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PROTEIN-LIPID INTERACTIONS:

THE ROLE OF DIVALENT CATIONS

IN

MIXED LIGAND COMPLEX FORMATION

by

ROBERT E. HAUSER

A dissertation submitted to the Graduate Faculty in the field of Chemistry in the partial fulfillment of the standard requirements for the degree of Doctor of Philosophy, the City University of New York.

1 9 7 6

This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/5/76
date

Professor Bernard J. Bulkin
Chairman of Examining Committee

12/18/75
date

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Executive Officer

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Professor Maria Tomasz

The City University of New York

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To my wife, Margaret, whose encouragement and sacrifices made these years worthwhile, for typing this thesis and to both the U.S. Marine Corps Reserves and the Hunter College Rugby team who supplied the necessary diversion from academia, I am indebted.

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To Margaret, Mr. and Mrs. Albert J. Hauser, Sr., my Brothers and family, this thesis is affectionately dedicated.

". . . There is not any least effect in Nature which can be fully understood by the most speculative minds in the world."

GALILEO GALILEI

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PROTEIN-LIPID INTERACTIONS:

THE ROLE OF DIVALENT CATIONS

IN

MIXED LIGAND COMPLEX FORMATION

by

Robert E. Hauser

Advisor: Professor Bernard J. Bulkin

* * * * * * * * * * * * * * *

A possible mode of lipid-protein interaction in cell membranes is via mixed ligand complex formation. In such complexes, divalent cations would link a protein ligand, coordinated via NH_2, COO^-, PO_4^-, or the amide carbonyl, to an acidic phospholipid, e.g., Phosphatidyl-L-Serine (PS) coordinated via, COO^-, PO_4^- or at higher pH, $-NH_2$. Mixed ligand complexes of this type have been prepared with Ca^{+2}, PS, and a wide variety of amino acids, several di and tri peptides and three proteins. These mixed ligand complexes were analyzed using analytical chemical methods, Infrared and visible spectroscopy.

Results will be presented describing the time, pH, specific ligand and the specific cation dependence towards these lipid-protein interactions. Further results will describe the effect of this mixed ligand complex

formation on the permeability of mixed phospholipid vesicles to glucose and inorganic phosphate. Together they will focus more attention on this mode of lipid-protein interaction in cell membrane function and physical structure.

INTRODUCTION

BIOLOGICAL MEMBRANES AND MOLECULAR COMPLEXES

When one ponders the idea of life itself or more generally, on independent existence, one must at least concede the fact that the individual "life" must be separated from its environment.

Cells exist by virtue of a surface barrier or membrane and thus this membrane must play a crucial role in the physiological functions associated with that cell. Membranes, as they are presently conceived, were first suggested to exist in the late 1800's as a result of experiments performed on plant cells.[1,2,3] However, it was not until quite recently[4,5,6] that the role of the biological membrane was considered to be more than a demarcator of protoplasmic space or a mere diffusion controlled static barrier. It is now believed that regardless of which membrane model is in vogue, the membrane is a dynamic structure in continual compositional flux about which numerous biochemical and physiological reactions of life occur in, on and through. Such processes as oxidative phosphorylation, vision, nerve impulse conductance, ion transport, protein and lipid biosynthesis, and the Krebs cycle are all membrane phenomena.

Ironically, it is this diversity of both function and structure coupled with the numerous experimental and logistical problems of handling natural biological membranes which pose a challenge to the biological sciences to elucidate both the structure and functional mechanisms of cell membranes.

a) Membrane Composition and Models

In the attempt to elucidate the dynamic structure of a cell membrane, a primary point of attack would be the total molecular composition of that envelope. However, it is a well-characterized fact that membrane composition varies from one class of cells to another [7,8,9] as to the amount and nature of the lipids and proteins present. Generally, biological membranes are found to contain four major classes of molecules: 1) lipids, 2) proteins, 3) sterols, and 4) water.

The lipids, essentially all present as phospholipids, make up from 25 to 50% by weight composition of the specific membrane.[10] Normally, the remaining 50% of the total weight of the membrane is made up of either structural, metabolic or transport proteins, or a combination of each.

Steroids, such as cholesterol are present in all membranes to varying degrees with the exception of

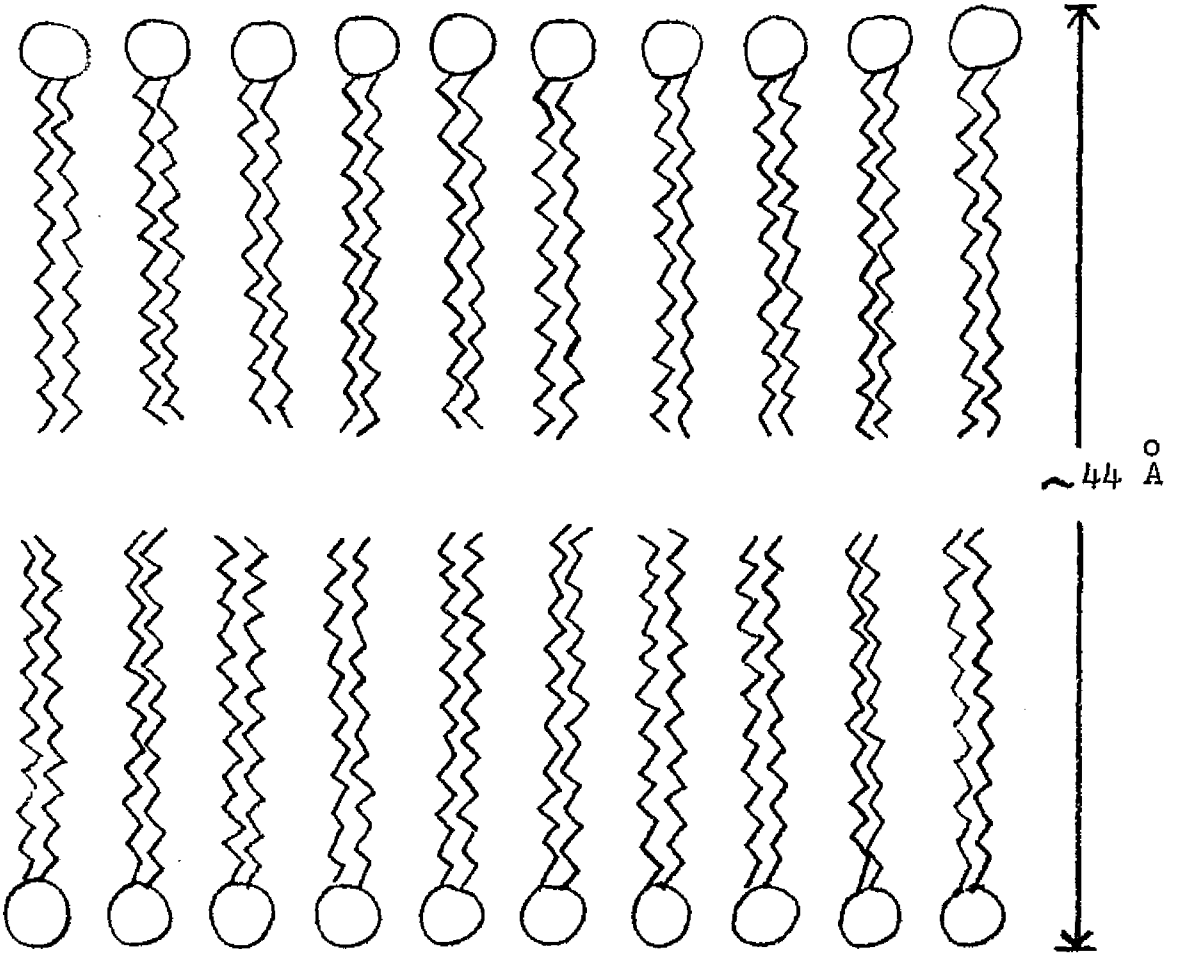
bacterial membranes. It is believed to play a significant role in both the structure of the membrane and in the selective permeability of certain organic solutes. Water, on the other hand, is associated with the polar head groups of all species at the lipid/water interface. Yet knowing the exact composition of a specific cell membrane does not shed light on the structure of that membrane nor does it directly relate to the molecular interactions which are ever present.

To compensate for this lack of structural insight, a number of model lipid-protein systems have been advanced to simulate a cell membrane structure. Figure 1 demonstrates the foremost suggested model, the lipid bilayer system. The idea emanated from the classical experiment of Gorter and Grendel in 1925. Knowing that lipid molecules arrange themselves at an air/waterinterface with their polar substituents in the water phase and the hydrocarbon tails in the air, they estimated the thickness of an erythrocyte membrane by extracting the lipid and spreading them over such an interface. Even though their calculated surface area and total lipid composition were incorrect, their conclusion that the lipids form a double layer was correct. Figure 1 shows this arrangement delineating both the hydrophilic polar head groups (the circles) and the hydrophobic hydrocarbon chains (tails). Experimental evidence such

Figure 1

Bilayer Arrangement of Lipids

The circles represent the polar head groups, the tails are the hydrocarbon fatty acid chains.


~44 Å

as X-ray diffraction,[12] electron microscopy,[13] electrical,[14] and surface tension measurement [15] supports this arrangement. In fact, it was the surface tension experiments which led to the Danielli and Davson [16] model which improved the bilayer model. This system incorporates the lipid bilayer leaflet but also adds a layer of hydrophilic protein to the polar surfaces above the membrane. Figure 2 shows such an arrangement with water acting as an intermediary between the lipid and protein surfaces. Subsequent membrane investigations supported the existence of such a protein sheath.[17, 18]

Various other models have been proposed [19,20,21] in accord with experimental evidence but only that of Vanderkooi/Green and S.J. Singer included a dynamic molecular flux to their model. Known as the protein liquid crystal model, Figure 3, it incorporates the lipid bilayer concept with proteins either in a helical or globular form distributed above and in the membrane itself. Most of the direct evidence comes from the X-ray diffraction studies of untreated retinal disc/membranes [22,23] which indicated a constant thermal motion in the plane of the membrane. It is this model which satisfactorily accounts for the experimental observations of the properties of cell membranes and the interrelationships of lipids and proteins.

With this model insight into membrane structure at hand, scientists can better recognize, understand and predict the numerous lipid/protein interactions which are continually present and which comprise the membrane

Figure 2

The

Danielli and Davson

Lipid Bilayer Model

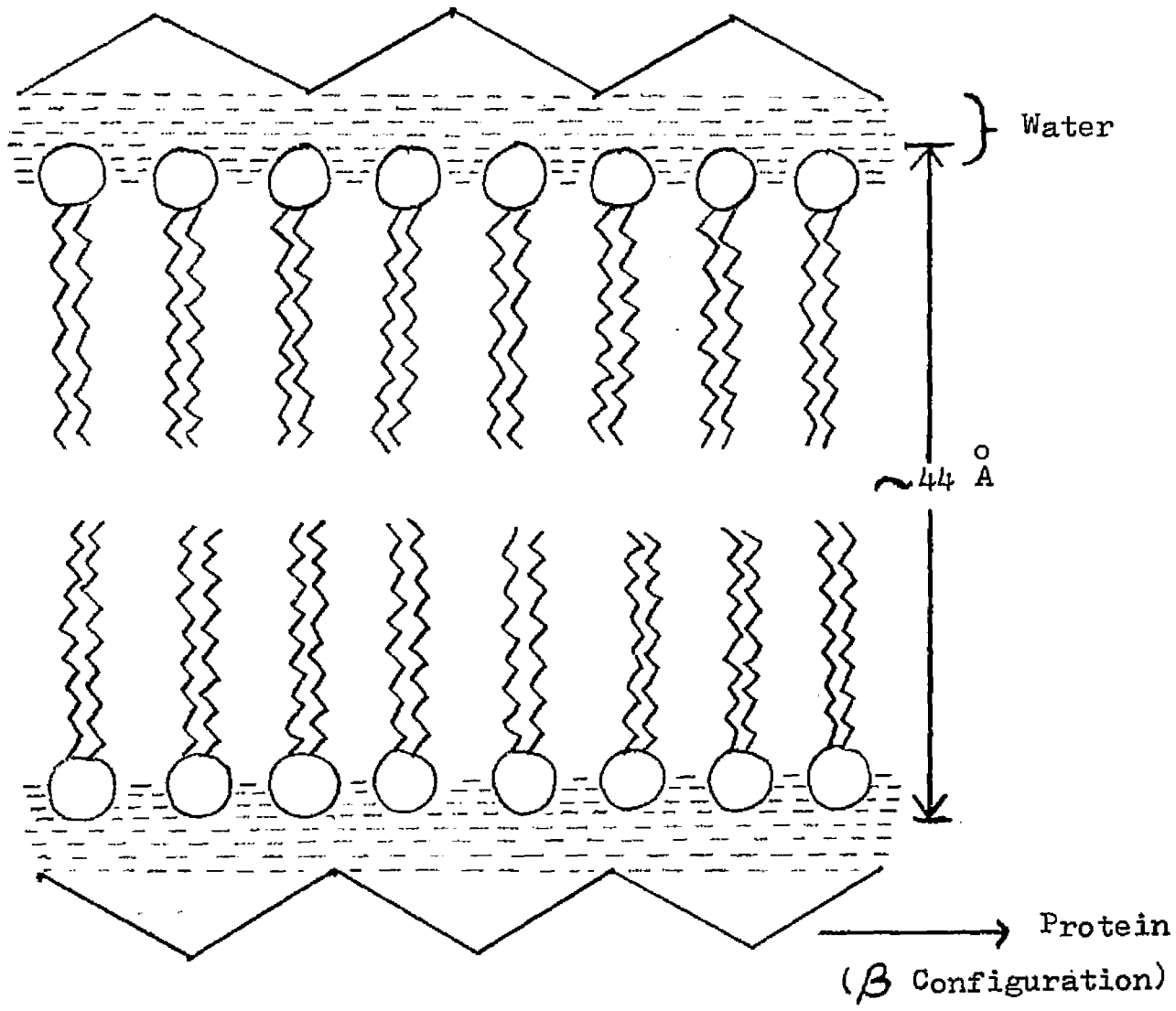
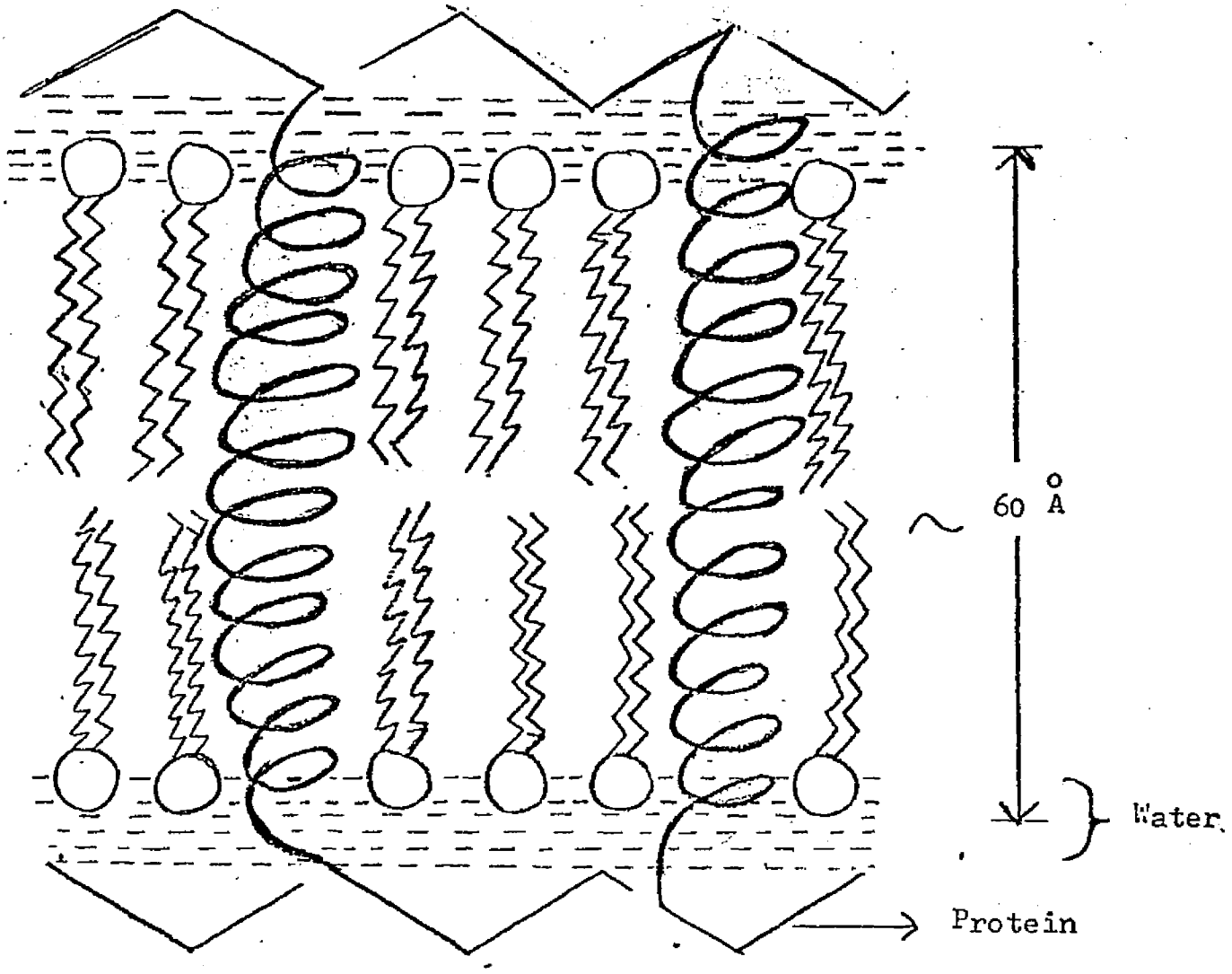

Water
~44 Å
Protein
(β Configuration)

Figure 3

Lipid Bilayer Conception
of the Liquid Crystal Membrane Model

The outer membrane protein is expected to be in the β configuration while the internal protein may be in a random coil or α helix as depicted.


60 Å
Water
Protein

environment. It can be argued from a purely biochemical point of view that the physiological functions and physical structure of such biological membranes can to a great degree be reduced to the interactions of proteins, peptides, amino acids with phospholipids, metal ions, and water. These interactions would lead to the formation of molecular complexes, e.g., Lipo-proteins,[24,28] which have been found to play a significant role in various biological processes. Generally speaking, these complex molecular associations, both the lipid/lipid and lipid/protein interactions can be summarized as follows:

1) Electrostatic Interactions:

The direct attraction of oppositely charged or dipolar species. This interaction would include hydrogen bonding, Van der Waals forces, normal ionic bonding and metal bridging of anionic functional groups or species.

2) Hydrophobic Interactions:

The attractive force experienced by the respective non-polar alkyl chains of both the lipid and protein species.

