however due to the low yields of purified enzyme a kinetic approach was adopted.

(f) Effect of LPE on the Kinetic Parameters of the CTP:phosphocholine Cytidylyltransferase

The cytidylyltransferase catalyses the reaction between phosphocholine and CTP to produce CDP-choline and pyrophosphate. From Fig. 27 it is obvious that LPE is having a pronounced effect on the activity of the enzyme. An initial question to be answered was, what effect in this lysolipid having on the ability of the two substrates to bind (or interact)
Figure 27. Lysophosphatidylethanolamine activation of purified CTP: phosphocholine cytidylyltransferase from rat liver.

Purified cytidylyltransferase was assayed at optimal substrate concentrations with increasing LPE concentrations. Enzyme activities are expressed as nmols of CDP-choline formed per minute x 10^-2.
Figure 27.
Figure 28. Double inverse plot of initial velocity of CDP-choline synthesis at saturating concentrations of phosphocholine and CTP and increasing LPE concentration.
Figure 28.

The graph shows a plot of velocity (in nmol·min⁻¹ x 10⁻²) against LPE⁻¹ (in mM⁻¹), with a line indicating that $K_a = 0.3$ mM.
Figure 29. Lysophosphatidylcholine inhibition of purified CTP:phosphocholine cytidylyltransferase from rat liver.

Purified cytidylyltransferase was assayed at optimal substrate concentrations with increasing LPC concentrations. Each assay had 20 μg LPE added to raise the basal level of activity. 'No lipid addition' indicates the activity of the enzyme when assayed with no LPE or LPC present. '0.55mM LPC' indicates the enzyme activity in the presence of 0.55mM LPC with no added LPE. Enzyme activities are expressed as nmols of CDP-choline formed per minute x $10^{-2}$. 
no lipid addition

0.55 mM LPC

Figure 29.
with the cytidylyltransferase? To this end, the effect of LPE on the $K_m$ for CTP was studied.

The enzyme was assayed at various concentrations of CTP and constant (saturating) phosphocholine concentration, as well as at various concentrations of LPE. A double reciprocal plot of the data revealed a change in the $K_m$ for CTP in such a way as to cause an increase in the affinity of the cytidylyltransferase for CTP (Fig. 30).

The effect of LPE on the $K_m$ for phosphocholine was then studied. In performing a similar experiment except varying the phosphocholine concentration at saturating CTP concentration, the inverse plot indicated little effect on the $K_m$ for this substrate (Fig. 31). From these two results it would appear that the activation of the cytidylyltransferase by LPE is primarily due to the decrease in the $K_m$ for CTP.

(g) Effect of LPC on the Kinetic Parameters of the CTP:phosphocholine Cytidyltransferase

With the above result it was of interest to investigate what effect LPC was having on the affinity of the substrates for the enzyme. Initially the effect of LPC on the $K_m$ for CTP was studied. The purified enzyme was assayed at various concentrations of CTP and constant (saturating) phosphocholine concentration, as well as increasing the LPC/LPE ratio. The inverse plot of the data revealed an interesting trend (Fig. 32). With an increasing concentration of LPC, the $K_m$ for CTP was shifted to a larger value, indicating that this lysolipid was decreasing the substrates
Figure 30. Double reciprocal plot of initial velocity of CDP-choline synthesis at saturating phosphocholine concentration, varying CTP concentrations, and increasing amounts of LPE. The amounts of LPE were 5 (●), 10(▲), and 20 (○) µg.
Figure 30.
Figure 31. Double reciprocal plot of initial velocity of CDP-choline synthesis at saturating CTP concentration, varying phosphocholine concentrations, and fixed amounts of LPE. The fixed amounts of LPE were 0 (●), 10 (▲), and 20 (○) µg.
Figure 31.

\[
\text{PHOSPHOLIPASE} \\
\text{PHOSPHOCHELINE-}^{-1}
\]

\[
\left(\frac{\text{mM}}{\text{L}}\right)
\]

\[
\text{VELOCITY}^{-1} \\
(\text{nmol} \cdot \text{min}^{-1} \cdot \text{L}^{-1})
\]

