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NMR AND FLUORESCENCE STUDIES OF
THE INTERACTION OF NEUROHYPOPHYSEAL HORMONES
AND PEPTIDE ANALOGUES WITH MODEL MEMBRANES

JANET F. JOHNS

submitted to fulfil the
requirements for an M.Sc.

Carleton University

April, 1990

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Studies acceptance of the thesis:

NMR AND FLUORESCENCE STUDIES OF THE INTERACTION OF
NEUROHYPOPHYSEAL HORMONES AND PEPTIDE ANALOGUES
WITH MODEL MEMBRANES

submitted by

Janet F. Johns, B.A. (Queen's), B.Sc.(Carleton)

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degree of Masters of Science.

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ABSTRACT

The effect of phosphatidylcholine (PC) and phosphatidylserine (PS) SUV on neurohypophyseal hormones and peptide analogues was examined using fluorescence emission and polarization and ^{13}C NMR relaxation. Intrinsic binding of the peptides to PS was concluded to be peripheral, a primarily electrostatic interaction. No effect of PC was observed at 10^{-4}M peptide. Arginine vasopressin and to a lesser degree, oxytocin reduce the mobility of the PS phosphodiester, but vasopressin has no effect on the conformation of the headgroup, or order of the PS bilayer as indicated by ^{31}P NMR. However, the divalent peptides, but not the monovalent or neutral peptides cause aggregation of PS SUV at low concentrations, enhance Ca^{++} mediated bilayer-hexagonal phase changes and fusion of PE (phosphatidylethanolamine)/PS SUV (studied using ^{31}P NMR and fluorescence energy transfer). These activities result from binding of the divalent peptides to the bilayer surface, arginine being more efficient than lysine in position 8.

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of PS SUV, 100 mM Na

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LIST OF SYMBOLS AND ABBREVIATIONS

$[\]$	concentration (solution)
$[]_{\text{outer}}$	concentration of lipid in the outer shell of vesicle
$\Sigma \Sigma$	surface concentration
$\sqrt{}$	square root
%	percent
α	delay before acquisition
δ	chemical shift
Δ	difference
$\Delta \sigma$	chemical shift anisotropy
$\Delta \sigma_{\text{eff}}$	effective chemical shift anisotropy
$\Delta \nu_{\frac{1}{2}}$	linewidth at half-height
ϵ	extinction coefficient
ϵ_R	dielectric constant
ϵ_0	permittivity of free space
η	viscosity
$\eta + 1$	nuclear Overhauser enhancement
λ	wavelength
$\lambda_{(\text{ex})}$	wavelength of excitation
$\lambda_{(\text{em})}$	wavelength of emission
$\sigma_{\parallel}$	chemical shift of parallel orientation
$\sigma_{\perp}$	chemical shift of perpendicular orientation
σ	surface charge density
σ_{max}	maximum surface charge density

τ	fluorescence lifetime
τ_R	correlation time
ψ_0	surface potential
ω	Larmor frequency
A	absorbance
a	area under emission spectrum
Å	Angstrom
ΔA	hyperfine splitting (ESR)
ACQ	acquisition
AEP	aminoethylphosphonate
Ala	alanine
Arg	arginine
Asn	asparagine
ATP	adenosine triphosphate
b	bound
BLM	black lipid membrane
°C	degrees Celsius
Ca	calcium
CaCl ₂	calcium chloride
CA9C	cholesteryl anthracene 9-carboxylate
cm	centimetre
CNS	central nervous system
CSA	chemical shift anisotropy
Cys	cystine
D	dextro

D ₂ O	deuterium oxide
DHNDB	4-dihexadecylamino-7-nitrobenzo-2-oxa-1,3-diazole
DPG	diphosphoglycerol
e	exponent
e	electronic charge
EDTA	ethylenediamine tetraacetate
em	emission
ESR	electron spin resonance
EtOH	ethanol
ex	excitation
f	free
FID	free induction decay
g	gram
Gln	glutamine
Gly	glycine
GlyNH ₂	glycine amide
H	horizontal
HCl	hydrochloric acid
H ₂ O	water
HOAc	acetic acid
Hz	Hertz
I	amplitude
Ile	isoleucine
IR	infrared
k	Boltzmann constant

K	kilo (10^3)
K	association constant
K_{int}	intrinsic association constant
K_{app}	apparent association constant
Kg	1000 x gravity
KHz	kiloHertz
KWK	Lys-Trp-Lys
L	levo
Leu	leucine
ln	natural logarithm
LUV	large unilamellar vesicles
Lys	lysine
M	molar
MeOH	methanol
Mg	magnesium
$MgCl_2$	magnesium chloride
mg	milligram
MHz	megaHertz
min	minutes
MLV	multilamellar vesicles
ml	millilitre
mm	millimetre
mM	millimolar
mol	mole
N	Avogadro's number
n	stoichiometry

Na	sodium
NaCl	sodium chloride
Na ₄ EDTA	sodium ethylenediamine tetraacetate
Na[(CH ₃) ₂ AsO ₂]	sodium cacodylate
NaCac	sodium cacodylate
NH ₄ OAc	ammonium acetate
nm	nanometre
NMR	nuclear magnetic resonance
nOe	nuclear Overhauser effect
ns	nanosecond
obs	observed
OD	optical density
OT	oxytocin
p	polarization
p ₀	intrinsic polarization
p'	uncorrected polarization
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
pep	peptide
Phe	phenylalanine
PI	phosphatidyl inositol
ppm	parts per million
Pro	proline
PS	phosphatidylserine

Q	quantum yield
r	anisotropy
r'	uncorrected anisotropy
rf	radiofrequency
r_f	distance compound travelled/distance solvent travelled for TLC
R	gas constant
R_{Sc}	ratio of light scattering increase
R_{Tyr}	ratio of quantum yield of peptide to the quantum yield of tyrosine
rpm	revolutions per minute
S	order parameter
s	slope
SDS	sodium dodecyl sulfate
SUV	sonicated unilamellar vesicles
T	temperature
T_1	spin lattice relaxation time
T_2	spin-spin relaxation time
t	time
t_{floc}	time to flocculation
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
TNS	2-p-toluidiny1 naphthalene-6-sulfonate
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet

v	vertical
V	volume
(v)	variable
z	valence

CHAPTER ONE: INTRODUCTION

I. Model Membranes and Neurohypophyseal Hormones

The interaction of biologically-active molecules and ions with phospholipids has been an active area for many years. As phospholipids provide the 'fluid' barrier between the cell or organelle and its immediate environment, this is not surprising. Whether molecules interact primarily with the headgroup, a peripheral association, or become 'dissolved' in the hydrophobic core, an integral association, all have the potential for affecting or reporting on cellular mechanisms.

Phospholipids associate with each other to form macromolecular assemblies. The thermodynamic behaviour of these assemblies depends on how the headgroups (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), diphosphoglycerol (DPG) and sphingomyelin (SPM) being the major animal groups) and the fatty acyl chains pack together. Phospholipids form bilayers with a distinct phase transition between the relatively rigid gel phase and the liquid crystalline phase (1). Mixtures of phospholipids exhibit

lateral phase separations, with areas of gel and fluid lipid co-existing in the bilayer (2). They may also form a non-bilayer hexagonal phase or assemble as micelles (3).

In addition to the thermodynamic behaviour of lipids, it is important to consider the electro-osmotic properties of bilayers. The bilayer provides a dipolar barrier to transport across it, and a surface which when charged and in an ionic environment has a surface potential which influences the activity of species close to the bilayer surface (4). These surface properties play a role in aggregation and fusion of phospholipid vesicles (5) which may be important for biological activities such as membrane fusion and exocytosis.

Model membranes systems (6) may be small (SUV) or large (LUV) unilamellar or multilamellar (MLV) vesicles. Bilayers can be oriented on flat glass plates. In some cases micelles may be the most convenient preparation. Black lipid films (BLM) are generally used for electro-osmotic studies. Monolayers have also been used to study surface properties. Model membrane systems have been well studied by a variety of spectroscopic means; NMR, ESR, fluorescence, IR and Raman spectroscopy (7). Such studies look at the conformation, the degree of anisotropy and the dynamic behaviour of molecules.

To relate the interactions of biological molecules with model membranes to biological activity (whether pharmacological or physiological) one can divide the interacting molecules into two groups; those that are ubiquitous and affect the phospholipid organization in situ, and those less concentrated species upon which the phospholipids act. For example, association of ions with charged headgroups, or cholesterol with acyl chains can alter the 'fluidity' (the two-dimensional viscosity of the bilayer) or the surface potential (8). These changes in the bilayer, in turn, could affect the ability of enzymes (9) or receptors (10) to adopt the necessary conformation for binding or expression of activity (11). They could limit the availability of substrate or alter the transport of molecules across the bilayer. Molecules which promote or inhibit the formation of non-bilayer phases may play a role in fusion events or transport also (12). Some interactions may be more specific in which a lipid may be used as a co-factor for a particular activity, and lipids may also be substrates for enzymic reactions (13).

In this study the interaction of the neurohypophyseal hormones, oxytocin and vasopressin and their analogues with model membrane systems are investigated.

Neurohypophyseal hormones, naturally occurring in the vertebrate animal kingdom, are secreted by the posterior pituitary gland, the neurohypophysis. They are synthesized in neurons as prohormones and encapsulated in neurosecretory granules. During the voyage of the granules down the axon to the terminal, the prohormone is degraded to the non-covalent hormone-neurophysin complex. Neurophysin is often called a carrier protein. On stimulation the granules undergo exocytosis, fusing with the membrane and releasing the hormones into the blood stream (14).

The hormones are cyclic nonapeptides with a wide range of biological activities. They are grouped into two classes, oxytocin-like and vasopressin-like. The cyclic portion is made up of 6 amino acids with a disulfide bridge from 1-Cys to 6-Cys. The remaining amino acids constitute the tripeptide tail. Natural variations occur at positions 3, 4 and 8. In mammals, oxytocin stimulates labour contractions and milk ejection during lactation. Vasopressin is an antidiuretic and a vasoconstrictor. Vasotocin has activities between these (see Table 1). Both peptides and fragments of the peptides are putative neurotransmitters (15). CNS activities affect firing behaviour of neurons, behaviour and memory. Other biological activities include aggregation of platelets and mitogenic

TABLE 1
 REPRESENTATIVE NATURALLY OCCURRING NEUROHYPOPHYSEAL HORMONES
 AND MAJOR BIOLOGICAL ACTIVITIES (14)

	Anti- diuretic (%)	Pressor (%)	Oxytocic (%)	Milk- Ejecting (%)
<u>Arginine Vasopressin</u>				
<u>Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH₂</u>	100	100	4	15
<u>Arginine Vasotocin</u>				
<u>Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-GlyNH₂</u>	56	63	27	49
<u>Oxytocin</u>				
<u>Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH₂</u>	1	1	100	100

effects (16). Because of their small size and relative ease of synthesis, many analogues have been prepared for structure-activity studies (17). This has led to the discovery of analogues that are very selective with respect to the variety of activities.

The hormones express activity by binding to a membrane-bound receptor which in turn activates a secondary system intracellularly (16). Overall activity is determined by receptor binding, level of transduction achieved by the receptor complex and the resistance of the hormone to enzymatic degradation in the biological system. In at least one case, there is evidence that phospholipids are required for binding of oxytocin to its receptors in the mammary gland as phospholipase action reduces binding (18). While this indicates that phospholipids are essential for binding, it does not necessarily imply that oxytocin binds to these lipids. They may be required for receptor conformation.

II. Previous Studies of Neurohypophyseal Hormone - Lipid Interaction

There is evidence that these peptide hormones are surface

active. Monolayer studies (19,20) indicate that both oxytocin and vasopressin adsorb to stearic and lauric acid and cholesterol monolayers with a 6:1 ratio. Both peptides reduce ^{45}Ca uptake on monolayers of stearyl monophosphate. Vasopressin at 10^{-5} M, but not oxytocin facilitates the release of bound ^{45}Ca .

Graziane and Livne (21,22) have observed dramatically increased water permeability with the addition of lysine vasopressin at nanomolar concentrations to the solution on one side of black lipid bilayers composed of egg PC and decane at pH 5.8, 0.3 M NaCl 26-27°C. The temperature dependence of the permeability was altered in comparison to the unmodified bilayer. A small increase in permeability was seen with the addition of 10^{-7} M amino acid mixture and 10^{-8} M oxytocin. Calcium at 10^{-5} M and prostaglandin at 10^{-9} M added prior to vasopressin inhibited the increase in water flow. When added subsequently, neither showed an inhibitory effect. Addition of vasopressin at nanomolar and micromolar concentrations indicated little change in electrical conductance of these BLM. However conductance changes were found by Fettiplace et al (23) with lecithin/decane bilayers at low pH (3.0). Lecithin/hexadecane BLM responded to 10^{-5} M vasopressin with a considerable conductance increase at pH 5.6, 37°C.

They also report an increase in conductance and water permeability at pH 2.0, 37°C for a lecithin/cholesterol decane system. BLM composed of the uncharged lipid, glyceryl monooleate, did not respond unless sodium dodecyl sulfate (SDS) was added to the medium.

Studies with PS/decane BLM indicate (from current vs voltage curves) that both lysine vasopressin (24) and oxytocin (25) decrease resistance with a deviation from linearity; conductance increases with the increase of the voltage drop across the BLM above. It was concluded that the peptides form channels across the bilayer (composed of 4-5 molecules for vasopressin). No specificity for Na^+ or Cl^- was observed.

Comparative studies were done by Fettiplace et al (26) who observed compensation potential changes across monolayers and electrophoretic mobility of lipid/decane droplets. They observed strong adsorption of vasopressin at 10^{-7} M, pH 4.9 to SDS/glyceryl monooleate, PS, and PS/PC bilayers. K was estimated at 10^{-7} M using the Stern equation (27) with area/molecule of 700 to 4300 Å². Less adsorption was noted to dioleoyl phosphate and alamethicin. No adsorption was observed to PC or PE. Oxytocin was also shown to interact similarly but no binding data were

presented. At pH 8.9, when the terminal amino group is uncharged, no changes were observed in either compensation potential of PS monolayers or surface potential of PS/decane droplets.

A study (28) using a drop mercury electrode in contact with the hydrophobic region of monolayers investigated the interaction of poly Lys, poly Lys-Phe, oxytocin, vasopressin and desaminoxytocin with PS and PC. They concluded that the PS monolayer undergoes fluctuating indentations allowing polar groups to contact the electrode. All the peptides caused an increase in these indentations for PS. PC layers were more stable and only poly Lys-Phe deformed the monolayer although all the peptides were absorbed to the PC surface.

ESR studies using labelled phospholipids (29) demonstrated that PS but not PC MLV were affected by lysine vasopressin, 3-Phe oxytocin and 2-Ala oxytocin. Interpretation of the data suggested that the phospholipid motion was slowed by the interaction of vasopressin. Vasopressin was able to equilibrate across the multilayers of PS MLV but not PC/PS MLV for which a freeze-thaw technique was necessary for dispersing the peptide throughout the sample. Lateral phase separation was also

observed using PC/PS MLV. Vasopressin had a greater effect than the other peptides.

The BLM studies indicate that there is some interaction between PC and the peptides. However other studies did not detect such an interaction. This could be due to the presence of decane in the bilayer. Differences have been noted between the effect of Ca^{++} on BLM and bilayers formed from two monolayers which have been attributed to the presence of remaining solvent (decane, hexadecane) in the BLM (30). It is also possible that it is the transmembrane potential difference across the BLM which causes such an effect. These studies were related to the action of vasopressin on toad bladder, however it is now thought that this is mediated via the adenyl cyclase system (31).

For PS, the results indicate that there is a substantial interaction between PS and the peptides with or without a potential difference across the bilayer. However, the binding constant for lysine vasopressin may be overstated as the electrophoresis results assumed that the surface potential was equal to the potential at the plane of shear which may not be the case. In addition, specific binding of Na^+ to PS was not considered (32).

**FIGURE 1: The Structure of Lipids and the Amino Acid
Sequence of Peptides Used in This Study Indicating Charged
Species**

pH studies (26) indicate that the terminal amino group, but not the lysyl amino group of lysine vasopressin is the interacting species. ESR studies indicate a greater effect on PS by lysine vasopressin, which contains both charges, than by 3-Phe oxytocin or 2-Ala oxytocin which have the terminal amino charge only. As these hormones can undergo ring fusion via the cystine bridge at high pH (33), it is not clear whether the pH results are valid.

III. Statement of the Problem

This study investigates these discrepancies using NMR and fluorescence spectroscopy to observe both the lipids PS, PC and PE and the peptides arginine vasopressin, lysine vasopressin, oxytocin, oxypressin, 2-Gly oxytocin, 2-Leu oxytocin, desamino lysine vasopressin and desamino oxytocin (Figure 1) within experimental limitations. Previous studies observed only the lipid portion of the interaction. By determining the requirements and specificity of binding it may then be possible to ascertain whether these effects can be related to biological activity. The experiments were completed in the first quarter of 1986. References dated from 1986 were unavailable at the time of experimentation but are used to interpret the data obtained.

As most of these peptides are positively charged at the pH values used, they will be affected by the surface potential, and will affect the surface potential when bound. The surface potential influences the concentration, C , of charged species at the surface. For a negatively charged surface, the concentration of positive ions at the surface is:

$$C_{\text{surface}} = C_{\text{bulk}} e^{-z \psi_0 e/kT} \quad (1)$$

where ψ_0 is the surface potential, z the valence of the ion, e is the electronic charge, k the Boltzmann constant and T the temperature. This is reflected in the apparent binding constant, K_{app} ,

$$K_{\text{app}} = K_i e^{-z \psi_0 e/kT} \quad (2)$$

where K_i is the intrinsic binding constant. When bound within the Stern layer, a charged species will alter the surface potential as the charge density of the surface will be altered. These points have been addressed by Eisenberg et al (32) for the binding of monovalent ions to PS. This approach has been used by Sargent and Schwyzer (34) as part of a model for the catalysis of peptide-receptor interaction by membrane lipids. In addition to the

accumulation of peptide at the membrane surface by the Boltzmann factor, the intrinsic interaction of the peptide with the membrane and the subsequent interaction of the membrane-bound peptide with the receptor is a three-step model in which the overall free energy of binding of a peptide in the aqueous phase to its receptor is composed of the free energy of each step. Kaiser and Kezdy (35) have pointed out that interaction of a peptide hormone with the membrane reduces a three-dimensional search for receptor sites to a two-dimensional one, can orient the peptide for productive interaction with its receptor and could protect the peptide from proteolytic degradation.

The opioid receptor appears to be a prime example of these phenomena (36). Correlation between site selective activities and predictions of peptide-membrane interactions indicate that the receptor sites are compartmentalized within the membrane core (the K site), at the negatively charged membrane surface (the μ site), and in the aqueous phase (the δ site). The ability of the peptides to interact with the membrane sites, or not in the case of δ sites, is a major factor in their activity.

The interaction between membranes and molecules can be electrostatic or hydrophobic (37). Peripheral interactions

are primarily electrostatic, occur on the membrane surface, are easily reversible and respond to the bulk ionic concentration. Binding constants for interactions of this nature are on the order of $1 - 40 \text{ M}^{-1}$ as observed for monovalent (32) and divalent (38) ions, the biological amines, gentamicin and spermine (39) and enkephalins binding to PC (40) and PS (41). Amphiphilic molecules such as 2,6-toluidinylnaphthalene sulfonate (TNS), which have both hydrophobic and hydrophilic groups, bind on the order of $10^3 - 10^4 \text{ M}^{-1}$ (42). Intrinsic interactions involve substantial hydrophobic interactions with the membrane core and are not easily reversible. However, molecules which interact this way appear to be amphipathic. The hydrophilic groups are necessary for insertion into the core and subsequent stability of the membrane-bound molecule. Peptides can have amphipathic primary structure in which hydrophobic and hydrophylic side chains are segregated in domains. This is the case for adrenocorticotropin-(1-24)-tetracosapeptide (ACTH) (43) and dynorphin (1-13) (44). The latter peptide has a binding constant of $9 \times 10^4 \text{ M}^{-1}$ to PC membranes. Amphiphilicity can also arise from the segregation of hydrophobic and hydrophilic side chains by the formation of the secondary α helix and β structures (35).

IV. Introduction to Techniques: NMR and Fluorescence Spectroscopy

Fluorescence and NMR spectroscopy are good choices for this study and provide information on both the peptides and the model membranes.

A. Peptides

The small size of the peptides has made them ideal candidates for detailed high resolution ^1H , ^{13}C and ^{15}N NMR analysis (45). Larger proteins which rotate more slowly are not observed with the resolution required. For this reason SUV are used for membrane-peptide studies as large vesicles also have a slow tumbling rate. Chemical shifts respond to the local environment of the nuclei and these, together with ^1H coupling constants can indicate conformational restraints. The conformations of oxytocin, vasopressin and analogues have been studied using NMR techniques. While evidence suggests that the conformation of oxytocin in dimethyl sulfoxide is restricted with a β (1-4) turn and at least one hydrogen bond between side chains, in water, the conformation of oxytocin exhibits flexibility. When bound to neurophysin, large ^{13}C chemical shift differences are observed for the side

chains, indicating environmental differences between the bound and free states, and in the peptide backbone which suggests conformational change. Relaxation data provide information on peptide dynamics. While ^1H NMR can give more detailed information than ^{13}C , the stringent requirements for resolution and the complexity of the spectra due to overlap of the lipid resonances overweigh its utility in this study. ^{15}N NMR was a relatively new technique at the time of experimentation. ^{13}C is the preferred nucleus.

^{13}C T_1 NMR relaxation is usually via a dipole-dipole interaction with attached hydrogens. Relaxation times are a function of intramolecular motions. The most effective motions for spin lattice relaxation will be those determined by the Larmor frequency, ω , as the process is a resonance transfer. Thus the relaxation data reports on motions occurring on the time scale of ω . T_2 spin-spin relaxation will report on slower motions as well (46). A molecule will have a 'global' correlation time as it undergoes isotropic rotation. Two correlation times may describe anisotropic rotation. In addition, there may be segmental motion between bonds and flip-flop or rotation between different conformations. Correlation times can be extracted from spectral density functions describing the motional model. Relaxation studies on oxytocin and

vasopressin indicate that the carbons of the ring are relatively rigid, reflecting the global correlation time of 5×10^{-10} s when an isotropic model is applied to the data. Side chains and the tripeptide tail have more flexibility (47).

Tyrosine fluorescence is well documented (48). The wavelength of the fluorescence signal is rather insensitive to the environment, however the quantum yield responds to the environment of the chromophore such as the proximity of local quenching groups and the dielectric of the solvent. There is little in the literature specifically on these peptides. Quantum yields relative to tyrosine have been reported (49,50,51). Fluorescence decay for the tyrosine of oxytocin and desamino oxytocin is doubly exponential. By comparing data from NMR combined with fluorescence decay data for an analogue in which the disulfide bond is replaced by a carbon linkage, Ross et al (51) propose that the multiexponential decay arises from different ground state rotamer populations, one of which is statically quenched by the proximity of the disulfide bond.

Studies using polarization and anisotropy decay have determined peptide dynamics. For these techniques the loss of polarization in the fluorescent signal after irradiation

of the sample with polarized light gives dynamic data similar to those obtained by NMR methods. The way in which depolarization occurs for fluorescence polarization studies depends on the global and internal motions of the molecule imposed on the chromophore and the lifetime of the excited state. The lifetime for oxytocin is on the order of 1.0 nanoseconds. Correlation times were found to be 4×10^{-10} s using polarization methods (52). Two decay times were fitted to the exponential anisotropy intensity decay data, 4.5×10^{-10} s and 3×10^{-9} s (53). The time scales of NMR relaxation and fluorescence are similar.

If there is an exchange of molecules between two sites on a slower time scale than the above motions, the fluorescence and NMR techniques can give different results. For NMR, if there is a chemical shift difference between the two sites, the spectrum can appear as a narrow line (fast exchange), two lines (slow exchange), or a broadened line (intermediate exchange) (54). This occurs because the rate of exchange may be faster or slower than the chemical shift difference. If faster, the line reports on the average of the two sites. If slower, the two lines report on each site, but relaxation information may be modified by the exchange rate. The linewidth of the resonance of intermediate exchange is complicated by the exchange rate.

The same argument can be applied to other parameters, coupling constants and relaxation rates, and it is possible that the same rate of exchange affects the spectral parameters differently depending on the time frame of the difference between the two sites. For fluorescence spectroscopy, however, the lifetimes are on the order of nanoseconds. For slow exchange rates, the spectra give a snapshot of the ensemble of populations and are not complicated by the exchange rate which is much slower than the lifetime. In this case, the two techniques complement each other.

In this study, ^{13}C NMR and tyrosine fluorescence were used to observe the effect of the lipids, PC and PS on the peptides.

B. Model Membranes

The motion of the phospholipid assembly is anisotropic. In the bilayer phase, the lipids rotate rapidly about a director axis parallel to the acyl chains, perpendicular to the plane of the bilayer. For hexagonal phase, the lipids rotate rapidly about a cylindrical axis. Spectroscopy can examine the flexibility of molecules with respect to the director axis (the order of the system, S) and the rate of

motion of the lipids about the director. In some cases, translational or lateral diffusion can also be determined.

ESR methods (55) involve the use of a free radical and rather bulky nitroxide probe. It has the advantage that anisotropy can be observed in both small and large assemblies depending on the rate of motion of the lipids about the director. It is also sensitive, giving useful spectra in a short space of time. However, the reporter group may disturb the lipid organization or report on the local environment only and not reflect the overall state of the bilayer.

Fluorescence methods (56) also depend on the addition of a reporter chromophore. These groups are generally hydrophobic, but rather bulky. The interpretation of the polarization data is complex as the anisotropy and rate of motion cannot be easily separated. An advantage is its higher detection sensitivity than resonance methods.