However, these interactions can be broken down to the more specific nature of the reactants. In the case of lipid/lipid interactions, numerous experiments have been performed in order to prove the existence of the hydrophobic center array which all membranes are expected

to contain[29-32] as well as experiments investigating the primary mode of association. Briefly, it has been found by O'Brien[33] that the area occupied by lipids in vesicles, mono and bilayers is determined by the nature of the fatty acid chains present. Long chain saturated fatty acids form more compact films while unsaturated chains lead to more expansive films. The addition of cholesterol also influences the area of monolayers by contracting them proportionately through increasing the hydrophobic interactions within the system. These data only approximate the molecular lipid/lipid effects in the membrane but do substantiate the hydrophobicity of the "core" of the membrane. Lipid/protein interactions on the other hand are known to play a more determinate role in membrane organization. Most of the model systems previously presented represent a β or extended configuration protein above the surface of the lipid presumably associated by electrostatic attractions. However, it is also widely known that other proteins are indigenous to the membrane. It is now believed that these intra membrane lipid/protein interactions are mainly hydrophobic in nature due to the relative ease of lipid and protein isolation from membranes under mild conditions.[34,35] In fact, Wallach has shown that the tertiary structure of the protein in contact with a membrane is determined to a great degree

by the amount of hydrophobic attraction it is subjected to. This does not, however, rule out electrostatic interactions as a major adhesive or cohesive force.

In the pursuit of the lowest free energy state, it is agreed by a number of investigators[36-38] that multiple forces must be involved as the "organizing membrane forces." Therefore, it is the combination of the various lipid and protein interactions, hydrophobicity, dipole/dipole and electrostatic attractions, which lead to the formation of the ubiquitous physiological envelope. Yet throughout the above quoted experiments, one aspect of electrostatic interactions, the metal bridging of a protein and lipid, has entirely been intuitive speculation.[39-47] However, it is a well-documented fact that biological fluids are comprised of varying amounts of several mono and multivalent cations, K^{+}, Na^{+}, Ca^{++} and Mg^{++} being the primary constituents. It is also known that such cations as Fe^{+3}, Mn^{+2}, Cu^{+2} and Co^{+2} are both present and are completely necessary for diverse biological, biochemical and physiological functions. It is this universal presence of these various ions and the experimental evidence of both lipid and peptide ion exchange and metal chelation properties which directs speculation towards the possibility of a mixed ligand moiety.

It was and is the point of this thesis to investigate, examine and hopefully characterize this

possible and often conjectured mode of protein/lipid interaction, a metal chelated mixed ligand complex.

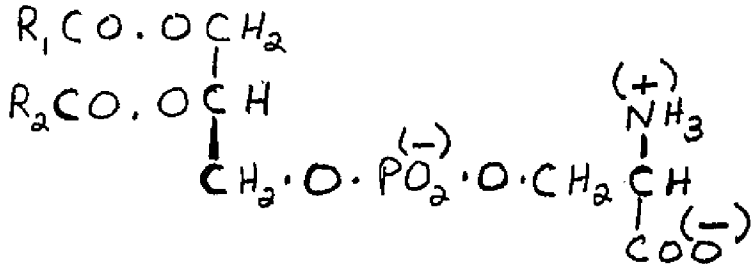
Results will be presented on the formation of a mixed ligand complex utilizing the anionic phosphatidyl-L-serine, (Figure 4) and various amino acids, di and tri peptides, fatty acids, organic dyes and proteins. Results will be presented on the particular divalent metal, pH and ligand specificity of these reactions. Work will also be presented on mixed vesicle systems and the effect of mixed ligand formation on glucose and phosphate release from these vesicles.

Figure 4

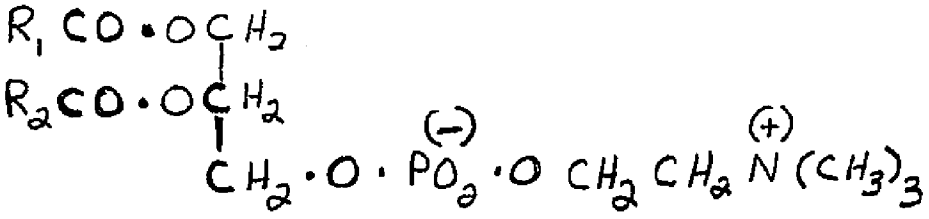
Structural Formulae of the
Phospholipids used in this work

The R's represent hydrocarbon chains, usually $C_{17}H_{35}$ in this case, some of which may be unsaturated.

1. Phosphatidyl-L-Serine (PS)



2. Phosphatidyl-Choline (PC)



EXPERIMENTAL TECHNIQUES

A) Infrared Spectrometer:

Infrared spectra were obtained on a Perkin-Elmer Model 521 dual grating spectrometer with a range of 4000 cm^{-1} to 250 cm^{-1}. The instrument was run at a spectral slit width of about 1 cm^{-1}. The chart paper used to record the spectra had an ordinate which represented percent transmittance and an abscissa which was linear in wave number. Samples were either a thin film about 10 um thick, held on IR tran-2 plates or a Nujol mull placed on 1 cm thick CsBr salt plates.

B) Ultra Violet/Visible Spectrometer:

Absorption spectra were obtained on a Cary 14R with a range of 1860 A^{o} to 26,000 A^{o}. The instrument was run with slit width of 1.0 mm. Ninhydrin samples were prepared normally and were contained in Luminon 21 Quartz (10 mm) cells.

C) Visible Spectrophotometer:

Visible region absorbances were recorded on a Beckman Model B spectrophotometer. The instrument was equipped with a red sensitive phototube which gave the instrument a range of 675 nm through 1000 nm. Runs were performed with slit width of .1 mm.

Samples were contained in Luminon 21 Quartz cells (10 mm).

D) PH Meter:

The apparent pH of all samples was obtained utilizing a Brinkmann E-512 with a Thomas combination electrode (glass/Ag-AgCl) with a temperature range of 0° - 75° and a pH range of 0 - 12.

E) Sonicator:

A sonifier cell disruptor from Heat Systems - Ultrasonics, Inc., Model W140 was used to prepare lipid-water gels and mixed phospholipid vesicles. Weighed amounts of lipid were dispersed in an accurately measured amount of glass distilled, doubly deionized water in a cooling cell, supplied by the above company. A microtip was immersed in the suspension and the material sonified from 2 through 15 minutes at setting 4 or 5. Optimum conditions warrants this procedure to be done in an N_2 atmosphere to prevent oxidation of the lipids. However, a stream of N_2 applied to the surface of the suspension will suffice.

F) Nuclear Magnetic Resonance:

The proton NMR spectra of the reactants and the dilipid complexes were obtained on a Model A-60 NMR Spectrometer from Varian Associates, Inc. Solvents used were D_2O, $CDCl_3$ and CD_3OD.

CHEMICALS

Phospholipids: All phospholipids used were chromatographically pure (TLC and Column).

A) Phosphatidyl-L-Serine:

This was supplied by Schwarz/Mann Chemical Co. as a [illegible] (TLC-silica gel w/CH_3CL-ETOH-H_2O [65:25:4 - V/V] solvent system) showed contaminants at Rf 0.12 and 0.58 which could easily be separated from the product (Rf 0.66) as described in the experimental. The molecular weight was approximately 788 and % P = 3.93. The lipid probably contains some degree of unsaturated fatty acids.

B) L-Phosphatidyl-Choline (EGG):

The lipid was obtained from Sigma Chemical Co. in an n-hexane solution Type 111-E. Chromatographically pure, molecular weight approximately 780.

Amino Acids, Peptides, Proteins:

All amino acids and peptides were chromatographically pure (TLC) and metal free. (EDTA treated). The proteins were obtained as pure as possible and metal free.

C) L, Alanine; Arginine; Asparagine; Aspartic Acid; Cysteine; Cystine; Glutamic Acid; Glycine-Ethyl Ester; Glycine; Histidine; Leucine;

Lysine; Methionine; Ornithine; Proline; Phenylalanine; Ortho-Phospho-Serine; Sarcosine; Serine; Threonine; Tryptophan; Tyrosine and Valine:

All were supplied by the Sigma Chemical Company.

D) GlyGly-Glycine and Glycylglycine:

This was supplied by Schwarz Mann Chemical Company and was TLC pure.

E) Gly-Gly-Ethyl-Ester:

Prepared from (D) by refluxing (4 hrs.) in ETOH/HCl solution of Gly-Gly.

F) Glycine-L-Phenylalanine; Glycyl-Glycyl-Phenylalanine; Glycine-L-Proline; Phenylalanyl-Phenylalanine; reduced Glutathione; Alanyl-Glycyl-Glycine; Glycyl-Glycyl-Alanine; Glycyl-Glycyl-Leucine; Leucyl-Glycyl-Glycine:

All supplied by Sigma Chemical Company and were TLC pure.

G) Albumin (Egg):

Grade Vl: Salt free and lyophilized. This was supplied by Schwarz/Mann Company as a (5X crystallized) white powder. The molecular weight is unknown but is approximately 43,500 with 5-6 moles mannose/mole and 1%

extraneous protein detected by electrophoresis on cellulose acetate in barbital buffer; pH 8.6 ionic strength 0.075. The isoelectric point is 4.9.

H) Albumin (Bovine):

This was supplied by Sigma Chemical Company (Fraction V) 15.4% N content. Essentially fatty acid free (less than .005%).

I) Phosvitin (Egg):

This was also supplied by the Sigma Chemical Company - 11.9% phosphorous molar N/P ratio of 2.7. Protein treated with EDTA to insure the absence of bound metals. Molecular weight = 33,910.

J) Equine Hemin:

(Molecular weight is 652); This was supplied by Sigma Chemical Company.

K) Insulin-Chain-A-Oxidized (Glycyl):

(Molecular weight is 11,466); This was supplied by Mann Research Chemical Company.

L) Poly-L-Alanine; Poly-Glycine; Poly-L-Hydroxy Proline:

These also were supplied by the Sigma Company. Molecular weights are high for each (10-25,000) but the actual value was not established.

M) All other chemicals used such as Triketohydrindene Hydrate (Ninhydrin) or TRIS-(Hydroxymethyl-Aminomethane) were supplied by Aldrich Chemical Company with 99% + purity.

N) All the salts used such as $CaCl_2$ were supplied by Fisher Scientific Company as reagent grade or higher purity.

O) The glucose, phosphate and Ca^{++} diagnostic reagent kits were supplied by Pierce Chemical Company.

P) The water used throughout these experiments was normally glass distilled twice and deionized using a commercial resin deionizer and treatment with EDTA followed by a third glass distillation. The amounts of divalent metals remaining in the water did not interfere with the complexation reactions. This water was boiled before using to remove excess CO_2.

PROCEDURAL TECHNIQUES

A) Preparation of the Mixed Ligand Complex:

Three methods may be used in the preparation of mixed ligand complexes.

Procedure 1:

A weighed amount of pure phosphatidyl-L-Serine is suspended in a measured amount of glass distilled, doubly deionized water.* Dispersion of the lipid may be accomplished by sonication and/or manual or mechanical agitation. In separate vessels equimolar amounts of the prospective ligand and the metal chlorides are dissolved in a measured amount of water. The protein/peptide/amino acid solution or solid, which works as well, is then added to the lipid dispersion with stirring. Finally, the salt either as the solid or the solution, is added, usually accompanied by immediate precipitation. This precipitation is determined by the molar quantity of the lipid. Usually, a lipid concentration of 10^{-4} molar will precipitate out.

The two other procedures are only variations of Procedure 1.

Procedure 2:

It is basically the same as Procedure 1 except that the metal chloride is added to the peptide solution

* All glassware was treated with EDTA, rinsed, soaked in deionized water and oven dried before use.

which then forms a metal-peptide (1:1) complex, which is then added to the lipid suspension.

Procedure 3:

A weighed amount of the peptide ligand is dissolved in a measured amount of water. To this solution, an equimolar amount of lipid is added with subsequent dispersion. An equimolar amount of the metal chloride is now added as the solid or solution. The one drawback to this method is that at molar concentrations (lipid) in the range of $10^{-1}M$ - $10^{-3}M$, the lipid may form a multilamellar liposome or vesicle which may entrap the peptide within the liposome thereby reducing the effective peptide concentration and increasing the likelihood of dilipid complex formation on addition of the metal chloride.

It is important to note here, that if the metal chloride is added to the lipid before the addition of the peptide ligand, only the dilipid complex will form. Analysis will show absolutely no mixed ligand formation.

Overall, Procedures 1 and 2 give identical results while Procedure 3 gives a slightly higher dilipid complex formation. A schematic of the above preparatory reactions are shown below:

Procedure 1/3: $PS + Peptide + M^{+2} \rightleftharpoons PS - M - Pep + Pep\text{-}M^{+} + M(PS)_2$

Procedure 2: $PS+Pep\text{-}M^{+} \rightleftharpoons PS\text{-}M\text{-}Pep+M(PS)_2$ + Peptide

$PS+M^{+2} \longrightarrow M(PS)_2$

$M(PS)_2+Peptide \not\longrightarrow$ N.R.

B) pH Adjustment of the Lipid/Peptide Solutions before Complexation:

The natural pH of the solutions of the reactants used in this work ranged from 3.5 $\longrightarrow$ 5.0. Adjustments to the pH of these solutions before complexation may be accomplished by the addition of NaOH and the appropriate buffer system. For desired pH's below 7.0, NaOH is added with either a phthalate or phosphate buffer. However, both these buffer systems will compete for the divalent metal halide and may precipitate. Experimental evidence has shown that more of the metal is necessary to complete the complexation with these buffer systems. However, mixed ligand complexes will be formed under buffered conditions or as was predominately used in this thesis, in the case where the buffers were omitted and only NaOH was added. For the desired pH's above 7.0, NaOH and/or the appropriate TRIS buffer system can be used without any interference in the complexation reaction.

C) Separation and Isolation of Products:

After complexation, the products are centrifuged down (clinical centrifuge is adequate). The supernatant

is decanted off and the precipitate is washed with glass distilled, doubly deionized water at least three times to insure the removal of all uncomplexed peptide ligand. The precipitate may be washed with an EDTA solution, if the experiment so warrants. After each washing the precipitate is centrifuged down, supernatant decanted and the washing repeated. After the last water washing, the precipitate is washed with a solution of chloroform - ethanol-water (65:25:4 - V/V). This will remove any dilipid complex. Two or three ml. of water is now added with the formation of a biphasic system. If a mixed ligand complex is present, it will remain as a precipitate at the interface of the dual system. The organic layer is removed, the precipitate centrifuged down and the water layer decanted off. The precipitate is now dried for at least three hours on a high vacuum line equipped with an Hg diffusion pump and a liquid N_2 trap (approximately 0.3 mm Hg).

D) Anion Analysis (Chloride): [67]

A) Gravimetric - 5.0 ml of the supernatant liquid (approximately 0.1 M solution) is treated with 3 ml of 6F nitric acid and diluted to 100 ml in a 200 ml beaker. A 5% solution of $AgNO_3$ is added to the cold solution. The solution is heated to near boiling and the precipitate digested for 10 minutes. After standing

overnight in a dark place, the supernatant is filtered through a weighed filtering crucible. The precipitate is washed several times with 2 ml of 6F HNO_3/liter H_2O, dried at 105° C and the chloride reported as % of AgCl.

B) Volumetric - 5.0 ml of the reaction supernatant is diluted to 100 ml with distilled water. 10 drops of dichlorofluorescein solution (0.1%) and 0.1g dextrin are now added. Immediately, the solution is titrated using a standard $AgNO_3$ (0.1F) solution until permanent appearance of pink color is developed. Report chloride directly.

Both of the above analytical techniques were utilized in this thesis to ascertain the amount of chloride remaining after complexation. The volumetric procedure was the method of choice, since it was noted that the gravimetric procedure at times gave higher values for the remaining chloride presumably due to the presence of precipitated peptide. The information obtained was used in conjunction with the following cation analyses to help substantiate the stoichiometry of the complexes formed.

E) Cation Analysis:

This analysis can be carried out in three distinct manners.

1) Ca^{++} -

a) Gravimetric - 5 ml aliquot of supernatant diluted to 50 ml with distilled/deionized water and treated

with 5.0 ml of 6N HCl. The solution was heated for 15 minutes to drive off excess CO_2. When the mixture cooled to 75° C, 25 ml of a 10% ammonium oxalate solution is added with 3 drops of methyl orange. A 50% NH_3 solution is now added dropwise until a yellow color formation. A white precipitate forms and after standing for at least one hour, the mixture is filtered through a weighed crucible and the precipitate washed and dried. Ca^{++} is reported as the % Ca^{+2} per the oxalate complex. However, this technique can now be expanded to an oxalate determination as follows. The crucible is transferred to a reaction flask which contains 100 ml d/d H_2O and 50 ml 6N H_2SO_4. The solution is heated (precipitate dissolves). The solution is now titrated with 0.1 N $KMnO_4$ until the slightest permanganate color remains (i.e., $2MnO_4^- + 6H^+ + 5H_2C_2O \rightleftharpoons Mn^{+2} + 10CO_2 + 8H_2O$). Results are reported as amount oxalate present.

b) Complexometric Titration:

1) Erio T indicator (substitution titration)

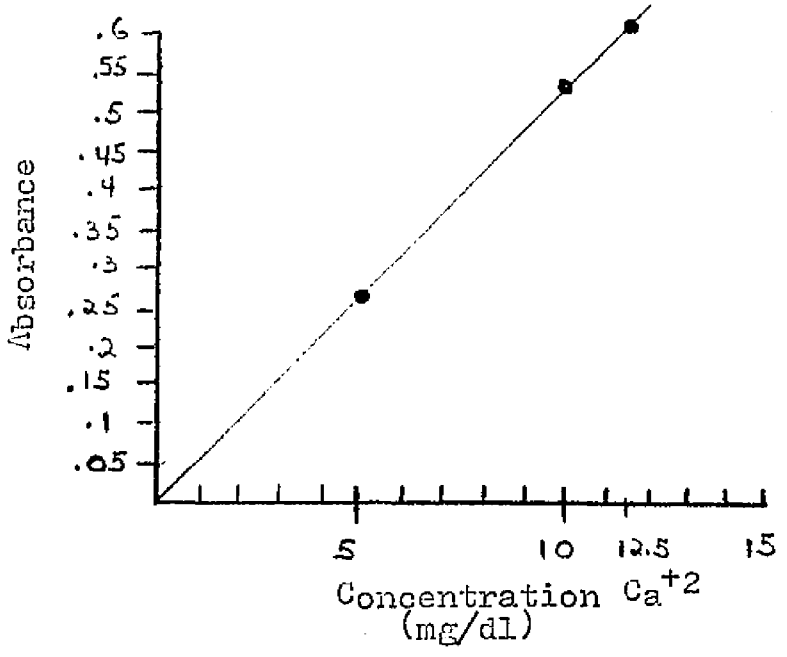
5.0 ml of the supernatant is neutralized with NaOH and diluted to 50 ml. 1 ml pH 10 buffer, 0.5 ml of 0.1 M Mg-EDTA complex and 2 drops Erio T indicator added. This is now titrated with standardized 0.01M EDTA with a color change of red to blue.