Points:
- ▲ 20 μg Lipase
- ▼ 10 μg Lipase
- ● 0 μg Lipase
Figure 32. Double reciprocal plot of initial velocity of CDP-choline synthesis at saturating phosphocholine concentration, varying CTP concentrations, and increasing amounts of LPC. The fixed amounts of LPC were 0 (●), 5 (○), 10 (▲), and 15 (□) μg. Each assay also contained 20 μg (0.4mM) LPE to raise the basal level of activity.
Figure 32.
affinity for the enzyme. This would account for the inhibition of the cytidylyltransferase which is observed. However, the interpretation of this data is complicated by the fact that two enzyme:lysolipid complexes are present. Since each point is assayed in the presence of 0.4 mM LPE, as mentioned in the figure legend, the assay will contain a CT:LPE complex, which has a low $K_m$ for CTP, and a CT:LPC complex which appears to have a high $K_m$ for CTP. In addition the $V_{max}$ of each of these two forms are not the same. The presence of both of these forms in the assay leads to an experimental velocity equation yielding square terms associated with the velocity and $K_m$. The result of this is one finds curved plots rather than classical Michaelis-Menten kinetic plots (i.e. straight lines). It is obvious in Fig. 32 that as the LPC/LPE ratio is increased there is deviation from linearity providing evidence for the presence of these two lysolipid/enzyme complexes.

(h) **Aggregation of the CTP:phosphocholine cytidylyltransferase**

Another interesting phenomenon associated with this enzyme is its ability to aggregate from a low molecular weight form to a high molecular weight form. As mentioned previously, this aggregation can be seen to take place over several days in rat liver cytosol stored at 4°C (191). Reports from Weinhold et al.(214) studying the rat lung form of the cytidylyltransferase also noted this aggregation process and implicated phosphatidylglycerol (PG) as the aggregation and activating factor. We were interested to see what effect this lipid had on the rat liver form of the enzyme.
Fresh rat liver cytosol (3 ml) was incubated with 1 ml (0.15 mg) of a phosphatidylglycerol suspension (Fig. 33) for 24 h at 4°C. A control sample of rat liver cytosol (3 ml) was incubated with 1 ml saline for the same time period. Although PG is able to aggregate the cytidylyltransferase in vitro, the role of this lipid in the aggregation of the enzyme in rat liver cytosol still remained unknown. A major reason for believing that PG is not involved in the aggregation of the cytidylyltransferase in rat liver is because PG is not present in rat liver cytosol (<0.6 µM, P.C. Choy). This is not the case with rat lung where PG is a major component of the lipids. It is possible however that the amount of PG necessary to cause aggregation over a period of days is very small and possibly undetectable by conventional isolation procedures. In an attempt to ensure complete removal of any PG in rat liver cytosol, a PG-specific phospholipase A was isolated from rat red cells (215). The isolated enzyme has a specific activity of 0.3 µmol·min⁻¹·mg protein⁻¹ (P.C. Choy). Its activity was approximately 100-fold higher with PG as substrate than with either phosphatidylcholine or phosphatidylethanolamine. The product of the reaction, lysophosphatidylglycerol, was ineffective in causing aggregation of the cytidylyltransferase (P.C. Choy).

The effect of this phospholipase A on the aggregation of the cytidylyltransferase was studied with freshly prepared rat liver cytosol. Four ml aliquots of cytosol were incubated with either 0.5 ml (2 mg) of the phospholipase A or with 0.5 ml (2 mg) of albumin solution as control, for 48 h at 4°C. As depicted in Fig. 34, slightly less H-form was generated in the phospholipase A treated cytosol compared to control, but the ratio
Figure 33. Effect of phosphatidylglycerol on the aggregation of CTP:phosphocholine cytidylyltransferase in rat liver cytosol.