NMR methods depend on the size of the vesicle. If the vesicle is small, and lateral diffusion fast, the director axis itself rotates isotropically. This gives rise to the narrow NMR resonances observed for SUV preparations. If the vesicle is large, the resonance lines broaden and

.detailed information is lost. However, there are two NMR techniques in common use which exploit this phenomenon for large MLV. The NMR parameters measured, the ^2H quadrupole splitting and the ^{31}P chemical shift anisotropy (CSA) are dependent on the orientation of the bonds from the observed to the neighbouring nuclei with respect to the magnetic field. High power probes and echo techniques which refocus the free induction decay (FID) are required to obtain the spectra due to the large spectral widths involved.

The ^2H quadrupole splitting (57) measures the time averaged angular fluctuations of a ^2H -C bond with respect to the director axis, or S, the order parameter. This is comparable to the order parameter obtainable from ESR spectra. The ^2H technique is not used in this study as ^2H labelled phosphatidylserine was not readily available at the time of experimentation. ^{31}P has the distinct advantage of being naturally abundant.

The chemical shift anisotropy (CSA) (58,59) of the ^{31}P nucleus arises from the anisotropic distribution of electron clouds around the nucleus due to bonding patterns. If a single immobilized crystal is rotated in a magnetic field, single lines are observed for each orientation with

different chemical shifts. The spectrum of a powder has the overlapping spectra of all orientations. An axis system is chosen to define the CSA in any direction - these are termed the principal axes and have specific chemical shifts associated with them. In the powder pattern these are reflected in the outer limits of the spectrum and the peak in the centre. They vary according to the structure of the molecule under investigation. As previously mentioned, if the nucleus moves fast enough in an isotropic manner, the CSA collapses and the average chemical shift is reported. If however, as in large vesicles there is averaging in one plane only the CSA is only partially averaged. The shape of the spectrum depends on the orientation of the principal axes with respect to the director axis about which the headgroup rotates and the rate of the rotation. Thus the spectral shape depends on the conformation of the head group as well as the phase of the lipids. For very fast axial rotation the bilayer phase for PS, PE and PC gives rise to a spectrum for which the outer edges can be used to estimate $\Delta\sigma_{\text{eff}} = (\sigma_{\parallel} - \sigma_{\perp})$. These values are 55, 41 and 47 ppm respectively. For the hexagonal phase the $\Delta\sigma$ is exactly half. If there is fluctuation of the nucleus with respect to the director, further averaging will occur and $\Delta\sigma$ will be less than that expected for the rigid rotor, somewhat comparable to S.

However, it should be noted that a change in the conformation of the headgroup can also give rise to a reduced $\Delta\sigma$ even for a rigid rotor. If the rate of rotation slows, the lineshape changes as the lineshape of the individual orientations are broadened. T_1 measurements, or the linewidths of depaked spectra (for which the spectrum is mathematically reduced to one orientation) can give dynamic information.

Membrane events other than phase changes, such as lateral phase separation and fusion are more easily studied using interacting probes and fluorescence or ESR techniques. Surface charges can also be determined using these methods. Ions that partition between the water and membrane in response to the surface charge are available for both fluorescence and ESR use. The spectra of the bound and free probes must be very different and quantifiable for success. Fluorescent energy transfer between two matched probes is particularly useful for observing lipid or vesicle content mixing during the fusion process. For this technique one probe, the donor, is irradiated. Its energy is transferred by a non-radiative process to second probe, the acceptor, which then emits at its characteristic wavelength. If the probes are in separate populations of vesicles, energy transfer cannot take place. The extent of

intermixing of vesicles can be followed by the decrease in the emission of the irradiated probe, or the increase in the emission of the probe excited by energy transfer.

Rayleigh scattering at the exciting wavelength is conveniently studied on the fluorimeter used for fluorescence experiments as most machines are in the 90° configuration for excitation and observation. The scattering of light is an elastic process in which the wavelength is not changed - photons are scattered without a change in energy. If the particles are less than $1/10$ of the wavelength, then they behave as Rayleigh scatterers. If measured at constant wavelength, angle of observation and path length, changes in the amplitude may be due to a change in refractive index, the volume or concentration of particles. Large particles for which the size is equal or larger than the wavelength scatter the light in a manner that causes interference effects and subsequent decrease of the scattered light amplitude relative to that of a Rayleigh scatterer (60). Light scattering measurements are useful for measuring aggregation and flocculation in solutions.

In this study, ^{31}P NMR was used to study the effects of peptides on PS MLV and the phase behaviour of PE/PS MLV

mixtures. Light scattering was used to monitor the aggregation of PS SUV. Fluorescence energy transfer techniques were used to observe the effects of peptides on fusion of PE/PS SUV. An attempt to determine the effect of peptides on the surface potential of PS SUV using fluorescence methods is outlined in the Appendix.

CHAPTER TWO: EXPERIMENTAL

1. MATERIALS AND METHODS

A. Buffer Systems

Only two buffer systems were employed throughout these experiments; 1) ammonium acetate (Anachemia)/ HOAc (Anachemia), and 2) sodium cacodylate (Sigma)/ HCl (Anachemia). The former facilitated lyophilization to recover peptides, the latter was useful particularly for fluorescence experiments, but used for other experiments to minimize differences between techniques. These were prepared with double-distilled water using volumetric flasks, graduated pipettes, a Mettler H20T balance, and one of the following pH meters; Orion Research Digital pH Meter/ Ionalyzer Model 501, a Fisher Accumet, or a Radiometer Model PHM-26. They were bubbled with a gentle stream of nitrogen for 10 to 20 minutes prior to use. The ammonium acetate (NH_4OAc) buffers were prepared as required and not stored. The sodium cacodylate ($\text{Na}[(\text{CH}_3)_2\text{AsO}_2]$ or NaCac) buffers were stored in the refrigerator (sometimes in concentrated form) for one to

three weeks, but filter-sterilized and re-bubbled with nitrogen before re-use. Na_4EDTA (BDH), NaCl (Anachemia or Fisher Scientific), CaCl_2 (Anachemia) or D_2O (MSD Isotopes, filter-sterilized before use) were added to these preparations when necessary. Additions to the buffer, concentration of the buffer salt, and the pH are noted for each experiment.

For some early experiments, peptide and vesicle solutions were prepared in double-distilled water or D_2O using HCl or HOAc to adjust the pH.

B. Peptides

1. Source and Storage

Synthetic peptides were obtained as lyophilized powders (acetate counter ion). The following were a kind gift of the late Professor Roderich Walter;

lysine vasopressin,
desamino lysine vasopressin,
2-Gly oxytocin,
2-Leu oxytocin,
oxypressin, and
desamino oxytocin.

The following were purchased from Vega Biochemicals (Tucson, Arizona);

oxytocin,
arginine vasopressin, and
lysine vasopressin.

The powders were stored in sealed vials which were refrigerated in plastic jars containing dessicant.

2. Preparation of Solutions and Analysis

Solutions were prepared in buffer (see individual experiments for details). Vials were allowed to come to room temperature while sealed. The contents were transferred into acid washed 1 ml or 2 ml volumetric flasks. The flasks were gently rotated to dissolve the peptides while buffer was added. Aliquots of the solutions were withdrawn under a gentle stream of nitrogen. These solutions were frozen when not in use.

Thin layer chromatography was used to detect oxytocin in lipid-oxytocin mixtures and in fractions from column chromatography of such mixtures. Microlitre aliquots were spotted on analytical silica gel plates 2 or 3 times, and dried with nitrogen between applications. The plates were developed in a chloroform:methanol:water, 65:25:4 (volume

ratio), mixture until the solvent front reached a few centimeters from the top. They were dried with nitrogen and placed in a jar containing iodine crystals for visualization. Oxytocin appeared as a single spot with an r_f of 0.4. Fresh and previously frozen solutions of arginine vasopressin were subjected to thin layer chromatography using a cellulose plate, and a butanol:acetic acid:water, 4:1:1, solvent mixture. These gave identical spots with an r_f of 0.7.

Peptide solutions of oxytocin, 2-Leu oxytocin and 2-Gly oxytocin were examined using ^{13}C NMR spectroscopy (see II.B.3(c)(i)). Initially oxytocin was lyophilized in the NMR tube from the NH_4OAc buffer, and rehydrated for further use. This led to changes in the NMR spectrum indicating some degradation. Solutions frozen for up to a month did not exhibit such changes.

All tyrosine-containing peptides were examined in solution by UV and fluorescence spectroscopy. Spectral details are noted in that section (III.D.1,2).

3. Peptide Concentration

(a) Weight and Volume

The concentrations of all stock solutions were determined by the weight of peptide. A volumetric flask was weighed three times to 0.01 mg using a Mettler H20T balance. The flask was removed with tongs and peptide added. It was replaced carefully and reweighed three times. The average weight of the peptide was determined. Concentrations were calculated using the known volume and the molecular weight of the peptide. This was calculated using the molecular weights of the constituent amino acids and elements as given in (61). The peptide molecular weights used are listed in Table 2 (which include the weight of the acetate counter ion). Volumetric capillary pipettes were used to deliver 10 to 100 microlitre aliquots of the stock to a known volume of buffer. Concentration of the diluted solution was then determined using the appropriate dilution factor.

(b) UV Analysis

The UV spectra were obtained as outlined in III.B.2 using a Cary 219 spectrophotometer. The absorbance at 270

TABLE 2
CONCENTRATION DATA FOR PEPTIDES

Peptide	Molecular Weight (acetate ion) g/mol	A(270) /0.1 mM
arginine vasopressin	1144	0.111
lysine vasopressin	1116	0.138
oxytocin	1067	0.136
oxypressin	1101	0.105
2-Gly oxytocin	961	
2-Leu oxytocin	1017	
desamino oxytocin	1051	0.151
desamino lysine vasopressin	1100	0.135

nm was read from the recorded spectrum, or the spectrometer was set at 270 nm and the absorbance read directly. A conversion factor was used to calculate concentrations. These are listed in Table 2.

C. Lipids

1. Source and Storage

All lipids were Grade I natural extracts obtained from Lipid Products (Nutfield, England). Bovine brain phosphatidylserine (PS), egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) were obtained in chloroform/methanol solution in vacuum sealed vials. These were stored in the freezer. Stock solutions were prepared with methanol previously bubbled with nitrogen. An acid washed 10 ml volumetric flask was placed under a stream of nitrogen. The sealed vial was broken and placed under another stream of nitrogen. The contents of the vial were transferred to the volumetric flask with several washings of methanol. The volumetric solution was sealed with a glass stopper and teflon tape and stored in the freezer. Measured aliquots were withdrawn from the still cold flask under a stream of nitrogen. All solvents were spectroscopy grade (Fisher Scientific).

2. Lipid Analysis

Thin layer chromatography was performed on every aliquot withdrawn from the stock solution. Analytical silica gel plates were used to check purity generally with a chloroform:methanol:water, 65:25:4, solvent mixture. Two or three applications of diluted stock were made to the plate using a capillary. These were treated as previously described (I.B.2). One spot for a single lipid was the criterion for purity. R_f values obtained are listed in Table 3B together with r_f values using different solvent mixtures and those obtained from vesicles rather than solvated lipid.

Three PS lots were subjected to fatty acid analysis using gas chromatography. Fatty acids were isolated in hexane using the following method (62). Chloroform solutions containing 5 mg of PS and 200 micrograms of a C17:0 fatty acid standard were placed in a sidearm flask and dried with a stream of nitrogen. 4.5 ml methanolic HCl were added, and the mixture was refluxed for one hour. After cooling, a few ml of a methanol: water, 9:1, mixture was added to fill the sidearm. 5 ml of hexane was added. The mixture was shaken vigorously. The hexane layer was then removed, diluted, and subjected to gas chromatography

TABLE 3
LIPID ANALYSIS

A. Fatty Acids of Bovine Brain PS

Fatty Acid	% Content		
	Sample # 1	2	3
16:0	1.0	0.7	0.9
18:0	38.5	37.4	42.2
18:1	38.7	37.5	43.4
18:2	1.2	1.4	0.8
20:0	0.9	1.1	ND
18:3	12.0	12.1	12.7
20:2	1.4	1.7	ND
20:3	0.5	0.7	ND
22:0	1.2	1.4	ND
20:4	2.3	2.4	ND
Other	2.3	3.6	ND

B. R_f Values for Lipids in Solvent Systems

Solvent	PS	PC	PE
A. 65:25:4:0	0.2-0.3	0.2-0.3	0.3
65:25:5:5	0.6-0.5		
65:25:5:2	0.5		
65:25:2:2	0.4		
25:15:4:2	0.5	0.3	
B. 46:18:3	0.2		

A - Chloroform:Methanol:Water:Acetic Acid

B - Chloroform:Methanol:7M ammonium hydroxide

by Dr. Keith Butler. A series of fatty acid standards were run under similar conditions, from which the PS fatty acids were identified. The major species are shown in Table 3A.

3. Preparation of Vesicles

(a) General Preparation

All operations were carried out under nitrogen. Purity of the vesicle preparation was checked before use either by TLC as for lipid solutions, and/or by the fluorescent KWK analysis (see III.C). The following method was used for all vesicle preparations. Aliquots of stock lipid solution were placed in 50 ml or 100 ml round-bottomed flasks. Lipid mixtures were vortexed at this point. The lipids were then dried using a stream of nitrogen. The flask was rotated during this process to ensure that the lipids were deposited in even layers rather than in clumps (which were difficult to hydrate). The flask was then covered with foil, attached to a lyophilizer, and evacuated overnight. Just before vesicle preparation the vacuum was broken with nitrogen and a measured volume of buffer (previously bubbled with nitrogen) was added. The flask was sealed with a glass stopper and teflon tape.

(b) Multilamellar Vesicles (MLV)

The buffer and lipid mixture was vortexed vigorously. When opaque and homogeneous it was frozen in an ethanol-dry ice bath, thawed and vortexed again. This procedure was repeated at least three times for PS and six times for PE/PS mixtures. During the final freeze-thaw cycle, the temperature of the PE/PS mixture was raised to 37°C and then allowed to come to room temperature. All mixtures were opaque.

Early MLV preparations were made with distilled water or D₂O rather than buffer. These were prepared in the same way as above. Subsequent additions of peptide solutions and/or acid to adjust pH were made with several rounds of freezing, thawing and vortexing to ensure the dispersion of peptide or acid throughout the layers.

PS preparations were examined by electron microscopy, column chromatography and ³¹P NMR spectroscopy (see I.C.4). PE/PS preparations were examined using NMR only. All data confirmed well-hydrated MLV of varying but large size.

(c) Sonicated Vesicles (SUV)

These were prepared using two different methods. The first involved inserting a sonicating microtip (Sonifier Cell Disruptor, Model 350, Branson Sonic Power Co.) into the round-bottomed flask which was placed in an ice bath under a stream of nitrogen. Sonication was usually for 20 to 30 minutes. This method was extremely efficient, but necessitated a centrifuge step to remove contaminating metal particles lost from the tip during the sonication process. It was difficult to maintain a nitrogen atmosphere during this step. Centrifuging at maximum speed using a Sorvall Superspeed RC2B for 20 minutes was sufficient to pellet the metal. If an ultracentrifugation step was necessary to remove large vesicle contamination (see I.C.4) the metal was removed at that time. The supernatant was retained for use.

The second method of vesicle preparation used one of two sonicating water baths. A Cole-Palmer Ultrasonic Cleaner, Model 8845-3 was used for small volumes and long sonication times (2 to 3 hours). A Bransonic bath-type sonicator, Model 220 was more efficient ($\frac{1}{2}$ to 1 hour). The sealed round-bottomed flask was suspended in the bath using a clamp. The bath temperature was monitored and kept cool

phosphorus analysis performed at a later date. They were thawed and vortexed before analysis. Several determinations were made for the same sample and the results were averaged.

(b) Bligh-Dyer Extraction

The method of Bligh-Dyer (69) was used to extract the lipid of MLV or SUV preparations from its buffer. A measured volume of MLV or SUV was placed in an acid-washed test tube. Double distilled water was added to make a 0.8 ml volume. 1 ml chloroform and 2 ml methanol were added, and the mixture was vortexed. A further 1 ml of chloroform and 1 ml water was added, the mixture was once again vortexed and then left to stand (covered) until two layers separated. The methanol layer was carefully removed for phosphorus analysis.

(c) Phosphorus Assay

The method of Duck-Chong (70) was used with minor modifications. The procedure was carried out in a fumehood with acid-washed glassware. Reagent A was prepared in the following manner 12 to 24 hours prior to use. Solutions of 4.2% ammonium molybdate (Anachemia) in 4.5 N HCl and 0.37%

4. Analysis of Vesicle Size

(a) Direct or Indirect Measurement

These techniques were used to examine preparations only.

(i) Electron Microscopy

Mr. Lee Sowden kindly performed this technique using the negative stain method (1% phosphotungstic acid). Plates were made of representative samples at 100K or 50K magnification. The vesicles were measured and sizes determined.

(ii) Optical Density: Turbidity

The Barrow and Lenz method (63) can detect MLV contamination of an SUV preparation. Essentially, it distinguishes between vesicles that act as Rayleigh scatterers (500 Å and below) and those that do not (above 500 Å). The OD of a 2 mM SUV preparation was recorded from 325 nm to 225 nm using the Cary 219 spectrophotometer (see III.B). The OD was plotted as a function of $1/\lambda^4$. An uncontaminated SUV preparation had a linear graph with intercept at 0.

(iii) ^{31}P NMR

Vesicle preparations can be examined using ^{31}P NMR spectroscopy. The spectral shape is dependent on the orientation and the dynamics of the phospholipid headgroup. For the well studied lipids used here, PS, PC and PE, the shape of the spectra depend on the extent of hydration (64), the phase (lamellar or hexagonal) (65) and the size (66) of the vesicles at constant temperature. Thus their spectra can give information about the preparations. A good SUV preparation should have a narrow linewidth. Larger widths at room temperature may indicate the presence of large vesicles. MLV contamination may show up as baseline distortions. Alternatively, the MLV signal may not be detectable using high resolution running conditions and MLV contamination may appear as a loss in signal. A good MLV preparation has a well defined shape the $\Delta\sigma_{\text{eff}}$ in the range of 40 to 50 ppm. A poorly defined shape (assuming adequate collection parameters and sufficient decoupling power) may indicate some unhydrated phospholipid ($\Delta\sigma_{\text{eff}}$ about 200 ppm) or the presence of a population of vesicles with a size less than 5000 Å. Contamination with SUV results in a peak at the isotropic chemical shift of the spectrum. As the width of the SUV and MLV spectra are so different, a very small level of contamination can

be observed in the MLV spectrum. ^{31}P spectra of vesicles samples were recorded as outlined in II.B.4 and II.C.

(b) Physical Separation

These techniques were used to purify vesicle preparations and to analyze size distribution.

(i) Sepharose 4B Column Chromatography

The molecular sieve method of Huang (67) was used both to analyze MLV or SUV preparations and to separate contaminating MLV from SUV preparations. MLV and large vesicles were eluted with the void volume. Small vesicles were retained on the column and eluted in a later fraction.

A 1.6 cm x 26 cm column was prepared using Sepharose 4B (Pharmacia) and equilibrated with a 26 mM NH_4OAc buffer, pH 5.4. The column was connected to an LKB automatic fraction collector equipped with a pump and a Uvicord II detection apparatus. 1 ml fractions were collected (measured by the number of drops) with a flow rate of 28 to 30 ml per hour. The void volume was determined using a solution of Blue Dextran. Samples of PS MLV or SUV were

filtered with a 1.2 micron Millipore filter to prevent blocking the column with very large vesicles. 0.5 ml (analytical) or 1 to 2 ml (preparative) samples were subsequently layered onto the column. Generally, 60 ml were collected per run. A stream of nitrogen was directed over the collecting tube, and tubes sealed as soon as possible after collection. Generally fractions 15 to 17 were collected for preparative SUV samples. Fractions were subjected to UV detection (280 nm) and TLC to locate PS. The column was stored with 26 mM NH_4OAc buffer and 0.02% sodium azide (Sigma) between runs. This was removed before re-use.

Electron microscopy of the void volume, fraction 11, and the mid SUV fraction 15 revealed vesicle sizes of 8000 Å to 1000 Å and 600 Å to 100 Å respectively.

(ii) Ultracentrifugation

This technique was used to further purify insufficiently sonicated SUV preparations (68). 5 ml to 0.6 ml samples were transferred to polycarbonate tubes which were placed in a Swing 50 bucket rotor with appropriate adaptors. These were spun for 20 to 30 minutes at 45K rpm (200 - 300 K g) on a Sorvall Ultracentrifuge at 20°C. The

supernatant was recovered for use, the pellet discarded. These preparations were clear and gave excellent ^{31}P NMR spectra.

5. Lipid Concentration

(a) General Methods

It was found that calculating aqueous lipid concentrations from the stock solution (using dilution factors) was successful only when preparing very dilute aqueous solutions. A molecular weight of 802 was used for PS. With high lipid concentrations the mixing volume was very different from the original, and making up to a known volume was impractical in many cases. Thus phosphorus analysis was done for every sample with a few exceptions.

SUV prepared for fluorescence studies in 25 mM NaCac buffer, pH 5.2 were found to have a constant optical density at 270 nm, 0.16 OD/mM, when subjected to UV analysis. This figure was used as a conversion factor in some cases as these samples required Bligh-Dyer extraction prior to phosphorus analysis.

Samples were usually frozen after the experiment and

phosphorus analysis performed at a later date. They were thawed and vortexed before analysis. Several determinations were made for the same sample and the results were averaged.

(b) Bligh-Dyer Extraction

The method of Bligh-Dyer (69) was used to extract the lipid of MLV or SUV preparations from its buffer. A measured volume of MLV or SUV was placed in an acid-washed test tube. Double distilled water was added to make a 0.8 ml volume. 1 ml chloroform and 2 ml methanol were added, and the mixture was vortexed. A further 1 ml of chloroform and 1 ml water was added, the mixture was once again vortexed and then left to stand (covered) until two layers separated. The methanol layer was carefully removed for phosphorus analysis.

(c) Phosphorus Assay

The method of Duck-Chong (70) was used with minor modifications. The procedure was carried out in a fumehood with acid-washed glassware. Reagent A was prepared in the following manner 12 to 24 hours prior to use. Solutions of 4.2% ammonium molybdate (Anachemia) in 4.5 N HCl and 0.37%

malachite green oxalate (Anachemia) in double-distilled water were prepared and mixed in the proportion of 1 to 3 respectively. The mixture was placed on a magnetic stirrer for 2 hours at room temperature. It was then filtered through Whatman filter paper into a clean flask. The mixture was refiltered prior to use. Remaining reagent was discarded after 3 days. A solution of 12% magnesium nitrate (Anachemia) in methanol was sealed with a glass stopper and stored in a refrigerator. A standard solution of potassium phosphate (Fisher Scientific) was prepared (22 micrograms/ml) and also stored in the refrigerator.

This procedure was applied to lipids in organic solution, MLV and SUV preparations and the methanol-lipid fraction of the Bligh-Dyer extraction. For each assay two blanks, three standard phosphorus samples (0.10 to 0.40 micrograms) and several volumes of the lipid samples to be analyzed (20 to 100 microlitres of aqueous solutions, 0.50 to 1.0 ml of organic solutions) were placed in test tubes. 25 microlitres of methanolic magnesium nitrate was delivered to the bottom of each test tube using a Hamilton syringe. These were vortexed and transferred to a rack which was placed in a boiling water bath to evaporate solvents. The rack was withdrawn and each tube was removed in turn and placed just above the blue tip of a Bunsen

burner flame. After a few seconds the sample was seen to bubble and orange smoke appeared. The tube was kept over the flame for a further 45 seconds, then replaced in the rack to cool. This was repeated for each tube. 1.0 ml of 1N HCL was added, and a marble (Canadian Tire) was placed on the top of each tube. The rack was returned to the boiling water bath for 15 to 30 minutes. The tubes were allowed to cool. At timed intervals, 2.0 ml of the filtered Reagent A was added to each sample with vortexing. The mixtures were left for 30 to 35 minutes. The absorbance was recorded at 640 nm in timed intervals on a Coleman Spectrophotometer Model 295 using good quality test tubes as cuvettes. The amount of phosphorus was calculated with reference to the standard. The assay was rather hard on glassware which was inspected for coloration and cracks and replaced on a regular basis.

High salt concentrations, the addition of NaOH to buffers and the NaCac buffer itself interfered with the phosphorus assay. MLV or SUV prepared with these additions were subjected to Bligh-Dyer extraction (see this section (ii)) prior to analysis. The test is extremely sensitive and great care had to be taken to ensure that glassware was clean and solvents (e.g. distilled water) contained no interfering contaminants.

(d) Calculation of Lipid on the Outer Surface of SUV

$$[\text{Lipid}]_{\text{outer}}$$

Sonicated vesicles do not have an equal distribution of lipid in the outer and inner layer. The outer layer contains more lipid than the inner layer due to the radius of curvature. This value is 2:1 for lipids (71) and

$$[\text{lipid}]_{\text{outer}} = 2/3 [\text{lipid}]_{\text{total}} \quad (3)$$

For PC/PS mixtures, the PS prefers the inner layer possibly due to the difference in hydration between the PC and PS headgroups. For PC/PS (4:1) mixtures Barsukov et al (72) have determined the inner/outer ratio for PS to be 2 when cosonicated at a pH similar to that used in this study. The calculation of PS concentration in the outer layer is therefore

$$[\text{PS}]_{\text{outer}} = 1/3 [\text{PS}]_{\text{total}} \quad (4)$$

for PC/PS (4:1) mixtures.