2) Murexide Indicator (direct titration)

5.0 and 10.0 ml aliquots of the reactions supernatant are diluted to 50 ml and neutralized with NaOH. 5.0 ml 1.0M NaOH is added followed by the indicator with immediate titration with standard 0.01M EDTA. The color change is from red to violet.

c) Colorimetric Determination:

(Pierce Chem. Rapid Stat. Kit). 0.05 ml sample of the supernatant is mixed with 3 ml of a 1.1 solution of a dye reagent (0.018% methylthymol blue, 0.036% 8 - quinolinol) and base reagent (sodium sulfite, monoethanolamine). The color develops virtually instantaneously and the absorbance is read on a spectrophotometer set at 612 nm. This procedure follows Beer's Law from 0 ⟶ 12.5 mg/dl of sample and the curve is shown below.



The amount of calcium present is determined through use of a standard (10 mg/dl) and the following equation:

$$Ca^{++} \text{ con unknown} = \frac{\text{Absorbance of Unknown}}{\text{Absorbance Standard } (.550)} X \text{ 10 mg/dl}$$

There is no interference from the presence of peptide or other cation species.

2) Mg^{+2} - Complexometric Titration - direct titration. Acidic 5 ml samples are first neutralized with 0.1 M NaOH followed by dilution to 50 ml. 1.0 ml pH 10 buffer (TRIS) and 5 drops Erio-T indicator added followed by titration with standard 0.01 M EDTA, color change is from red to blue.

3) $Sr^{+2}(Ba^{+2})$ - Complexometric Titration - substitution and back titration. 5 ml of supernatant neutralized with 0.1 M NaOH and diluted to 50 ml with H_2O and 5 ml 0.1M Mg^{+2} EDTA, 1 ml pH10 buffer and 2 drops of Erio-T indicator. Solution is now titrated with 0.01 M EDTA until color change of red to blue.

4) Mn^{+2} - Complexometric Titration - direct titration - 5.0 ml of supernatant treated with 5 ml triethanolamine solution (20%) and one spatula full ascorbic acid, followed by neutralization with NaOH and adjusted to pH 10 by addition of buffer. Erio-T indicator added with titration with 0.01 M EDTA until color change of red to blue.

5) Fe^{+3} - Direct Complexometric Titration -

5 ml of the supernatant is first acidified and then neutralized if necessary with NaOH to the first change of Congo red paper (pH2-3). Approximately 0.2 gm glycine added with a few drops of variamine blue. Titrate with 0.01M EDTA, color change is blue-violet, gray-yellow.

6) Al^{+3} -

The entire supernatant is treated with 1.0M KOH with the precipitation of $Al(OH)_3$. The mixture is centrifuged down and separated. The precipitate is dissolved in 0.1M HCl and EDTA is added to slight excess. The pH is now adjusted to 6 by the dropwise addition of NH_4OH (check with methyl red paper). Boil and cool mixture. Dilute to 100 ml and add 3 ml 1M Na acetate. Add 0.2 gm salicylic acid and titrate with standard 0.1 M $FeCl_3$ until red-brown color persists for a short time.

7) Cu^{+2} - Electrogravimetric -

The entire supernatant (10-15 ml) is treated with 0.1 M $AgNO_3$ until all free chloride is precipitated. The solution is diluted to 50 ml and 2 ml con. H_2SO_4 and 2 ml 6F HNO_3 is added. A weighed platinum electrode is placed on the negative electrode of a variable amperage electrolysis apparatus. Allow 2 amps to pass through the cell until all Cu^{+2} is deposited. Remove electrode, wash with absolute ETOH and dry. Reweigh and report amount Cu^{+2} deposited.

F) Total Phosphorus Assay: [81]

2.0 ml of the sample to be analyzed (suspension or as a weighed solid complex in 2 ml H_2O) and 0.5 ml H_2SO_4 placed in 12 ml conical centrifuge tube and heated in an oven at 150° C for 3 hours. After this time, 2-3 drops of 30% H_2O_2 is added and the mixture returned to oven for 2 more hours. 4.6 ml of 0.22% ammonium molybdate and 0.2 ml of the Fiske-Subba Row reagent (0.5 gm, 1-amino-2-napthol-4-sulfonic acid and 1.0 gm anhydrous sodium sulfite in 200 ml 15% sodium bisulfite) is added, mixed thoroughly and heated in boiling water for 7-10 minutes. The optical density at 830 nm was recorded on a photometer with a red sensitive phototube. This method is primarily used for phosphorus bound as phosphate esters in the original sample.

G) Liposome Preparation:

1) Sonication method - This method is basically described in the instrumental section of these techniques. It should be noted here that this procedure is a well-documented scheme of producing uniform vesicles of both pure and mixed lipid systems. [74,97-99] The average diameter of these vesicles is approximately 160 A° (range 125 - 250 A°) [88] and have an average molecular weight of 4×10^6. [48] The typical methods of analysis for these vesicles is either via electron micrograph or gel filtration

on Sepharose 4B Column. However, no vesicle size analysis was performed on the vesicles of these experiments since the preparative procedures were followed exactly to those referenced and the actual liposome size did not play a critical role in the experiments performed.

The following figures give the amounts of lipid (2×10^{-4} mole total) used in the mixed liposome experiments:

	PC (gm)	PS (gm)
9/1	- 0.1418	0.0158
3/1	- 0.1181	0.0394
1/1	- 0.0788	0.0788
1/3	- 0.0394	0.1181
1/9	- 0.0158	0.1418

2) Mechanical or manual method - This procedure is self-explanatory. The lipid is added to a aqueous medium and agitated. Actually the lipid will disperse on its own forming a multi-lamellar liposome with particle sizes ranging from 0.1 to 15 um (95% from 0.1 → 2 um) as compared to the average sonicated vesicle size of 16 nm. This system has been criticized by a number of investigators[97] but past work[100,162] has shown that heterogeneity of these preparations is not of great importance. This method was used primarily in the mixed ligand formation experiments.

3) Non-sonication techniques:[101]

An ethanol solution of the lipid(s) is rapidly injected with a syringe into a 0.16 M KCl solution. All solutions are purged with N_2. The suspension can be concentrated by ultrafiltration but larger liposomes may form if the volume is taken below 2 ml. Infrared spectral analysis of these vesicles showed no evidence of oxidation and a homogeneous preparation of liposomes with an assumed average diameter of 26.5 nm. These vesicles were of the single bilayer type and are, according to the authors, (Ref. 101) indistinguishable from those prepared by sonication. This author did not observe any difference in the vesicles produced by this method or by those of the sonicated procedure. Both vesicles reacted in the exact same manner in the mixed ligand reactions and in the solute efflux experiments and could be used interchangeably. Infrared and H^1 NMR 60 MHz spectra did not show any difference in the vesicles from the sonicated or the injection methods.

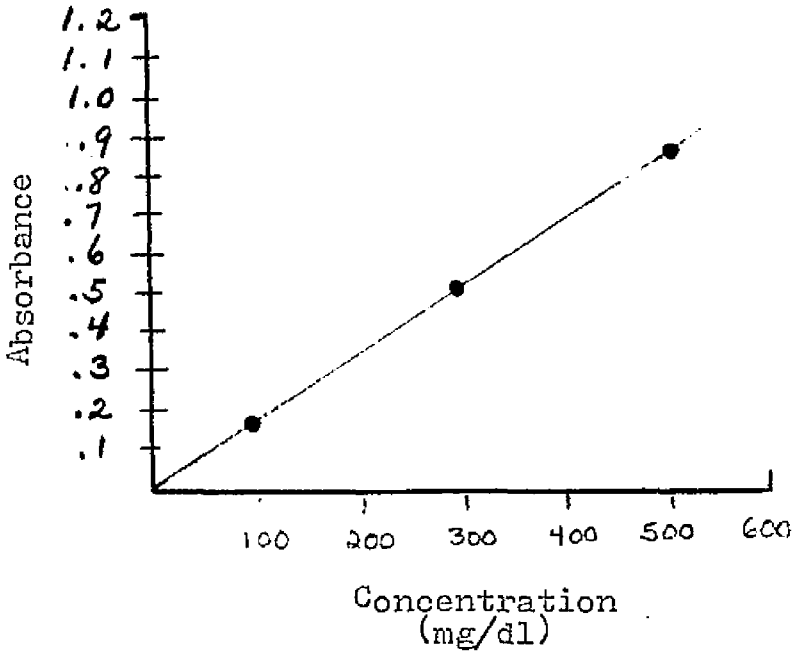
H) Glucose Analysis:

This procedure used the single reagent quantitative colorimetric determination of glucose (Pierce Chem. Co.). 0.0 5 ml of the sample is mixed with 5.0 ml of a color reagent (active ingredients: acetic acid, 95.1%; O-toluidine, 4.6%; Thioureu 0.14%).

The mixture is heated for 10 minutes at 100° C. The solution is cooled for 2-3 minutes and the color intensity is read at 630 nm. The calculation of the glucose concentration is determined by the use of a standard and the following equation:

$$\text{Glucose con Unknown} = \frac{\text{Abs. Unk x 100 mg/dl}}{\text{Abs. Standard (0.182/100 mg/dl)}}$$

The reported precision of this method is 0.4%. Beer's Law holds from 0 through 500 mg glucose/dl.



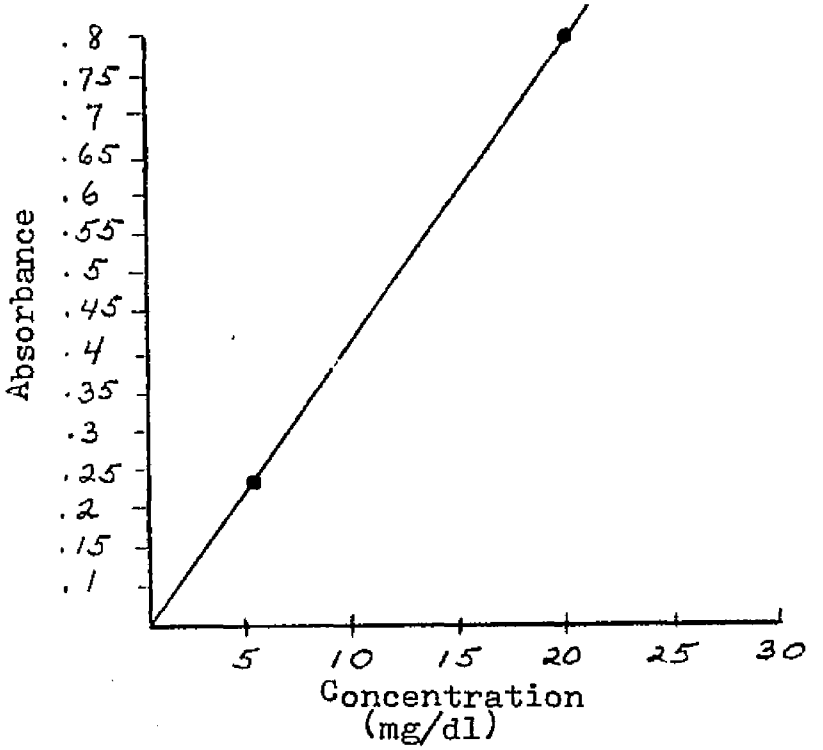
Note: The above graph and those used in the Ca^{+2} and phosphorous determinations were prepared from data given by the Pierce Chemical Company. Personal spectrometric analysis before and during each experiment produced identical results with that given in these graphs.

I) Phosphorus Analysis as Inorganic Phosphate-Quantitative Colorimetric Determination (Pierce Chem. Co.):

0.0 5 ml sample is mixed in a test-tube with 1.5 ml of a reducing reagent (p-methylammonium-phenol sulfate, 0.5%; sodium metasulfite 1.5%.) Now 0.5 ml of the molybdate solution is added (ammonium para molybdate, 1.41%; sulfuric acid 2.65%) followed by 0.2 ml of a base reagent (no active ingredients, 67.5% of a non-reactive organic base). The mixture is allowed to color develop for 5 minutes and the intensity is read at 690 nm on a spectrophotometer. Phosphorus concentration is determined by use of standards and the following equation:

$$\text{Phosphorus con Unk} = \frac{\text{Abs. Unk. x 5.0 mg/dl}}{\text{Abs. Standard (0.245)}} \text{ for 5.0 mg/dl}$$

Beer's Law is observed over the range of 0 through 20 mg/dl phosphorus. The reported precision is 0.8% for this method.



EXPERIMENTS AND RESULTS

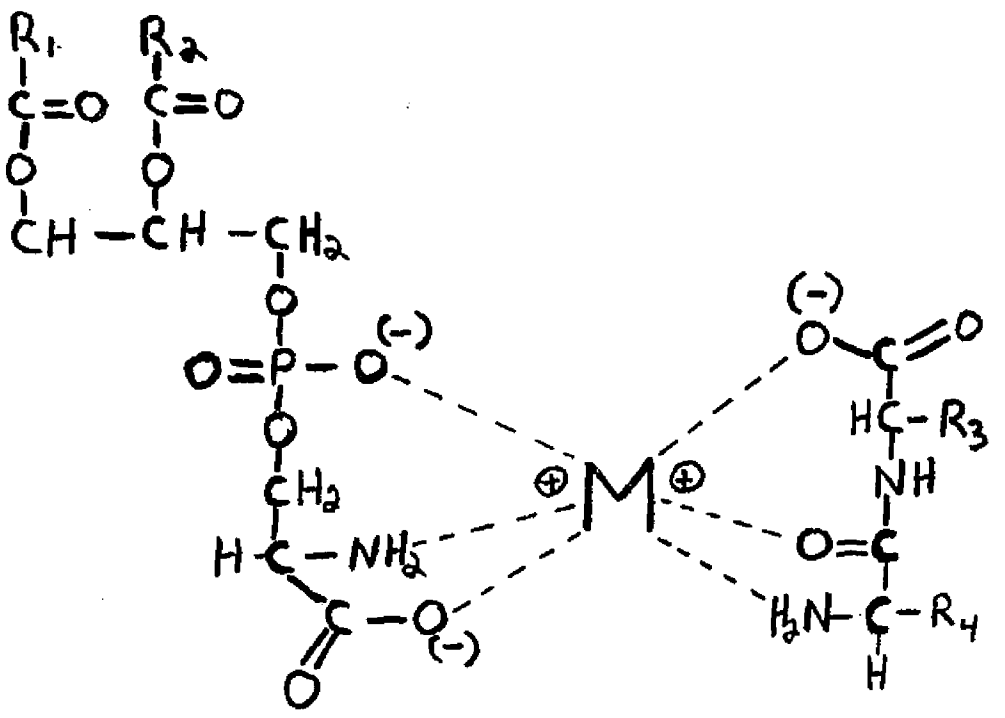
Anionic phospholipids have for a number of years been considered to be the major ion exchange medium in biological membranes.[48,50,62] Numerous studies on the acidic phospholipids as monolayers,[49-51] bilayers[52,53] and as multilamellar vesicles[54-56] and experiments investigating the effects of divalent metals[48,51,57-60] on these same lipid systems have greatly increased the overall knowledge of possible cell surface reactions.[61] Notwithstanding, these and the few experiments on metal induced protein/lipid interactions,[43,44,47,119] direct evidence of a mixed ligand complex in an aqueous medium was lacking if existent at all. The initial thrust of this work was to make, isolate and characterize such a mixed ligand complex or "tricomplex" as it has been sometimes referred to in the literature.[39,40] Figure 5, shows graphically this mixed ligand complex and also shows the possible binding sites for metal induced chelation. The following experiments and the results listed in Table 1 and 2 coupled with the preparative procedure listed under Experimental Techniques will be indicative of the overall complex formation reactions.

A) Mixed Ligand Preparation and Analysis:

0.0788 gm PS (1×10^{-4} Mole) is added to 10 ml distilled/deionized water or a solution containing the

Figure 5

Representation of a mixed ligand complex depicting all possible metal chelation sites.



Possible Chelation Sites

PS -- P-$O^{(-)}$
-- C-$O^{(-)}$
-- NH_2

Peptide -- C-$O^{(-)}$
--(amide) C=O
-- NH_2

appropriate buffer system and a standard of 0.1 Molar KCl. The PS can either be manually or mechanically agitated forming a dispersion or multilamellar liposome. It may also be sonicated if uniform vesicles are desired, however, this adds to the possibility of oxidation of the polar head groups or the fatty ester side chains. Now an equimolar amount of Glycylglycine (0.0132 gm) is added to the lipid dispersion either as a solid or in solution. The PS/Glygly mixture is agitated again to insure equal distribution. The pH may be checked at this point and adjusted to the required level by the addition of 0.1000 N NaOH solution. After this has been completed, an equimolar amount of the metal chloride in this case $CaCl_2 \cdot 2H_2O$ is added to the mixture either as a solid or as a solution. A precipitate will form immediately on the salt addition. As stated in the preparation techniques, a definite precipitate formation is dependent on the overall lipid concentration. The precipitate in this case may either be the mixed ligand complex ($PS^- - Ca^{++} -$ GlyGly) or the well-known and characterized di-lipid complex.[64-66] The dipeptide - Ca^{++} complex is soluble in this aqueous system. The precipitate is centrifuged down and the supernatant is decanted off. The isolation and separation techniques are listed in the experimental section and are the same for all the species shown in Tables 1 and 2. Analysis of the complexation reactions now follows a three pronged attack: 1) a protein/

Table I

Binding of Amino Acids to

Phosphatidyl Serine and Ca^{+2}

in a Mixed Ligand Complex

Amino Acid	% Mixed Ligand Complex Formation pH 7.0	pH 7.3
D,L, Alanine	35 %	63%
L, Arginine	0 .1	0
Asparagine	0	0
D,L, Aspartic Acid	40	51
Cysteine	5	4
Cystine	0	0
D,L, Glutamic Acid	0	10
Glycine	68	75
Histidine	54	40
Leucine	65	72
Lysine	5	11
Methionine	0	0
Ornithine	35	43
L, Proline	52	53
ortho-Phospho-Serine	65	60
Sarcosine	53	56
D,L, Serine	38	45
L, Threonine	28	22
Tryptophan	0	0
D,L, Tyrosine	15	15
D,L, Valine	74	72

Table II

Binding of Peptide and Proteins to Ca^{+2} and Phosphatidyl Serine as Mixed Ligand Complexes

Peptide	% Mixed Ligand Complex at pH 7.0	% Mixed Ligand Complex at pH 7.3
gly-L phenylal	25%	13%
gly-L-pro	43	40
phenylal-phenylal	3	1
glutathione	88	75
glyglygly	25	50
glyglyala	35	60
glyglyL-leu	49	50
leuglygly	83	83
glygly-phenylal	68	67

Protein	Moles PS Bound at pH 6.0	Moles PS Bound at pH 7.0	Moles PS Bound at pH 7.5
Phosvitin	0	29-31	29-31
Egg Albumin	0	4.1-4.5	4.1-4.5
Bovine Albumin	0	6.9-7.1	6.9-7.1
Equine Hemin	0	0	0
Insulin-Chain a oxidized (Glycyl)	0	0	0

peptide analysis utilizing a Ninhydrin test of the supernatant; 2) a cation/anion test of the supernatant; 3) a TLC and Infrared Spectrum of the solid complex.