Fresh rat liver cytosol (3 ml of 20% homogenate in isotonic saline) was incubated with 1 ml (0.15 mg) of a phosphatidylglycerol suspension (■—■) or with 1 ml saline (○—○) for 24 h at 4°C. The H-form and L-form of the cytidylyltransferase were resolved by chromatography on a Sepharose 6B column (2.6 cm x 40 cm) equilibrated with Buffer A. Fractions (7 ml) were collected and 40 μl aliquots from each fraction was assayed for enzyme activity in the presence of rat liver lipid. The H-form of the enzyme was eluted near the void volume (V₀) of the column.
Figure 33.
Figure 34. Effect of a phosphatidylglycerol-specific phospholipase A on the aggregation of the CTP:phosphocholine cytidylyltransferase.

Fresh rat liver cytosol (4 ml of a 20% homogenate in isotonic saline) was incubated with 0.5 ml (2 mg) of phosphatidylglycerol-specific phospholipase A (■—■) isolated from rat red blood cells (215), or with 0.5 ml albumin (2 mg) (□—□), for 48 h at 4°C. The H-form and L-form of the enzyme were resolved by Sepharose 6B chromatography as described in Fig.
Figure 34.
of the H-form/L-form remained the same. Since the rate of aggregation of
the cytidylyltransferase was not significantly affected by such treatment,
it was concluded that PG is not important for the aggregation of the
cytidylyltransferase in rat liver cytosol.

The only lipid isolated from rat liver which was capable of
aggregating the cytidylyltransferase was diacylglycerol (192).
DISCUSSION

A. Lipid-Protein Interactions in the Polar Headgroup Region of Semliki Forest Virus

The biological membrane has been, until recent time, a relatively unknown quantity. Even though the components of the membrane have been identified, the actual details of the physical interactions between these components still remain unclear. One of the reasons for this is due to the extreme complexity of the intact biological membrane, with most membranes consisting of a wide variety of lipids and numerous proteins in various stages of immersion into the bilayer. The number of interactions taking place within these systems does not allow the study of specific lipid-protein interactions, rather one is studying an average lipid-protein interaction, which is an oversimplified interpretation of the actual interactions that take place within the membrane. The solution to such a problem would seem to be the creation of a model system consisting of only a few components. Most of the useful information on lipid-protein interactions has come from the study of these model systems as previously described. However, these systems are not without problems. When proteins are added to phospholipid mixtures it is difficult to know whether they are regaining their natural conformation and interactions with the lipids.
Therefore it would be useful to find an intact biological membrane system that contains only a few components which will reduce the number of interactions within the membrane.

Semliki Forest virus offers a number of attractive features over most intact biological membranes and reconstituted model systems. As mentioned in the introduction, Semliki Forest virus is formed by 'budding' from the plasma membrane of the host cell. The lipid envelope of the virus can effectively be considered a pure plasma membrane of a cell except that only virally coded membrane proteins are found in the membrane. This reduction in the different types of proteins will substantially reduce the number of lipid-protein interactions. Another convenient feature of the virus is its size. The virus particles are spherical and of uniform size (65 nm) (Fig. 7). This is of significance when studying the membrane using proton NMR (129). The only drawback is the great difficulty in producing large quantities of virus which are needed for NMR experiments.

The study of intact biological membranes by proton NMR has generally resulted in the failure to determine any useful information as to the dynamic organization of the membrane. This is in part due to the large number of interactions in the biological membrane but also to the contribution of many nuclei to the proton NMR spectrum which causes problems in analysing the spectrum. Although the spectrum of Semliki Forest virus is complex, the resonance of the choline methyl groups is quite distinct from the remaining portion of the spectrum (Fig. 11). This makes it considerably easier to monitor changes in the linewidth of the choline methyl resonance.
What factors can influence the linewidth of the choline methyl resonance in a biological membrane? The proton magnetic resonance signal from the methyl groups of the choline moiety of the phospholipids is broadened by magnetic dipolar interactions from other protons within the methyl group, from protons on the neighbouring two methyl groups, and from protons on neighbouring molecules. In the absence of motion, the spectrum for a non-orientated phospholipid choline would be over ten kHz in linewidth (239), however, when the phospholipid is part of a membrane, the choline undergoes considerable motion which causes extensive motional narrowing of the proton NMR resonances. The details of this headgroup motion in the membrane of Semliki Forest virus may be very complex. [The dynamics of the phosphatidylcholine headgroup in a model membrane is discussed by Seelig et al. (93)]. The choline proton NMR signal in the virus is narrowed by a number of motions including:

(a) rotation of each methyl group about its symmetry axis, (b) rotation of the three methyl groups about their common symmetry axis, (c) fluctuations in the orientation of the headgroup moiety about the plane normal to the bilayer, (d) rotation of the headgroup (or the entire molecule) about the plane normal to the bilayer, (e) rotational Brownian tumbling of the virus in the suspending medium, and (f) lateral diffusion of the phospholipid molecules around the spherical viral membrane.(216).

The effect of thermolysin digestion of Semliki Forest virus is to remove the surface glycoprotein spikes while leaving the hydrophobic regions of the proteins in the membrane (210,211). It is unlikely that interactions between the phospholipids and the glycoproteins can inhibit motions (a) and (b) appreciably. We anticipate, therefore, that proteolysis
of the virus would affect only the motional narrowing mechanisms (c), (d), and (e) for the phospholipid headgroups on the outside of the membrane and only mechanism (e) for the choline headgroups on the inside of the membrane. When the glycoprotein spikes of the virus are removed, the effective radius of the virus decreases from a value of not more than 32 nm to about 25 nm as determined by electron microscopy (210). For structures on the order of 50 nm in diameter and assuming a lateral diffusion constant of $D_t = 2.6 \times 10^{-8} \text{ cm}^2/\text{s} (241)$, the contribution of both $D_r$ and $D_t$ (Equation 3) to the rotational correlation time, $t_c$, is significant. The removal of the glycoprotein spikes leads to a 1.3-fold reduction in the virus radius. This reduction in particle size will lead to a larger contribution of $D_r$ on $t_c$ than $D_t$ due to its cubic relationship. The result of this reduction in the rotational correlation time will lead to a reduction in the linewidth of all proton NMR resonances.

After thermolysin treatment (Figs. 12 and 14) the single choline resonance for the intact virus splits into two peaks—presumably one from the outside and one from the inside of the virus membrane (the narrow component of the choline resonance in Fig. 12 comprises about 40% of the total choline resonance). Since the thermolysin would digest protein only on the outside of the viral membrane, the broad peak is assigned to the choline containing lipids on the inside of the membrane and the narrow peak to the choline containing lipids on the outside of the membrane. This dramatic narrowing cannot be attributed only to an increase in Brownian tumbling since a narrowing of at most a factor of about two can be expected by the decrease in the virus radius.
We undertook several experiments using the virus lipid extract, a mock virus lipid sample, and egg phosphatidylcholine. The choline resonance obtained with the virus lipid liposome sample (10 Hz, Fig. 15) and the egg phosphatidylcholine vesicles (7 Hz, Fig. 16) would indicate that the choline resonance of the thermolysin-treated virus may not be unreasonably narrow. The result obtained using the mock virus lipid sample (Fig. 16) is complicated by the fact that the sample does not have the same fatty acid composition and does not have glycolipid and phosphatidylinositol. However, this information is not inconsistent with increased motion of the choline headgroups as a result of thermolysin digestion of the viral glycoprotein spikes.

The only other system to be studied with regard to the effect of protease digestion of the glycoprotein spikes has been Vesicular Stomatitis virus. Two groups have been investigating this virus using $^{31}$P NMR (141) and $^{13}$C NMR (139,140). From $^{13}$C spin-lattice relaxation ($T_1$) data, Stoffel et al. concluded that the motion of the phospholipid choline headgroups of Vesicular Stomatitis virus are more restricted by the digestion of the viral glycoproteins with trypsin. On the other hand, Moore et al. using $^{31}$P spin-lattice relaxation data concluded that this same treatment of the virus leads to an increase in the motion of the phosphate of the headgroup and presumably to an increase in the mobility of the headgroup itself. That the two groups reached opposing conclusions is a manifestation of the difficulties in interpretation of nuclear spin-lattice relaxation data in such heterogeneous systems.
To this point however the nature of the interactions between the polar-headgroups of the phospholipids and the membrane protein remain unknown.