II. NUCLEAR MAGNETIC RESONANCE

A. Equipment and Special Handling

All spectra were obtained with a Bruker CXP-300 multinuclear spectrometer equipped with tuned probes for each nucleus, as well as high power probes for solid-state studies. A variable temperature controller was used to maintain or change temperature using a stream of air or nitrogen. The data were plotted with the Bruker Aspect-2000 computer and subsequently transferred to a Nicolet 1280 data processor for further analysis (73).

For high resolution studies, high quality acid-washed flat-bottomed 10 mm NMR tubes were used with teflon vortex plugs. Sealed capillary tubes containing thermometric solutions or chemical shift references were held in place by the vortex plug. For the high power studies, 7 mm acid-washed tubes were used; the coil was in the transverse arrangement for these probes.

D₂O (MSD Isotopes) was filter-sterilized before use as an internal lock for all NMR experiments. For solutions containing less than 25% D₂O (volume percent), the reported pH is that read from a pH meter. In cases where

90% to 100% was used, the pH was read from a pH meter and converted to pD (74) using the equation;

$$\text{pD} = \text{pH} + 0.4 \quad (5).$$

B. High Resolution Studies

1. General Data Collection

8K or 16K ^{13}C and ^{31}P FID's were obtained at 75.47 MHz and 121.57 MHz respectively with broadband proton decoupling at 300 MHz. Quadrature phase-sensitive detection was used, generally with a Bruker four phase cycling routine (x,y,-x,-y) to minimize artefacts. Where noted an add-subtract routine was used instead.

A strong standard sample was used to tune the probe, shim, determine the 90° pulse length (by increasing the pulse width to find the 90° , 180° , and 360° width) and check that the phase-cycling programs and decoupler were working efficiently. Subsequent samples (at lower strength) were fine tuned and shimmed using the lock signal. Where the 90° pulse length was critical (e.g. measuring T_1 's, T_2 's etc.) and observed by analysis to be different from that of the standard sample, the method of Haupt (75) was

used to determine the 90° pulse length for the particular sample.

2. Pulse Sequences and Data Processing

(a) One Cycle Sequence [90° - α -ACQ-DELAY]

90° pulsewidths were used with appropriate DELAYS ($5 \times T_1$) for the resonance(s) of interest. Occasionally pulsewidths less than 90° were used and the DELAY was shorter, but adjusted to avoid saturation. A small α delay of a few points was inserted before acquisition to minimize artefacts arising from transmitter pulse breakthrough. For ^{31}P and ^{13}C FID's, either continuous broadband proton decoupling was applied, or a gated decoupling sequence was used. For this sequence, the decoupler was turned on for a time of at least T_1 before the 90° pulse and turned off at the end of acquisition. This ensured that the full nOe was obtained for decoupled spectra.

The FID's were transformed using either the Bruker or the Nicolet computer. The beginning of the FID was observed for spurious points. These were either corrected or removed. The baselines were corrected, and the FID was then '0-filled' (from 8K to 16K or 16K to 32K).

were sonicated for a few seconds while still sealed. Vortex plugs and capillaries were then replaced and spectra recorded.

Five titrations were performed, three separately and two concurrently to minimize experimental differences. Chemical shifts and linewidths were recorded for each PS addition. Half-saturation values were determined graphically. During and after the titrations the temperature was varied to observe its effect on the titration curve. T_1 and T_2 measurements were made at the end of the runs to compare with those of peptide alone.

One titration was performed with l-D-Cys oxytocin in an 18 mM NH_4OAc buffer, 23% D_2O , pH 5.4. A drop of dioxane was added as an internal reference. 16K spectra with a 20 kHz sweepwidth were collected using an add-subtract routine and continuous broadband decoupling. Two separate titrations were performed with l-D-Cys oxytocin and oxytocin in 25 mM NaCac buffer, 10% D_2O , pH 5.3. Two concurrent titrations were made for l-D-Cys oxytocin and oxytocin in the same buffer, but including 0.1 mM EDTA. 16K spectra (from 1000 to 8000 scans) were collected using a 15 kHz sweepwidth, 90° pulse widths from 10 to 17 microseconds and a gated decoupling sequence.

$\eta+1$, was determined by comparing the intensities of the resonances of S1 and S2;

$$n0e = I(S1)/I(S2). \quad (6)$$

(c) T_1 Sequence [$180^\circ - t(v) - 90^\circ - \alpha - \text{ACQ} - \text{DELAY}$]

The inversion-recovery method (77) was used for all T_1 measurements. Continuous broadband decoupling was used for ^{13}C and ^{31}P measurements. A rough estimate of the T_1 of interest was made, and an automatic sequence was run to obtain FID's with variable delays, $t(v)$. The first and last FID's of the sequence were obtained with $t(v) = \text{DELAY}$ to act as controls. For long runs with many points, an additional control FID was inserted in the middle of the sequence. Subsequent $t(v)$'s were alternately short and long to avoid artefacts due to progressive changes in conditions.

The FID's were treated identically and plotted using the Bruker computer. The intensities of the resonance(s) of interest were measured, $I(t)$. Data were plotted by hand for initial observations according to ;

$$\ln[I(\text{DELAY}) - I(t)] = \ln[2xI(\text{DELAY})] - (t/T_1). \quad (7)$$

The T_1 was determined by applying the T_1 3IR Nicolet program to the data. This is a three-parameter fit with a factor, W , as an indication of the accuracy of the 180° pulsewidth, 1.0 being a perfectly set pulse (78).

(d) T_2 Sequence [$90^\circ(x) -t(v) -180^\circ(y) -2t(v) -180^\circ(y) -ACQ$
-DELAY]

The T_2 's were determined using the Carr-Purcell-Meiboom-Gill method (79). Broadband decoupling was used for all experiments. An automatic sequence was run to collect FID's with a series of $t(v)$'s alternately long and short. The $t(v)$ was lengthened until the signal-to-noise fell below that measurable in a reasonable time. One $t(v)$ was repeated throughout the sequence as a monitor of changing conditions. The FID's were treated as for T_1 experiments and the T_2 's determined using the Nicolet program T_2 CP.

3. ^{13}C NMR

(a) Standards and References

A freshly prepared mixture of dioxane (Anachemia) and D_2O , 2:1, was used as a standard sample for ^{13}C

experiments. The 90° pulses ranged from 10 to 20 microseconds for this probe.

Chemical shifts are reported referenced to a capillary of TMS in 10 mM NaCac, pH 5.2, at 27°C. The chemical shifts of cacodylate, 17.15 ppm (27°C), or dioxane, 66.70 ppm (27°C) were used as internal references.

In some cases, where broadband decoupling was used for solutions of high ionic strength, the temperature of the sample was higher than that recorded by the temperature controller (noted by introduction of a thermocouple into the NMR tube immediately following the experiment). This was probably caused both by the position of the temperature controller's thermocouple with respect to the sample in the probe, and the localized nature of the heating. For these experiments, a capillary containing a thermometric solution was used to monitor the internal temperature. The experimental collection parameters were set, decoupling and rf power introduced and the temperature adjusted until constant as monitored by the thermometric solution. The temperature was subsequently monitored throughout the experiment, or at the end of the experiment.

The thermometric solution was composed of a 5:1 (volume

proportions) mixture of diiodomethane:cyclooctane freshly prepared for each day's experiments. Gold label 99% cyclooctane was used without further purification. 50 ml diiodomethane were washed with 10 crystals of sodium thiosulphate in 25 ml of distilled water in a separatory funnel. The diiodomethane fraction was dried with sodium sulphate, filtered through glass wool and distilled under a vacuum. The distillate was collected at 70°C, cooled and stored in the fridge under nitrogen in a sealed flask. This preparation was used repeatedly.

The temperature was determined by the difference in chemical shift, $\Delta\delta$, between the cyclooctane resonance (about 27 ppm) and the diiodomethane resonance (about -58 ppm) used in the following equation (80);

$$1/T = 0.0220027 - (0.000223362 \times \Delta\delta). \quad (8)$$

(b) ^{13}C -labelled Peptides

These experiments detail the comparison of [1-hemi-D-(^{13}C) cystine] oxytocin (1-D-Cys oxytocin) and [1-hemi-(^{13}C) cystine] oxytocin and their interaction with PS. The synthetically ^{13}C -labelled peptides were a gift of Dr. Roxanne Deslauriers. Their preparation has been reported previously (81).

(i) General NMR

The ^{13}C resonances of the peptides were monitored under a variety of conditions. Chemical shifts and linewidths were recorded. $n\text{Oe}$, T_1 and T_2 measurements were made.

(ii) PS Titrations

All operations were performed under a stream of nitrogen. A concentrated solution of PS SUV was prepared using the water bath method with ultracentrifugation as necessary. A measured volume of peptide solution (about 1.5 ml of a 1 mM peptide solution for these experiments) was placed in an NMR tube with a vortex plug and capillary. The tube was sealed with teflon tape, and a spectrum was recorded. The tube was opened and aliquots (10 to 50 microlitre portions) of the SUV preparation were introduced through the vortex plug (which was raised slightly to facilitate the operation) using volumetric micropipettes. The vortex plug and capillary were replaced and the NMR tube resealed. This was repeated until PS/peptide was at least 10 (molar ratio). In some cases, the capillary and vortex plug were removed and the final solution was frozen in the sealed NMR tube for further use. Thawed samples

were sonicated for a few seconds while still sealed. Vortex plugs and capillaries were then replaced and spectra recorded.

Five titrations were performed, three separately and two concurrently to minimize experimental differences. Chemical shifts and linewidths were recorded for each PS addition. Half-saturation values were determined graphically. During and after the titrations the temperature was varied to observe its effect on the titration curve. T_1 and T_2 measurements were made at the end of the runs to compare with those of peptide alone.

One titration was performed with 1-D-Cys oxytocin in an 18 mM NH_4OAc buffer, 23% D_2O , pH 5.4. A drop of dioxane was added as an internal reference. 16K spectra with a 20 kHz sweepwidth were collected using an add-subtract routine and continuous broadband decoupling. Two separate titrations were performed with 1-D-Cys oxytocin and oxytocin in 25 mM NaCac buffer, 10% D_2O , pH 5.3. Two concurrent titrations were made for 1-D-Cys oxytocin and oxytocin in the same buffer, but including 0.1 mM EDTA. 16K spectra (from 1000 to 8000 scans) were collected using a 15 kHz sweepwidth, 90° pulse widths from 10 to 17 microseconds and a gated decoupling sequence.

(iii) Competition with Ca^{++} and Na^+

Previously frozen PS-titration samples in NaCac buffer were used for competition studies. Aliquots of NaCl (Anachemia) or CaCl_2 (Anachemia) in NaCac buffer were introduced to the NMR tube in the same manner as the SUV outlined in the previous section. NMR collection parameters were identical to those for PS titrations. CaCl_2 was used to titrate a 12:1 PS/oxytocin mixture (using the NaCac buffer without EDTA). NaCl was used to titrate 10:1 mixtures of both PS/oxytocin and PS/1-D-Cys oxytocin in buffer containing EDTA.

(iv) Effect of pH (Peptide/PS MLV)

PS MLV were prepared in D_2O . Solutions of oxytocin or 1-D-Cys oxytocin were added together with HOAc (using the freezing and thawing method) to make 4:1 PS/peptide (molar ratio) mixtures, at pD 5.4. A drop of dioxane was added to each sample. Spectra were recorded using an add-subtract routine, continuous broadband decoupling and 10 kHz sweepwidth in 3000 scans. The pD was subsequently lowered with HCl until the sample precipitated. Spectra were recorded at pD 5.4, 4.0, 3.5 and 2.2 for oxytocin and at pD 5.4, 4.1 and 2.4 for 1-D-Cys oxytocin. T_1

measurements were made on the 1-D-Cys oxytocin/PS sample at pD 5.4 and pD 2.4.

(c) Natural Abundance Studies of Peptide and PS

(i) Peptides

All spectra were recorded with 16K data points using a 16kHz sweepwidth, a gated decoupling sequence, a 90° pulse and 2 second DELAY.

Spectra for peptide analysis were obtained of 3 mM oxytocin, 20 mM NH₄OAc buffer, 100% D₂O, pD 5.5, and 14 mM oxytocin, 7 mM 2-Gly oxytocin and 8 mM 2-Leu oxytocin in 250 mM NaCac buffer, 10% D₂O, pH 5.3. The latter three spectra were recorded in 19K to 23K scans. The spectra were checked for anomalies.

(ii) Oxytocin/PS SUV

The 16K spectra were acquired with a 15K sweepwidth at 42°C. A gated decoupling sequence was used with a 90° pulse length and a 2 second DELAY.

A spectrum was recorded of a 7.2 mM oxytocin sample in

a 25 mM NaCac buffer, 10% D₂O, 1 mM EDTA, pH 5.2 in 15K scans. A sample of PS SUV (19mM) in the same buffer was recorded in 3K scans. An aliquot of PS SUV was added to the oxytocin sample to make a 4:1 (molar ratio) PS/oxytocin mixture. The mixture became cloudy and was sonicated in a water bath for three minutes until clear. A spectrum of this sample was recorded in 27K scans. It appeared cloudy again at the end of the experiment. The sealed samples were frozen. At a later date, the samples were thawed and NaCl solution added to 24 mM. The samples were sonicated briefly. The PS sample was clear, but the PS/oxytocin sample was still cloudy. Spectra of both the PS SUV and the PS/oxytocin mixture were recorded in 24K and 38K scans respectively. After transforming, the PS spectrum was weighted and subtracted from the PS/oxytocin spectrum using a Nicolet program.

4. ³¹P NMR

(a) Standards and References

Either 85% phosphoric acid or a concentrated sample of sodium phosphate was used as a standard sample. Chemical shifts are reported with respect to an external capillary of 85% phosphoric acid at 0.00 ppm or 0.25 or 0.10 M

amino-ethyl phosphonate (AEP) at 18.60 ppm (82). For these experiments broadband decoupling was necessary and internal heating was observed as for ^{13}C experiments. A capillary was employed using a thermometric solution of 10 mM ATP, 30mM MgCl_2 , pH 7.1 and NaCl to 0.2 M ionic strength (83). An equilibration time was allowed and spectrum recorded using a 45° pulse and a 0.2 second DELAY with 2000 to 2500 scans. The capillary was present before, removed during, and replaced after the experiment. These precautions were taken because the thermometric solution itself generated heat if left in the tube for a long period. The temperature was measured by the difference in ppm between the α and β resonances of ATP. A difference of 8.48 ppm was taken to be 25°C as monitored by the variable temperature controller with a variation of 0.012 ppm/ $^\circ\text{C}$.

(b) Effect of Oxytocin and 2-Gly Oxytocin on PS MLV

PS MLV were prepared in D_2O . Four 6.2 mM PS samples were prepared, two containing 3mM NaCl, one with 1.5 mM oxytocin and the other with 1.5 mM 2-Gly oxytocin. The pH was adjusted to 5.4 with HOAc. One of the NaCl samples was sonicated in a water bath.

8K FID's were collected from all samples at ambient temperature (39°C) with a sweepwidth of 10kHz using a 50° pulse, a 0.5 second DELAY and continuous broadband decoupling. Spectra were collected with and without an 85% phosphoric acid insert. The MLV spectrum was collected in 138K scans, the SUV in 14.6K and the peptide-MLV in 65K scans. These were examined for linewidths and lineshapes. and lineshapes.

(c) T_1 Studies: Peptide/PS SUV

(i) Oxytocin/PS

A PS/oxytocin sample was prepared with 2.3 mM PS and 2.0 mM oxytocin in 20 mM NH_4OAc buffer, 16% D_2O , 10 mM EDTA, pH 5.4. PS samples were prepared from the stock solution at the same time and run under the same conditions at 40°C. 8K spectra were collected in 360 scans using a 20K sweepwidth and continuous broadband decoupling with a 7 second DELAY. The T_1 was measured together with linewidths and chemical shifts.

(ii) Arginine Vasopressin/PS

A stock solution of PS SUV was prepared in 50 mM NaCac

buffer, 10% D₂O, 0.2 mM EDTA, pH 5.3. Two 10 mM PS samples were prepared. To one of these two successive aliquots of arginine vasopressin were added giving arginine vasopressin/PS of 0.06 and 0.12. 8K spectra of 36 scans were recorded at 29°C using a 10K sweepwidth, 7 second DELAY with continuous broadband decoupling. T₁'s were measured, and chemical shifts and linewidths were recorded for the PS and peptide/PS samples.

C. ³¹P High Power Studies

1. Data Collection and Processing

A high power probe with the coil in the transverse arrangement was used for these studies. A standard sample of 200 mg per ml PC MLV in D₂O was used as a reference sample. Appropriate offsets, and the 90° pulse (4 to 7 microseconds) were determined. The efficiency of decoupling and the reliability of the complex pulse sequence were also checked. Finally, the top of the echo was set using the reference phase. A spectrum of PC was obtained and the lineshape judged to be excellent before proceeding to the more demanding experiments. Subsequent samples were fine tuned for both the observe and decoupling frequencies.

The methods advised by Rance and Byrd (84) were used with minor modifications to obtain good spectral shapes. All spectra were obtained using a Hahn echo sequence (85)

$$90^\circ - t(1) - 180^\circ - t(2) - \text{ACQ-DELAY}$$

and 8 step phase cycling as follows:

Scan	90°	180°
<hr/>		
1	-y	+y
2	-x	+x
3	+y	+y
4	+x	+x
5	-y	-y
6	-x	-x
7	+y	-y
8	+x	-x.

The top of the echo occurs at the end of $t(2)$ if $t(1) = t(2)$. $t(1)$ was set at 60 microseconds, $t(2)$ at 60 microseconds for one experiment, 20 to 30 microseconds for subsequent experiments. Acquisition before the top of the echo was reached allowed the first few points to be

discarded as these were occasionally poor due to spurious noise from switching or transmitter breakthrough. 4K FID's were obtained with 125 kHz sweepwidths and a DELAY of 2 to 4 seconds. Gated broadband decoupling was employed during the acquisition time. Using the gated decoupling sequence allowed the sample temperatures to be monitored by the variable temperature controller. When temperatures were changed, an equilibration time of at least 10 minutes was allowed before the FID was collected.

The data were transferred to the Nicolet computer and processed. Although the appropriate phases were adjusted to set a data point at the top of the echo with the PC sample, this was not possible for subsequent samples because of low signal-to-noise ratios. The point closest to the expected top of the echo was determined and the FID left shifted to that point. The FID was then transformed and a 0-order phase correction was applied to phase correct the resulting spectrum. This spectrum was erased. The FID was recalled, the 0-order phase correction applied, and an interpolation program used to set the point exactly at the top of the echo. Points collected before this were discarded and the FID transformed. No further phase corrections were made to the spectrum obtained. The centre of the powder pattern was determined using the Nicolet M1 command.

The spectra were dePaked using a program available on the Nicolet computer written by Dr. Eric Dufourc using the algorithm of Sternin (86) which calculates the 90° orientation of the spectrum. Linewidths of the dePaked spectra were determined using the Nicolet line-fitting programs. $\Delta\sigma_{\text{eff}}$ was determined by measuring the difference between the edges of the pattern and/or from the difference between the dePaked spectrum and centre of the undePaked spectrum.

2. PS MLV and Arginine Vasopressin

The MLV were prepared in the NMR tube, rather than the usual round-bottomed flask. The dry lipids were hydrated with buffer alone or with buffer and arginine vasopressin to a predetermined volumetric mark on the NMR tube. A sample of PS and PS/arginine vasopressin were prepared and analyzed concurrently. A 25 mM PS sample was prepared in 50 mM NaCac buffer, 10% D₂O, 2 mM EDTA at pH 5.1. A 20 mM PS sample was prepared in a 98 mM NaCac buffer with the same additions at pH 5.2. Arginine vasopressin/ PS samples were prepared with a 0.38 peptide/PS ratio, 6.3 mM PS in the first buffer, a 0.46 ratio, 3.7 mM PS in the second.

The temperature was recorded at 23°C to 24°C for all

samples. The more concentrated PS samples required only 1500 to 3500 scans, but those less concentrated required 11K to 12K scans. The FID's were processed with 50 Hz line broadening. The resulting spectra were dePaked.

3. PE/PS - Ca^{++} Induced Phase Transitions: The Effect of Peptides

PE/PS (4:1, weight ratio) MLV were prepared in 100 mM NaCac buffer, 10% D_2O , pH 7.0. Aliquots of buffer or peptide in buffer were added to the stock solution with freezing and thawing and the samples were sealed. Spectra were recorded, the samples were unsealed and aliquots of CaCl_2 (to prepare samples with different Ca^{++} /PS molar ratios) were added under a nitrogen atmosphere. The samples were resealed and subjected to a few freezing and thawing cycles to disperse the additions.

Initially, the phase behaviour of the lipid mixture was examined with respect to temperature and calcium concentration (as suggested by Tilcock and Cullis (87)). FID's were recorded of the following samples:

2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Sample	T(°C)	Total Lipid (mM)	Ca ⁺⁺ :PS
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1	39	25	0
	29	25	0
2	27	25	1:1
	39	25	1:1
	44	25	1:1.

Subsequent samples were run at 44°C. Spectra were recorded for the lipid or lipid and peptide sample. Additions of CaCl₂ were made and the sample was allowed to equilibrate before another spectrum was recorded. The experiment was completed in two sessions, using different lipid concentrations, but the same temperature and Ca⁺⁺/PS molar ratios. The sample additions of both sessions are listed in Table 4. During the first session, the spectra were recorded as the samples appear on the list. At the end of the session a sample identical to 1(1) was prepared with 0.5:1 Ca⁺⁺:PS and its FID recorded to ensure that changes were due to peptide added, not the age of the sample. During the second session, the samples were analyzed alternately to ensure that the addition of CaCl₂ and the recording of the spectra were made at approximately the same time for each of the samples.

TABLE 4
NMR SAMPLE CONTENT
FOR Ca^{++} INDUCED PHASE TRANSITIONS

Session 1

Sample and Addition	[Lipid] mM	[Peptide] mM	Peptide:PS	Ca^{++} :PS
1(1) no addition	12.5	-	-	-
	12.5	-	-	0.5:1
	12.5	-	-	1:1
2(1) arginine vasopressin	12.5	2.2	0.9:1	-
	12.5	2.2	0.9:1	0.5:1
3(1) oxytocin	12.5	2.2	1:1	-
	12.5	2.2	1:1	0.5:1

Session 2

Sample and Addition	[Lipid] mM	[Peptide] mM	Peptide:PS	Ca^{++} :PS
1(2) no addition	18.7	-	-	-
	18.7	-	-	0.2:1
	18.7	-	-	0.4:1
2(2) arginine vasopressin	18.7	0.66	0.2:1	-
	18.7	0.66	0.2:1	0.2:1
	18.7	0.66	0.2:1	0.4:1
3(2) lysine vasopressin	18.7	0.69	0.2:1	-
	18.7	0.69	0.2:1	0.2:1

The FID's were transformed in the usual manner. The ⁷² spectral shapes were observed and apparent phases noted.

III. FLUORESCENCE (AND UV) SPECTROSCOPY

. Equipment and Special Handling

All fluorescence and light-scattering measurements were taken with a Perkin Elmer MPF44A spectrophotofluorimeter equipped with a Perkin Elmer DCSU-2 spectral correction unit. The fluorimeter was attached to an analogue-to-digital converter which was interfaced to a Commodore Pet 32K computer for data handling. All programs were written by Dr. A.G. Szabo. A variable temperature controller was used to keep the sample compartment at a constant temperature (22°C unless otherwise noted) and the cell holders were equipped with a magnetic stirrer. A Cary 219 spectrophotometer was used to obtain UV data.

Spectrosil quartz (HELMA) cuvettes with a 1 cm pathlength filled with at least 2 ml of solution were used for all experiments. They were bubbled gently with a stream of high quality nitrogen for 5 to 7 minutes prior to use. All transfers to the cuvettes were also made under high quality nitrogen. Samples were allowed to equilibrate

in the fluorimeter chamber for 10 minutes before a spectrum was recorded. Each cuvette contained a small magnetic stir bar to stir the solutions throughout the experiment.

It was found that the peptide fluorescence of samples containing unsaturated lipids were sensitive to quenching effects from contaminated lipid preparations. Quenching of fluorescent signals was observed at a level of impurity lower than that required for TLC detection. For this reason, every precaution was taken to ensure that preparations were maintained under high quality nitrogen. All SUV were prepared with the Branson water bath (see I.C.3(c)). Unfortunately, the small volumes prepared were often reduced and splattered on the sides of the round-bottomed flask when a stream of nitrogen was directed at the surface. To avoid this problem, stock lipid solutions were placed in the bottom of a glass-stoppered flask which had a glass sidearm joint at the top. This was connected to a glass tube which delivered nitrogen. In this way the stock solution was kept under a nitrogen atmosphere at all times. The KWK assay was devised to avoid running experiments with contaminated lipids (see III.C).

Organic solvents used were Spectroscopy grade (Fisher

Scientific). To remove fluorescent contaminants, glass or teflon equipment was first heated in dimethyl formamide then acid-washed before use.

B. General Methods of Obtaining Spectra

1. UV Spectra

The Cary 219 spectrophotometer was equipped with an automatic baseline feature and automatic slit adjustment for scanning. Two cuvettes, a reference and a sample cuvette, were filled with buffer (or solvent), placed in the spectral chamber and an automatic baseline was determined over the range of 380 nm to 240 nm. A spectrum was recorded to ensure that the baseline was flat. If more than one sample cuvette was to be used, the others were checked at this time against the reference. The sample cuvette was filled with the sample (in the same buffer or solvent) to be analyzed and the UV spectrum scanned and recorded over the appropriate range. If the absorbance, $A(\text{nm})$, at a single wavelength was required, the same procedure was used, but the spectrophotometer was set at the wavelength of interest and the absorbance either recorded on paper, or read from a digital meter.