I) Ninhydrin Analysis:

The Ninhydrin test is a sensitive analytical test for proteins, peptides and free amino acids. A typical test would be the addition of 1 or 2 ml of a 0.1 M ninhydrin solution (triketohydrindene hydrate) to 1 or 2 ml of the unknown sample followed by gentle heating. If the unknown solution contains a free amine (NH_2) group and a free carboxyl (-COOH) group the test will register positive by the formation of a color ranging from deep blue to violet pink in a few minutes. (Proline and Hydroxyproline will produce a yellow color). This test can be made quantitative by observing the absorption spectra at 590 nm either on a Cary 14 or the Beckman Spectrophotometer. This analysis will reveal the concentration of unreacted amino acid or peptide present. The buffer systems will not interfere with this test reaction.

II) Anion and Cation Analysis:

The cation/anion analysis is primarily used, in the simple complex formation reactions, to verify the stoichiometry of the products formed. Since the anion throughout these experiments was the chloride species,

either a standard gravimetric or a volumetric analysis[67] may be used. Both methods were employed in these and subsequent experiments (see Experimental Techniques) giving identical results for overall chloride concentration. Typical results for these stoichiometric investigations[68] are listed in Table 3.

The cation analysis depended on the specific species under investigation. As Table 3 demonstrates, various divalent and trivalent cations were used in the mixed ligand complex formation reaction. As can be readily seen Ca^{+2}, Mg^{+2}, Sr^{+2}, Mn^{+2}, Al^{+3} and Fe^{+3} were the only ions that would form the mixed ligand complex. Analysis of each will be briefly described. The Ca^{+2} determination can be carried out in three manners, a gravimetric analysis utilizing the oxalate method,[67] a colorimetric determination utilizing the metallochromic dye methylthymol blue[69] and a complexometric titration using EDTA and murexide or ERIOCHROME Black T as an indicator.[70] The method of choice is the colorimetric determination since the presence of peptide, lipid or other divalent cations such as Mg^{++} will not interfere with the analysis. The oxalate procedure as per Skoog and West[67] gave consistently low values for Ca^{++}, possibly due to the fact that the oxalate reaction was in competition with the peptide ligand or the ligand itself contaminated the oxalate precipitate. The complexometric

Table III

Stoichiometric Analysis -
Reaction of Metal Ions with
Phosphatidyl-Serine and Glycyl-Glycine -
pH 7.0 -- 7.3

Salt	% Glycyl-Glycine Reacted	Complex(es) Formed
$AlCl_3$	8	Al^{+3}-$(PS^-)_2$-$Cl^{(-)}$ and Al^{+3}-PS^- - Gly-Gly-$2Cl^{(-)}$
$BaCl_2$	0	Ba^{+2} - $(PS^-)_2$
$CaCl_2$	> 99 (Max.)	Ca^{+2} - PS^- - Gly-Gly - $Cl^{(-)}$
$CdCl_2$	0	Cd^{+2} - $(PS^-)_2$
$CoCl_2$	0	Co^{+2} - $(PS^-)_2$
$CrCl_2$	0	Cr^{+2} - $(PS^-)_2$
$CuCl_2$	0	Cu^{+2} - $(PS^-)_2$
$FeCl_2$	0	Fe^{+2} - $(PS^-)_2$
$FeCl_3$	7	Fe^{+3}-$(PS^-)_2$-$Cl^{(-)}$ and Fe^{+3}-PS^- - Gly-Gly - $2Cl^{(-)}$
$HgCl_2$	0	Hg^{+2} - $(PS^-)_2$
$MgCl_2$	58	Mg^{+2} - PS^- - Gly-Gly - $Cl^{(-)}$ and Mg^{+2} - $(PS^-)_2$
$MnCl_2$	24	Mn^{+2}-$(PS^-)_2$ and Mn^{+2} - PS^- - Gly-Gly - $Cl^{(-)}$
$NiCl_2$	0	Ni^{+2} - $(PS^-)_2$
$PbCl_2$	0	Pb^{+2} - $(PS^-)_2$
$SnCl_2$	0	Sn^{+2} - $(PS^-)_2$
$SrCl_2$	8	Sr^{+2} - $(PS^-)_2$ and Sr^{+2} - PS^- - Gly-Gly-$Cl^{(-)}$

Table IIIa

Salt	% Glycyl-Glycine Reacted	Complex(es) Formed
$ZnCl_2$	0	$Zn^{+2} - (PS^-)_2$

Notes:

1) Initial concentration of all reagents $10^{-3}M$

2) Ionic strength maintained by use of 1.0 M KCl

3) Complexes formed arrived at through compilation of quantitative ninhydrin, IR, TLC, cation and anion analysis.

titration[71] is an accurate quantitative determination for Ca^{++} but the results can be affected by the presence of other divalent cations.

The remaining mixed ligand forming cations (Mg^{+2}, Mn^{+2}, Sr^{+2}, Al^{+3}, Fe^{+3}) were all analyzed by complexometric titrations, after the quantitative ninhydrin test indicated less than 100% peptide left in the reaction mixture. Since the other cations in Table 3 gave a 100% peptide ninhydrin test, and a confirming dilipid complex infrared spectrum, no further analysis for these cations was necessary. The complete procedures are listed in the Experimental Techniques section.

III) Thin Layer Chromatography and Infrared Absorption Spectra:

The final analytical mode for the complexation reactions was the analysis of the insoluble precipitate. This analysis was accomplished in two phases: a qualitative TLC and an infrared spectrum.

It is well-known that lipids can readily be solubilized in a solvent of $CHCl_3$/ETOH/H_2O, (65:25:4 - V/V) or $CHCl_3$/MEOH/H_2O, (5:3:1 - V/V).[72] It is also known that the metal dilipid complex is soluble in these mixtures.[43,44,47] However, the mixed ligand complex is not soluble in either, but has been found soluble in a solution of $CHCl_3$/MEOH/H_2O-HCl, (3:1:0.1 (10 N HCl) - V/V).

This fact is true only when the peptide ligand is an amino acid, di or tripeptide. The protein/metal/lipid system is not soluble at all in these solvent systems. Therefore, one can use this selective solubility as a spot test for mixed ligand formation or one can use the acidic solvent system on a thin layer alumina or silica gel sheet. Since the precipitate had been washed to remove all water soluble impurities, two spots on the thin layer sheet would indicate a mixture of dilipid and mixed lipid complex. This test is non-conclusive in itself but it is useful in conjunction with the ninhydrin/cation and infrared analysis.

The infrared spectra of these products are more informative as to the overall mixed ligand formation. Figure 6 shows the infrared absorption spectra (KB_r) of three different forms of phosphatidylserine.[72] Table 4 displays the appropriate band assignments for the phosphatidylserine species. The structures have been assigned to these spectra in view of the knowledge that the metal free form corresponds to the isoelectric point of the PS species (pH 1.2). The presence of the 1639 cm^{-1} band for the mono-sodium salt indicates that the sodium must be bound to the carboxyl group and not the phosphate. The amount of ionization of the carboxyl group can be determined by comparing the peak height of the 1639 cm^{-1} to any CH band. Note also that the

Figure 6

Infrared absorption spectra (KBr) of four different forms of Phosphatidyl-serine. [74]

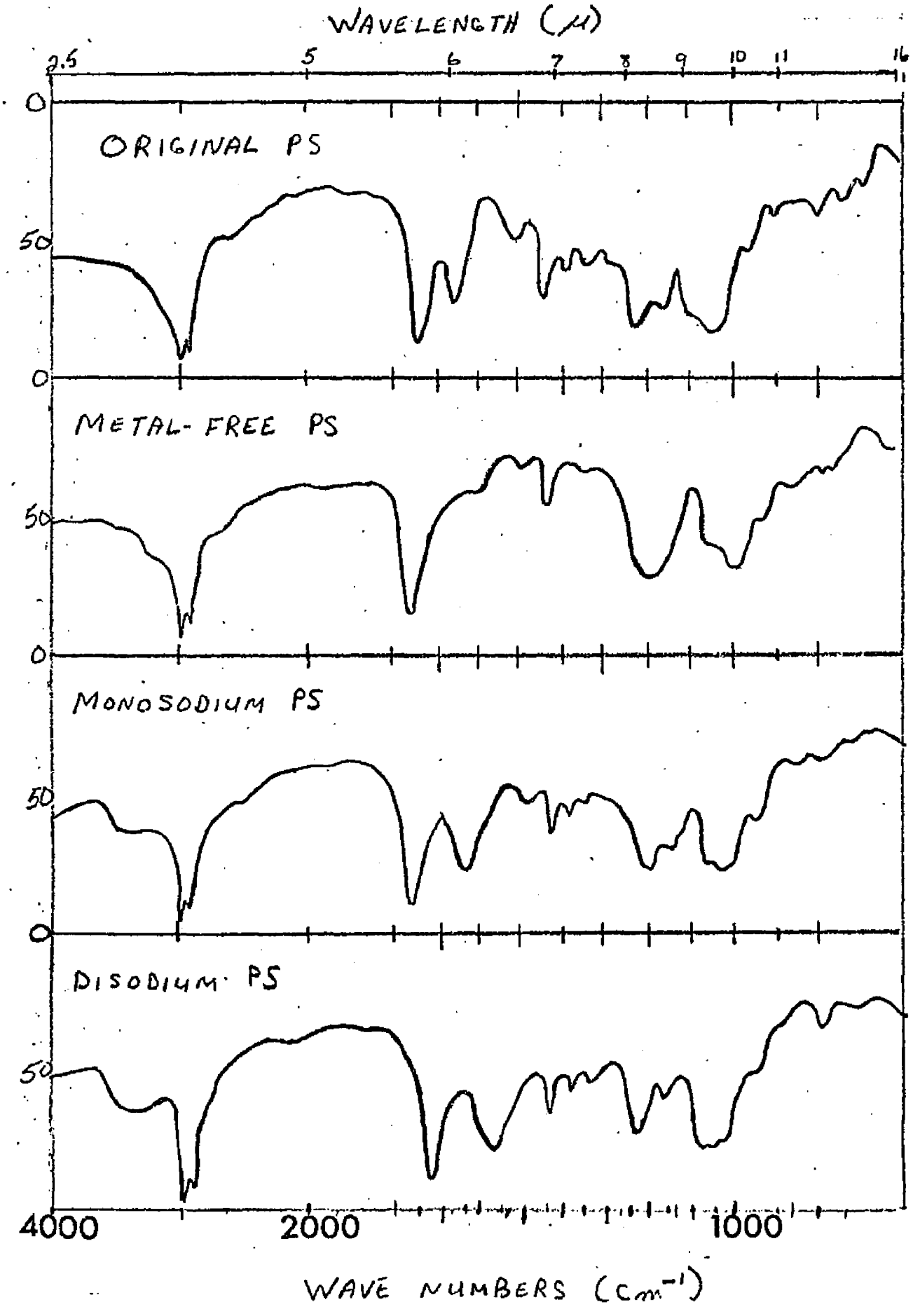

WAVELENGTH (μ)
2.5
5
6
7
8
9
10
11
16
ORIGINAL PS
METAL-FREE PS
MONOSODIUM PS
DISODIUM PS
0
50
4000
2000
1000
WAVE NUMBERS (cm⁻¹)

Table IV

Infrared Absorption Band Assignment
for Ionized Phosphatidyl-L-Serine

Wave Number (cm^{-1})	Group Vibration	Strength
1739	Ester + COOH	Strong
1639	$COO^{(-)}$	Strong
1555	NH_3 Deformation	Medium
1420	Anti-Symmetrical $COO^{(-)}$	Medium
1245 ± 5	P = O	Strong
1100	$P - O^{(-)}$	Strong
1050	P - OH	Medium
- - -	- - -	- - -
1645	$COO^{(-)}$ -- Ca^{++}	Strong
1115	$P - O^{(-)}$ -- Ca^{+}	Strong

1739 cm^{-1} (ester + COOH) band has also been equally reduced.

Thus, with the knowledge of the assigned bands of the spectra, it is now possible to analyze for mixed ligand complexation. Figure 7 shows the Nujol Mull spectra of native metal free PS and the Ca^{++} dilipid complex. The band assignments are analogous to those of the KBr spectrum and are virtually the same spectra. However, in the case of a mixed ligand complex one would also expect the representative bands of the peptide ligand to be present. What will be demonstrated now is the spectra of a typical mixed ligand moiety, the (glycyl-glycine - Ca^{++} - PS), Figure 9 and one which is more dramatic (PS^{-} - Ca^{++} - N - Gly-Gly - L-Phenylalanine), Figure 13. It must be kept in mind that these spectra are representative of the spectra obtained for each of the reactants in Tables 1 and 2 which formed a mixed ligand complex. Incorporation of each spectrum would be cumbersome and would not add significantly to the overall effectiveness of this method of analysis. Figure 8 shows the infrared spectrum and band assignment for the Nujol Mull spectrum of Glyclyglycine. Of particular importance are the absorptions at 2760 cm^{-1}, 1665 cm^{-1}, 1598 cm^{-1} and 960 cm^{-1}, for these absorption bands will stand unique in the spectrum of the mixed ligand complex as shown in Figures 9 and 10. On examination of the spectrum in Figure 10 and comparison

Figure 7

Infrared spectra of A) PS (Nujol Mull) and B) PS - Ca^{+} - PS Dilipid Complex (Nujol Mull).

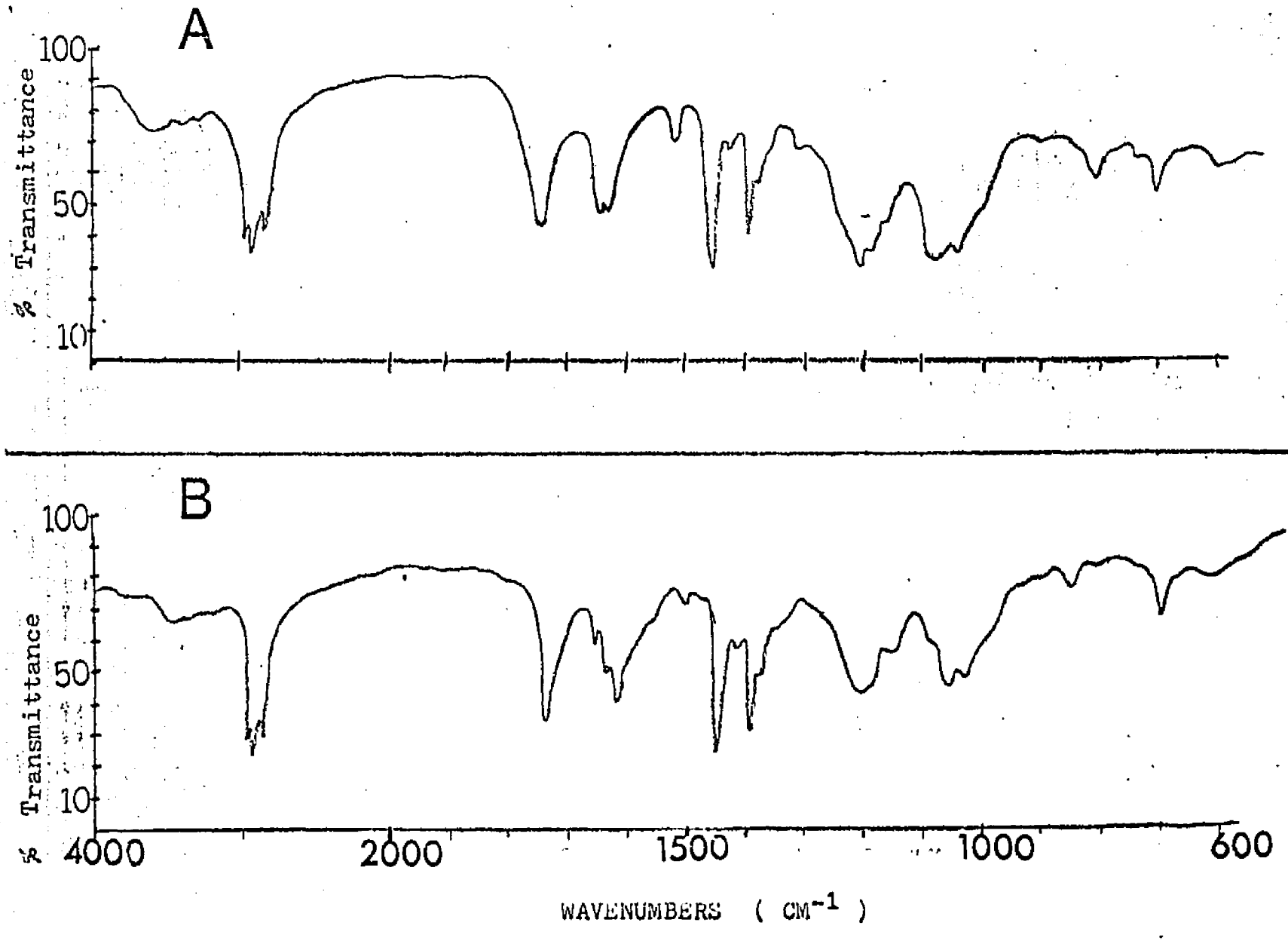
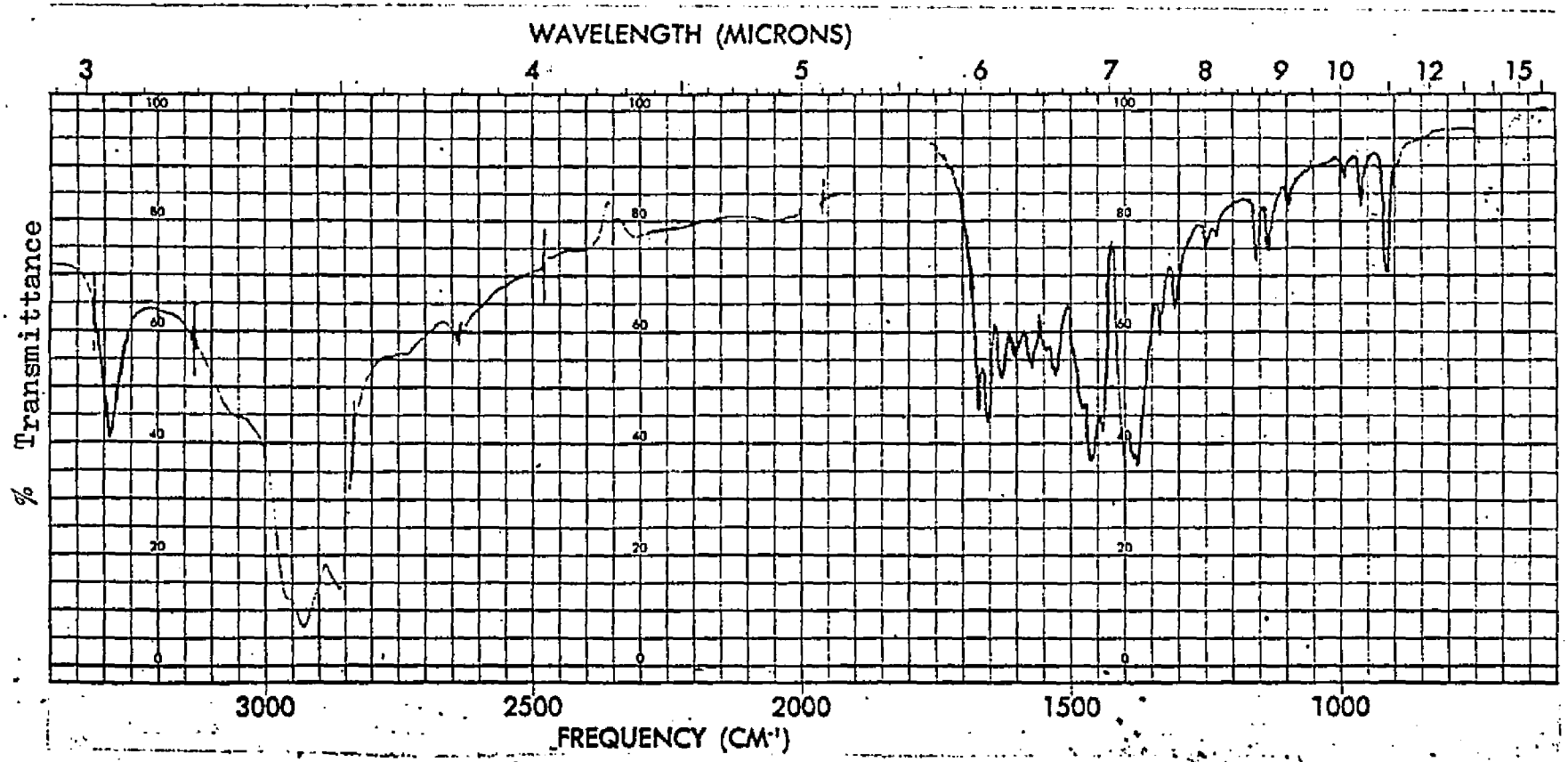

A
100
50
10
% Transmittance
B
100
50
10
% Transmittance
4000
2000
1500
1000
600
WAVENUMBERS (CM-1)

Figure 8

Infrared spectrum of Glycylglycine (Nujol Mull) with major band assignment.