B. Incorporation of Deuterated Cholines into BHK-21 Cells

Proton magnetic resonance is helpful when studying a resonance which is resolved from the bulk of the proton resonances. However, it does not easily lend itself to a quantitative evaluation of the changes in the motion of a particular nucleus. Therefore it would be advantageous to select a nucleus which is sensitive to anisotropic motion and gives quantitative information on the local order experienced by the nucleus. Deuterium is such a nucleus. It also has the advantage of having a low natural abundance (0.015%), therefore by selectively deuterating a certain area of a molecule one can obtain information about the local order experienced by the nucleus only at that site in the molecule.

The results obtained using proton NMR yield only a qualitative evaluation of changes in the motion of the choline methyl groups. In order to confirm the result obtained using proton NMR and to also obtain a more quantitative evaluation of the increased motion of the choline headgroup we chose to grow the virus host cell on medium supplemented with tri-trideuteromethylcholine in the hope of labeling a high percentage of the choline containing lipids. As described in the Results sec. B(b) (Table 6) this labeled species was not incorporated into phosphatidylcholine. Two other deuterated species, mono-trideuteromethylcholine and di-trideuteromethylcholine, were synthesized and shown to be easily incorporated into the choline containing lipids. The fact that both of the latter two deuterated species were incorporated and not the
tri-trideuteromethylcholine was thought to be anomalous. This species has been shown to be incorporated into rat liver choline containing lipids as well as into mouse LM cell choline containing lipids (217).

It was of interest to determine the site of blockage of incorporation. Several possibilities exist: 1) It is possible that the molecule cannot be transported across the plasma membrane of the cell, or, 2) the molecule may not be utilized by the enzymes of the phosphatidylcholine biosynthetic pathway. The results of Table 7 show that tri-trideuteromethylcholine is an effective substrate for choline kinase, the first enzyme in the pathway leading to phosphatidylcholine. Therefore, unless the subsequent two enzymes in this pathway are unable to utilize the deuterated phosphocholine, then the only alternative for the lack of incorporation would be due to inhibition of transport.

To investigate this aspect, tri-trideuteromethyl-[1,2-³H]-choline was chemically synthesized for use in transport studies. Unfortunately this product was contaminated with [1,2-³H]-ethanolamine. If tri-trideuteromethyl-[1,2-³H]-choline is not able to be transported then there should be no uptake of the label into the cell. Due to the contamination of the labelled choline with [1,2-³H]-ethanolamine we did expect to observe some uptake of radioactivity but it should all be associated with phosphatidylethanolamine and its presursors. As shown in Fig. 19 there was an initial loss of radioactivity from the medium but at 37 min this uptake plateaued leaving radioactivity in the medium. The uptake of [³H]-choline did not show this behaviour. Analysis of the organic soluble fraction from cells incubated with tri-trideuteromethyl-[1,2-³H]-choline showed that all of it was associated with phosphatidylethanolamine. No radioactivity was found to be incorporated into phosphatidylcholine (Fig. 20). Although the aqueous soluble fraction
was not analysed, since only 10% of the total absorbed radioactivity was contained in this fraction (Table 8), it is most likely that the labeled materials are radioactive precursors of phosphatidylethanolamine. It is concluded therefore that tri-trideuteromethylcholine is unable to be incorporated into cellular phosphatidylcholine because it cannot be transported.