2. Emission and Excitation Spectra

The sample solution was placed in the fluorimeter chamber together with a 'blank' solution (buffer or solvent without the sample of interest), and left to equilibrate. For emission spectra, the exciting light was set at the chosen wavelength of absorption, $\lambda(\text{ex})$ with the appropriate slit width, and the light emitted from the sample was scanned over the expected range (in corrected mode) with the appropriate slit width and often using a cut-off filter. This was repeated in identical fashion for the blank solution. The spectra were recorded on paper and generally stored in the computer in digitized form (points at 0.5 nm intervals). The blank spectrum was subtracted from the sample spectrum to give the 'blank-subtracted' spectrum which is the one reported and the one on which all calculations were made. Excitation spectra were obtained in exactly the same way, but observing the intensity of the emitted light at one wavelength, $\lambda(\text{em})$, while scanning over the expected range of the exciting light. Wavelength maxima, $\lambda(\text{max})$ were determined by the computer. Fluorescent intensities at a specific wavelength, $I(\text{nm})$, were either obtained from the computer, or calculated from the paper record. Details of filters, slit widths and wavelengths used are noted for each experiment.

C. KWK Assay for SUV Preparations

In view of the problem with quenching of peptide fluorescence by lipid SUV, and the difficulty of finding methods to assess the preparations, this assay was devised as a practical solution to the problem.

The peptide, Lys-Trp-Lys (KWK), was chosen for this assay as on the addition of lipid, the tryptophan spectrum undergoes both a blue shift and an intensity enhancement (88). Thus the compound presented two parameters with which to check the lipid. KWK (Research Laboratories Inc) was purified by Mr. Don Krajcarski. It was found that on addition of excess PS, the maximum blue shift was always 15 nm, but the intensity enhancement varied for different preparations (see Figure 2). The variation was linked to the quenching observed when lipid was added to peptide solutions; the lower the enhancement, the more quenching observed.

At 10:1 PS/KWK (molar ratio), a good preparation of lipid gave an enhancement of at least 1.7 at 337 nm for a solution of KWK in 1 mM NaCac buffer, pH 7.0 (89). This was experimentally confirmed as the enhancement criterion for experiments using a 25 mM NaCac buffer, pH 5.3.

FIGURE 2. The Use of KWK to Monitor the Purity of PS SUV

The relationship of signal enhancement to wavelength maximum of the spectra of KWK at 22°C for different PS preparations.

- 1 mM NaCac, 1 mM NaCl, 0.2 mM EDTA, pH 7.0
- Δ 25 mM NH_4OAc buffer, pH 5.3
- 25 mM NH_4OAc buffer, pH 5.3, PS briefly exposed to air

Subsequently all lipid preparations were checked in the following manner. Preparations that did not meet the expected enhancement were discarded.

The emission spectrum of a 0.02 mM KWK solution was obtained from 300 nm to 450 nm with λ (ex) of 290 nm. Slits for excitation and emission were 3 nm and 4 nm respectively. An aliquot of the stock lipid solution was added to make a minimum of 20:1 lipid/KWK solution and a spectrum was obtained in identical fashion (using a buffer and lipid solution as the 'blank'). The enhancement was measured as the ratio of the blank-subtracted intensities at 335 nm before and after lipid addition. For samples containing PS, an enhancement over 1.7 (at 22°C to 25°C) for a 15 nm blue shift was required before proceeding with the lipid preparation.

D. Fluorescence (and UV) of Tyrosine-Containing Peptides

1. UV Absorption, Fluorescence Emission and R_{Tyr} Calculations

UV spectra were obtained for the peptides. Scans were recorded from 350 nm to 245 nm. Emission spectra were

obtained using an excitation wavelength of 270 nm and scanning the emission from 275 nm to 400 nm. Excitation and emission slits were 3 nm and 4 nm respectively. These were all recorded in 25 mM NaCac buffer, pH 5.3 at 22°C for fluorescence measurements, ambient temperature for UV spectra.

R_{Tyr} (90) was calculated for oxytocin, arginine vasopressin and 1-D-Cys oxytocin (the ^{13}C -labelled peptide used for NMR see II.B.3(b)) using the following formula;

$$R_{\text{Tyr}} = [a(\text{pep})/a(\text{Tyr})] \times [A(\text{Tyr})/A(\text{pep})]. \quad (9)$$

$a(\text{Tyr})$ refers to the area under the corrected blank subtracted spectrum of a solution of tyrosine in 10 mM NaCac buffer, pH 7.0, at 22°C. $a(\text{pep})$ refers to the area under the emission spectrum of the peptide in 25 mM NaCac buffer, pH 5.3 at the same temperature, and collected under the same spectral conditions. The computer was used to calculate the relative areas from the spectra (275 nm or 280 nm to 400 nm) obtained with $\lambda(\text{ex})$ of 270 nm or 275 nm. Slit widths were 2 nm for exciting light and 2 or 3 nm for emitted light. $A(\text{Tyr})$ and $A(\text{pep})$ refer to the absorbance of the solutions at the exciting wavelength. R_{Tyr} was also calculated using the following formula;

$$R_{\text{Tyr}} = [I(310, \text{pep})/I(310, \text{Tyr})] \times [A(\text{Tyr})/A(\text{pep})] \quad (10)$$

where $A(\text{Tyr})$ and $A(\text{pep})$ have the same meaning as above, but are multiplied by the reciprocal of the emission intensities at 310 nm, rather than the reciprocal areas. Calculations performed using the latter formula gave R_{Tyr} values within the error of those calculated using areas.

R_{Tyr} values were estimated for arginine vasopressin, oxypressin, desamino oxytocin and desamino lysine vasopressin with reference to R_{Tyr} for oxytocin calculated as above, $R_{\text{Tyr}}^{\text{OT}}$,

$$R_{\text{Tyr}} = [I(310, \text{pep})/I(310, \text{OT})] \times [A(\text{OT})/A(\text{pep})] \times R_{\text{Tyr}}^{\text{OT}}. \quad (11)$$

The intensities were taken from spectra obtained in exactly the same way. Excitation was at 270 nm, using 3 nm slits. Emission slits were 4 nm. Concentrations were in the 0.1 mM to 0.3 mM range in 25 mM NaCac buffer, pH 5.3 at 22°C.

2. Polarization Experiments

(a) Basic Description of Titrations

Stock solutions of lipid SUV were prepared. The KWK assay was performed before the experiment took place. In addition, emission spectra were obtained ($\lambda(\text{ex})$ 270 nm, 3 nm (ex) slits, 4 nm (em) slits) and a measure of the UV absorbance at 270 nm was made for each peptide. A polarization value was then determined. All measurements were made at 22°C. Aliquots of SUV were then added to the cuvette containing a measured volume of peptide solution, and to one containing an equal volume of buffer. The UV absorbance of both solutions was recorded. They were allowed to equilibrate in the fluorimeter chamber and a new polarization value was obtained. This was repeated for each lipid addition until there was no significant change in polarization values for three consecutive additions (the plateau region). At the end of the experiment emission spectra were recorded. The stock solutions were subjected to phosphorus analysis. The lipid concentration of each sample was determined by a dilution factor from the known concentration of the stock.

(b) Polarization Measurements

Polarization measurements were made using UV transmitting polaroid film HNBPB for Polaroid Inc. Samples were excited at 270 nm using a 4 nm slit width. Intensities were sampled at 310 nm for a fixed period (30 to 60 seconds) using emission slits from 6 nm to 10 nm (15 nm in one experiment) depending on the lowest emission signal. Slits were kept constant during one experiment but were varied from one experiment to another. A 290 nm cut-off filter was employed to minimize artefacts due to scattered exciting light. The average intensities at 310 nm, $I(310)$, were stored in the computer. Calculations were made using the computer record. Intensities were also recorded on paper for visual observation.

The sample of interest was illuminated with the exciting polarizer in the vertical position, V, and $I(310)$ was measured with the emission polarizer in first V, and then in H, the horizontal position, giving $I(VV,obs)$ and $I(VH,obs)$. This was repeated for a solution of buffer, or a solution of lipid and buffer with the same lipid concentration as the peptide-lipid solution. These values are labelled $I(VV,blank)$ and $I(VH,blank)$. The values used to calculate the polarization were

	B	C	D
			3 DHNDB
% MIX	2 CA9C	6 DHNDB	+1 CA9C
0	1.0	1.0	0.0
20	0.8	0.8	0.4
40	0.6	0.6	0.8
60	0.4	0.4	1.2
80	0.2	0.2	1.6
100	0.0	0.0	2.0

A sample containing 1 ml of B (CA9C) and 1 ml of A (SUV only) was prepared to compare with the 0% sample. A slight difference in the I(460) was noted between these. This was taken as 'background fusion', and subtracted from the final values calculated. With this exception, a graph of I(460) as a function of % mix was linear. 2 ml of sample A (SUV) was used as a blank. Theoretically, the % mix of a fused sample could be determined from this graph.

Unfortunately the samples did not behave well once calcium was added. A period of apparent fusion was observed, followed by a period of flocculation during which emission signals from all samples dropped. The addition of

Scattering by the SUV solutions was a problem. This limited the concentration ranges that could be used for the experiments. The polarizers tended to reduce the intensity of the tyrosine emission, but increasing the concentration of the peptide resulted in having to increase the amount of lipid added to attain the same molar ratios. At high lipid concentrations, the scatter became more intense than the emission signal, and the blank subtracted values were meaningless.

Checks were made to ensure that the blank subtract method was valid for these experiments. The light scattering of each sample and blank was compared to ensure that the scattering properties were the same for peptide-lipid and lipid samples. This measurement was made for each lipid addition, after the polarization measurements were made. The polarizers were left in the VV position. Intensity measurements with emission and excitation wavelengths both at 270 nm and slits at 2 nm were recorded for samples of buffer, A, buffer and peptide, B, buffer and SUV, C, and buffer and lipid and peptide, D.

$$I(\text{lipid}) = I(C) - I(A), \quad (17)$$

and

$$I(\text{lipid,peptide}) = [I(D) - I(B)] / 10^{-A}, \quad (18)$$

where A is the absorbance of the peptide at 270 nm. This equation accounts for the loss in intensity at 270 nm due to absorbance rather than scattering. A ratio, $I(\text{lipid,peptide})/I(\text{lipid})$ or R_{SC} , of 1.0 indicated that the scattering properties were similar and the blank-subtraction valid.

In addition to the direct effects of light scattering on the measured polarization values, indirect effects can be seen as a decrease in polarization caused by the scatter of the exciting or emitted photons (91). A method of correcting this problem has been applied to membrane systems (92) by measuring the anisotropy as a function of the turbidity of the lipid solution, $OD(\text{lipid})$, such that

$$r(\text{obs}) = -r'K OD(\text{lipid}) + r' \quad (19)$$

where r' is the anisotropy extrapolated to $OD = 0.0$ and K is a constant. Usually, this is tested by diluting the sample to effect a change in $OD(\text{lipid})$. Due to the nature of these titrations, an increase in OD was used. At the end of the experiment, the final sample contained a certain ratio of lipid:peptide. The concentration of lipid and

peptide were increased, keeping the ratio the same.

Polarization values and OD's were measured in the usual way. Generally, three measurements were made, K and r' determined and p' calculated from

$$p' = 3r'/(r' + 2) \quad (20)$$

for each titration step assuming K was constant over the entire titration.

(c) Experimental Details

(i) SUV/Peptide Titrations at pH 5.3

Initial experiments were performed with PS/oxytocin using an 18 mM NH_4OAc buffer. These required correction as $r(\text{obs})$ was not equal to r' . When the 25 mM NaCac buffer was substituted such corrections were unnecessary. The NaCac buffer was used for further experiments. Arginine vasopressin, lysine vasopressin, oxypressin, 1-D-Cys oxytocin, desamino oxytocin and desamino lysine vasopressin were titrated using PS SUV. PS was added to at least a 15:1 PS/peptide molar ratio. Peptide concentrations were in the 0.1 to 0.3 mM range. Similar titrations were performed on the same peptides using PC SUV. Oxypressin

and arginine vasopressin were titrated with PC/PS (4:1 weight ratio) SUV as well. 4 nm excitation slits were used at 270 nm. Emission slits ranged from 6 nm to 10 nm with a 290 nm cut-off filter with the exception of experiments with 1-D-Cys oxytocin.

A comparison of PS titrations of oxytocin and 1-D-Cys oxytocin was performed at 0.1 mM peptide. The emission slit had to be opened to 15 nm, and the excitation slit to 5 nm because the latter peptide has a very low emitting efficiency. For this reason the samples were run in the same session and under exactly the same conditions in order to compare the results.

Plateau polarization values, p_b , and initial values, p_f , were determined and averaged for each titration. Half-saturation values were determined graphically.

A measure of the change in quantum yield between free peptide and PS-bound peptide (in the plateau region) was made for oxytocin and oxypressin by calculating $R_{Tyr}(\text{bound})/R_{Tyr}(\text{free})$ from equation (10) (Section D.1) from spectra collected using excitation and emission slits of 3 and 4 nm respectively before and after titration. A standard tyrosine sample in the same buffer was used as a

reference. The same measurement was made for oxytocin and 1-D-Cys oxytocin using excitation and emission slits of 2 and 3 nm respectively from both equation (9) and equation (10). The absorbance of the final solutions was very small in comparison to the turbidity due to scatter, therefore, the absorbance was calculated by dilution from the initial solution. The absorbance of the peptide did not appear to be affected by PS in the initial steps of the titration, where it was possible to subtract the turbidity.

(ii) PS/Oxytocin Titrations: Effect of Buffer Strength and pH

Oxytocin (0.1 mM) was titrated with PS at pH 5.3 with a 12.5 mM NaCac buffer, and at pH 6.2 and 7.0 with the 25 mM NaCac buffer. Half-saturation values and plateau polarization values were obtained as above.

E. Energy Transfer: Ca^{++} -Induced Fusion of PE/PS SUV

1. Introduction

The fusion process was initiated by adding aliquots of CaCl_2 to PE:PS vesicles. The fusion process was terminated by the addition of Na_4EDTA . Mixing of vesicle

contents was followed by the method of Uster and Deamer (93) with some modifications. This technique observes the transfer of energy between two populations of vesicles. One population contains a donor probe (CA9C) which, when excited, transfers its energy via a non-radiative pathway to an acceptor probe (DHNDB) located in the second population. The efficiency of transfer depends (among other things) on the distance between the two probes. Theoretically, transfer can only occur when the lipids of the two vesicle populations mix. The extent of mixing can be measured with reference to artificially mixed vesicle populations.

2. Preparation of Probe - SUV Populations

The probes cholesteryl anthracene 9-carboxylate (CA9C) and 4-dihexadecylamino-7-nitrobenzo-2-oxa-1,3-diazole (DHNDB) were obtained from Molecular Probes. The probes were examined in THF, EtOH and H₂O solutions using UV, excitation and emission spectra and used without further purification. Spectra were also obtained of the probes in PE:PS SUV.

All results were obtained using a 100 mM NaCac buffer at pH 7.0. PE/PS (4:1 or 3:1 weight ratio) were prepared.

Stock solutions of CA9C and DHNDB were prepared in THF. The probes were originally introduced into the SUV as in (88), outlined as follows. Aliquots of the stock SUV were removed to four 50 ml or 25 ml volumetric flasks wrapped in foil and containing magnetic stir bars. These were diluted with buffer and stirred. Microlitre quantities of the stock probe solutions were added to each of the four flasks which were then stirred for 10 to 20 minutes at room temperature. The probes represented 0.8 to 1.2% of the total lipid (mole percent). The four flasks contained SUV and probes in the following proportions:

- A. SUV
- B. SUV and 2[CA9C]
- C. SUV and 6[DHNDB]
- D. SUV and 3[DHNDB]+[CA9C]

The spectra recorded of these preparations indicated that the DHNDB probe was not well incorporated in the vesicles (an emission maximum was observed at 560 nm, similar to that in water). As little transfer took place between the probes in buffer at the concentrations used for these experiments (experimental observation), the transfer observed for experiments using vesicle populations prepared in this way was assumed to arise from probes that were incorporated into the bilayer.

Later experiments employed a different method of incorporation. Probes and/or THF were added to four equal methanol-lipid mixtures prior to the overnight lyophilization step (see I.C.3(a)). All four samples were hydrated with buffer, sonicated and diluted in volumetric flasks to make the same solutions as listed above. The spectra of these samples indicated that a greater proportion of the DHNB was incorporated in the bilayer using this method (emission maximum located at 525 nm, with a shoulder at 560 nm).

3. General Method

A calibration curve for fusion was prepared. The decrease in CA9C donor emission was monitored. $I(460)$ was measured for the following 2 ml samples using a 430 cut-off filter and a 4 nm emission slit. Excitation was at 385 nm or 370 nm using a 3 nm slit. The samples were prepared from the stock SUV-probe solutions. Solution D contains the probes in the same vesicle population. By varying the proportion of this 'mixed' solution from 0% to 100%, all fusion possibilities are modelled.

	B	C	D
			3 DHNDB
% MIX	2 CA9C	6 DHNDB	+1 CA9C
<hr/>			
0	1.0	1.0	0.0
20	0.8	0.8	0.4
40	0.6	0.6	0.8
60	0.4	0.4	1.2
80	0.2	0.2	1.6
100	0.0	0.0	2.0

A sample containing 1 ml of B (CA9C) and 1 ml of A (SUV only) was prepared to compare with the 0% sample. A slight difference in the I(460) was noted between these. This was taken as 'background fusion', and subtracted from the final values calculated. With this exception, a graph of I(460) as a function of % mix was linear. 2 ml of sample A (SUV) was used as a blank. Theoretically, the % mix of a fused sample could be determined from this graph.

Unfortunately the samples did not behave well once calcium was added. A period of apparent fusion was observed, followed by a period of flocculation during which emission signals from all samples dropped. The addition of

EDTA (to 3 times the Ca^{++} concentration) with stirring after flocculation redispersed the lipid and stabilized the signal.

The emission I(460) of samples of 0% mix (CA9C), 100 % mix (CA9C + DHNDB), and the fusion sample ((CA9C)+(DHNDB)) were recorded after flocculation and EDTA addition as a function of CaCl_2 concentration. Increases were observed for all three samples as a function of increasing calcium. However the transfer efficiency appeared to decrease with increasing calcium. The largest change occurred in the 100% mix sample. A large sample of C and A (DHNDB and SUV)) was prepared. CaCl_2 was added and the sample stirred continuously. At timed intervals 2 ml samples were withdrawn and placed in cuvettes containing EDTA. The probe was excited at 485 nm and full emission spectra were recorded. It was clear by the decrease in the emission of DHNDB at 570 nm, that this probe was excluded from the bilayer during the flocculation period. Therefore transfer efficiency was much reduced after flocculation. While CA9C emission was relatively unaffected by calcium, it was affected slightly by the addition of peptide.

For these reasons, the I(460) of three samples was monitored for every addition:

1. 1 ml A and 1 ml B - SUV and (CA9C), 0% mix
2. 1 ml B and 1 ml C - SUV and (CA9C) + (DHNDDB), sample
3. 2 ml D - SUV and (CA9C + DHNDDB), 100%

mix.

Excitation was at 370 nm or 385 nm using 3 nm slits (a 4 nm slit was used for emission). Occasionally full spectra were recorded at the end of the experiment. Each sample was incubated for 5 to 10 minutes in the fluorimeter chamber. An aliquot of buffer, or peptide was added and the sample was incubated again for 3 to 8 minutes. An aliquot of CaCl_2 was added followed by an aliquot of EDTA 2 to 10 minutes later. The reaction was monitored either continuously or at timed intervals until 2 or 3 minutes after EDTA addition when the signal had stabilized. A paper record was maintained. The three samples were treated identically.

% mix was measured in the following manner using the $I(460)$'s recorded at exactly the same time, t , after being placed in the fluorimeter chamber. The 'background' fusion was calculated from the apparent % mix at time $t=0$. This was subtracted from the % mix calculated for time t .

$$\begin{aligned} \% \text{ mix} = & ([I(1) - I(2)]/[I(1) - I(3)]_{t=t} \times 100) - \\ & ([I(1) - I(2)]/[I(1) - I(3)]_{t=0} \times 100) \quad (21) \end{aligned}$$

The 'kinetics' of the process could also be followed when the I(460) was monitored continuously. The time to flocculation, $t(\text{floc})$, was measured as the time from calcium addition to the intercept of the initial and final slope of the I(460) at flocculation.

4. Experimental Details

(i) PE:PS, 4:1

The time course of flocculation of a 0.3 mM SUV preparation was measured as a function of CaCl_2 concentration from 1 mM to 7 mM. The effect of 0.01 and 0.05 mM arginine vasopressin was observed for the 4 mM CaCl_2 addition. % mix was measured for a 0.4 mM SUV preparation with 7 mM CaCl_2 5 minutes after initiation (post-flocculation). This was repeated for samples containing 0.01 and 0.05 mM arginine vasopressin.

(ii) PE:PS, 3:1

% mix was measured at 5 minutes after initiation (post-flocculation) for samples containing 2 to 4 mM CaCl_2 and 0.01 or 0.05 mM arginine vasopressin. The THF method was used to introduce the probes to 0.3 mM SUV.

A 0.15 mM SUV preparation (for which the probes were introduced prior to sonication) was used to follow the time course of a sample initiated with 1 mM calcium. % mix was measured two minutes after initiation (pre-flocculation). Similar measurements were made for samples containing 0.05, 0.1 and 0.2 mM arginine vasopressin and 0.2 mM oxytocin.

F. Light Scattering: The Effect of Divalent Peptides on SUV

1. General Methods

For these experiments samples of SUV were titrated with aliquots of peptide. In order to compare the effect of peptides, titrations were run concurrently with aliquots added to each sample alternately. This avoided the possibility of differences due to the age or preparation of the SUV. Concentrations of the stock solution were measured using phosphorus analysis. The sample concentration was determined by dilution.

Light scattering was measured at 325 nm or 355 nm as noted using excitation slits of 1 or 2 nm and emission slits of 2 nm. Paper records were kept of the intensities measured, I . These were sampled for 30 to 60 seconds. During the experiments there were occasions of flocculation

when the measured intensities fluctuated wildly. To account for this, several readings were made over a period of time and these were averaged.

R_{SC} was obtained as described for the polarization experiments (III.D.2(b)) but without the use of polarizing film. I is measured for samples containing buffer, A, buffer and peptide, B, buffer and lipid, C, and buffer and lipid and peptide, D. The intensities of samples A and B are subtracted from samples C and D. In these experiments the intensities of the blank samples A and B were small enough to be neglected. At 270 nm, the absorbance of the peptides was large, and correction was required. However, at 325 nm this was reduced to a 5% required correction at the highest peptide addition, and was not necessary for measurements at 355 nm.

2. Experimental Details

The effect of arginine vasopressin, lysine vasopressin and oxytocin on PS SUV was observed at 325 nm. R_{SC} was determined and plotted as a function of peptide added. PS concentration was 0.3 mM. Two samples at two different additions of arginine vasopressin were subjected to electron microscopy along with a PS-buffer sample to act as a control.

A series of titrations were performed to observe the effect of NaCl on this phenomenon. A stock solution of PS SUV prepared with the 25 mM NaCac buffer at pH 5.3 was divided into five portions. Aliquots of NaCl in the same buffer were added to four of these samples to make them 25 mM, 75 mM, 175 mM and 375 mM in NaCl. The volumes of all five samples were adjusted to be equal. This gave samples of 25 mM, 50 mM, 100 mM, 200 mM and 400 mM in total Na⁺ including that from the 25 mM NaCac already present. 0.07 mM PS samples of these were titrated with arginine vasopressin. Scattering ratios were monitored at 325 and 355 nm.

CHAPTER THREE: RESULTS AND DISCUSSION

I. General Behaviour of Peptide-Lipid Systems and Experimental Limitations

The techniques used in this study are all dependent on the size of the vesicle observed. For NMR spectroscopy, high resolution studies require SUV, broad line studies require MLV. Fluorescence studies require the emission to be considerably greater than the scattering of the solution. The physical consequences of the binding of peptides to pure PS membranes at the pH values and ionic strengths used presented a major experimental limitation. Divalent peptides caused flocculation of the SUV which could be followed using light scattering techniques. This limited the use of techniques requiring SUV structures. Conversely, the addition of oxytocin (monovalent) to MLV promoted formation of small vesicles. This was discovered when binding studies using centrifuge methods to separate the MLV/peptide mixtures from unbound peptide indicated increasing lipid in the supernatant with the addition of oxytocin. This phenomenon was observed for 2-Leu oxytocin also, and to a lesser extent with 2-Gly oxytocin. The presence of small vesicles was confirmed by electron

microscopy and ^{31}P high resolution examination. This limited the use of the monovalent peptide for MLV studies. Mixed lipid systems were not as sensitive.

The type of binding and the stoichiometry were examined by observation of the peptides using high resolution NMR and fluorescence methods. This was mainly limited to the monovalent peptides interacting with PS and PC. The effect of divalent peptides, compared to that of oxytocin was noted by observation of the lipids. ^{31}P NMR was used to examine the phosphodiester moiety of PS. Enhancement of Ca^{++} induced hexagonal phase changes for PE/PS mixtures was examined with ^{31}P NMR. Correlated to this, enhanced fusion of PE/PS vesicles was examined using fluorescence energy transfer and the aggregation of PS SUV was studied using light scattering techniques. Initial attempts to determine the effect of peptides on the surface potential of PS SUV are outlined in the Appendix.