3300 - NH stretch

1673 - R-C(=O)-NH

1636 - C=O stretch

1595 - C=O(-)

1540 (Amide II) NH deformation

1408

Figure 9

Infrared spectrum of the glycylglycine/PS mixed ligand complex.

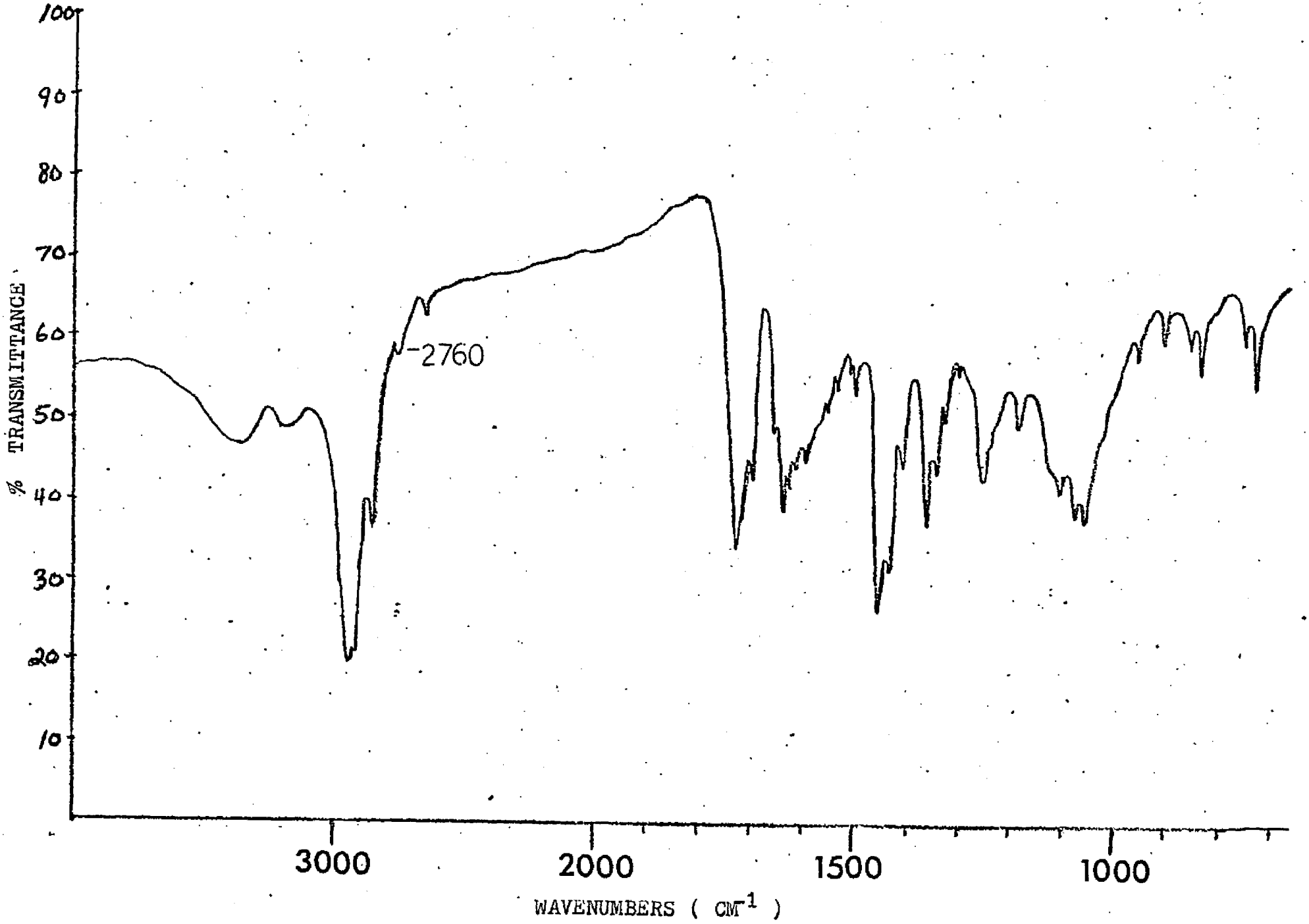

100
90
80
70
60
50
40
30
20
10
% TRANSMITTANCE
2760
3000
2000
1500
1000
WAVENUMBERS (CM^{-1})

Figure 10

1800 $\rightarrow$ 700 cm^{-1} region of the infrared spectrum of the glycylglycine mixed ligand complex.

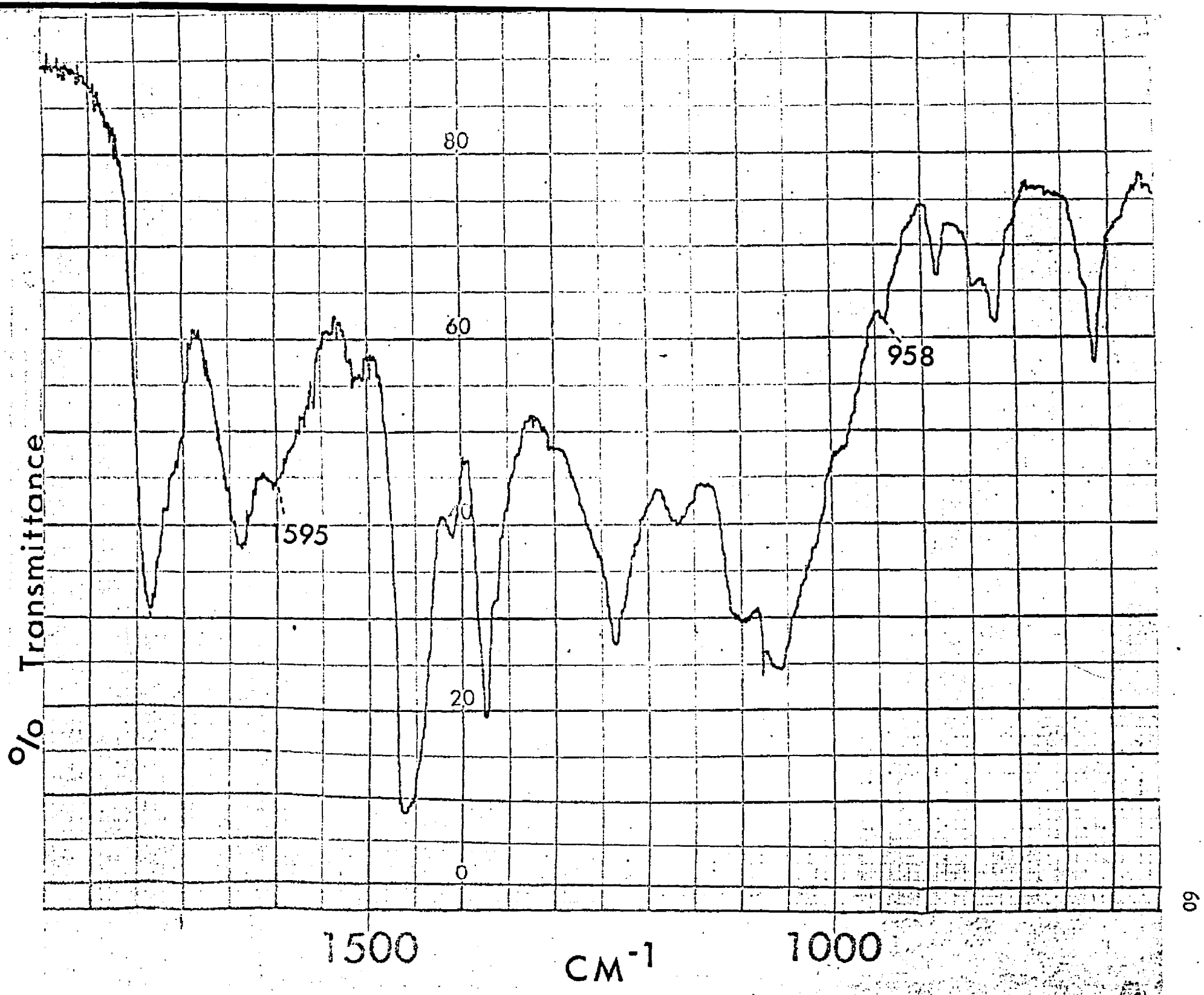
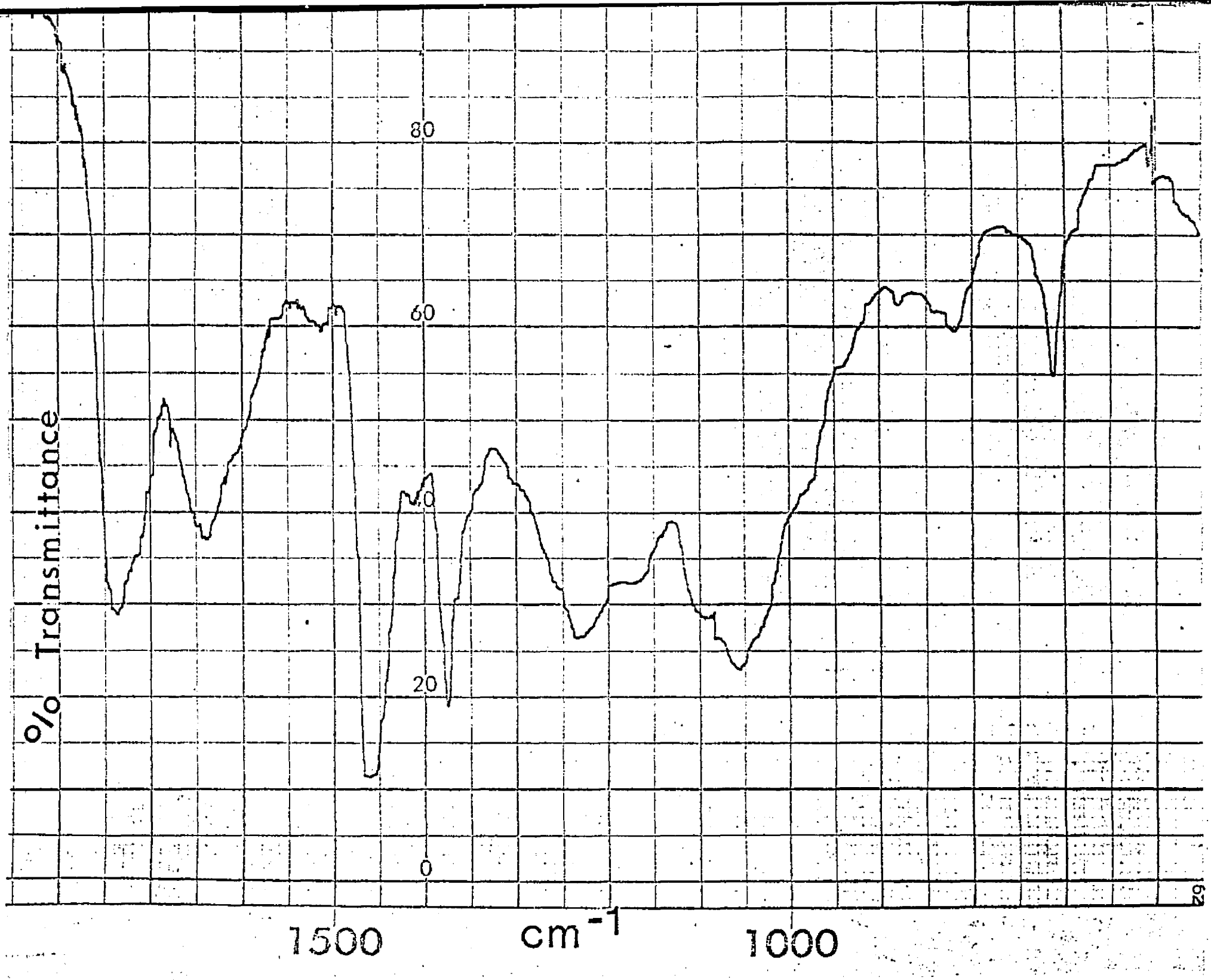

80
60
40
20
0
% Transmittance
1595
958
1500
1000
CM-1

Figure 11

1800 $\longrightarrow$ 600 cm^{-1} region of the infrared spectrum of the $Ca(PS)_2$ (Dilipid) complex. (Nujol Mull - CsBr 1 cm salt cell)


80
60
40
20
0
% Transmittance
1500
cm⁻¹
1000
62

of it to either the PS dilipid complex in Figure 7 or the abbreviated spectra of the PS spectra shown in Figure 11, one can readily see the obvious difference in the spectra and can also compute the amount of peptide ligand which is present in the complex by comparing the increase in the 2760 cm^{-1} and the 960 cm^{-1} band to the ionized phosphate band at 1100 cm^{-1} using the standard base line technique. The band at 1598 cm^{-1}, Figure 8 is thoroughly indicative of a metal - Gly-Gly carboxyl chelate.

The above spectra of both Gly-Gly and the Gly-Gly mixed ligand complex like the majority of the spectra taken hinge on the enhancement or appearance of a new band. Complete analysis of each spectrum was complicated by the overlapping of numerous bands of both the PS and peptide species and by the fact that no solution spectra could be obtained. However, the analysis did compare well with the data received from the other modes of analysis. The next set of spectra was slightly more easy to interpret. Figures 12 and 13 show the spectra of N-Glycylglycine-L-Phenylalanine and the mixed ligand complex of the same. Although the absorption bands have not been completely assigned for this species, one can on inspection observe the number of new bands which are now present in the mixed ligand species. Such a

Figure 12

Infrared spectrum (Nujol Mull) of N-Glycylglycyl-Phenylalanine.