C. CTP:phosphocholine Cytidylyltransferase: Lipid-Protein Interactions

The majority of the studies on lipid-protein interactions deal with the effects of the membrane protein in the behaviour of the bulk lipid. Studies on systems such as the cytochrome oxidase (108,114-116), the Ca\(^{++}\) ATPase (117), and rhodopsin (118) all deal with the structure of the lipid surrounding the protein. However in a biological membrane the situation is not one sided. Although proteins may be able to order or disorder membrane lipids, these lipids are also capable of affecting the performance of an enzyme which is associated directly or peripherally with the membrane lipid. Very little study has been done in this area presumably due to the complications associated with the insolubility of lipids in aqueous solution. It is clear however, that some proteins have specific requirements for the presence of lipid for enzymatic activity.

Often the activation of an enzyme by the addition of exogenous lipid merely reflects the proteins need for a hydrophobic environment. Even though most of these proteins can be isolated in a so-called 'soluble' state, the majority of them are associated, at least peripherally, with the membranes of the cell. But some of these proteins, such as pyruvate oxidase (175-177), L-lactate dehydrogenase (174), \(\beta\)-hydroxybutyrate
dehydrogenase (181-183), malate oxidase (178), and malate-vitamin K reductase (171) respond in a very specific manner to added lipid. Only one of the above enzymes however responds to a specific lipid class and fatty acid composition. β-hydroxybutyrate dehydrogenase is shown to require unsaturated phosphatidylcholines in order to bind NADH, which is essential for enzyme activity.

The early work of Fiscus and Schneider (190) on the rat liver CTP:phosphocholine cytidylyltransferase suggested that this enzyme may be dependent on lipid for activity. Further investigation by Choy et al. (212) implicated LPE as the activating factor in rat liver cytosol stored at 4°C for several days. They also noted that LPC was a potent inhibitor of cytidylyltransferase activity in vitro. With these results we thought that this system might be ideal for the study of lipid-protein interactions as well as a possible model for enzyme regulation by lipids. Since we were unable to obtain large amounts of purified protein, which would be required for physical biochemical studies, a classical kinetic approach was adopted to investigate the effect of these two lysolipids on the behaviour of the protein.

Our results indicated that the activation of the cytidylyltransferase by LPE was due to a reduction in the $K_m$ for CTP (Fig. 30). This lysolipid showed little effect on the $K_m$ for phosphocholine (Fig. 31). LPC on the other hand exhibited the exact opposite effect of LPE, by increasing the $K_m$ for CTP (Fig. 32). The effect of LPC inhibition could not be attributed to competitive inhibition with phosphocholine.
These results would seem to suggest that some type of enzyme regulation could be taking place involving the two lysophospholipids. However, before such a conclusion can be reached, it must be determined if the events being observed in vitro are actually taking place in vivo. Further in the discussion evidence will be presented which supports a physiological role for LPE in the regulation of the CTP:phosphocholine cytidylyltransferase.

(a) Aggregation of the CTP:phosphocholine Cytidylyltransferase

The aggregation of the cytidylyltransferase for a low molecular weight L-form to a heavier molecular weight H-form is an interesting phenomenon in itself as well as being the key to the purification of the enzyme (191). A similar process takes place in the rat lung (214). The fetal rat lung contains predominately the L-form of the enzyme, however, with lung maturation there is an increase in the amount of H-form as well as an increase in the phosphatidylglycerol concentration. In the rat lung, phosphatidylglycerol has been implicated as being both the activating and aggregating factor (214).

Since the rat liver form of the cytidylyltransferase is activated by LPE (212), it appeared that the lipid requirements of the two enzymes are different. Although phosphatidylglycerol will accelerate the in vitro
aggregation of the enzyme (Fig. 33), our results indicate that this lipid is not important in the aggregation of the cytidylyltransferase when stored at 4°C for several days. Studies with the rat lung and rat liver enzymes indicate that the aggregation requires between 0.05-0.5mM phosphatidylglycerol and since no phosphatidylglycerol could be detected in rat liver cytosol (lower detection limit < 0.6 µM) its role in cytidylyltransferase aggregation was unlikely. This conclusion was further supported by the lack of effect on aggregation when rat liver cytosol was treated with a phosphatidylglycerol-specific phospholipase A (Fig. 34). If trace quantities of phosphatidylglycerol were present and responsible for the aggregation of the cytidylyltransferase in the cytosol, the phospholipase A should have removed it and prevented aggregation. The fact that no changes were observed in the rate of aggregation negated the role of this lipid in the aggregation of the rat liver form of the enzyme. A report by Choy et al. has implicated diacylglycerol as the only lipid capable of aggregating the rat liver cytidylyltransferase (192).