II. Binding and Stoichiometry Using Fluorescence and NMR Techniques to Observe Peptide Behavior

A. Introduction

Fluorescence spectroscopy was used to examine the general behaviour of the tyrosine-containing peptides arginine vasopressin, lysine vasopressin, desamino lysine vasopressin, oxypressin, oxytocin, [1-hemi-D (3 ^{13}C) cystine] oxytocin (1-D-Cys oxytocin) and desamino oxytocin when free and bound to PS SUV at pH 5.3. Variations of concentration, ionic strength and pH were examined as well as the effect of PC and PC/PS mixed vesicles on some of the analogues.

NMR was used to examine ^{13}C -labelled peptides for a more detailed analysis of the binding of oxytocin and the 1-D-Cys analogue. These have the carbon label in the backbone of the ring structure. Binding of these peptides to neurophysin has been studied (81). Oxytocin exhibits changes in chemical shift and T_1 on binding, but 1-D-Cys oxytocin does not bind. The effect of pH, NaCl and CaCl_2 concentrations were also examined as the technique is not as sensitive to scattering problems as fluorescence. The natural abundance spectrum of oxytocin, PS and the

oxytocin/PS complex was also observed for an overall view of the molecule, but without the advantages of the signal-to-noise enhancement and lack of complexity of the labelled material. These results were compared with fluorescence emission and polarization experiments.

B. Fluorescence Spectroscopy of Tyrosine-Containing Peptides

1. Peptides: Fluorescence and Polarization.

The peptides all exhibited the tyrosine fluorescence maximum of 302 ± 1 nm. However, the emission amplitudes varied. R_{Tyr} values were determined for all peptides with the exception of lysine vasopressin and are shown in Table 5. Quantum yields of the peptides are sensitive to pH (ionization of the terminal amino group). Literature values (R_{Tyr}) for oxytocin range from 0.26 (49,50) at pH 7.8 and in water, to 0.17 (51) at pH 3. It is clear that the increased value for the desamino peptides with respect to the charged peptides is due to this phenomenon. At pH 5.3, oxytocin is completely ionized. The experimental value of 0.16 is acceptable. The very low value for the 1-D-Cys analogue may be due to a difference in the spatial arrangement of tyrosine with respect to the disulfide bond.

As pointed out by Cowgill (94), the tyrosine of oxytocin does not show the quenching expected due to the proximity of the disulfide bond. This is thought to be due to steric strain preventing tyrosine from approaching the bond to within a Van der Waals distance. Ross et al (51) has shown some static quenching for oxytocin and desamino oxytocin that is not evident when the disulfide bond is replaced with a carbon analogue. However, the arrangement in the 1-D-Cys analogue most likely allows a closer approach leading to the expected quenching and low R_{Tyr} value. A comparison of the quantum yield and lifetime with respect to tyrosine would indicate this possibility. This low emission caused some difficulty in the fluorescence experiment.

Polarization values (p) were also determined for the peptides and are shown in Table 5 together with τ_R , the rotational correlation times for oxytocin and desamino oxytocin estimated from the simple form of the Perrin equation (95):

$$\left(\frac{1}{p} - \frac{1}{3} \right) = \left(\frac{1}{p_0} - \frac{1}{3} \right) \left(1 + \frac{RT\tau}{\eta v} \right) \quad (22)$$

TABLE 5

FLUORESCENCE DATA FOR PEPTIDES

	R_{Tyr} (± 0.01)	p	τ_R (10^{-10} s)
arginine vasopressin	0.14	0.09 $\pm$ 0.01	
desamino lysine vasopressin	0.25	0.058 $\pm$ 0.005	
oxyressin	0.14	0.088 $\pm$ 0.009	
oxytocin	0.16	0.069 $\pm$ 0.008	2.5 $\pm$ 0.4
desamino oxytocin	0.21	0.057 $\pm$ 0.006	2.4 $\pm$ 0.3
[1-hemi-D (3^{13}C) cystine]			
oxytocin	0.05	0.13 $\pm$ 0.01	
lysine vasopressin		0.08 $\pm$ 0.01	

data obtained at 22°C, in 25 mM NaCac buffer, pH 5.3

where p is the observed polarization, p_0 is the intrinsic polarization, τ is the fluorescence lifetime, R the gas constant, T the temperature, η the viscosity and V is the molar volume. In this case

$$\tau_R = (V\eta/RT) \quad (23)$$

assuming the molecule to be a rigid sphere. Correlation times were determined using the lifetimes of 0.99 ns for oxytocin and 1.2 ns for desamino oxytocin found by Ross et al (51), and the intrinsic polarization as 0.31 at 270 nm extrapolated from the data of Lackowicz and Maliwal (52) and listed in Table 5. The values are similar to that measured for the Cys-1 β carbons of oxytocin (81), 2.6×10^{-10} s.

2. Effect of PS on Peptide Fluorescence and Polarization

No change was observed in the emission wavelength for any of the analogues on the addition of PS. There was a very slight decrease in emission amplitude (to 20%) which is insignificant given the dilutions and effects of scattering. Both tyrosine and tryptophan have been shown to exhibit large changes in amplitude when inserted into the hydrophobic core of membranes (88) due to the change in

the dielectric of the solvent. The fact that none of the peptides exhibit such a change is significant and suggests that the interaction does not have a hydrophobic element and that the peptides bind at the surface of the bilayer- a peripheral or extrinsic interaction.

By their polarization profiles as shown in Figure 3, the peptides can be grouped into three classes; the divalent peptides, arginine and lysine vasopressin, which have positively charged lysyl or argininy1 side chains and a charged terminal amino group; the terminal α amino monovalent peptides, oxytocin and oxypressin; and the desamino peptides, desamino oxytocin which is uncharged, and desamino lysine vasopressin which is monovalent, with a positively charged lysyl side chain, but no terminal amino group. The divalent peptides appear to be affected the most by PS, however, these values were found to be artefactual as the divalent peptides caused flocculation and thus increased scattering in the sample. This was easily detected by the light scattering method (Experimental, III.D.2.(b)). The polarization profile of the desamino peptides indicates only a slight increase in response to PS addition. This could be attributed to the increased viscosity of the solution as PS vesicles are added, and suggests that the desamino peptides do not bind.

FIGURE 3: Polarization Profiles of Peptides Titrated with
PS SUV

Peptides were 0.1 mM in 25 NaCac buffer, pH 5.3 at 22°C

□ arginine vasopressin

■ lysine vasopressin

△ oxypressin

▲ oxytocin

○ desamino lysine vasopressin

● desamino oxytocin

However, it could also be possible that they do bind, but that the tyrosine chromophore is not as affected by binding as in the α amino peptides. Detailed studies could be performed on the terminal α amino monovalent peptides only.

3. Polarization Profiles of Oxytocin, Oxypressin and 1-D-Cys Oxytocin

The results for a series of experiments are outlined in Table 6. When p is plotted as a function of $[PS]_{\text{outer}}/[peptide]$, the profiles are similar to that shown in Figure 4 for PS. The profiles all show an increase in polarization with a plateau region at ratios of +10 for oxytocin and +7 for oxypressin. The final polarization values reflect an increase in error with respect to the initial values due to the increased scattering of the PS solution. This is also reflected in the half-saturation values.

For oxypressin, a variation of 0.1 to 0.2 mM in concentration does not lead to a significant change in half-saturation values. Thus the profile represents a titration with virtually no free PS until the plateau is reached. On this basis it is possible to estimate that there are 3 to 4 PS molecules bound per oxypressin

TABLE 6
FLUORESCENCE DATA FOR POLARIZATION CURVES OF α -AMINO PEPTIDES TITRATED WITH PS (22°C)

[Peptide] (mM)	[NaCac] (mM)	pH	p _f	p _b	$\frac{1}{2}$ Saturation [PS] _{outer} [Peptide]	Slit Width (exc,em) (nm)	$\frac{T_R(b)}{T_R(f)}$	$\frac{R_{Tyr}(b)}{R_{Tyr}(f)}$ $\pm 0.2^1$
Oxytocin								
0.08	25	5.3	0.060	0.20	4 $\pm$ 1	4,8	7.6	0.8
0.13	25	5.3	0.073	0.21	2.7 $\pm$ 0.3	4,8	6.8	
0.15	25	5.3	0.060	0.20	2.7 $\pm$ 0.6	4,8	7.6	0.8
0.19	25	5.3	0.076	0.22	2.2 $\pm$ 0.4	4,6	7.5	
0.29	25	5.3	0.074	0.19	1.7 $\pm$ 0.5	4,10	5.1	
0.10	25	6.2	0.078	0.22	2.5 $\pm$ 0.5	4,7	7.3	
0.10	25	7.0	0.078	0.22	3.0 $\pm$ 0.5	4,8	7.3	
0.10	12.5	5.3	0.084	0.23	1.7 $\pm$ 0.3	4,7	7.7	
Oxypressin								
0.11	25	5.3	0.101	0.24	1.5 $\pm$ 0.2	4,8	7.8	0.8
0.11	25	5.3	0.086	0.23	2.2 $\pm$ 0.4	4,7	7.5	
0.22	25	5.3	0.081	0.24	1.7 $\pm$ 0.2	4,8	9.7	0.8
0.24	25	5.3	0.085	0.21	1.8 $\pm$ 0.3	4,8	5.6	
[1-hemi-D (3 ¹³ C) cystine]								
Oxytocin								
0.10	25	5.3	0.131	0.20	1.8 $\pm$ 0.3	5,15	2.5	0.9 (1.2)
Oxytocin								
0.10	25	5.3	0.079	0.17	3.8 $\pm$ 0.8	5,15	3.6	1.0 (1.1)

1. Calculated using Equation 9, brackets denote calculation using equation 10, see Chapter III Experimental III.D(c)(i)

molecule. It also indicates that the apparent binding constant is on the order of or greater than 10^4M^{-1} .

The half-saturation values for oxytocin do show more variation than those for oxypressin. There is a significant difference between the low and high concentration range. The half-saturation value for the lower buffer concentration (12.5 mM) is similar to that of oxypressin. For a peripheral association, binding would be expected to increase with decreasing salt concentration. This suggests that the number of PS molecules bound to oxytocin is similar to that of oxypressin, but that the apparent binding constant may be lower than that of oxypressin but at least the same order of magnitude. The lack of response to the increase of pH does not suggest that the unprotonated amino group binds. The pH at the surface of the vesicle can be expected to be somewhat lower due to the negative surface potential. The pK for the amino group is 6.4 (96).

The plateau polarization value thus reflects the bound species. As there is little change in the quantum yield (see Table 6) between the bound and free species, it is reasonable to assume that the lifetimes of the peptides in the free and bound states are similar. On this basis,

equation (22) can be substituted and rearranged to give the ratio of the correlation time in the bound state (b) to that of the free state (f).

$$\tau_R(b)/\tau_R(f) = \left(\frac{1}{p_f} - \frac{1}{p_0} \right) / \left(\frac{1}{p_b} - \frac{1}{p_0} \right) \quad (24)$$

These were calculated for each experiment using the intrinsic polarization value of 3.1 as before, and are shown in Table 6. At pH 5.3, 25 mM NaCac buffer, average values were estimated at 7 ± 1 for oxytocin, and 8 ± 2 for oxypressin respectively. Thus binding to PS imposes slower motions on the tyrosine chromophore and the effect is similar for both oxytocin and oxypressin.

The profile of the [1-hemi-D-(3¹³C) cystine] oxytocin was compared to that of oxytocin in a separate experiment in which the excitation and emission slits had to be widened because of the low quantum yield of the 1-D-Cys analogue. This was performed to compare with NMR data outlined in a following section. The increased slit width caused some depolarization in the plateau region as evidenced by the low p_b for oxytocin. This is most likely due to the increased levels of scatter in the emission spectrum. However, the half-saturation value is

comparable to the profiles obtained with the narrower slits. The profile of the 1-D-Cys analogue is similar with a half-saturation value equal to that of oxypressin. The correlation time ratios of the bound and free species using the p values obtained for this experiment were found to be 3.6 and 2.5 for oxytocin and 1-D-Cys oxytocin respectively. The reduced value is due to the depolarization observed on widening the emission slits. The low value for oxytocin precludes comparing these results. It is clear, however, that the 1-D-Cys analogue binds in a similar manner to that of oxytocin and oxypressin.

3. Effect of PC on Polarization Profiles

The effect of PC vesicles on the polarization of arginine vasopressin, oxytocin, oxypressin and desamino oxytocin were examined. These were all similar to the profiles shown by the desamino analogues with the addition of PS, and the same arguments apply. The slight increase could be attributed to the increase in viscosity as lipid is added.

Oxypressin, which showed less variation in its polarization profile was used to observe the effect of mixed lipid vesicles. Profiles of oxypressin titrated with

FIGURE 4: Polarization Profiles: Titration of Oxypressin
with PS, PC and PC/PS (4:1) SUV

- PS
- PC/PS, $[\text{lipid}]_{\text{outer}} = [\text{PC} + \text{PS}]_{\text{outer}}$
- PC/PS, $[\text{lipid}]_{\text{outer}} = [\text{PS}]_{\text{outer}}$
- ▲ PC

0.1 mM oxypressin in 25 mM NaCac buffer, pH 5.3 at 22°C

PC and PS and PC/PS, 4:1, are shown in Figure 4 (closed symbols). The PC/PS profile is between that of the pure lipid vesicles. This might suggest that the final polarization value is 0.14, with a half-saturation value of 2.2. However, when the PC/PS data are plotted as a function of $[PS]_{\text{outer}}$ only (open symbols), the data follow that of the pure PS profile indicating that the polarization value of 0.14 does not represent the fully bound peptide. Further addition of vesicles was not possible due to the increasing scatter. Whether the peptide responds specifically to PS, or to a charged lipid surface is not clear. The K_{app} for binding to mixed vesicles would be reduced due to the decrease in surface charge density.

C. NMR of ^{13}C -Labelled Peptides, 1-D-Cys Oxytocin and Oxytocin.

1. The Peptides

The chemical shifts of the resonances were 40.2 ppm and 39.5 ppm for [1-hemi-(^{13}C) cystine] oxytocin and [1-hemi-D-(^{13}C) cystine] oxytocin respectively in 25 mM NaCac, pH 5.3 at 25°C. These are consistent with those reported by Deslauriers et al (81). The shifts are sensitive to pH and buffer due to the titratable terminal

amino group. At this pH oxytocin is fully ionized while 1-D-Cys oxytocin is not quite, and its resonance exhibits an upfield shift to pH 4.5. The shifts are also temperature-sensitive, going downfield as the temperature is raised. Linewidths of the resonances were between 2 and 5 Hz. T_1 , T_2 and nOe data are shown in Table 7.

The T_1 values are slightly higher than those reported by Deslauriers, 93 ± 8 msec which may be due to differences in buffer content and pH. However, the full nOe was obtained for both peptides (maximum expected is 2.988 (97)) indicating that relaxation is via a dipolar mechanism in the extreme narrowing limit. The correlation time, τ_R , calculated using the isotropic model (98) would be between 2.0 and 1.5×10^{-10} s from T_1 measurements and between 2.4 and 2.5×10^{-10} s from T_2 measurements. While large differences between these measurements would indicate that the isotropic model is invalid for these data, these small differences are most likely due to an underestimation of the experimental error. These figures are in excellent agreement with those found using fluorescence polarization methods (Table 5).

TABLE 7

NMR RELAXATION DATA FOR
¹³C-LABELLED OXYTOCIN AND 1-D-CYS OXYTOCIN

	T_1	T_2	nOe
	(s)	(s)	
Oxytocin	0.22±0.01 (55°C)		
	0.14±0.01 (31°C)	0.10±0.01	2.9±0.2
1-D-Cys Oxytocin	0.14±0.01 (35°C)		
	0.13±0.01 (31°C)	0.10±0.01	2.8±0.2

data obtained in 25 mM NaCac buffer, pH 5.3

2. Addition of PS SUV

When PS vesicles were added to solutions of the peptides at pH 5.4, a dramatic increase in the linewidth, $\Delta\nu_{\frac{1}{2}}$, of the resonances was observed for both peptides. This could be reversed by lowering the pH of the sample below pH 2.5 (Figure 5) or by adding aliquots of CaCl_2 or NaCl (Figure 6). These phenomenon suggest a peripheral rather than integral association of the peptides to PS vesicles.

The resonance linewidths appeared to be insensitive to the state of the PS vesicles. After refrigeration, the PS/peptide samples exhibited some aggregation. This was apparent in both the physical appearance of the samples and in the natural abundance spectrum of the PS resonances. After a brief sonication, the spectrum of PS exhibited relatively narrow lines again, but the peptide resonance was unaffected (Figure 7). This could indicate that the motion of the bound species is independent of the vesicle, or that the motions are already so slow, that the vesicle size does not affect the linewidth.

The linewidths increase with increasing PS reaching a plateau level for both the oxytocin and 1-D-Cys oxytocin (Figure 8) at a PS:peptide ratio of approximately 5. The

FIGURE 5: The Effect of pH on the Interaction of
 ^{13}C -Labelled Oxytocin with PS MLV

1.4 mM oxytocin, 6.2 mM PS MLV in 100% D_2O titrated with
HCl, ambient temperature 33°C.

Spectral Details: 75.47 MHz - 8K spectrum collected in
3000 scans with a 10kHz sweepwidth, a one-cycle
add-subtract routine, 90° pulse and 3 s DELAY

FIGURE 6: Effect of CaCl_2 and NaCl on Linewidths of ^{13}C -Labelled Peptides, Oxytocin and 1-D-Cys Oxytocin.

For NaCl titration, solutions were 0.7 mM peptide, 7 mM PS SUV in 25 mM NaCac , 0.1 M EDTA, 10% D_2O buffer at pH 5, 42°C

For CaCl_2 titration, solution was 0.8 mM oxytocin, 9 mM PS SUV in above buffer at 26°C .

▲ oxytocin

● 1-D-Cys oxytocin

FIGURE 7: Effect of Aggregation and Sonication on the ^{13}C
Spectrum of ^{13}C -Labelled 1-D-Cys Oxytocin Bound to PS SUV

- A. Spectrum of sample just removed from the refrigerator.
- B. The same sample after brief sonication.

Sample was 0.7 mM 1-D-Cys oxytocin, in 25 mM NaCac buffer,
10% D_2O , pH 5.3.

Spectral Details: 75.47 MHz - 16K spectrum, collected in
5600 scans, with a 15kHz sweepwidth, and a one-cycle
sequence with a 90° pulse and a 0.8 s DELAY.

half saturation values for two titrations were 2.2 ± 0.1 for oxytocin and 2.7 ± 0.1 for l-D-Cys oxytocin. These are similar to those found for polarization studies at a ten-fold lower concentration and confirms that the binding curves represent titrations. However, the plateau linewidth is greater for oxytocin in comparison to that of l-D-Cys oxytocin. A very small downfield shift was observed for the oxytocin resonance, while the l-D-Cys oxytocin resonance shifts upfield with increasing PS (Figure 8). T_1 , T_2 data and the temperature dependence of the resonance linewidths in the plateau region are shown in Table 8.

It can be seen that the T_1 values appear to be unchanged in comparison to those of the free species given slight temperature variations, but the T_2 values are very much shorter. These are consistent with the linewidths measured.

The behaviour of the two analogues are similar with respect to PS with two exceptions, the chemical shift behaviour and the difference in plateau linewidths. The chemical shift behaviour of l-D-Cys oxytocin can be attributed to the different pK of the α amino group (81). As the pH at the surface of the vesicle is lowered by the

FIGURE 8: Comparative Titrations of ^{13}C -Labelled Oxytocin and 1-D-Cys Oxytocin with PS SUV as Observed by Linewidth, $\Delta\nu_{1/2}$, and Chemical Shift, δ , Changes

Solutions were 1 mM peptide in 25 mM NaCac buffer, with 0.1 mM EDTA, pH 5.3 at 31°C.

▲ oxytocin

● 1-D-Cys oxytocin

TABLE 8
RELAXATION DATA FOR ^{13}C -LABELLED OXYTOCIN AND 1-D-CYS OXYTOCIN BOUND TO PS

	Oxytocin	1-D-Cys Oxytocin
T_1 (s)	0.10 ± 0.02	0.17 ± 0.02 (35°C)
T_2 (ms)	4.1 ± 0.2	5.1 ± 0.5 (27°C)
$\Delta \nu_{1/2}$ (Hz)	88 ± 7	65 ± 5 (27°C)
Temperature Dependence of $\Delta \nu_{1/2}$		
Temperature	(28 - 42) °C	(28 - 47) °C
$\Delta \nu_{1/2}$	(90 - 46) Hz	(65 - 25) Hz

data obtained in 25 mM NaCac buffer, pH 5.3

negative surface potential, the aqueous phase and the double diffuse layer have a different pH. The steady decrease in the chemical shift of 1-D-Cys oxytocin is due to the fast exchange of the peptide between the bulk and the surface environment. Oxytocin is unaffected because it is fully ionized at the bulk pH 5.3 and its chemical shift is constant with respect to pH below this level. The slight downfield shift observed is a function of binding.

The T_2 of the plateau region reflects the T_2 of the bound species. If exchange were contributing to the linewidth, a maximum would be observed at a point where the fraction of peptide bound is less than 0. Titrations to a ratio of 10 $[PS]_{outer}/[peptide]$ do not show any such maximum. The similarity of the profiles to those obtained by fluorescence which are uncomplicated with respect to exchange confirm this. The difference in linewidth between the two peptides translates to a very small difference in T_2 . Using an isotropic model, these correspond to a T_R of $2-3 \times 10^{-8}$ s. For this type of analysis, the T_1 values would be expected to be 300 - 400 msec, which is not observed. The large difference between the T_2 and T_1 values, given that exchange is not a factor, suggests that there are very slow motions associated with binding, and that the isotropic model is not appropriate. This could

arise from anisotropic motion, or macromolecular associations (formation of dimers etc.) in which there is freedom of motion that can give rise to the longer T_1 values. The rate of exchange must be faster than $(1/T_{2b} - 1/T_{2f})$ which is 240 s^{-1} and 190 s^{-1} for oxytocin and 1-D-Cys oxytocin respectively.

A more detailed analysis would be required to determine a model for the motional characteristics of binding. As the temperature dependence could be due to both binding and the type of motion, it would be necessary to establish the former for such an analysis. A combination of this type of information together with fluorescence data could be used.

It can be seen from the fluorescence and NMR experiments that slower motions are felt by the peptide close to the amino group on binding. In order to establish whether this is a localized effect, natural abundance ^{13}C NMR of the oxytocin/PS interaction was observed.

C. Natural Abundance ^{13}C NMR of Oxytocin/PS SUV

The spectra of PS and oxytocin are shown in Figures 10 and 11. The chemical shifts for oxytocin are in agreement with those of Walter et al (99) and Hruby et al (100) and

FIGURE 9: ^{13}C NMR Spectrum of PS SUV

19 mM PS in 25 mM NaCac buffer, 25 mM NaCl, 1 mM EDTA, 10%
 D_2O , pH 5.2 at 42°C

Spectral Details: 75.47 MHz - 16K spectrum, collected in
24K scans with a 15 kHz sweepwidth using a one-cycle
sequence with a 90° pulse and a 2 s DELAY, processed with 5
Hz line-broadening

FIGURE 10: ^{13}C NMR Spectrum of Oxytocin

7.2 mM oxytocin in 25 mM NaCac buffer, 1 mM EDTA, 10%
 D_2O , pH 5.2 at 42°C

Spectral Details: 75.47 MHz - 16K spectrum, collected in
15K scans with a 15 kHz sweepwidth using a one-cycle
sequence with a 90° pulse and a 2 s DELAY, processed with 5
Hz line-broadening

assignments were made from these papers. Assignment of PS resonances were made from the studies of Levine et al (101) and Stoffel et al (102) for the fatty acids, with headgroup assignments from Holwerden et al (103). The spectrum of a 2.6:1, PS/oxytocin mixture is shown in Figure 11. It is compared with a simulated spectrum of the addition of a spectrum of PS and a spectrum of oxytocin line-broadened and weighted to approximate that of the bound spectrum of the furthest upfield peak. The comparison illustrates that the increased linewidth observed for the labelled peptide (previous section) reflects a global rather than local phenomenon.

A more detailed analysis of the spectrum of the bound oxytocin molecule was made possible by subtracting the PS spectrum using the $(CH_2)_n$ peak as a reference (Figure 12). As the PS spectrum is also affected by binding to oxytocin, residual peaks due to PS are observed in the subtracted spectrum.

In the 0-80 ppm region, most of the α CH resonances have disappeared (Ile, Gln, Cys-1) or are within the baseline (Pro, Cys-6, Asp, Tyr) and shifts cannot be determined. The α CH resonances of Leu and Gly are the only ones observable and appear at the same chemical shift as those

FIGURE 11: Comparison of ^{13}C NMR Spectrum of Oxytocin Bound to PS (A) with Simulated Spectrum of Free Oxytocin and PS (B)

A. PS-Oxytocin (Bound) 19 mM PS and 5.9 mM oxytocin in a 25 mM NaCac buffer, 25 mM NaCl, 10% D_2O , pH 5.2 at 42°C.

Spectral Details: 75.47 MHz - 16K spectrum, collected in 38K scans with a 15 kHz sweepwidth using a one-cycle sequence with a 90° pulse and a 2 s DELAY, processed with 5 Hz linebroadening

B. PS and Oxytocin (Free). Simulated spectrum in which a spectrum of PS (Figure 9) was added to the spectrum of oxytocin (Figure 10). The latter was line-broadened by 22 Hz to approximate the uncorrected linewidth of the furthest upfield oxytocin resonance (Ile δCH_3) and weighted to approximate the relative amplitudes of A.

of the unbound peptide. The two βCH_2 resonances for Cys-1 and Cys-6 which are part of the backbone of the ring are reduced, but observable. The Cys-1 resonance is shifted slightly downfield (as observed for the labelled peptide), but the shift of the Cys-6 resonance cannot be determined due to the overlap of the βCH_2 Leu resonance. β resonances are observable for Leu, Tyr, Asn, Ile, Pro and Gln. All but the Pro βCH_2 resonances appear reduced. Chemical shifts are unchanged. Other side chain resonances are all observable. The linewidths are fairly narrow with the exception of the Pro δCH_2 resonance. The resonances appear at the same chemical shift as in the unbound spectrum.