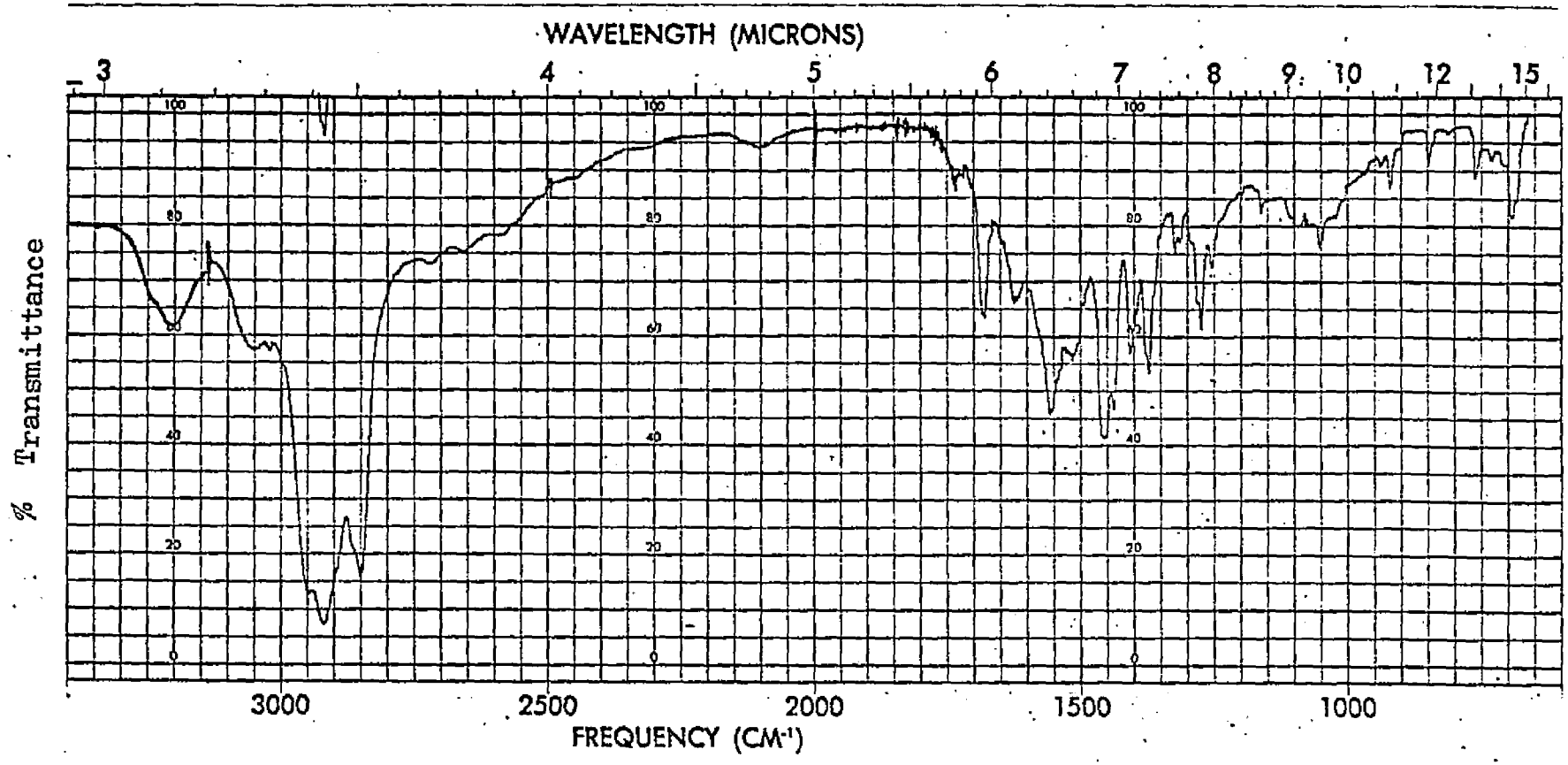
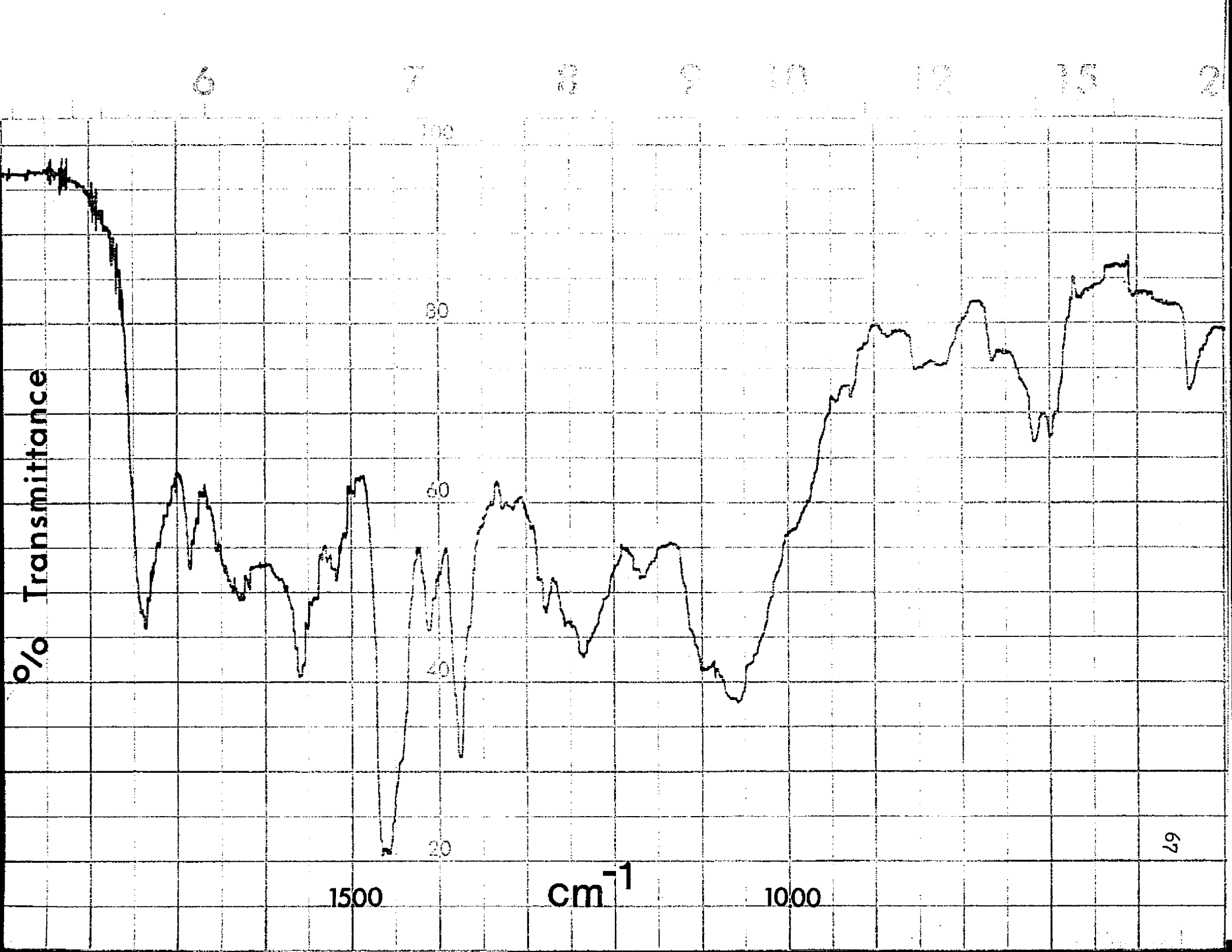

WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
12
15
% Transmittance
100
80
60
40
20
0
3000
2500
2000
1500
1000
FREQUENCY (CM-1)

Figure 13

Infrared spectrum (1800 $\longrightarrow$ 700 cm^{-1}) of the mixed lipid complex of PS, Ca and N-Glycylglycyl-Phenylalanine (Nujol Mull).


6
7
8
9
10
12
15
100
80
60
40
20
% Transmittance
1500
cm-1
1000
67

spectrum makes analysis of the complex precipitate more straightforward and easier to interpret.

B) Point of Chelation:

The primary goal of this endeavor had now been achieved - the formation of the mixed ligand complex. Analysis has shown that this complex was in a stoichiometric ratio of 1:1:1 and that there was a noticeable specificity for both the cation and the peptide ligand. However, the point of chelation was still in doubt. Previous work on PS has indicated Ca^{++} binding to the carboxyl, [64] the phosphate, [48,74] and both the carboxyl and the phosphate group simultaneously. [61,65] Therefore, it was imperative that the point of chelation in these mixed ligand systems be known.

Strictly from the interpretation of the infrared spectra of all the complexes formed, it appears that the ionized phosphate ($P\text{-}O^{(-)}$ - 1100 cm^{-1}) band is unchanged throughout. The band is not shifted in anyway as is the ionized carboxyl group of the PS ($COO^{(-)}$ - 1639 cm^{-1} in the pH range studies (6.0 $\longrightarrow$ 8.0). The carboxyl band is shifted to higher wavenumbers indicating that the primary point of chelation is at the carboxyl site and not at the phosphate. However, further evidence was necessary and was obtained through the negative results of three experiments. The first experiment was the reaction of lecithin (Figure 4) in an analogous

complexation reaction. Since the lecithin has no carboxyl group, mixed ligand formation could only occur through the ionized phosphate. The results were that: 1) very little precipitate was formed and 2) the precipitate complex was only the dilipid species. No mixed ligand complex was formed no matter what the ligand (Alanine, Aspartic Acid, Glycine, Glycylglycine, Glycylglycylglycine, reduced Glutathione, Glutamic Acid, Histidine, Leucine, Proline, Sarcosine, Serine, Valine, Egg Albumin and Phosvitin), pH (5.5 $\longrightarrow$ 8.0) or cation (same as Table III).

The second experiment was to react an esterified amino acid (glycine, ethyl ester) and an esterified peptide (glycylglycine-ethyl ester HCl) with the PS system. It has been shown that the amide group may coordinate to a metal/lipid species,[77] thus the carboxyl group of the peptide ligand may not be the primary chelation site on the peptide ligand. The results showed that there was no mixed ligand formation. Attempts to esterify the PS moiety led to hydrolysis of the fatty acid esters and thus this experiment could not be performed. The third experiment was the reaction of PS at pH 3 (isoelectric point pH 1.2) which contains only an ionized phosphate with various peptide ligands and cations. The results again showed no mixed ligand formation which indicates that for mixed ligand formation the chelation site must

be the carboxyl group. (This fact is also borne out in analytical chemistry with the carboxyl group having a greater affinity for Ca^{++} than phosphate.)

c) Attempted Complexation:

Various other molecules were used in the mixed ligand reaction. These included acetic acid, sodium acetate, NaH_2PO_4, methyl red and cresolphthalein all in an aqueous medium, and the following in a biphasic solution of $H_2O/CHCl_3/MEOH$: Stearic, Decanoic, Oleic and Lauric acids, Poly Glycine, Poly-L-Alanine and Poly-L-Hydroxy-Proline. The results of the aqueous reactants showed no mixed ligand formation, the dilipid complex was the sole product in the pH range studied (6.5-7.5). For the biphasic reaction a complex precipitate formed at the interface of the mixture on the addition of $CaCl_2$. This method of complexation was utilized by Hendrickson and Fullington[43] in their experiments on protein/lipid interactions, but their work was carried out at much higher pH ranges (9 - 14). In their experiments, as was also true for those carried out in this thesis, the interface precipitate would only form after addition of the metal species. Two possibilities now exist: 1) the divalent cation binds to the lipid reducing its surface potential, thus favoring a combined hydrophopic and coordination bonding interaction with the peptide or fatty acid ligand

(favored by the authors of reference)[43], or 2) a mixed ligand complex is formed at the interface through chelation of the respective carboxyl groups. Suffice it to say at this time that this author's analysis of the complex product(s) was inconclusive.

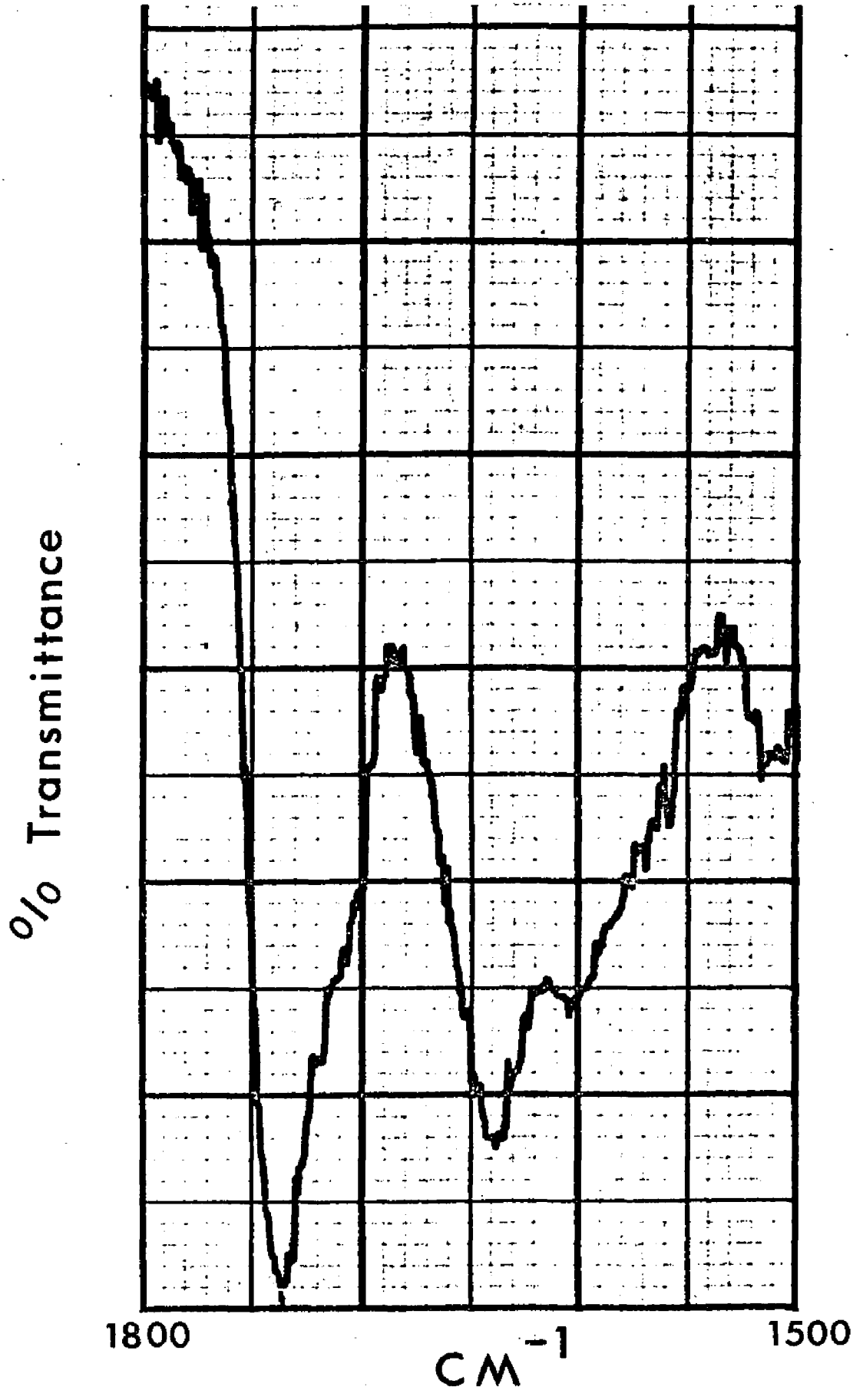
The infrared spectrum for the fatty acid reaction products showed a different appearance in the 1700 - 1600 cm^{-1} region (Figure 14), but a quantitative analysis was not possible because of the gross overlapping of the respective bands. The TLC for the fatty acids ($CHCl_3$/ETOH/H_2O-HCl-3:1:0.1) displayed two spots but this too was inconclusive because the two products could be the dilipid complex, a mixed ligand complex or a metal fatty acid complex. As for the poly amino acids, the infrared was totally unenlightening while the TLC of the product(s) formed at the biphasic interface showed three spots while some precipitate would not dissolve. These results will be re-evaluated in the discussion section of this thesis.

D) Protein Complexes:

As can be seen from Table 2, a number of proteins have been used with varying success in mixed ligand complex formation. The amount of protein complexation is reported differently from that of the previous species and will now be explained. The preliminary reactions for

Figure 14

$1800 \longrightarrow 1500\ cm^{-1}$ Infrared spectrum of the Stearic Acid/PS/Ca^{+2} complex (Nujol Mull).


% Transmittance
1800
1500
CM -1

each of the proteins was conducted in a 1:1:1 molar ratio of protein/lipid/Ca^{++}. Precipitates were formed slowly and were isolated, purified and analyzed as previously described. Generally, it was found that a mixed ligand complex was formed, but not necessarily in a 1:1:1 stoichiometric reaction scheme. This phenomenon was borne out by the total analysis of the reaction products. The stoichiometry of these complexes was determined in the experiments reported below:

1) Phosvitin:

This is a phosphoglycoprotein obtained from hens' egg yolks. It has been chemically characterized [78] and its conformation has also been studied [79] with the following facts being discovered: Molecular weight - 33,910; Phosphorus content = 11.8% ⟶ 12.5%; Nitrogen content = 13.0% ⟶ 13.4%; Amount Ca^{++} and Mg^{++} bound [80] (pH 6.5-6.8) = 127 Ca^{++} or 103 Mg^{+2}; Number of ionized phosphate/carboxylate groups per molecule (pH 6.8) = 136 phosphates, 31 carboxylates.

With this knowledge at hand two major experiments were performed in the attempt to reveal the stoichiometry of the mixed ligand formation. The preliminary reaction of a 1:1:1 preparative technique produced a mixed ligand complex/dilipid mixture with the bulk of phosvitin remaining in the solution. Therefore, subsequent reaction schemes followed the exact preparative procedures outlined in the

Experimental section with the following changes in the reactions. The concentration of PS and Ca in the first set of reactions was 100 times that of phosvitin. The second experiment had PS and Ca at a 40 times concentration. Both experiments were conducted at pH 7.0. The isolation and analysis was done on each as previously described but with the addition of a total phosphate analysis of the product using the Bartlett[81,82] method also described in the Experimental section. Analysis of each set of reactions produced the following:

1) 100 Times Concentration PS/Ca:

The infrared spectrum of the crude product depicted a mixture of a mixed ligand and a dilipid complex. Treatment of the crude product with a 3:1:0.1 solution of $CHCl_3/ETOH/H_2O$ dissolved only a small fraction of the precipitate indicating that only a small amount of the dilipid complex was present. A TLC of the crude product with the acidified 3:1:0.1 solution produced two distinct spots while the TLC of the product after workup showed only one spot which is consistent with the previous observations.

Analysis of the supernatant using both a ninhydrin and a biuret test for proteins gave negative results indicating that all of the protein had been precipitated out of solution. A colorimetric Ca^{+2}

determination discovered that approximately 33-37% of the original amount of Ca added remained in solution.

The phosphate analysis of the precipitated complex (after workup) resulted in an increase of 15-31 additional phosphates per mole of phosvitin. The average value for the additional phosphates was 29. These numbers were arrived at by assuming total carboxylate binding (31 sites) with phosvitin which would increase the molecular weight of the PV complex to 59,581.* Utilizing this number in the calculations in the equimolar comparison of PV to the precipitated complex produced the additional range of phosphates reported. A Ca^{+2} determination on the product was analogous to the phosphate results.

The phosphate analysis of the dilipid mixture resulted in approximately 70% of the original PS being present as the dilipid complex. The Ca^{+2} analysis supported the above results with 34-36% of the original amount of Ca^{+2} added being found in this species.

2) 40 Times Concentration PS/Ca:

The infrared spectrum of the crude product of this reaction showed only a mixed ligand complex. Subsequent treatment with a 3:1:0.1 solution of $CHCl_3$/ETOH/H_2O and a TLC using the acidified above solution showed that the

* The molecular weight of 59,581 is derived from the molecular weight of PV plus 31 moles of Ca^{+2} and 31 moles of PS.

precipitate was primarily one product with a slight amount of impurity which proved to be the dilipid complex (Rf values and solution properties).

Analysis of the supernatant for protein (ninhydrin and biuret test) and Ca^{+2} produced negative results.

The phosphate analysis of the isolated mixed ligand complex resulted in an increase of 25 through 31 additional phosphates per mole of PV. The average increased value of phosphates was 30.1. The comparison analysis again assumed total carboxylate binding. Ca^{+2} determination of the product resulted in a range of 29 through 32 moles of Ca^{+2} per mole of PV.

The phosphate and Ca^{+2} analysis of the dilipid complex supported the above reported results.

Each of the above sets of experiments were performed three times in addition to that reported above, each with almost identical analysis of the product with the exception of the 100 times concentration PS/Ca whose range was found to be 26-31 in the subsequent reactions. The average additional phosphate was 29.5.

It is extremely interesting to note that no more than 31 PS were complexed with the PV even though there are numerous ionized phosphate binding sites available.

It appears that this is a supporting case for specific carboxylate binding in mixed ligand complexes since PV itself has only 31 ionizable carboxylate groups.