Several questions still remain to be answered with regards to the physiological significance of the activation and aggregation phenomena. Work recently completed within our laboratory by Lim and Vance (223) suggests a physiological role for LPE activation and possibly diacylglycerol aggregation of the cytidylyltransferase.

Previous studies with rats (218) and swine (219) fed on high cholesterol diets exhibited markedly altered concentrations and distributions of plasma lipoproteins as well as a several fold elevation of plasma cholesterol and phospholipid. By feeding female wistar rats a high cholesterol/cholate diet, Lim and Vance observed a 2-fold increase in
cytidylyltransferase activity compared to control which was correlated with a 3-fold increase in cytosolic LPE. However, to this point an increase in phosphatidylcholine biosynthesis has yet to be demonstrated. They also noted that the hypercholesterolemic rat liver cytosol had 25-30% of the total cytosolic cytidylyltransferase in the H-form whereas only 10% of the enzyme in the control rat liver cytosol was in the H-form. This 2.5-fold increase in the H-form was correlated with an approximately 2.5-fold increase in the diglyceride content of the hypercholesterolemic rat liver cytosol. Considering these findings it is possibly not unreasonable to consider the physiological relevance of CTP:phosphocholine cytidylyltransferase control by LPE and diglyceride.

(b) Control of the CTP:phosphocholine Cytidylyltransferase by LPE and LPC

The initial studies on the rat liver cytidylyltransferase in crude cytosol reported that lipid extracts from fresh rat liver cytosol had little activating potential unless they were oxidized under an air stream for 15 h causing the formation of a higher proportion of lysolipids (190). The regulation of the cytidylyltransferase by lysolipids suggests a positive feedback mechanism whereby degraded phospholipids regulate the synthesis of phosphatidylcholine.

A simplistic model for such a regulation mechanism may be viewed as follows. The growth of a cell requires that phospholipids be synthesized to form new membrane structures as well as for other functions. Since most phospholipids are in a continual state of turnover, the ratio of the amount of a phospholipid to its degraded component(s) (i.e. lysolipids)
should be relatively constant. Assuming this to be true, then a buildup of phosphatidylcholine in a cell will also lead to an increase in LPC concentration which will interact with the cytidylyltransferase. In this case, the overproduction of phosphatidylcholine will lead to a reduction of cytidylyltransferase activity and a subsequent reduction in phosphatidylcholine biosynthesis. The role of LPE in the regulation of this system may be envisaged as helping to maintain a relatively constant ratio of phosphatidylcholine/phosphatidylethanolamine. If phosphatidylethanolamine synthesis is stimulated for some reason, then the levels of LPE will also increase causing stimulation of the cytidylyltransferase. In this way phosphatidylcholine biosynthesis will be stimulated and the phosphatidylcholine/phosphatidylethanolamine ratio will be maintained.

The regulation of the cytidylyltransferase is based on changes in the $K_m$ for CTP by the action of these two lysolipids. It must be determined whether the \textit{in vivo} concentration of CTP is in the proper range to allow changes in the $K_m$ for this substrate to regulate the enzyme. Measurement of the CTP level in rat liver (220) indicate an average concentration of about 0.07mM. The true Michaelis constant for CTP, as determined by Choy \textit{et al.} (191) and Ansell \textit{et al.} (209) is approximately 0.3mM. The levels of CTP within the rat liver should be low enough to permit changes in the $K_m$ for this substrate to regulate the enzyme.