Carbonyl and the tyrosine phenol resonances are observed in the 100 - 180 ppm region. The phenol resonances are observable with the exception of C_4 and unshifted. The carbonyl region is overlapping and unclear with the exception of the resonances for the two Cys residues. The Cys-1 carbonyl is observable and unshifted. However, the Cys-6 resonance appears shifted. As the serine carbonyl of PS also resonates at this chemical shift, the peak may be due to PS rather than the Cys-6 carbonyl, which would suggest that the Cys-6 resonance is missing. There may be other changes in the carbonyl envelope.

The lack of significant shifts for those carbons observed indicates that no gross conformational change occurs on binding to PS as might be expected for a specific interaction as occurs with neurophysin. Shifts for the Cys-1 carbons can be attributed to local environmental effects due to the proximity of the terminal amino group. The apparent increase in linewidth for most resonances indicates that the molecule is affected by overall slower motions. However, the linewidths follow the same pattern obtained for a T_1 analysis of oxytocin (Table 9) (47) indicating that the molecule retains its pattern of flexibility although slower motions are imposed by binding. The tripeptide tail and the sidechains retain their degrees of freedom with respect to the backbone ring structure.

The PS spectrum also exhibits changes on binding to oxytocin. Figure 13 illustrates these using the $(CH_2)_n$ envelope as a reference. No changes in chemical shift are observed. The resonances for the three carbons at the end of the fatty acyl chain appear increased as well that of the standard, NaCac. These could be due to a decrease in the $(CH_2)_n$ resonance, or an increase in the resonances themselves. Amplitudes will be affected by changes in linewidth, nOe and T_1 if the T_1 's are altered and the peak becomes less or more saturated with respect to recycle

TABLE 9
COMPARISON OF T_1 DATA FOR FREE OXYTOCIN
AND LINEWIDTH DATA FOR PS-BOUND OXYTOCIN

	T_1 (47) (s)	$\Delta\nu_{\frac{1}{2}}$ (Hz) ± 3
Ile δCH_3	1374	7
Ile γCH_3	825	2
Cys-1 βCH_2	87	18
Gly αCH_2	376	8
Leu αCH	151	13
Tyr C_2C_6	132	24
Tyr C_3C_5	138	26

FIGURE 13. Comparison of the ^{13}C NMR Spectrum of PS SUV
bound to Oxytocin with that of Free PS SUV

- PS SUV-Oxytocin as in Figure 11
- PS SUV (Figure 9) weighted so that the amplitude of
the PS $(\text{CH}_2)_n$ resonance is equal to that of the PS
SUV-Oxytocin spectrum

time. The NaCac resonance should only be affected by inhomogeneity changes between the two samples. Its linewidth increased from 14 to 18 Hz on the addition of oxytocin to the sample. The fatty acid resonances are narrow, and the linewidth should reflect the homogeneity of the sample. Therefore, NaCac can be used as a standard for these resonances to determine whether the amplitude changes are significant. These are listed in Table 10. They indicate that there is no significant change in amplitudes for the acyl chain peaks. The T_1 of the tCH_3 carbon is on the order of 1.8 seconds (104) and its resonance should be saturated at a recycle time of 1.5 seconds. The other resonances also have long T_1 s. It can be assumed, therefore that there are no changes in T_1 , T_2 or nOe for these resonances. However, the resonances for carbons closer to the surface in the glycerol and headgroup all show a considerable decrease in amplitude with the exception of the NH_3CH serine resonance. Reliable linewidths can only be assessed for those peaks with no underlying peptide resonances. They indicate a considerable increase; from 36 to 60 Hz for the $CH_2C=O$ and from 56 to 70 Hz for the CH_2OPO resonances. The NH_3CH resonance also exhibits an increased linewidth. It does have an underlying peptide peak which would artificially increase the linewidth, but this should be very small. It is also

TABLE 10
 RELATIVE AMPLITUDES OF ACYL CHAIN RESONANCES
 OF PS IN ^{13}C NMR SPECTRA OF PS AND OXYTOCIN-PS

		$\Delta \nu_{\frac{1}{2}}$	$\Delta \nu_{\frac{1}{2}}$
	I(PS-OXYTOCIN)/	PS-OXYTOCIN	PS
	I(PS)	(Hz)	(Hz)
tCH_3	0.9 ± 0.1	6	6
CH_2CH_3	0.91 ± 0.009		
$\text{CH}_2\text{CH}_2\text{CH}_3$	0.97 ± 0.09		
$(\text{CH}_2)_n$	1.07 ± 0.07	78	82

possible that the increase in linewidth is balanced by a decrease in T_1 which would lead to an increase in amplitude if saturated. The increasing linewidths observed for the glycerol and headgroup region would indicate a decrease in the mobility of this region with no change recorded for the acyl chain region. However, it is possible that the entire molecule undergoes a decrease in mobility and that this is simply not reflected in the acyl chain region. T_1 relaxation is thought to be due to both a fast and slow component, the latter dependent on operating frequency and order parameter. If changes are seen in the slow component, rather than the fast component depending on the balance of these two components, the change may not be registered in the overall T_1 . T_2 or linewidth measurements should reflect the slower motions - but could also be due to slow exchange between bound and unbound lipids. Therefore one can only submit that there are changes in the PS spectrum which indicate a change in mobility between the bound and unbound species.

D. Summary and Discussion

1. Binding and Stoichiometry of the Monovalent Peptides

The lack of enhancement of tyrosine fluorescence and the

lack of chemical shift changes for the observable carbons of oxytocin other than those near the terminal amino group on binding, the easy reversal by the addition of NaCl and CaCl_2 and at low pH where the serine carbonyl is protonated all suggest a peripheral, primarily electrostatic association. The binding profiles appear to be titrations. Therefore, differences in binding constants between the peptides cannot be assessed, but the stoichiometry can be estimated using half-saturation values. The variations in half-saturation values for the low concentrations in the fluorescence data for oxytocin may be attributable to experimental error, as the NMR data at a ten-fold higher concentration indicate a half-saturation value of 2.2. Thus the stoichiometry of the association for oxytocin and 1-D-Cys oxytocin can be assessed at 3 to 5 molecules of PS per peptide. Data for oxypressin indicate 3 to 4 molecules per peptide. The differences can be attributed to experimental error and are not particularly significant. Using a value of $70 \text{ \AA}^2$ for the area of a PS molecule, the surface area of the interaction can be determined as 210 to $350 \text{ \AA}^2$. This is larger than the area expected for a sphere of $14 \text{ \AA}$ diameter ($150 \text{ \AA}^2$), but the area of these peptides has been measured as $300 \text{ \AA}^2$ (28). This is consistent with the peptide covering the surface in a peripheral association.

B. ^{31}P NMR: Oxytocin and Arginine Vasopressin - PS

1. PS SUV

Data for oxytocin and arginine vasopressin T_1 studies are shown in Table 11. While the data were obtained in different buffers, at different temperatures, a similar trend is observed. The T_1 's decrease and the resonances shift upfield as either oxytocin or arginine vasopressin is added. At this frequency (121 MHz), the chemical shift anisotropy will contribute to relaxation of both T_1 and T_2 . This is evident in the linewidths. At lower field strengths two resonances are seen separated by a few Hz. This has been exploited to measure the asymmetry of phospholipids in sonicated vesicles (71). In this study, the two lines are coalesced. The linewidth is strongly dependent on size as can be seen by the increase in linewidth of the PS vesicles to which arginine vasopressin is added. As aggregation begins, the linewidth increases. Changes in T_1 can be due either to a change in chemical shift anisotropy, and/or a change in mobility. As arginine vasopressin has little effect on the CSA, (see next section), it can be assumed that the decrease in T_1 is due to a decrease in mobility as increasing the temperature of these SUV caused an increase in T_1 values. This was

$$\frac{\sum \text{PS}_n \text{OT}}{\sum \text{PSNa}} = \frac{K_{\text{OT}}[\text{OT}]}{K_{\text{Na}}[\text{Na}]} \quad (28)$$

Thus as the left hand side approaches 0, so must the right and

$$K_{\text{OT}}[\text{OT}] \ll K_{\text{Na}}[\text{Na}]. \quad (29)$$

Using 10% as a measurable limit and a binding constant of 0.8 M^{-1} for sodium (5), K_{OT} cannot be more than 30 M^{-1} . This is consistent with the titration data, as at 25 mM NaCl, the surface charge can be estimated at -120 mV at 40°C. This gives a factor of 85 for the Boltzmann term, thus K_{app} would be $2.6 \times 10^3 \text{ M}^{-1}$. This would be in the titration range for the concentrations used. It is also evident that binding at such a low association to PC would not be apparent at the concentrations employed in this study, nor would the binding of desamino oxytocin to PS, as this peptide is neutral and is not affected by the surface potential. Thus, the issue of whether the binding is specific for PS, or generally to phospholipids is not evident from this study. Further NMR studies using higher concentrations of PC, or calorimetric studies clearly showing lateral phase separation might answer this question.

The binding constant suggests the involvement of interactions other than ionic as the binding constant for NH_4^+ is 0.17 M^{-1} , less than Na^+ . The estimate is similar to that of the binding of enkephalins to lysoPC micelles and PS (40,41). For enkephalins hydrophobic and electrostatic elements are indicated. In this study there is no evidence for hydrophobic elements as evidenced by the lack of shifts for the observable carbons in the NMR spectrum and lack of enhancement of the tyrosine fluorescence. However, further studies might indicate changes in the resonances of those carbons unobservable in these experiments. If the electrostatic interaction is with the serine carboxyl, then one would expect specificity with respect to PS. If the interaction is with the phosphodiester moiety, then the intrinsic binding constants for PC and PS would be similar. There is no evidence in the ^{13}C PS spectrum for a specific interaction with the serine carboxyl, although there is overlap in this part of the spectrum. However, the effect of oxytocin on the glycerol headgroup region is evident.

The bound species exhibit decreased mobility. A seven-fold decrease in the correlation time of the tyrosine chromophore is indicated from oxytocin and oxypressin estimated with a simple isotropic model. While a useful

method for comparative purposes, this may not be adequate to describe the bound species. NMR relaxation data for oxytocin and 1-D-Cys oxytocin indicate that an isotropic model is not appropriate to describe the motion of the β -1-Cys carbons of the bound species. While T_1 remains unchanged, the T_2 indicates much slower motions. This would suggest anisotropic motion. However, the mobility of the bound species is at least of the same order of magnitude for oxytocin and 1-D-Cys oxytocin. Natural abundance ^{13}C NMR of oxytocin indicates that the decrease in mobility of the bound peptide is a global phenomenon, and not restricted to the terminal amino region, but the tripeptide tail retains a greater degree of freedom than the ring portion of the molecule. This would suggest that the ring portion is forced into contact with the surface, while the tripeptide tail may be extended.

Data from surface potential measurements (see Appendix) indicate that desamino lysine vasopressin does bind to PS vesicles, but in a different manner to the terminal amino monovalent peptides. The lack of change in the polarization of the tyrosine chromophore could indicate that either the binding constant for this peptide is much lower, or that the chromophore is relatively unaffected by binding. In this case, the tripeptide tail could be

immobilized by virtue of the lysyl charge, and the ring portion of the molecule could experience greater degrees of motional freedom. Natural abundance ^{13}C NMR of the interaction of desamino lysine vasopressin with PS could easily establish this.

From the similarity in the bound species of oxytocin and oxypressin, the side chain in position 3 does not appear to contribute to the binding process. The similarity of binding of oxytocin and 1-D-Cys oxytocin suggests that the chirality in position 1 is not a major factor either. Binding depends on the terminal α amino charge imposing interactions between the rest of the molecule and the surface. Data from surface potential measurements (see Appendix) suggest that the hydrophobicity of the amino acid in position 2 may be important. The observed increase in TNS binding follows the pattern oxytocin = oxypressin > 2-Leu oxytocin > 2-Gly oxytocin. It is not clear, however, whether the differences in this experiment arise from different binding constants, a different effect on surface charge, or a different ability to provide a new binding site for TNS on the vesicles surface. Further investigation could distinguish between these possibilities.

2. Comparison with the Divalent Peptides.

As the binding constant of the divalent peptides must be at least of the order of the monovalent peptides, the stoichiometry can be determined from data obtained in ESR experiments (29). This is found to be 3 PS molecules to peptide which is similar to that of the monovalent peptides. The binding constant for lysine vasopressin has been estimated at 10^7 M^{-1} (23). This would suggest a large contribution from the charge in position 8 when compared to the estimated binding constant for the monovalent peptides in this study. It is possible that the phenylalanine residue contributes to binding - the binding constant of oxypressin could be more than that of oxytocin, but the similarity between the bound species and the profiles of their effect on TNS binding to PS (see Appendix) suggests that this is not the case. The binding constant for lysine vasopressin was determined from surface charge using the Stern equation (27). However, the measurements were made on PS-decane droplets, specific binding of sodium was not accounted for, and the plane of shear was taken to be the surface plane. The figure may not be accurate or comparable to these studies. The lack of enhancement of tyrosine fluorescence on binding to PS for the divalent peptides suggests that the binding

of the monovalents and divalents are similar. The following section attempts to assess the contribution of charge in position 8.

III. Effect of Divalent Peptides and Oxytocin on Lipids

A. Introduction

High resolution ^{31}P NMR was used to compare the effects of oxytocin and arginine vasopressin on the phosphodiester moiety of PS SUV. Broadline ^{31}P NMR was used to observe the effect of arginine vasopressin on PS MLV. The monovalent peptides could not be studied using this technique. The data obtained were compared to the effects of Ca^{++} and Mg^{++} on PS.

The effect of lysine and arginine vasopressin and oxytocin on PE/PS Ca^{++} mediated bilayer-hexagonal phase changes were examined using broadline ^{31}P NMR. Ca^{++} mediated fusion of PE/PS SUV was monitored using fluorescence energy transfer techniques. These activities were correlated to the aggregating effect of the peptides on PS SUV measured by light scattering at the exciting wavelength.

B. ^{31}P NMR: Oxytocin and Arginine Vasopressin - PS

1. PS SUV

Data for oxytocin and arginine vasopressin T_1 studies are shown in Table 11. While the data were obtained in different buffers, at different temperatures, a similar trend is observed. The T_1 's decrease and the resonances shift upfield as either oxytocin or arginine vasopressin is added. At this frequency (121 MHz), the chemical shift anisotropy will contribute to relaxation of both T_1 and T_2 . This is evident in the linewidths. At lower field strengths two resonances are seen separated by a few Hz. This has been exploited to measure the asymmetry of phospholipids in sonicated vesicles (71). In this study, the two lines are coalesced. The linewidth is strongly dependent on size as can be seen by the increase in linewidth of the PS vesicles to which arginine vasopressin is added. As aggregation begins, the linewidth increases. Changes in T_1 can be due either to a change in chemical shift anisotropy, and/or a change in mobility. As arginine vasopressin has little effect on the CSA, (see next section), it can be assumed that the decrease in T_1 is due to a decrease in mobility as increasing the temperature of these SUV caused an increase in T_1 values. This was

TABLE 11

COMPARATIVE EFFECTS OF OXYTOCIN AND ARGININE VASOPRESSIN
ON ^{31}P PS RELAXATION AND CHEMICAL SHIFT

PEPTIDE/PS	OXYTOCIN ¹		ARG VASOPRESSIN ²		
	0.00	0.95	0.00	0.06	0.12
T_1 (s)	1.37±0.08	1.1±0.1	1.40±0.02	1.15±0.02	1.10±0.02
δ (ppm)	0.000	0.080	0.000	0.066	0.088
$\pm$ 0.005					
$\Delta\nu_{\frac{1}{2}}$ (Hz)	35	36	15	35	40
$\pm$ 3					

1. in buffer 197 NH_4OAc , 16% D_2O , 10 mM EDTA, pH 5.4, 2 mM PS, 30°C

2. in buffer 50 mM NaCac, 10% D_2O , 0.2 mM EDTA, pH 5.3, 9 mM PS, 29°C

*shifts relative to PS with no addition of peptide

measured on vesicles in NH_4OAc buffer. The fact that the T_1 's are similar at different temperatures for SUV in the two buffers may be due to differences in the buffer cations.

From the data presented, it appears that arginine vasopressin has a greater effect on these parameters than oxytocin. At the peptide/PS ratio for oxytocin, the PS should be fully bound. At the peptide/PS ratio for arginine vasopressin, PS is less than half bound assuming the stoichiometry is similar to that of lysine vasopressin, but the magnitude of the changes is equal to that of oxytocin. Further additions were impossible due to the flocculation of the PS vesicles. The greater effect of arginine vasopressin can be attributed to the additional charge on arginine in position 8.

The chemical shift and decrease in T_1 are similar to, but less than those observed for the divalent cation Mg^{++} . T_1 is decreased from 1.9 to 1.1 s for 2 mM Mg^{++} (30 mM PS) and a shift of 0.40 ppm is observed for 1 mM Mg^{++} (40 mM PS) as observed by Kurland et al at 40.5 MHz (105). Ca^{++} has a greater effect on these parameters. The chemical shift is in the direction of neutralization of the phosphate. This would indicate that the charged group

of the peptides is close to the phosphodiester moiety - already indicated by changes in headgroup mobility measured in the ^{13}C NMR spectrum of PS-oxytocin mixtures.

2. PS MLV - Arg Vasopressin

Previous data obtained from ESR studies (29) indicate a small change in ΔA , a parameter measuring order and/or mobility of doxyl-labelled probes on the addition of lysine vasopressin to PS MLV. The addition of arginine vasopressin to PS MLV was therefore examined by ^{31}P NMR. The data in Table 12 were drawn from several spectra on two different preparations for which an excess of the peptide was added. Representative spectra together with the dePaked spectra are shown in Figure 14. $\Delta\sigma_{\text{eff}}$ was measured from the bilayer spectrum, and linewidths from the dePaked spectrum uncorrected for the 50 Hz line-broadening that was applied to the spectra. The data indicate that while motions are slowed by arginine vasopressin, as evidenced by the increased linewidth of the 90° degree orientation (consistent with the T_1 data of the previous section), there is no measurable change in the conformation, or the order in the region of the phosphodiester.

Charged phosphatidylserine generally adopts the bilayer

TABLE 12

³¹P NMR DATA FOR PS MLV AND ARGININE-VASOPRESSIN/PS MLV

	PS	Arginine Vasopressin/PS
$\Delta\sigma_{\text{eff}}$ (ppm)	55 $\pm$ 2	54 $\pm$ 2
$\Delta\nu_{\frac{1}{2}}$ (Hz)	(2.7 $\pm$ 0.9)10 ²	(7.2 $\pm$ 0.3)10 ²

data obtained in 50 or 98 mM NaCac buffer, 2mM EDTA, 10%
D₂O, pH 5.3, 24°C

**FIGURE 14: The Effect of Arginine Vasopressin on the ^{31}P
NMR Spectrum of PS MLV**

Samples in 50 mM NaCac buffer, 2 mM EDTA, pH 5.1 at 23°C

Left Hand Side

Original Bilayer Spectra

**upper, PS-Arginine Vasopressin: 6.3 mM PS, 2.4 mM arginine
vasopressin, collected in 11K scans**

lower, PS: 25 mM PS, collected in 1600 scans

Right Hand Side

Corresponding dePaked spectra

**Spectral Details: 121.57 MHz - 4K spectra collected with a
125 kHz sweepwidth using a Hahn echo sequence with $t(1) =$
 $t(2) = 60$ microseconds, 4 s DELAY with high-power
decoupling, processed with 50 Hz line-broadening**

phase. The effect of arginine vasopressin can be compared to that of Mg^{++} and Ca^{++} . Addition of Ca^{++} to PS derived from egg yolk causes the ^{31}P PS spectrum to exhibit an anhydrous powder pattern (106). This is due to the formation of $CaPs_2$, a dehydrated crystalline complex. This complex is not apparent when Ca^{++} binds to PS SUV unless aggregation occurs and is thought to be due to binding in the trans mode, from one vesicle to another (107). While Mg^{++} causes flocculation of MLV (as does arginine vasopressin), it does not form a similar complex with unsaturated PS, and the ^{31}P spectrum of PS retains its bilayer pattern. Thus the interaction of arginine vasopressin with PS is not comparable to that of Ca^{++} , but is comparable to that of Mg^{++} . From data presented in the previous section, it appears that arginine vasopressin is less effective than Mg^{++} in affecting the phosphodiester moiety.

C. Enhancement of Ca^{++} Mediated Events: Bilayer-Hexagonal Phase Transitions and Fusion in PE/PS Mixtures

1. Introduction

One of the more active areas in lipid biochemistry is the modelling of dynamic events across bilayers - fusion and

transport. Cullis et al (12) have suggested that the ability of phospholipids to form non-bilayer structures in response to temperature, head-group composition and surface-active agents is an indication that these reversible phase changes, or the events that precede them are biologically relevant. It appears that many substances which affect the bilayer-hexagonal phase changes also affect fusion of phospholipid vesicles of the same composition. Fusion in a biological system operates on a faster time scale than that of model membranes. An excellent review of the current literature is given by Blumenthal (108).

As ions are involved in fusion processes of vesicles containing negatively charged lipids, it is of interest to see whether the hormones used in this study could be involved in such processes. The bilayer-hexagonal phase behaviour of PE/PS MLV was examined using ^{31}P NMR spectroscopy and fusion of PE/PS vesicles was observed using fluorescence energy transfer. Aggregation of PS SUV was measured using light scattering to correlate with data obtained on the Ca^{++} events.

2. Bilayer-Hexagonal Phase Transitions

(a) Conditions for Observing Phase Transitions

Conditions for effecting the Ca^{++} induced phase transitions for PE/PS, 4:1, were established in 100 mM NaCac buffer, pH 7.0 at 44°C (87). The temperature dependence is a function of the fatty acids of the lipids. This is similar to the expected transition temperature for bovine brain PS (109). The spectra of the lipids with 0.0, 0.5 and 1.0 Ca^{++} /PS additions indicate the expected transitions (Figure 15). The initial spectrum illustrates the overlapping bilayer patterns for PE and PS. The $\Delta\sigma_{\text{eff}}$ of PS is larger than that of PE, which gives rise to the odd shape of the spectrum. On the addition of 0.5 Ca^{++} /PS an intermediate stage is evident with both bilayer and hexagonal phase. At a 1.0 ratio, the bilayer has virtually disappeared, and the hexagonal phase is evident characterized by the $\Delta\sigma_{\text{eff}}$, half that of the bilayer.

(b) Effect of Peptides

Figure 16 shows the effect of incubating the PE/PS bilayers with 1:1 oxytocin or arginine vasopressin. The

FIGURE 15: Ca^{++} Induced Phase Transitions in PE/PS (4:1)
MLV as Monitored by ^{31}P NMR

Samples are 12.5 mM total lipid in 100 mM NaCac buffer, 10% D_2O , pH 7.0 at 44°C . Ca^{++} additions are as indicated.

Spectral Details: 121.57 MHz - 4K spectra collected with a 125 kHz sweepwidth using a Hahn echo sequence with $t(1) = 60$ microsecond, $t(2) = 20$ microseconds, 4 s DELAY with high power decoupling, in 5136, top, 1392, middle and 1408, bottom, scans processed with 100 Hz line-broadening

spectra appear similar until a 0.5 ratio of Ca^{++} is added. The sample to which oxytocin was added appears relatively unchanged, but with an additional resonance seen at the centre of the pattern - the isotropic shift. This is due to isotropic averaging of some PS or PE molecules. While these have been noted by other workers to be a feature of such transitions, it is only evident in the sample to which oxytocin was added for these experiments. If PS MLV are incubated with oxytocin, a great portion of the sample reverts to small vesicles which would give rise to such a spectrum. This is not evident in the PS/PE MLV without Ca^{++} addition. The sample to which arginine vasopressin was added indicates that there is apparently more hexagonal phase than bilayer, and that this peptide has enhanced the Ca^{++} induced event. This is more clearly demonstrated in Figure 17. In this case a ratio of 0.2 Ca^{++} /PS was added to samples incubated with arginine or lysine vasopressin. Once again, the samples before addition are unchanged. After Ca^{++} addition, the initial sample is between that of the 0.0 and 0.5 ratios. However, the sample to which arginine vasopressin was added indicates a much greater proportion of hexagonal phase. The sample to which lysine vasopressin is added also indicates more hexagonal phase, than with no addition, but less than with the arginine peptide. A quantitative

FIGURE 16: Effect of Arginine Vasopressin and Oxytocin on
PE/PS (4:1) MLV Ca^{++} Induced Phase Transitions as
Monitored by ^{31}P NMR

Samples are 12.5 mM total lipid in 100 mM NaCac buffer, 10%
 D_2O , pH 7.0 at 44°C . Right hand spectra without Ca^{++} ,
left hand spectra with 0.5:1.0 Ca^{++} /PS, peptide additions
as indicated

- Spectral Details: 121.57 MHz - 4K spectra collected with a
125 kHz sweepwidth using a Hahn echo sequence with $t(1) =$
60 microseconds, $t(2) = 20$ microseconds, 4 s DELAY with
high power decoupling in approximately 1K scans, processed
with 100 Hz line-broadening

FIGURE 17: Effect of Lysine and Arginine Vasopressin on
PE/PS (4:1) MLV Ca^{++} Induced Phase Transitions as
Monitored by ^{31}P NMR

Samples are 18.7 mM total lipid in 100 mM NaCac buffer, pH
7.0 at 44°C. Right hand spectra without Ca^{++} , left hand
spectra with 0.2:1 Ca^{++} /PS, peptide additions as
indicated

Spectral Details: 121.57 MHz - 4K spectra collected with a
125 kHz sweepwidth using a Hahn echo sequence with $t(1) =$
60 microseconds, $t(2) = 20$ microseconds, 2 s DELAY with
high power decoupling in approximately 1600 - 1700 scans,
processed with 100 Hz line-broadening

demonstration would require measuring the areas under the patterns, or the dePaked areas of the 90° orientations. The signal-to-noise ratio prevented such an analysis, but the effect is evident in a qualitative manner.