2) Egg Albumin (Ovalbumin):

A water soluble protein found in egg yolks with a molecular weight of 43,500 (sedimentation coefficient 3.66).[117] The amino acid analysis shows that there are 141 carboxyl side chain groups comprised primarily of aspartic acid. This protein was treated with a ten-fold excess of PS and Ca^{+2} at pH 7.0 in the normal mixed ligand complex formation reaction. An insoluble precipitate formed which was on analysis found to be a mixture of the Ca^{++} dilipid complex and a mixed ligand complex. The ninhydrin test of the supernatant was positive indicating the presence of ovalbumin in solution. Of the original 0.2250 gm ovalbumin (1×10^{-5} moles), 0.0373 gm remained in solution with approximately 30% of the Ca^{+2}; no PS remained in the supernatant. The dilipid complex was removed and a total phosphate analysis on that species showed the presence of 6.775×10^{-5} moles of phosphorus. This indicates that for the complexed ovalbumin (7.5×10^{-6} mole) there must be approximately 4.3 moles of phospholipid bound. The Ca^{+2} colorimetric analysis of the precipitate analyzed to 4.9 moles Ca/mole albumin. This reaction was carried out twice more at the same pH but with the PS and Ca^{+2} in a five-fold excess. The

subsequent analysis of each trial again showed approximately 25% of the ovalbumin remaining in solution after complexation (Experiment 1 - 27%; Experiment 2 - 24%) and the stoichiometry for the mixed ligand complex of Experiment 1: 4.5 PS/ovalbumin, 5.0 Ca/ovalbumin and Experiment 2: 4.1 PS/ovalbumin; 4.6 Ca^{+2}/ovalbumin. The complex is assumed to be the mixed ligand moiety chelated by the Ca^{+2} due to its behavior toward the $CHCl_3$/ETOH/H_2O system - it would not dissolve which is a consistent trait with a mixed ligand complex. The infrared absorption spectrum was inconclusive as to the type of complex but both species were detected.

3) Bovine Albumin:

A water soluble protein obtained from cow serum and bought essentially fatty acid free.[118] The molecular weight is between 65,400 (sedimentation coefficient = 4.31) for the fatty acid free and 67,000 (sedimentation coefficient = 4.41). The amino acid analysis shows 133 carboxylate side chain groups as aspartic and glutamic acids (not necessarily all exposed). The normal complexation reaction was followed with the exception of a ten-fold excess of PS and Ca^{+2} (1×10^{-4} mole) to the bovine albumin (1×10^{-5} mole). An insoluble complex was formed immediately with the addition of $CaCl_2$. This experiment was conducted in duplicate with the following

analysis: Experiment 1: a) supernatant = ninhydrin - positive test; 4.64×10^{-6} moles of bovine albumin remaining in solution = 46% of original amount added. b) precipitate = mixed ligand complex and dilipid complex mixture. Total phosphorus analysis of dilipid complex = 6.2740×10^{-5} mole PS. This indicates that 3.7260×10^{-5} moles PS is complexed to 5.4×10^{-6} mole bovine albumin which corresponds to 6.9 moles PS/mole bovine albumin. Experiment 2: a) supernatant: 4.71×10^{-6} mole bovine albumin remaining in solution (quant. ninhydrin test). b) precipitate: tricomplex/dilipid mixture. P analysis of dilipid complex = 6.2441×10^{-5} mole PS. Analysis shows that 3.7559×10^{-5} mole PS bound to 5.29×10^{-6} mole bovine albumin which is a ratio of 7.1 moles PS/mole bovine albumin. For both experiments the Ca^{+2} analysis on the precipitate gave the identical PS mole ratio/mole bovine albumin. Again, the mixed ligand complex was assumed due to its solvent properties. The infrared absorption spectrum was again inconclusive but did show mixed ligand complexation.

These three stoichiometric reactions will be discussed further in the Discussion section of this dissertation.

E) <u>Cation Competition</u>:

Up to this point, a number of mixed ligand complex species had been prepared with Ca^{++} being the principle chelator. A series of divalent and trivalent

cations were also examined to note their tendencies towards such mixed ligand formation (Table 3). Since biological fluids contain a number of cations simultaneously (Ca^{+2}, Mg^{+2}, Mn^{+2}, K^{+}, Na^{+}) and since it was known that Ca^{++} can be displaced from phospholipid monolayer systems by Mg^{+2}, Na^{+} or K^{+},[48,50,62] it now became of interest to note the competitive binding tendencies of various cations to Ca^{++}. Table 5 shows the results of these competition studies.

Both monovalent cations, Na^{+} and K^{+}, produced no effect on mixed ligand formation in the pH range observed (6.5 - 7.5) as was also true for the divalent Ba^{+2}, Ni^{+2} and the trivalent Al^{+3}. The remaining 4 cations produced varying amounts of mixed ligand complex. The experiment followed the same preparative procedure for tricomplex formation with the exception that equimolar amounts of both cations were added simultaneously. Isolation and analysis of the precipitate and supernatant was also accomplished in the same manner as per the techniques with the following exceptions: 1) Mn^{+2} - was determined by complexometric titration as per the experimental techniques except that the Ca^{++} was removed from solution by adding a slight excess of EDTA, alkalating that solution and then adding NaF which will mask the Ca^{+2}. [83] The mixture is back titrated with a standard Mn solution (with some hydroxylamine) - Ca is precipitated as the

Table V

Ca^{+2}/Metal Competition Reaction
with Phosphatidyl-Serine and Glycyl-Glycine -
(Simultaneous Addition of Metals)

$Ca/_X$ Metal	% Metal X Bound	pH	Ninhydrin	IR
$AlCl_3$	0.00 %	7.0	Negative	Tricomplex - Ca^{+2}
	0.00	7.3	Negative	Tricomplex - Ca^{+2}
$BaCl_2$	0.15	7.0	Negative	Tricomplex - Ca^{+2}
	0.00	7.3	Negative	Tricomplex - Ca^{+2}
$CuCl_2$	11.20	6.8	Positive	Dilipid/Tricomplex Mixture (Cu/Ca)
	0.00	7.0	Positive	Dilipid Complex (Ca^{+2})
$FeCl_3$	0.00	7.0	Negative	Tricomplex - Ca^{+2}
	0.00	7.3	Positive	Tricomplex/Dilipid Mixture (Ca^{+2})
$MgCl_2$	23.09	6.5	Negative	Tricomplex - (Ca/Mg)
	0.00	7.0	Negative	Tricomplex - Ca^{+2}
	23.39	8.0	Positive	Tricomplex/Dilipid Mixture (Ca/Mg)
$MnCl_2$	0.09	6.5	Negative	Tricomplex - Ca^{+2}
	0.17	7.0	Negative	Tricomplex - Ca^{+2}
$NiCl_2$	2.50	6.5	Negative	Tricomplex - Ca^{+2}
	0.00	7.0	Negative	Tricomplex - Ca^{+2}
$SrCl_2$	7.00	6.0	Negative	Tricomplex - Ca^{+2}
	0.00	7.0	Negative	Tricomplex - Ca^{+2}

Notes:

1) IR analysis supplemented with TLC and cation analysis of precipitate

2) Analysis performed 1 day after metal addition

3) Cation analysis described in Experimental Techniques section.

fluoride -- the procedure is now the same as stated in the experimental; 2) analysis of the Sr/Ca mixture was preceded in all cases by the removal of Ca^{++} by treatment of the supernatant with 1.2 M ammonium lactate followed by filtration of the mixture through a 19 cm long Dowex 50 resin column. At this point the filtrate could either be analyzed by the titration method or through a gravimetric procedure in which the Sr^{+2} is precipitated as the sulfate on addition of K_2SO_4 to the filtrate.

Nevertheless, each of the competitive experiments (Table V) (done in triplicate) was accompanied by a Ca^{++} colorimetric determination in which the competing metals did not interfere with the analysis. Results from both ion analysis were correlated and agreed within experimental error.

Inspection of Table V reveals Mg^{++} as being the principal competing ion in these experiments. A more thorough investigation of this competition was undertaken and will now be reported. The initial results are shown on Table VI. The analysis was done approximately 2 hours after complexation at the effective pH values shown. However, the possibility of kinetic control for this reaction was a likelihood, thus a time extension of this reaction was undertaken. The results of this work are shown on Table VII and displayed graphically on Figure 15. The pH range chosen (6.0 -- 8.0) was selected because

Table VI

Initial Ca^{+2}/Mg^{+2} Competition Results

pH	% Mg Bound	Ninhydrin	IR
4.0	0.00 %	Positive	Dilipid Complex
5.0	20.00	Positive	Dilipid Complex
6.0	9.52	Negative	Tricomplex
6.2	20.91	Negative	Tricomplex
6.5	23.09	Negative	Tricomplex
6.8	14.29	Negative	Tricomplex
7.0	0.00	Negative	Tricomplex
7.3	12.29	Negative	Tricomplex
7.5	7.88	Positive	Tricomplex/Dilipid Mixture
7.8	17.49	Positive	Tricomplex/Dilipid Mixture
8.0	23.39	Positive	Tricomplex/Dilipid Mixture
9.0	0.00	Positive	Dilipid Complex

Notes:

1) Nin: pH 8 > 7.8 > 7.5
2) Analysis Time = 1 → 3 hours after metal addition
3) Amount Dilipid Complex: pH 8 > 7.8 > 7.5
4) Ca^{+2} and M^{+2} concentration both 2 x 10^{-4} mole
5) pH adjustment 4.0→6.8 NaOH
   7.0→9.0 TRIS Buffer.

Table VII

Time/pH Dependence
for Ca^{+2}/Mg^{+2} Competition -
Simultaneous Addition

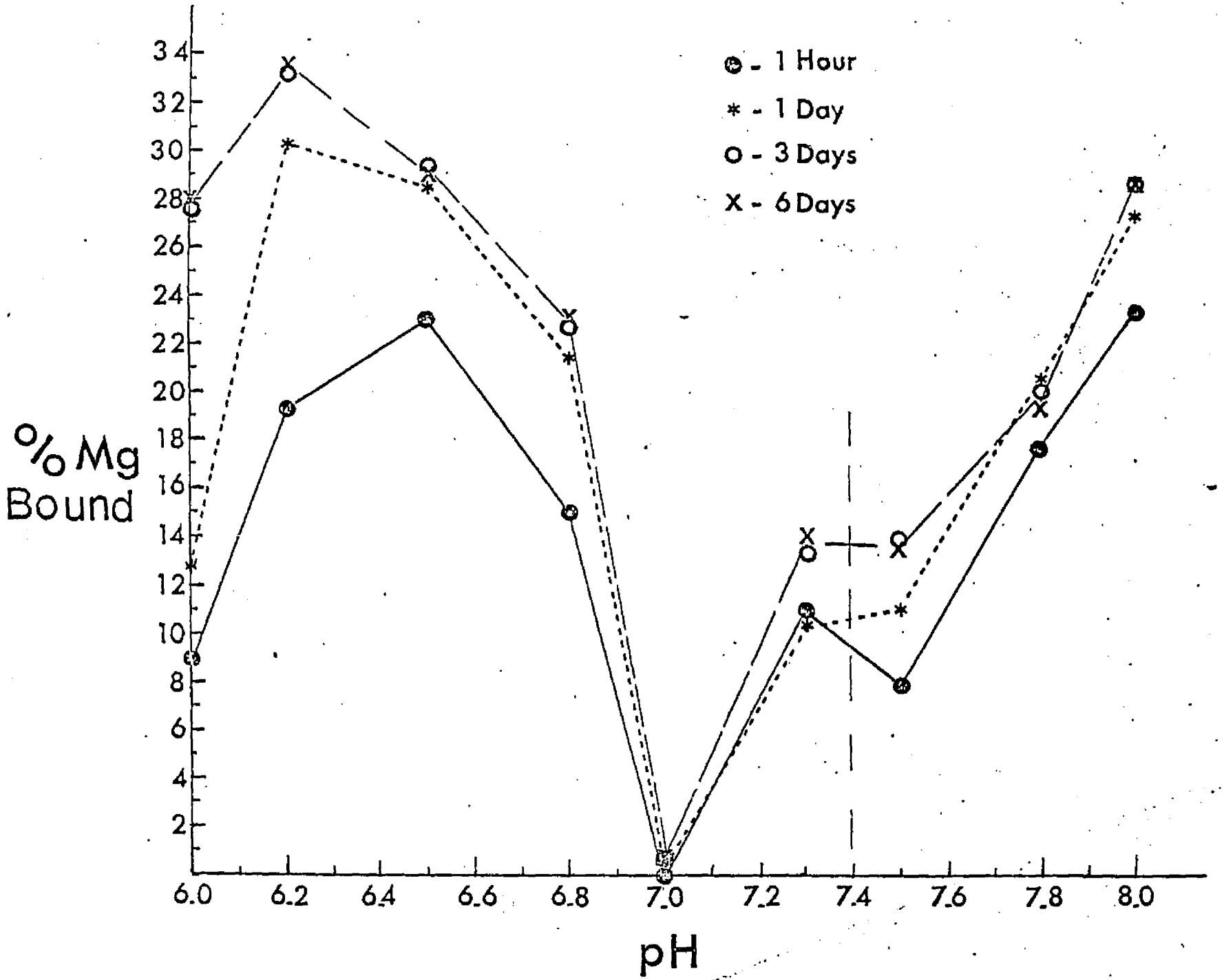
pH	% Mg Bound				Analysis Ninhydrin	IR
	1 - 3 Hrs.	1 Day	3 Days	6 Days		
6.0	8.91 %	12.86 %	27.50 %	28.00 %	Negative	Tricomplex
6.2	19.45	30.15	33.50	36.60	Negative	Tricomplex
6.5	23.10	28.62	29.31	28.91	Negative	Tricomplex
6.8	15.01	21.60	22.81	23.01	Negative	Tricomplex
7.0	0.00	0.41	0.50	0.64	Negative	Tricomplex
7.3	11.96	10.50	13.60	14.00	Negative	Tricomplex
7.5	8.00	11.08	14.00	13.96	Positive	Tri/Dilipid
7.8	17.79	20.50	20.00	19.60	Positive	Tri/Dilipid
8.0	23.60	27.60	28.90	28.70	Positive	Tri/Dilipid

Notes:

1) Tri = Tricomplex; Dilipid = Dilipid Complex
2) Ninhydrin and Infrared Analysis done after Day 6
3) Ninhydrin: pH $8 \succ 7.8 \succ 7.5$

Figure 15

% Mg bound as a function of time and pH in the simultaneous addition Ca^{++}/ Mg^{++} competition experiment.


⊙ - 1 Hour
* - 1 Day
O - 3 Days
X - 6 Days
% Mg Bound
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
pH

1) it is in the physiological pH range; 2) below pH 6.0 only varying amounts of the carboxyl are ionized and the major product is the dilipid species; 3) above pH 8.0, the possibility of hydroxide formation is present. The graph (Figure 15) demonstrates three points: 1) pH dependence for Ca/Mg binding; 2) a unique metal specificity at pH 7.0 and, 3) an obvious time dependence (measurements were conducted with the supernatant in contact with the precipitate). These results as seen in Table VII and Figure 15, were for the experiment in which there was simultaneous addition of both Ca/Mg. The exact experiment was carried out first adding Mg^{++} to form the Mg reaction products (mixture of tricomplex and dilipid complex) with the subsequent addition of Ca^{++}. If this order is reversed, Mg^{++} will not displace Ca^{++} from the complexes formed. The results of this experiment are shown in Table VIII* which, when compared to Table VII, are identical within experimental error. The Ca^{++}/Mg^{++} competition was further carried out with a tripeptide, reduced glutathione (γ- L glutamyl-L-cysteinylglycine) in both the simultaneous addition and the staggered addition methods. The results of these experiments are shown in Table IX and are graphed in Figure 17. The data for both methods is virtually the same above pH 6.8. Below 6.8 the predominant product is the Ca^{++} dilipid species with virtually 100% glutathione being present in the

*The results are graphically depicted in Figure 16.

Table VIII

Ca^{+2}/Mg^{+2} Competition -
Mg^{+2} Complex Formation
Followed By
Ca^{+2} Addition

pH	% Mg Bound				Analysis Ninhydrin	IR
	1 - 3 Hrs.	1 Day	3 Days	6 Days		
6.0	10.01 %	12.75 %	25.91 %	27.91 %	Negative	Tricomplex
6.2	20.23	29.55	34.31	36.44	Negative	Tricomplex
6.5	22.86	28.60	28.75	28.95	Negative	Tricomplex
6.8	14.91	20.96	22.09	23.06	Negative	Tricomplex
7.0	0.08	0.25	0.43	0.59	Negative	Tricomplex
7.3	11.81	11.99	13.51	13.60	Negative	Tricomplex
7.5	8.05	11.73	13.64	13.81	Positive	Tri/Dilipid
7.8	17.95	20.09	20.53	20.53	Positive	Tri/Dilipid
8.0	23.58	26.59	27.91	27.93	Positive	Tri/Dilipid

Notes:

1) Tri = Tricomplex; Dilipid = Dilipid Complex
2) Ninhydrin and Infrared Analysis done Day 6
3) Ninhydrin: pH $8 > 7.8 > 7.5$

Figure 16

% Mg bound as a function of time and pH in the Ca^{+2}/Mg^{+2} competition experiment in which the Ca^{+2} was added after the formation of the Mg^{+2} complexes.