The involvement, if any, of the aggregation process in the regulation of the cytidylyltransferase and therefore phosphatidylcholine synthesis, is obscure. Not enough information has been determined about the \textit{in vivo} levels of the H-form (it may possibly be an artifact associated with the homogenization of the liver), which makes it difficult to
speculate on the physiological significance of aggregation in the regulation of phospholipid synthesis.

This model as presented is very simplistic and contains several anomalies which cannot be solved until more knowledge about the overall scheme of phospholipid metabolism in the rat liver has been obtained. An initial problem is to determine the physiological significance of LPC, if any exists, in cytidylyltransferase regulation. To this point no *in vivo* role in cytidylyltransferase regulation has been demonstrated for this lysolipid. It does however occur within the rat liver cell (222) which at least gives it the capability of being involved in such a regulatory mechanism. A role for LPE in the regulation of phosphatidylcholine biosynthesis would seem to have some physiological significance in light of the results of Lim and Vance (223). Phosphoglyceride metabolism and regulation are very complex subjects in the rat liver cell. What is presented in this thesis may or may not be of physiological significance to the regulation of phosphatidylcholine biosynthesis, but at best it is an extremely small piece of a vast puzzle which at the present time is only beginning to be solved.

D. Suggestions for Future Work

(a) Semliki Forest Virus:Lipid Headgroup-Protein Interactions

When this project was started, we did not realize the difficulties associated with the preparation of 50-100 mg samples of pure Semliki Forest virus which are required for NMR experiments. This is one aspect of the project which must be improved before any future work can
be done. To this point in time the basic observation is that thermolysin
digestion of Semliki Forest virus appears to lead to increased motion of
the polar headgroups of the choline containing lipids. However before the
details of these interactions can be explained there are a number of areas
to be investigated. The nature of the thermolysin-treated virus particle
must be rigorously analysed with respect to composition, size, and fragility.
Although the intact virus appears to be stable to multiple centrifugations
and other manipulations, the thermolysin-treated virus may not be as stable.
It would also be informative to study the lipid asymmetry of the viral
membrane. To this point in time there have been no reports in this area.

Since we are removing the sugar and protein portion of these
'spikes' from the membrane surface it is not possible to distinguish whether
the interactions between the lipid and glycoprotein are mediated via the
sugar portion of the protein or the protein portion. Experiments involving
glycosidases may help to distinguish between the two possibilities.

The use of deuterium NMR to investigate this system is very
necessary. By specifically labeling the choline headgroups of the
phospholipids, a more quantitative determination of changes in the molecular
motion of the headgroups can be made. However the use of deuterium NMR
requires at least several fold increases in the amount of sample compared
to what is required for proton NMR. Therefore until methods are devised
to obtain vast quantities of Semliki Forest virus, it would not be
advisable to continue studies with this system.
(b) CTP:Phosphocholine Cytidylyltransferase:Lipid-Protein Interaction

The CTP:phosphocholine cytidylyltransferase has the potential of being an interesting system for the study of lipid-protein interactions as well as possibly being involved in the regulation of phospholipid regulation in the rat liver. The studies presented in this thesis only deal with the effect of LPE and LPC on the kinetic parameters of the enzyme. However there are many questions that can be asked concerned with the number of binding sites for each of the lysolipids, and whether or not they are binding at the same or different sites on the enzymes surface. Our initial observation (Fig. 33) suggests that they do not compete with each other for binding however the result is not entirely clear. The use of equilibrium binding studies using radioactively labeled lipids may help to determine the number of lipids binding sites/enzyme.

The specificity of binding is an aspect of interest. The only difference between oleoyl-LPE and oleoyl-LPC is in the headgroup region and yet each lysolipid has such an opposing effect on the enzymes activity. Studies involving modification of the headgroup region may help to determine the spatial requirements of the headgroup region. The effect of changes in the fatty acid composition could also be investigated to see what requirements are necessary to maintain the activity of the lipid.

But again the basic problem to be overcome is the production of large amounts of purified enzyme which would be required for such experiments. At this point in time only very small quantities of this protein can be obtained, but hopefully in the near future an improved purification scheme will be developed.
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