(c) Discussion

The concentrations of Ca^{++} were in the minimal 1 - 2 mM range required to elicit the phase changes. Phase transitions of this kind are thought to be due to the interaction of Ca^{++} with the PS molecules. As PE adopts the hexagonal phase naturally, the addition of PS stabilizes the bilayer configuration. Ca^{++} interacts with PS in such a way as to remove the stabilizing influence. This could be due to the formation of CaPs_2 complexes causing phase separation, or formation of complexes that adopt a different shape causing the destabilization. Intermolecular hydrogen bonding may also play a role in stabilizing the bilayer. It is possible that interaction with Ca^{++} disrupts these, leading to the phase change (37).

Both arginine vasopressin and lysine vasopressin affect the phase changes but to a different degree. Oxytocin does not, although the isotropic feature might be due to the

interaction of oxytocin with a PS phase separated by Ca^{++} . This suggests that the observed effect is due to the charged side chain in addition to the terminal amino charge and is an activity of the divalent peptides only. At these concentrations, the interaction should be in the titration range. Thus lysine is less effective than arginine in enhancing the hexagonal phase change. The divalent peptides must either alter the temperature dependence of the phase transition, or reduce the threshold of Ca^{++} required to elicit the response. These possibilities should be investigated.

This effect is similar to the effect of Mg^{++} which can reduce the Ca^{++} concentration required to effect a phase change for PE/PS/cholesterol MLV. For this system, Mg^{++} at high concentrations can induce a phase change, but this is not observed with PE/PS systems (110).

As the formation of such non-bilayer structures appears to be related to fusion events in some way, it was of interest to discover whether these peptides could affect fusion for PE/PS vesicles.

3. Ca^{++} Mediated Fusion: Fluorescence Energy Transfer

(a) General Behaviour of the System

The method of Uster and Deamer (93) was used to measure the extent of fusion of PE/PS vesicles as % mix as described in the experimental section. This is based on the overlap of the emission spectrum of the donor, cholesteryl anthracene-9-carboxylate (CA9C), to the excitation spectrum of the acceptor, 4-dihexadecylamino-7-nitrobenzo-2-oxa-1,3-diazole (DHNDB). The continuous trace (Figure 18) indicates the general behaviour of the system. Ca^{++} addition is followed by a period of flocculation during which the signal drops. Addition of EDTA disperses the flocculation and stabilizes the signal. During the period of flocculation, the DHNDB probe was extruded from the bilayer. This reduced the transfer efficiency. In addition, the emission of CA9C underwent some changes as a function of Ca^{++} addition. The measurement of fusion as % mix was corrected for these anomalies as outlined in Chapter Two: Experimental III.E.3. The time to flocculation, $t(\text{flocc})$, depends on the concentration of Ca^{++} added (Table 13) and on the concentration of vesicles.

FIGURE 18: Representative Trace of the Amplitude of CA9C Donor Probe as a Function of Time: Ca^{++} Induced Fusion of PE/PS (3:1) SUV

Sample contained 0.15 mM total lipid, 0.39% CA9C probe only
- no acceptor - in 100 mM NaCac bufer, pH 7.0 at 22°C.

Sample excited at 385 nm with a 3 nm slit width and monitored at 460 nm (I(460)) continuously with a 4 nm slit width and 430 cut-off filter. 4 mM CaCl_2 and 12 mM Na_4EDTA additions as indicated.

TABLE 13

TIME COURSE OF FLOCCULATION AND THE EFFECT OF
 Ca^{++} AND ARG VASOPRESSIN ON PE/PS (4:1) VESICLES

$[\text{Ca}^{++}]$ (mM)	[Arg Vasopressin] (mM)	t_{floc} (min)
1.0	0.00	6
2.0	0.00	6.0
4.0	0.00	2.3
	0.01	1.8
	0.05	1.0
7.4	0.00	1.5

TABLE 14

EFFECT OF ARG VASOPRESSIN ON FUSION IN THE
 POST-FLOCCULATION PERIOD

[Arg Vasopressin] (mM)	% Mix ± 5
0.00	21
0.01	24
0.05	25

1. measured with 7.4 mM Ca^{++} , PE/PS (4:1) vesicles at 5
 min after Ca^{++} addition

(b) Effect of Peptides

The incubation of the vesicles with arginine vasopressin before Ca^{++} addition resulted in some small changes in the CA9C spectrum, but no fusion as measured by % mix. However, the addition of hormone did affect the time course of the flocculation period (Table 14). There appeared to be no effect on the resultant fusion when measured after the flocculation period (Table 15). These experiments were performed with a PE/PS ratio of 4:1.

Before the flocculation period, arginine vasopressin did affect the extent of fusion in a concentration dependent manner. This was examined in PE/PS 3:1 mixtures (Tables 16 and 17). The level of fusion was measured after 5 minutes at a series of Ca^{++} and Arg vasopressin additions. For a 2 mM Ca^{++} addition, the flocculation period occurs at greater than 5 minutes. In this case, an increase in % mix is seen with increased arginine vasopressin concentration. At 3 and 4 mM Ca^{++} , flocculation has already occurred at the time of measurement. In these cases, there is little measurable change in % mix at these arginine vasopressin concentrations. Fusion data in Table 16 were obtained in the preflocculation period. In this experiment, % mix was measured 2 minute after Ca^{++} addition, well before

TABLE 15
TIME COURSE AND FUSION OF PE/PS (3:1) VESICLES

[Ca ⁺⁺] (mM)	Time to Flocculation	% Mix after 5 min [Arg Vasopressin] (mM)		
		0.00	0.01	0.05
2.0	5	10	15	19
3.0	3	20	19	
4.0	2	25	20	

TABLE 16
FUSION OF PE/PS (3:1) VESICLES IN THE
PRE-FLOCCULATION PERIOD AND THE EFFECT OF PEPTIDES

[Peptide] (mM)	% Mix ± 5
0.00	3
0.06 Arg Vasopressin	11
0.10 Arg Vasopressin	17
0.20 Arg Vasopressin	33
0.20 Oxytocin	1

1. measured after 2 min with 2 mM Ca⁺⁺ addition

flocculation. Fusion increases with increasing hormone. On the addition of 0.20 mM Arg vasopressin, flocculation occurred at the time of measurement. Oxytocin at a similar concentration had no effect.

(c) Experimental Limitations

The extrusion of the DHNDB probe reduced the usefulness of this assay. The PE-NDB probe, in which the NDB chromophore is attached to the PE headgroup, would most probably have a better chance of staying in the vesicles during these events. It has been suggested, by studies using the PE-NDB probe paired with another headgroup probe PE-Rhe, that aggregation can bring the chromophores close enough together to record a % mix for aggregation (111). In this study, arginine vasopressin may cause aggregation, but no increase of % mix was demonstrated without the addition of Ca^{++} . Thus in this experiment, it can be assumed that the % mix levels reflect fusion, not aggregation. This may be due to the location of the CA9C probe away from the surface of the vesicle contact area. The level of fusion measured is similar to that of PE/PS LUV (112).

(d) Discussion

The fusion of vesicles is of two parts: the aggregation of vesicles, followed by the fusion event

where V_1 denotes a single vesicle, V_2 an aggregated dimer and F_2 the fusion product (one large vesicle). The rate constants for aggregation are C_{11} and D_{11} , whereas that for fusion is f_{11} (113). As no fusion is observed on addition of vasopressin alone to the vesicles after 10 to 15 minutes, it is apparent that vasopressin cannot cause fusion. In these experiments, Ca^{++} is the fusogen. However, vasopressin can cause aggregation of PS vesicles. Before the flocculation period, the rate limiting step for fusion is the aggregation of vesicles. After flocculation, when large aggregates are formed, and there is nothing left to aggregate. The fusion process becomes rate limiting. The fact that vasopressin appears to have no effect on the post-flocculation fusion levels is evidence that it has no effect on the fusion process. Thus, it can be concluded that the effect of vasopressin is to increase aggregation, the first step in the fusion process. Aggregation during incubation of SUV with the peptide could be monitored to

confirm this. Oxytocin is ineffective in enhancing Ca^{++} fusion. The charge in position 8 appears to be necessary, as noted for the enhancement of bilayer-hexagonal phase transitions.

This synergistic effect is comparable to that of Mg^{++} with PE/PS LUV (112). At low concentrations, Mg^{++} does not promote the fusion of these LUV. However, when added together with Ca^{++} , the rate of fusion is increased over the rate measured with Ca^{++} alone. This is thought to be due to the appearance of new high affinity binding sites for Ca^{++} in aggregated vesicles, binding in the trans mode as previously mentioned (106,107). This is not apparent in unaggregated vesicles which have a lower affinity for Ca^{++} . As rates of aggregation and fusion depend on vesicle size, concentration, phospholipid composition and temperature, it is not possible to compare the activity of arginine vasopressin in this study with the activity of Mg^{++} as noted by Duzgunes et al (112). The experiments with PE/PS LUV were working with comparable levels of Ca^{++} , but 3 to 4 mM Mg^{++} . These experiments indicate appreciable fusion enhancement with 0.06 mM arginine vasopressin.

These results correlate well with the enhanced

bilayer-hexagonal phase behaviour outlined in the previous section. Further experiments (using a better fusion assay) to investigate the effect of lysine in position 8 in comparison to arginine would be useful as would be a comparison with Mg^{++} . They also correlate with the aggregation phenomenon observed with PS SUV.

4. Aggregation of PS SUV

(a) Comparison of the Effect of Peptides on PS SUV Aggregation

The effect of arginine vasopressin, lysine vasopressin and oxytocin on the aggregation of PS SUV at 25 mM NaCac, pH 5.3 is shown in Figure 19. The threshold for aggregation is 0.1 mM for arginine vasopressin and 0.2 mM for lysine vasopressin. It should be noted that the light scattering profile for arginine vasopressin is observed up to 400 mM NaCac with a threshold of 0.02 mM arginine vasopressin (0.07 mM PS), but with reduced scattering ratios. The light scattering method simply measures aggregation, but gives no information on fusion, or the mixing of vesicle contents. Electron microscopy of the aggregates formed by arginine vasopressin indicated massive structures with some bilayer intact, some multilamellar

FIGURE 19: Comparative Effects of Divalent Peptides and Oxytocin on the Aggregation of PS SUV: Light Scattering Profiles

Samples were 0.33 mM PS in 25 mM NaCac buffer, pH 5.3, at 22°C. Light Scattering was monitored at 325 nm using 2 nm excitation and 1 nm emission slit widths. The ratio of scattering of (PS + peptide)/PS samples, R_{Sc} , is plotted as a function of peptide addition

- arginine vasopressin
- ▲ lysine vasopressin
- oxytocin

formations and deformed vesicles. The effect of the peptides on the aggregation phenomenon is clearly arginine vasopressin > lysine vasopressin and oxytocin has little effect.

(b) Discussion

These results correlate well with the observed effects of the peptides on Ca^{++} mediated events. The formation of stable aggregates depends on the collision rate, and the ability of the bilayers to overcome the repulsive forces between them (114). Attractive forces are Van der Waals, while the repulsive forces are due to charge repulsion and due to the hydration shell of the headgroups. The hydration repulsion prevents bilayers from approaching closer than 12 Å for PE and 27 Å for PC (115), indicating a difference in the bound water of different headgroups. PC is therefore inhibitory to the formation of aggregates and subsequent fusion. In the case of charged vesicles, such as PS vesicles, it is the negative charge repulsion that keeps vesicles separate. Aggregation by divalent and monovalent cations is due to charge neutralization as the cations bind. It is possible to compare the results of these experiments with those of Portis et al (107) because the concentrations, vesicle size, lipid content and temperature

are similar. The threshold concentration for aggregation by Mg^{++} is 2 mM. In these experiments, the threshold is approximately 0.1 mM for arginine vasopressin and 0.2 mM for lysine vasopressin. At lower vesicle concentrations (0.7 mM PS) this value is reduced to 0.02 mM for arginine vasopressin. It is therefore clear that the peptides are more active than Mg^{++} . This suggests that either the binding constant or the aggregating ability of the peptides is greater than Mg^{++} . This could be due to a trans binding mode of the peptides. As the charges are well separated, it is possible that they prefer to bind to two different vesicles. The separation between the charges would prevent the close approach of the bilayers that occurs with Ca^{++} . In either case, it is clear that there is a difference between the activity of the peptides, due to a difference in the charged side chains. The guanidino group of arginine appears to be more effective than the amino group of lysine.

The persistence of activity of 0.02 mM arginine vasopressin in 400 mM Na^+ suggests that the molecule binds much more strongly than oxytocin, for which 250 mM Na^+ is enough to compete effectively with 0.7 mM oxytocin. Although there is a higher Boltzmann factor for the divalent peptide ($z = 2$), at 400 mM Na^+ the surface

potential could not be more than -47 mV. For a divalent peptide at 25°C, this translates to a Boltzmann factor of 39 for a divalent ion as opposed to 6 for a singly charged peptide. It cannot account for the large difference in Na^+ competition.

D. Summary

Both arginine vasopressin and oxytocin decrease the mobility of the phosphodiester moiety of PS as measured by ^{31}P NMR. The chemical shift indicates the proximity of the charged peptide to the ^{31}P nucleus. Arginine vasopressin appears to be more effective than oxytocin due to the extra charge on the arginine side chain. However, the magnitude of these effects is less than that noted by other workers for Mg^{++} (105). The order and conformation of the phosphodiester is not affected by arginine vasopressin as evidenced by the lack of effect on the PS ^{31}P CSA. The divalent peptides, arginine and lysine vasopressin, but not oxytocin enhance the Ca^{++} mediated bilayer-hexagonal phase transition of PE/PS MLV. Arginine vasopressin is more effective than lysine vasopressin. Arginine vasopressin, but not oxytocin also enhances the Ca^{++} mediated fusion of PE/PS vesicles as measured by fluorescence energy transfer. This is most likely due to

the promotion of aggregation of PE/PS vesicles by arginine vasopressin, rather than the promotion of fusion. These events were correlated with the aggregation of PS SUV as monitored by light scattering. Arginine and lysine vasopressin, but not oxytocin causes considerable aggregation of these SUV. Again, arginine vasopressin is more active than lysine vasopressin. The concentration of peptide required to cause PS aggregation is much lower than that of Mg^{++} as noted by Portis et al (107). This could be due to a higher binding constant of the peptides, or an enhanced ability to aggregate vesicles by cross-linking. While the peptides may bind to PE, the lack of effect of PC vesicles on the polarization of the tyrosine chromophore suggests that, like the monovalent peptides, binding to neutral surfaces is very low. The correlation between the activities of the peptides on PS vesicles and PE/PS vesicles suggests that the peptide-PS interaction effects the mixed vesicles.

From the persistence of arginine vasopressin binding at 400 mM Na^+ it is clear that the intrinsic binding constant of the divalent peptides must be greater than that of the monovalent peptides, however a reasonable estimate cannot be made from these data.

No hydrophobic association is observed for these peptides either by tyrosine fluorescence enhancement on binding to PS, or evidence of binding to PC vesicles. However, the divalent peptides appear to exert a profound effect at the surface of the PS bilayers. The forces that govern the separation of interacting vesicles also govern the separation between bilayers of MLV. Whether the divalent peptides' activities are due to charge neutralization, binding in the trans mode, vesicle to vesicle, or bilayer to bilayer, or to some other factor is not clear from these experiments.

It is clear, however, that the intrinsic binding association must be greater than 30 M^{-1} (as measured for oxytocin) and that the guanadino group of arginine is more effective than the amino group of lysine for the activities outlined in this study.

CHAPTER FOUR: SUMMARY AND CONCLUSIONS

I. Binding of Peptides to PS, PC and PE

The binding of monovalent peptides to phospholipid surfaces appears to be peripheral. There is no evidence for a hydrophobic association with acyl chains. At the concentrations used (to 10^{-4} M) there was no evidence of binding to PC. Binding of the monovalent peptides, oxytocin, and 1-D-Cys oxytocin is dependent on the charge of the PS serine carboxyl, and is easily reversed by Na^+ and Ca^{++} ions. Binding of oxypressin to PS appears to be reduced in the presence of PC, which reduces the surface charge of the vesicles. No evidence for binding to PC was found for any of the monovalent peptides. An estimate of K_i is not more than 30 M^{-1} for oxytocin and 1-D-Cys oxytocin, which is consistent with these observations. The binding profiles of TNS to PS vesicles to which oxytocin and oxypressin were added were similar (Appendix). This, together with fluorescence data indicating a similar titration and effect of PS on the bound peptide suggests that the K_i of oxypressin is similar to that of oxytocin. As the binding is greater than that expected for a simple cation, it would appear the molecule must have other

interactions, but the primary interaction is with the amino terminal group. The stoichiometry is 3 to 5 molecules of PS to one peptide. Fluorescence and NMR studies indicate that the ring portion of oxytocin is immobilized by the interaction, but the tripeptide tail has some freedom of motion. The interaction appears to be somewhat indifferent to changes other than charge in the 1 and 3 position. TNS studies (Appendix) indicate that the hydrophobicity of the amino acid in position 2 may be a factor in the interaction. These studies also indicate that desamino lysine vasopressin does bind to PS, however, the tyrosine chromophore is not affected by the binding. This could be due to a lower binding constant of the lysine amino group in comparison to the terminal amino group, or it could be due to a lack of effect of binding on the tyrosine chromophore.

Fluorescence studies do not indicate a hydrophobic association with PS for the divalent peptides, arginine and lysine vasopressin, nor is an interaction with PC evident at 10^{-4} M. Lysine vasopressin has a similar stoichiometry to the monovalent peptides. However, the divalent peptides have quite different activities which can be related to the additional charge in position 8. They enhance Ca^{++} mediated bilayer-hexagonal PE/PS phase

changes. Arginine vasopressin enhances Ca^{++} mediated PE/PS fusion. This appears to be due to enhanced aggregation of PE/PS vesicles. Arginine and lysine vasopressin also cause aggregation of PS vesicles. Comparative studies of arginine and lysine vasopressin indicate that the guanidino and amino groups contribute specifically to activity which must be interpreted as a difference in binding ability. ^{31}P NMR studies indicate that the phosphodiester moiety is less affected by these peptides than by Mg^{++} . However, the aggregating ability of the peptides is much greater. This may reflect a higher binding constant of the peptides, or an enhanced aggregating ability that manifests itself at a lower concentration of bound peptide than is required for charge neutralization. Lysine and arginine vasopressin have a higher intrinsic binding constant than the monovalent peptides, but no estimates could be made in this study.

The effect of the charge in position 8 suggests that this group does have the capability of binding to PS as indicated in the TNS studies (Appendix) with desamino lysine vasopressin. It is possible that desamino lysine vasopressin does bind, but that binding does not affect the mobility of the tyrosine chromophore. From this it is possible to suggest that in this case, the tripeptide tail

is attached to the surface, while the ring portion of the peptide to which the tyrosine is attached retains its flexibility. For oxytocin, the ring is immobilized by virtue of the terminal amino group which forces the ring into contact with the surface, leaving the tripeptide tail free. This would be consistent with the T_1 and T_2 data. For the divalent peptides, a similar model is possible. If the amino charge provides the primary attraction, the tripeptide tail may be sterically hindered from interacting with PS molecules on the same vesicle. Binding of the charged group in position 8 to another vesicle may be more energetically favourable and would provide stability to the aggregate by cross-linking.

Previous studies of the interaction of these peptides with stearyl monophosphate monolayers (20) is consistent with the studies reported here. ^{31}P NMR data indicate that this nucleus is sensitive to the binding of oxytocin and arginine vasopressin. While this may be a secondary effect, it is possible that the primary interaction is with the phosphodiester moiety, the carboxyl group being necessary for providing the attractive surface charge. No specific interaction with the carboxyl group was indicated in the ^{13}C NMR spectrum of PS, although the carbons in the glycerol region were sensitive to the interaction

indicating a headgroup interaction. As there is no evidence that any of the peptides enter the hydrophobic portion of the bilayer in these studies, the observation of increased water permeability and channel formation (21,22,24,25) is a mystery. It should be noted that the previous investigations use decane in the BLM and impose a potential across the BLM, either osmotic or electric. Under these circumstances, with the peptide added to only one side, defects could be formed where there is a local change in surface charge due to the binding of the molecule. It is possible that such events are not observed at the concentrations used in this study. It is also possible that the addition of decane to the lipid allows hydrophobic interactions. The experiments which measured the binding constant of lysine vasopressin also used a PS-decane system (26). It would be useful to determine the effect of decane on peptide binding to PS. Surface potential studies using the TNS probe (Appendix) indicate that the Stern equation may not adequately describe the binding of these peptides, but further work is required to establish this. However, this study has shown that the binding of divalent peptides to PS is higher than that of monovalents from the persistence of aggregating effects by 0.02 mM arginine vasopressin in 400 mM NaCl. This increase is ascribed to the charge of position 8.

II. Biological Implications

Circulating levels of hormone are very low (10^{-12} M). The binding constants for receptors are 10^9 M⁻¹ (16). Most negatively charged lipids appear to reside on the inner surface of the cell. This suggests that the effects noted in this study are not likely to be biologically significant per se. To date there appears to be no evidence that the peptides are internalized as other peptides are (116). Desamino oxytocin which shows no evidence of binding in these studies is more active than oxytocin for uterotonic and milk ejecting biological activities. The many activities of vasopressins are linked to two types of receptors, V_1 and V_2 (16). The latter activates the adenylyl cyclase system, for which desamino arginine vasopressin is more active than arginine vasopressin. The former activate a Ca^{++} mobilizing system during which the cytosolic concentration of Ca^{++} is increased. For these receptors, the divalent peptide is most active, and the monovalent peptides are partial agonists. For platelet aggregation, the addition of a Ca^{++} ionophore restores the activity of α agonists (117). However, the chirality of the peptide in position 8 is also important, the D-arginine analogue is inhibitory. It is doubtful whether this change would affect the activities noted in this

study, but a simple check of aggregating ability in comparison to one of the other divalent peptides would establish this. Therefore, in the case of the V_1 receptor alone, it is possible that binding to the receptor could be enhanced by a negative surface potential as outlined by Sargent and Schwyzer (34). If the D-arginine analogue aggregates PS as much as arginine vasopressin, it could be concluded that the lipid interaction could only affect receptor binding, and not activity.

Other than the possible effect of a negative surface charge at the V_1 receptor for vasopressins it is very difficult to reconcile the rather nonspecific effects noted here with biological activity that occurs on a much more specific level. However, this does not preclude the possibility that they are involved at some level in activity, or that there are other lipids with which the peptides interact more specifically. These types of interaction may be important for biological studies in which the peptide concentrations are artificially increased. They may also be a factor when cells are treated with phospholipases, which would alter the lipid headgroups.

III. Suggestions for Further Study

It is clear that the divalent peptides have a profound affect on the surface of PS vesicles. However, PS vesicles are highly charged, and while perhaps useful as models for peripheral peptide interactions, understanding the biological role of lipid-hormone interactions may best be studied using a more biological system. As V_1 receptors together with couplers and effectors have been recently isolated using lysophosphatidylcholine (118), this may provide a more appropriate system for study.

APPENDIX

MEASUREMENT OF SURFACE POTENTIALS USING A FLUORESCENT PROBE

I. Introduction

These experiments were pursued for two reasons, to measure binding at a lower concentration for the monovalents to determine whether the binding was affected by the 1 and 3 position, and to measure the binding for analogues substituted in the 2 position. As the results were preliminary, and ambiguous with the exception of indicating that desamino lysine vasopressin does bind to PS vesicles, they have been included in this Appendix.

The surface potential is a potent tool for measuring the binding of charged ions to surfaces. The modified Guoy-Chapman-Stern theory has been used to examine the binding of monovalents (32) and divalents (38) to negatively charged SUV and is well described.

The surface is seen as a uniformly charged surface (discrete charge effects are ignored). The electrostatic potential, ψ_0 , at the surface is related to the charge density, σ , by the Guoy equation:

$$\sinh (e \psi_0 / 2kT) = A \sigma \sqrt{C} \quad (31)$$

where C is the bulk phase ion concentration, e is the electronic charge, k is the Boltzmann constant, T is the temperature and

$$A = 1/(8N\epsilon_r\epsilon_0 kT)^{\frac{1}{2}} \quad (32)$$

Where N is Avogadro's number, ϵ_r is the dielectric constant and ϵ_0 is the permittivity of free space. For a PS molecule, which is assumed to have a surface area of $70 \text{ \AA}^2$, the maximum charge density is $-1/70 \text{ \AA}^2$. The distribution of ions at the surface under the influence of the surface potential is given in equation (1). Adsorption of ions occurs with specific binding of the ions to the surface which results in excess neutralization over that expected from the usual screening in the diffuse double layer. Assuming a 1:1 PS/C relationship, the adsorption is described by :

$$\sigma = \sigma_{\max} / (1 + KC_0) \quad (33)$$

If the surface potential is measured for a particular bulk concentration of ion, equations (31) and (33) can be combined to find K .

II. Experimental

The method of Eisenberg et al (32) was used to measure the surface potentials of PC and PS SUV in the presence of various peptides. 2-p-toluidiny1 naphthalene-6-sulphonate (TNS) has been shown to bind hydrophobically to these lipids and its binding can be described by the Stern equation (42). The fluorescence intensity of TNS (free) in buffer is much much less than that of TNS (bound) to lipid. Thus its fluorescent properties can be used to determine the concentration of TNS (bound), $[TNS]_b$. If conditions are arranged such that the $[TNS]_b$ is very small in comparison to the amount of surface lipid available, the surface potential of a negatively charged surface can be determined from the binding equation;

$$[TNS]_b / ([TNS]_f \times [PS]_f) = K(TNS) \times e^{(e\psi_0/kT)} \quad (34)$$

where K is the binding constant and ψ_0 is the surface potential.

The sodium salt of TNS (Molecular Probes, Inc.) was examined by UV spectroscopy in MeOH and in 25 mM NaCac buffer, 0.1 mM EDTA, pH 5.3. The ϵ for absorbance were calculated. An ϵ of 2.19×10^4 at 318 nm in MeOH was

used to calculate concentrations of stock TNS solutions. Emission and excitation spectra were recorded at 25°C for both solutions using a λ (ex) of 320 nm and λ (em) of 445 nm, both slits were 2 nm. Values obtained were checked with the literature (119) and TNS used without further purification. A check of I(445) obtained using 321 nm exciting light was determined as a function of TNS absorbance at 321 nm. All subsequent measurements were made using TNS solutions with absorbance less than 0.08 ([TNS] of about 0.004 mM). Above this limit, the fluorescence intensity was not linear with respect to TNS concentration.

Experiments were carried out at 25°C, using a 25 mM NaCac buffer, 0.1 mM EDTA, pH 5.3. A stock solution of TNS (0.1 mM) in buffer was prepared, wrapped in foil or frozen when not in use, and kept for 1 to 2 weeks. Aliquots of this solution were used for the experiments. A 50 microlitre aliquot of this was dissolved in 2 ml MeOH for each day's experiments. Its UV and fluorescence spectra were recorded and its concentration checked using the OD at 321 nm. Stock solutions of PS or PC SUV were prepared in the usual manner. All concentrations were determined by dilution from the known concentrations of the stock solutions. Instrument parameters were fixed for λ (ex) of

321 nm and λ (em) of 445 nm with 3 and 4 nm slits respectively. Intensities were sampled for 30 seconds or less. Full emission spectra were recorded for a few samples at the beginning and at the end of the experiment.

TNS-lipid titrations were carried out by varying either the lipid or the TNS concentrations. The former titrations were performed to check that TNS was not absorbed appreciably by the SUV. Titrations were repeated with various concentrations of peptide present. The data were obtained from titrations in which aliquots of TNS were added to SUV samples with or without peptide. For measurements on the lipid alone, three samples were prepared with buffer (A), buffer and lipid (B), or buffer and lipid (C). These, together with the MeOH-TNS reference were incubated in the chamber for 10 minutes. The intensity of the reference sample was obtained. Aliquots of TNS were added to samples A and B, and buffer added to C in quick succession. The fluorescence intensities were measured at timed intervals (30 to 45 seconds) after the addition. This was repeated for three TNS additions. The intensity of the reference sample was recorded again after the titrations were complete to check that there had been no instrumental change over the sampling period. For the measurements on the peptide-SUV, peptide was added to

sample B. Checks had been made previously to confirm that samples containing peptide and TNS without lipid were equivalent to sample A. $I(\text{TNS}(b))$ was determined in the following way for each TNS addition;

$$I(\text{TNS}(b)) = I(B) - I(A) - I(C). \quad (35)$$

The subtractions account for the background fluorescence of TNS in water and the background scatter due to the SUV.

$[\text{TNS}]_b$ for each TNS addition was determined using the following equation;

$$[\text{TNS}]_b = I(\text{TNS}(b)) / \alpha \quad (36)$$

where

$$\alpha = [I(\text{TNS-MeOH}) / [\text{TNS}]_{\text{MeOH}}] \times [Q(\text{TNS, SUV}) / Q(\text{TNS, MeOH})]. \quad (37)$$

$Q(\text{TNS, MeOH})$, $Q(\text{TNS, PC})$ and $Q(\text{TNS, PS})$ were taken as 0.632, 0.60 and 0.59 respectively from (32). $[\text{TNS}]_f$ was then calculated as $[\text{TNS}]_{\text{total}} - [\text{TNS}]_b$. Plots of $[\text{TNS}]_b$ as a function of $[\text{TNS}]_f$ were analyzed using a Canon Scientific Statistical Calculator, F-37P for each SUV and SUV-peptide sample. The slopes, s , obtained were divided by the $[\text{lipid}]_{\text{outer}}$ to give

$$R = [K_{TNS} e^{(e\psi_0/kT)}] \quad (38)$$

for each sample.

III. Results and Discussion

A. The Behaviour of the TNS Probe in PS and PC Vesicles

The probe exhibited a large increase in fluorescence when bound to vesicles. Fluorescence in buffer alone was not observable at these concentrations. K_{TNS-PC} was determined as $2.3 \times 10^3 \text{ M}^{-1}$. In this case, the surface potential is 0. This is somewhat lower than the values determined by Eisenberg et al (32), but when this value of K was used to determine the surface potential for PS vesicles in 100 mM Na^+ , the value found, $-69 \pm 2 \text{ mV}$, was comparable to the -70 mV they reported. The value obtained for 25 mM Na^+ was -97 ± 2 . These are lower than the expected values for K_{Na} of 0.6 M^{-1} as noted in their paper. The wavelength maximum for PS is slightly blue-shifted with respect to that of PC. Wavelength maxima for TNS in MeOH, PC and PS were found to be 443, 440 and 434 nm respectively. The TNS probe is sensitive to the viscosity as well as the dielectric constant which may account for these differences (119).

2. Effect of Peptides on Surface Potentials.

The addition of 1.1×10^{-3} M oxytocin had no effect on $K_{\text{TNS-PC}}$ nor was any fluorescence measured when this amount of oxytocin was present with TNS in buffer alone. However, when TNS was added to PS vesicles incubated with varying concentrations of peptide a dramatic enhancement of the fluorescence was observed with buffers containing both 25 mM Na^+ and 100 mM Na^+ . Wavelength maxima were further blue-shifted by 3 - 7 nm. R_0 (R as in equation 38) was measured for the PS sample without peptide addition, R_p for the PS sample containing peptide. Assuming K to be constant,

$$\ln (R_p/R_0) = e/kT(\psi_0(\text{PS-Peptide}) - \psi_0(\text{PS})) \quad (39)$$

Plots of $\ln R_p/R_0$ are shown in Figures 20 and 21 for oxytocin, oxypressin, 2-Leu oxytocin, 2-Gly oxytocin, desamino lysine vasopressin and desamino oxytocin in buffer containing 25 mM Na^+ and 100 mM Na^+ . The plots look very much like binding curves. It is possible to calculate the expected surface potentials arising from different situations assuming the peptide is all bound and assuming a stoichiometry of three. The concepts can be understood more clearly with the relationship;

FIGURE 20: Effect of Peptides on 'Surface Potential' of PS
SUV, 25 mM Na⁺

Samples were 1.5×10^{-4} M [PS]_{outer} in 25 mM NaCac
buffer, 0.1 mM EDTA, pH 5.3 at 25°C. Peptide additions as
indicated.

○ oxytocin

● oxypressin

△ 2-Leu oxytocin

▲ 2-Gly oxytocin

□ desamino lysine vasopressin

■ desamino oxytocin

FIGURE 21: Effect of Peptides on 'Surface Potential' of PS
SUV, 100 mM Na⁺

Samples were 1.5×10^{-4} M [PS]_{outer} in 25 mM NaCac, 75
mM NaCl, 0.1 mM EDTA, pH 5.3 at 25°C. Peptide additions as
indicated

○ oxytocin

● oxypressin

△ 2-Leu oxytocin

▲ 2-Gly oxytocin

$$\frac{\sigma}{\sigma_{\max}} = \frac{\sum \text{PS}^-}{\sum \text{PS}_{\text{tot}}} \quad (40)$$

where $\sum$ denote surface concentrations. If no peptide is present, the surface charge density and the surface potential for 25 mM Na^+ at 25°C using K_{Na} of 0.6 M^{-1} is -0.00572 Å^{-2} and -118.3 mV respectively. Thus,

$$\frac{\sum \text{NaPS}}{\sum \text{PS}_{\text{tot}}} = \frac{1 - \sigma}{\sigma_{\max}} \quad (41)$$

which is 0.60. If the peptide binds in a way to prevent the binding Na^+ to any PS molecule, completely competitively, then in the all bound condition, PS^- will be 2/3 of PS_{tot} . This translates to a surface charge density of -0.0095 Å^{-2} and a corresponding potential of -144 mV , which is in the opposite direction of that noted in this experiment. However, if it is assumed that the 2/3 of $\text{PS}_{\text{tot}} = \text{PS}^-$ is available for binding to Na^+ , using the same constants, it can be determined that the surface charge density would be -0.00476 Å^{-2} with a corresponding surface potential of -109 mV . This corresponds to an R_p/R_0 value of only 0.35. The values indicated in Figure 20 are considerably higher than this. Even if the

quantum yield of TNS increased to 1 when peptide is present, it could not account for the high ratios.

There are two possibilities for these observations. The TNS is reflecting a true surface charge, and the simple model cannot account for the binding. The peptide may bind in such a way as to change the charge plane. The assumption that the dielectric remains constant to the surface may not be appropriate. In addition, Na^+ and the peptides may bind to different sites which cannot be taken into account using this analysis. In this case, the difference in the values obtained for the peptides would be due to binding. This would indicate that the amino acid in position 2 is important for the interaction with PS, the less hydrophobic species being less active. While desamino oxytocin appears to bind, this is probably a reflection of the experimental error. Desamino lysine vasopressin does bind, but the very low value indicates that it either binds in a different manner, or has a much lower binding constant. Preliminary studies with ^{23}Na NMR to determine Na^+ binding indicated that the peptide either promoted Na^+ binding, or that the Na^+ was in a very different environment from that of PS with no peptide added.

The other possibility is that when bound to PS, the

peptide provides a hydrophobic binding site for TNS. TNS does not bind to melittin monomers, but does to melittin dimers. This is apparent in the fluorescence spectra (120). It is possible that when the peptide is bound to the surface it provides a binding site in much the same way. In this case, the R_p/R_0 values would represent a difference in K_{TNS} as well as possible surface charge. In this case the profiles in Figures 20 and 21 may not reflect a difference in the binding of peptide to PS, but a difference in the binding of TNS to peptide-PS sites. This would be consistent with the observed difference in position 2, the more hydrophobic species being more successful.

As the lifetime of TNS bound to PS, or to peptide-PS should be different, measurement of the lifetime should indicate whether the peptide provides a new binding site. In addition, surface charges measured using a cationic probe, rather than an anionic probe, or determined by electrophoresis methods might indicate whether the surface charge is as indicated by this experiment.

The experiment does provide proof that desamino lysine vasopressin does bind, but does not indicate the strength of the binding.

REFERENCES

- 1 Lee, A.G. in Membrane Fluidity in Biology, Volume 2, General Principles (Aloia, R.C.,ed) Academic Press: New York, 1983; pp 43-88
2. Jain M.K. in Membrane Fluidity in Biology, Volume 1, Concepts of Membrane Structure (Aloia, R.C. ed) Academic Press: New York, 1983; pp 1-37
3. Cullis, P.R.; deKruijff, B.; Biochim. Biophys. Acta 1979 (559) 399
4. McLaughlin, S.; Curr. Top. Membr. Transp. 1977 (9) 71
5. Nir, S.; Bentz, J.; Wilschut, J.; Duzgunes, N.; Prog. Surf. Science 1983 (13) 1
6. Szoka, F.; Papahadjopoulos, D.; Ann. Rev. Biophys. Bioeng. 1980 (9) 467
7. Grell, E. ed.; Molecular Biology, Biochemistry and Biophysics, Volume 31: Membrane Spectroscopy, Springer-Verlag: Berlin, 1981
8. Chapman, D. in 1; pp 5-41

9. Sandermann Jr., H.; *Biochim. Biophys. Acta* 1978 (515) 209
10. Loh, H.H.; Law, P.Y.; *Ann. Rev. Pharmacol. Toxicol.* 1980 (20) 201
11. Houslay, M.D.; Gordon, L.M.; *Curr. Topics Memb. Transp.* 1983 (18) 179
12. Cullis, P.R.; deKruijff, B.; Hope, M.J.; Verkleij, A.J.; Nayer, R.; Farren, S.B.; Tilcock, C.; Madden, T.D.; Bally, M.B. in 2; pp 39-81
13. Hanahan, D.J.; Nelson, D.R.; *J. Lipid Res.* 1984 (25) 1528
14. Soloff, M.S.; Pearlmutter, A.F.; *Biochem. Action of Hormones* 1979 (6) 265
15. deWied, D.; *Prog. Brain Res.* 1983 (60) 155
16. Jard, S.; *Curr. Top. Memb. Transp.* 1983 (18) 255
17. Manning, M.; Lowbridge, J.; Haldar, J.; Sawyer, W.H.; *Fed. Proc.* 1977 (36) 1848

18. Warr, J.L.; Branda, L.A.; Gynecol. Obstet. Invest. 1983
(15) 103

19. Snart, R.S.; Sanyal, N.N.; Biochem. J. 1968 (108) 369

20. Kafka, M.S.; Pak, C.Y.C.; Biochim. Biophys. Acta 1969
(193) 117

21. Graziani, Y.; Livne, A.; Biochem. Biophys. Res. Comm.
1971 (45) 321

22. Graziani, Y.; Livne, A.; Biochim. Biophys. Acta 1973
(291) 612

23. Fettiplace, R.; Haydon, D.A.; Knowles, C.P.; Proc.
Physiol. Soc. (1971) 18P

24. Bach, D.; Miller, I.R.; Biochim. Biophys. Acta 1974
(339) 367

25. Krasil'nikov, O.V.; Sabirov, R.Z.; Tashmukhamedov,
B.A.; Doklady Akademii Nauk SSSR 1983 (272) 220

26. Fettiplace, R.; Haydon, D.A.; Biochim. Biophys. Acta
1976 (436) 593

27. Stern, O.; Z. Elektrochem. 1924 (30) 508
28. Miller, I.R.; Rishpon, J.; Tenenbaum, A.;
Bioelectrochem. Bioenerg. 1976 (3) 528
29. Johns, J.F., 4th year Thesis, Carleton University 1978
30. Laclette, J.P.; Montal, M.; Biophys. J. 1977 (19) 199
31. Dousa, T.P.; Fed. Proc. 1977 (36) 1867
32. Eisenberg, M.; Gresalfi, T.; Riccio, T.; McLaughlin,
S.; Biochemistry 1979 (18) 5213
33. Florence, T.M.; Biochem. J. 1980 (189) 507
34. Sargent, D.F.; Schwyzer, R.; Proc. Natl. Acad. Sci. USA
1986 (83) 5774
35. Kaiser E.T.; Kezdy, F.J.; Ann. Rev. Biophys. Bioeng.
1987 (16) 561
36. Schwyzer, R.; Biochemistry 1986 (25) 6225
37. Boggs, J. M. in 1; pp 89-130

38. McLaughlin, S.; Mulrine, N.; Gresalfi, T.; Vaio, G.;
McLaughlin, A.; J. Gen. Physiol. 1981 (77) 445
39. Chung, L.; Kaloynides, G.; McDaniel, R.; McLaughlin A.;
McLaughlin, S.; Biochemistry 1985 (24) 442
40. Deber, C.M.; Behnam, B.A.; Proc. Natl. Acad. Sci. 1984
(81) 61
41. Jarrell, H.C.; Deslauriers, R.; McGregor, W.H.; Smith,
I.C.P.; Biochemistry 1980 (19) 385
42. McLaughlin, S.; Harary, H.; Biochemistry 1976 (15) 1941
43. Gysin, B.; Schwyzer, R.; Biochemistry 1984 (23) 1811
44. Schwyzer, R.; Biochemistry 1986 (25) 4281
45. Hruby, V.J. in Topics in Molecular Pharmacology
(Burgen, A.S.V.; Roberts, C.G.K. eds) Elsevier/North
Holland Biomedical Press: Amsterdam, 1981; pp 99 -126
46. Jardetzky, O.; Roberts, G.C.K.; NMR in Molecular
Biology; Academic Press: New York; 1981 Chapter 12

47. Deslauriers R.; Smith, I.C.P.; Walter, R.; J. Amer. Chem. Soc. 1974 (96) 2289
48. Cowgill, R.W. in Biochemical and Fluorescence; Concepts, Volume 2 (Chen, R.F.; Edelhoch, H. eds) Dekker: New York; 1976
49. Cowgill, R.W.; Arch. Biochem. Biophys. 164 (104) 84
50. Glickson, J.D.; Lenkinski, R.E.; Rama Krishna, N; Agresti, D.G.; in Peptides; Proceedings of the 5th American Peptide Symposium (Goodman, M.; Meienhofer, J eds) John Wiley and Sons Inc.: New York; 1977 pp 325-328
51. Ross, J.B.A.; Laws, W.R., Buku, A.; Sutherland, J.C.; Wyssbrod, H.R.; Biochemistry 1986 (25) 607
52. Lakowicz, J.R.; Maliwal, B.P.; J. Biol. Chem. 1983 (258) 4794
53. Lakowicz, J.R.; Laczko, G.; Gryczynski, I.; Biochemistry 1987 (26) 82
54. as in 46, Chapter 3

55. Marsh, D. in Techniques in the Life Sciences, B4/11
Lipid and Membrane Biochemistry- Part II (Hesketh, T.R.;
Kornberg, H.L.; Metcalfe, J.C.; Northcote, D.H.; Pogson,
C.I.; Tipton, K.F. eds) Elsevier Scientific Publishers
Ireland, Ltd: County Clare, Ireland; 1982 (B426)
56. Lee, A.G. in 55, (B422)
57. Seelig, J.; Quart. Rev. Biophys. 1977 (10) 353
58. Smith, I.C.P.; Ekiel, I.H in Phosphorus-31 NMR
Principles and Applications (Gorenstein, D.G. ed.) Academic
Press Inc.: New York; 1984 pp 447-475
59. Seelig, J.; Biochim. Biophys. Acta 1978 (515) 105
60. Oster, G. in Physical Techniques in Biological
Research, Volume 1: Optical Techniques (Oster, G;
Follister, A.W. eds) Academic Press: New York 1955 pp 51-71
61. Weast, R.C. ed. CRC Handbook of Chemistry and Physics,
55th Edition; CRC Press:Cleveland, Ohio 1974
62. Kates, M. Techniques of Lipidology in Laboratory
Techniques in Biochemistry and Molecular Biology, Volume 3,

(Work, T.S.; Work, E. eds) North Holland/ American
Elsevier:New York 1972

63. Barrow, D.A.; Lentz, B.R.; Biochim. Biophys. Acta 1980
(597) 92

64. Griffin, R.G.; J. Am. Chem. Soc. 1976 (98) 851

65. Cullis, B.R.; deKruijff, B.; Biochim. Biophys. Acta
1978 (507) 207

66. Burnell, E.E.; Cullis, P.E.; deKruijff, B.; Biochim.
Biophys. Acta 1980 (603) 63

67. Huang, C.; Biochemistry 1969 (2) 344

68. Barenholz, Y.; Gibbes, D.; Litman, B.J.; Coll, J.;
Thompson, T.E.; Carlson, R.D.; Biochemistry 1977 (16) 2806

69. Bligh, E.G.; Dyer, W.B.; Can. J. Biochem. Physiol. 1969
(37) 911

70. Duck Chong, C.G.; Lipids 1979 (14) 492

71. Hutton, W.C.; Yeagle, P.L.; Martin, R.B.; Chem. Phys.
Lipids 1977 (19) 255

72. Barsukov, L.I.; Victorov, A.V.; Vasilenko, L.A.;
Evstigneeva, R.P.; Bergelson, L.D.; Biochim. Biophys. Acta
1980 (598) 153
73. The software for transfer and modification of the
Nicolet program was written by R.A. Byrd and M. Rance.
74. Glasoe, P.K.; Long, F.A.; J. Phys. Chem. 1960 (64) 188
75. Haupt, E.J.; J. Mag. Res. 1982 (49) 358
76. Freeman, R.; Hill, H.D.W.; Kaptein, R.; J. Mag. Res.
1972 (7) 327
77. Vold, R.L.; Waugh, J.S.; Klein, M.P.; Phelps, D.E.; J.
Chem. Phys. 1968 (48) 3831
78. Levy, G.C.; Peat, I.R.; J. Mag. Res. 1975 (18) 517
79. Meiboom, S.; Gill, D.; Rev. Sci. Instrum. 1958 (29) 688
80. Vidrine, D.W.; Peterson, P.E.; Anal. Chem. 1979 (13) 78
81. Deslauriers, R.; Smith, I.C.P.; Stahl, G.L.; Walter,
R.; Int. J. Pept. Prot. Res. 1979 (13) 78

82. Deslauriers, R.; Byrd, R.A.; Jarrell, H.C.; Smith, I.C.P.; Eur. J. Biochem. 1980 (111) 369
83. Gupta, R.K.; Gupta, P.; J. Mag. Res. 1980 (40) 587
84. Rance, M.; Byrd, R.A.; J. Mag. Res. 1983 (52) 221
85. Hahn, E.L.; Phys. Rev. 1950 (80) 580
86. Sternin, E.; Bloom, M.; MacKay, A.L.; J. Mag. Res. 1983 (55) 274
87. Tilcock, C.P.S.; Cullis, P.R.; Biochim. Biophys. Acta 1981 (641) 189
88. Dufourc, J.; Faucon, J.F.; Maquet-Dana, R.; Pileni, M.P.; Helene, C; Biochim. Biophys. Acta 1981 (649) 67
89. Personal communication from Dr. Claude Helene
90. Cowgill, R.W.; Biochim. Biophys. Acta 1968 (168) 417
91. Teale, F.W.J.; Photochem. Photobiol. 1969 (10) 363

92. Lentz, B.R.; Moore, B.M.; Barrow, D.A.; Biophys. J.
1979 (25) 489
93. Uster, P.S.; Deamer, D.W.; Arch. Biochem. Biophys. 1981
(209) 385
94. Cowgill, R.W.; Biochim. Biophys. Acta 1967 (140) 37
95. Perrin, R.; J. Phys. Radium 1926 (7) 390
96. Campbell, B.J.; Chu, F.S.; Hubbard, S.; Biochemistry
1963 (2) 764
97. Kuhlman, K.F.; Grant, D.M.; Harris, R.K.; J. Chem.
Phys. 1970 (52) 3439
98. Doddrell, D.; Glushko, V.; Allerhand, A.; J. Chem.
Phys. 1972 (56) 3883
99. Walter, R.; Prasad, K.U.M.; Deslauriers, R.; Smith,
I.C.P.; Proc. Natl. Acad. Sci. USA 1973 (79) 2086
100. Hruby, V.J.; Deb, K.K.; Spatola, A.F.; Upson, D.A.;
Yamamoto, D.; J. Am. Chem. Soc. 1979 (101) 202

101. Levine, Y.K.; Birdsall, N.J.M.; Lee, A.G.; Metcalfe, J.C.; *Biochemistry* 1972 (11) 1416
102. Stoffel, W.; Sierenberg, O.; Tunggal, B.D.; Hoppe-Seyler's *Z. Physiol. Chem.* 1972 (354) 1962
103. Holwerda, D.L.; Ellis, P.D.; Wuthier, R.E.; *Biochemistry* 1981 (20) 418
104. Keniry, M.A.; Smith, R., *Biophys. Chem.* 1980 (12) 133
105. Kurland, J.R.; Hammoudah, M.; Nir, S; Papahadjopoulos, D; *Biochem. Biophys. Res. Comm.* 1979 (88) 927
106. Hope, M.J.; Cullis, P.R.; *Biochem. Biophys. Res. Comm.* 1980 (92) 846
107. Portis, A.; Newton, C.; Pangborn, W.; Papahadjopoulos, D.; *Biochemistry* 1979 (18) 780
108. Blumenthal R.; *Curr. Top. Membr. Transp.* 1987 (29) 203
109. Cullis, P.R.; Verkleij, A.J.; *Biochim. Biophys. Acta* 1979 (552) 546

110. Bally, M.B.; Tilcock, C.P.S.; Hope, M.J.; Cullis, P.R.; Can. J. Biochem. Cell Bio. 1983 (61) 346
111. Duzgunes, N.; Allen, T.M.; Fedor, J.; Papahadjopoulos, D.; Biochemistry 1987 (26) 8435
112. Duzgunes, N.; Wilschut, J.; Fraley, R.; Papahadjopoulos, D.; Biochim. Biophys. Acta 1981 (642) 182
113. Bentz, J.; Duzgunes, N.; Nir, S.; Biochemistry 1983 (22) 3320
114. Rand, R.P.; Ann. Rev. Biophys. Bioeng. 1981 (10) 277
115. Lis, J.; McAlister, M.; Fuller, N.L.; Rand, R.P.; Parsegian, V.A.; Biophys. J. 1982 (37) 657
116. Goldfine, R.D.; Biochim. Biophys. Acta 1981 (650) 53
117. Thomas, M.E.; Osman, A.H.; Scrutton, M.C.; Thrombosis Res. 1983 (32) 557
118. Aiyar, N.; Bennett, R.; Nambi, P.; Valinski, W.; Anqioli, M.; Minnich, M.; Crooke, S.T.; Biochem. J. 1989 (261) 63

119. McLure, W.O.; Edelman, G.M.; Biochemistry 1966 (5)

1908

120. Condie, C.C.; Quay, S.C.; J. Biol. Chem. 1983 (258)

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