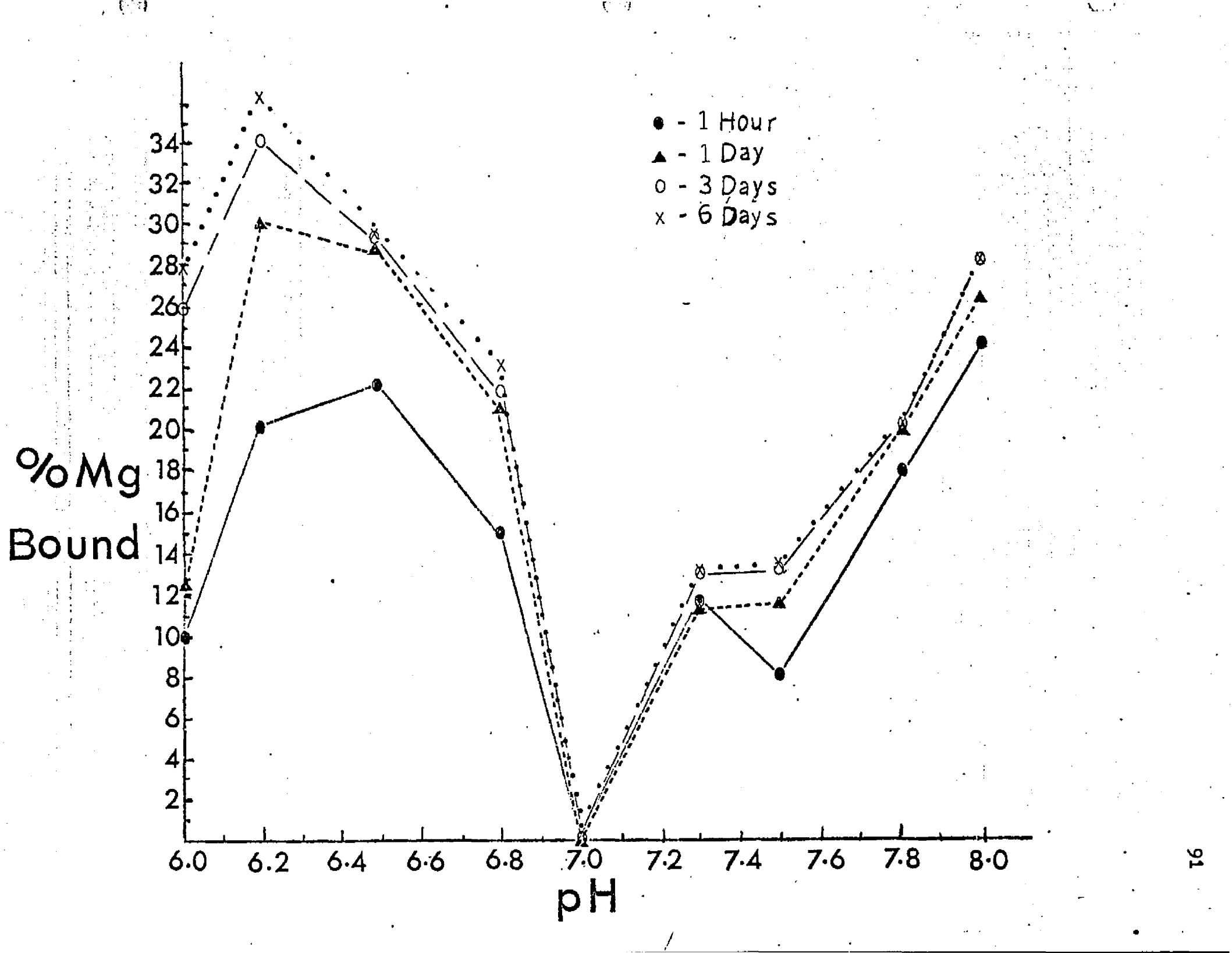

● - 1 Hour
▲ - 1 Day
o - 3 Days
x - 6 Days
%Mg Bound
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
pH

Table IX

Glutathione Mixed Ligand Complex

Ca^{+2}/Mg^{+2} Competition

a) Simultaneous Addition

b) Mg^{+2} Complex, Ca^{+2} Addition

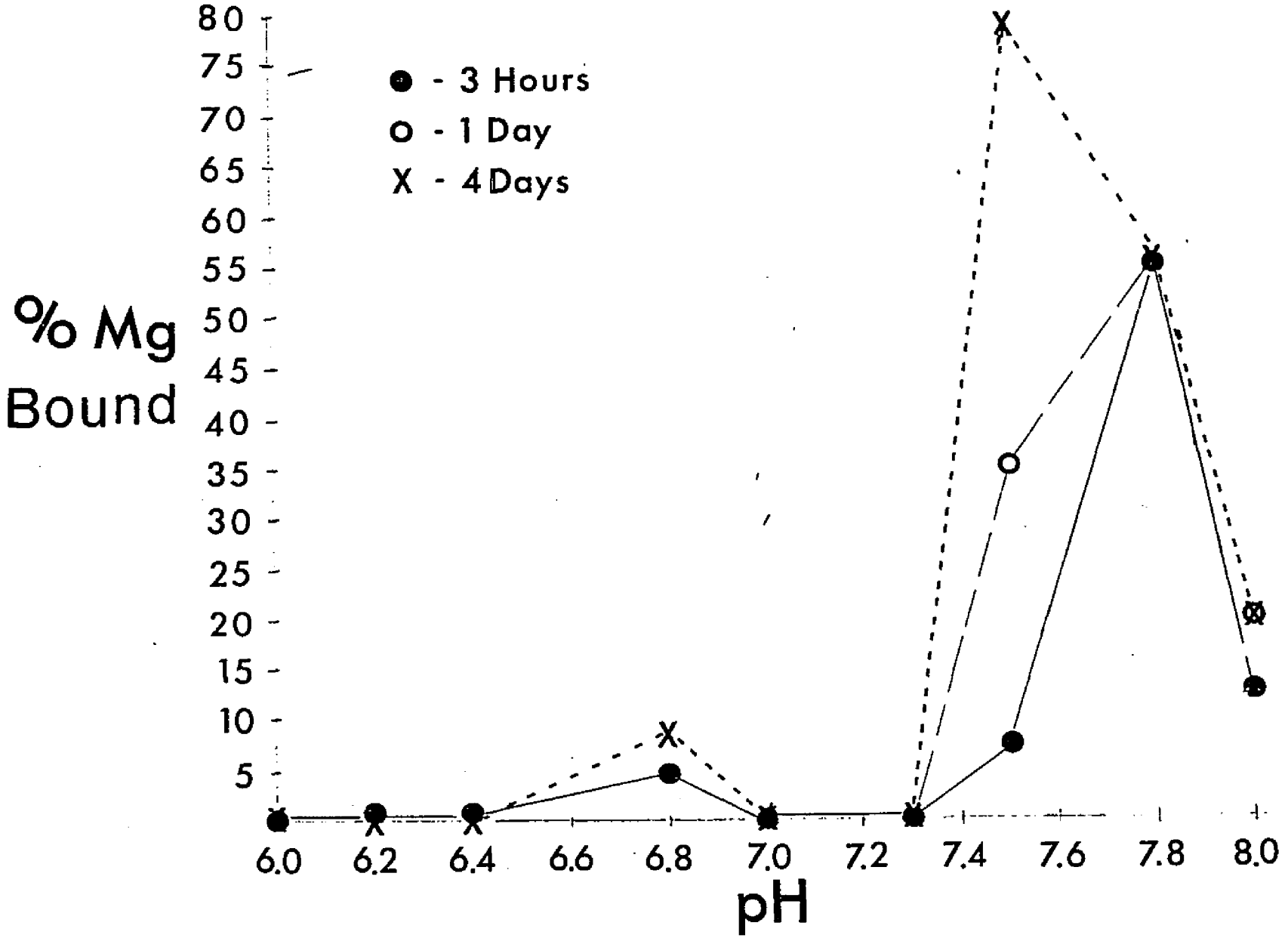
	% Mg Bound						Analysis Ninhydrin		IR	
	3 Hrs.		1 Day		4 Days					
pH	a	b	a	b	a	b	a	b	a	b
6.0	0.00%	1.40%	0.00%	0.00%	0.00%	0.00%	Positive	Positive	Dil	Dil
6.2	1.31	1.70	0.00	0.00	0.00	0.00	Positive	Positive	Dil	Dil
6.5	1.46	3.81	1.01	2.16	0.00	1.91	Negative	Positive	Tri	Tri/Dil
6.8	4.67	7.93	4.65	8.13	4.66	8.10	Negative	Negative	Tri	Tri
7.0	0.30	0.40	0.81	0.85	1.54	1.54	Negative	Negative	Tri	Tri
7.3	0.64	0.57	0.53	0.61	0.48	0.63	Negative	Negative	Tri	Tri
7.5	7.38	5.92	35.40	41.68	78.42	63.04	Negative	Positive	Tri	Tri/Dil
7.8	54.17	54.81	54.81	54.97	55.38	55.40	Positive	Positive	Tri/Dil	Tri/Dil
8.0	12.62	13.10	20.17	20.09	21.53	21.53	Positive	Positive	Tri/Dil	Tri/Dil

Notes:

1) Tri = Tricomplex; Dil = Dilipid Complex
2) Ninhydrin: pH 6.0 = 6.2 > 8.0 > 7.8 > 7.5
3) Ca^{+2}, Mg^{+2} initial concentration 1×10^{-2} molar
4) Analysis done on Day 4

Figure 17

The Ca^{+2}/Mg^{+2} (simultaneous addition) competition experiment with PS and reduced glutathione as a function of time and pH.


80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
● - 3 Hours
o - 1 Day
X - 4 Days
% Mg Bound
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
pH

supernatant analysis. Since Ca^{++} is known to have a greater affinity for the lipid in the formation of the dilipid complex, the results only bear out this fact and do not bear any relationship to mixed ligand formation which was the purpose of this experiment. These results and those of the other competition experiments will be reviewed in the discussion portion of this thesis.

F) pH Dependence of Mixed Ligand Formation and Stability:

Besides the obvious pH effect on mixed ligand formation as demonstrated by the results in Tables I, II, V - IX and Figures 15 and 16, there is also an effect on the product after formation.[68] The Ca/Gly Gly/PS system is stable from pH 5.0 → 7.6. Above and below these limits the complex begins to dissociate as is evidenced by a positive ninhydrin test. Dissociation of the mixed ligand complex is complete above pH 8.0 and below pH 4.0. However, if the pH is adjusted in either direction into the pH stability limits, the mixed ligand complex will reform completely. The Mg^{++}/Gly Gly/PS system needs a more detailed description. Whereas, the Ca complex was virtually 100% mixed ligand complex, the maximum mixed ligand complex was 68% of the reaction product and actually was a mixture with the dilipid complex from pH 7.0 - 8.0. Thus at pH 7.0, Mg^{+2} does not completely bind either PS or GlyGly in an insoluble complex. When the pH

was raised to 8 by the dropwise addition of 0.1 M NaOH, all of the PS was complexed as the mixed ligand and dilipid complex mixture. 42% of the original GlyGly remained in solution. If the pH is raised above this point, hydroxide formation is encountered, but if the pH is lowered to pH 6, complete dissociation of all complexes occurs. Careful reassociation to neutral pH by the dropwise addition of 0.1 M NaOH led to a complex which was now only 22% mixed ligand complex with 88% of the GlyGly in solution. Allowing the mixture to stand for a number of days (under N_2 atmosphere), heating, or further pH variation did not alter the mixed complex/dilipid ratio.

G) Membrane Formation:

A most interesting property of the Ca^{+2} tri-complex was discovered quite by accident. If the precipitate complex is allowed to remain in contact with its supernatant, it will form a thin multi-layer membrane which showed remarkable stability to swirling and treatment with EDTA. The membrane mimicked the pH behavior of a normal Ca mixed ligand complex but did not dissociate until the pH was above 8.0. This phenomenon was not shown by any other of the tricomplex species including the Mg complex. Thus, Ca^{+2} must stabilize long range molecular interactions in such a system. Infrared spectral evidence indicated that the GlyGly was incorporated within the membrane.

H) Liposome Investigations:

The phenomenon of mixed ligand complex formation has been demonstrated in the previous section. However, these reactions were carried out on aqueous dispersions of PS or as multi-lamellar structures depending on the total volume of solvent used. In order to expand and extend the use of the data presented to pertain to biological systems, the experimental thrust had to proceed to a model membrane system which would eliminate both the problems of the use of natural membranes and of aqueous dispersion. Thus, experiments were performed on mixed phospholipid liposomes.[63,84-85] The exact methods of preparation and precautions are listed in the Experimental Techniques section. Throughout these investigations the following lipid mole ratios of PC/PS were used: 9/1, 3/1, 1/1, 1/3, 1/9 with the total lipid being 2×10^{-4} moles.

The preliminary investigation was to note and observe the effect of both Ca^{+2} and Ca^{+2}/GlyGly on these mixed lipid vesicles. Numerous investigations have studied the effects of Ca^{+2} on pure lipid (PC and PS) vesicles and monolayers[48,50,61-66,87,88], as well as mixed lipid systems.[89-92] It should be noted that some of the above investigations gave conflicting reports as to the binding of Ca^{+2}, size of the liposome, site of chelation and the effects of the bound Ca^{+2} to the vesicle itself.

The results of this thesis does, however, correlate with the majority of investigations. For pure PS liposomes or aqueous dispersion, depending on the molar concentration of the PS, either a precipitate is formed (dilipid complex) or a 1:1 Ca^{+2}/PS complex is suspended in the dilute solution. (The 1:1 complex can be collected through centrifugation or by concentrating the solution at which time the dilipid complex will form as is confirmed through analytical and spectrometric analysis).

Pure PC will not complex as did the PS on the addition of Ca^{+2} even to molar concentration of 10^{-2}. A very small amount of precipitate can be collected after centrifugation but the bulk of the lipid remains as the liposome or dispersion. Ca^{+2} analysis showed that over 95% of the available Ca^{+2} remained in that dispersion. The effects of Ca^{+2}/GlyGly has already been discussed. Recall that, at pH 7.0 all the PS/Ca/GlyGly is complexed in a mixed ligand moiety, while for the PC system all the GlyGly remains in solution with only a small amount of the lipid being complexed ($<$ 5%) as a Ca/lipid complex.

The mixed phospholipid liposome systems showed varying properties toward Ca^{+2} and the Ca^{+2}/GlyGly systems. The results are summarized in Table X. It is interesting to note that in the more concentrated solutions, (10 ml total volume) the PS seems to "fall out" of the liposome forming a separate precipitate while at the higher

Table X

General Properties and Observations
of the Variable Lipid Vesicles
When Treated With Ca^{+2} or Ca^{+2}/GlyGly

- -

1) Amount of Ca^{+2} and Ca^{+2}/GlyGly complexed to the variable lipid vesicles was equimolar to the amount of PS present.

2) Addition of Ca^{+2} or Ca^{+2}/GlyGly to a concentrated vesicle (2×10^{-2}) M solution caused the PS to precipitate out of the vesicles.

3) The supernatant of the solution from (2) contained PC and 50% of the original Ca^{+2} which was added in the Ca^{+2} (only) liposome experiments.

4) A mixed ligand complex would form on the addition of GlyGly to a dilute vesicle solution which was already Ca^{+2} chelated. This phenomena was not observed when the lipid molar concentration was 2×10^{-2} M.

5) Addition of other cations (K^{+}, Na^{+} and Mg^{+2}) did not displace Ca^{+2} from either the Ca^{+2} or Ca^{+2}/GlyGly complexed vesicles at pH 7.0. Small amounts of Ca^{+2} (approximately 1×10^{-6} mole) were displaced by Mg^{+2} at pH 6.5 and 7.5.

6) Ca^{+2} will totally displace, K^{+}, Na^{+} and Mg^{+2} to form the Ca^{+2} and Ca^{+2}/GlyGly complex liposome systems at pH 7.0.

7) GlyGly is not complexed to the liposomes until the addition of Ca^{+2}.

volume (30 ml total volume) the PS remains in the vesicle system with either reacting system. Reducing the volume of the 30 ml system to 10 ml does not have an effect on the vesicle itself. It remains intact and the analysis shows that even in the higher percentage PS liposome, the PC remains in the vesicle and is not dissociated out into solution as was seen in the lower volume experiment. Again, it appears from the results that the complexation is facilitated through the carboxyl of the acidic phospholipid since only equal amounts of PS and GlyGly complexed with Ca^{+2} while the remaining Ca^{+2} and GlyGly remained in the supernatant solution. Figures 18 through 20 show the typical infrared spectra for three of the five concentrations of the mixed liposomes studied. Note the differences in the spectrum of the Ca^{+2} (only) and the Ca^{+2}/GlyGly systems. These spectra show that mixed ligand formation does occur in mixed lipid vesicles. The H^1 Nuclear Magnetic Resonance spectrum although used by a number of investigators in the analysis of such mixed lipid systems,[32,93-96] did not prove useful in these cases because the peptide peaks were totally enveloped by the predominant phospholipid peaks. The intergration of the peak area for the N-H specie of the complexed liposome did, however, show an increase consistent with the complexed specie stoichiometry while no acidic protons were observed in the spectra. C^{13} Fourier Transform NMR was contemplated but not used because

Figure 18

The infrared spectra of the 9/1 mixed ligand PC/PS vesicle after treatment with a) Ca^{+2} (only) and b) Ca^{+2}/GlyGly.

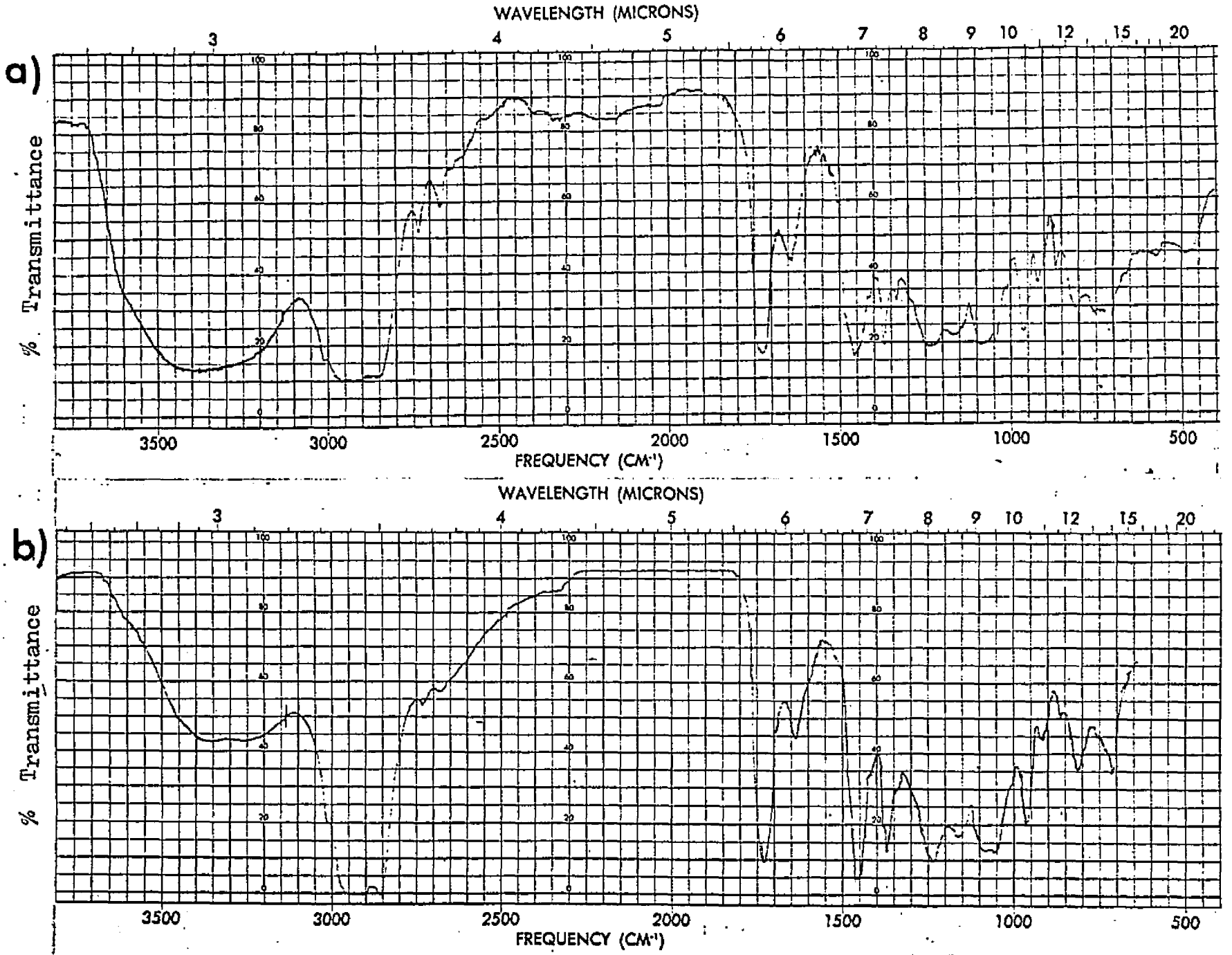
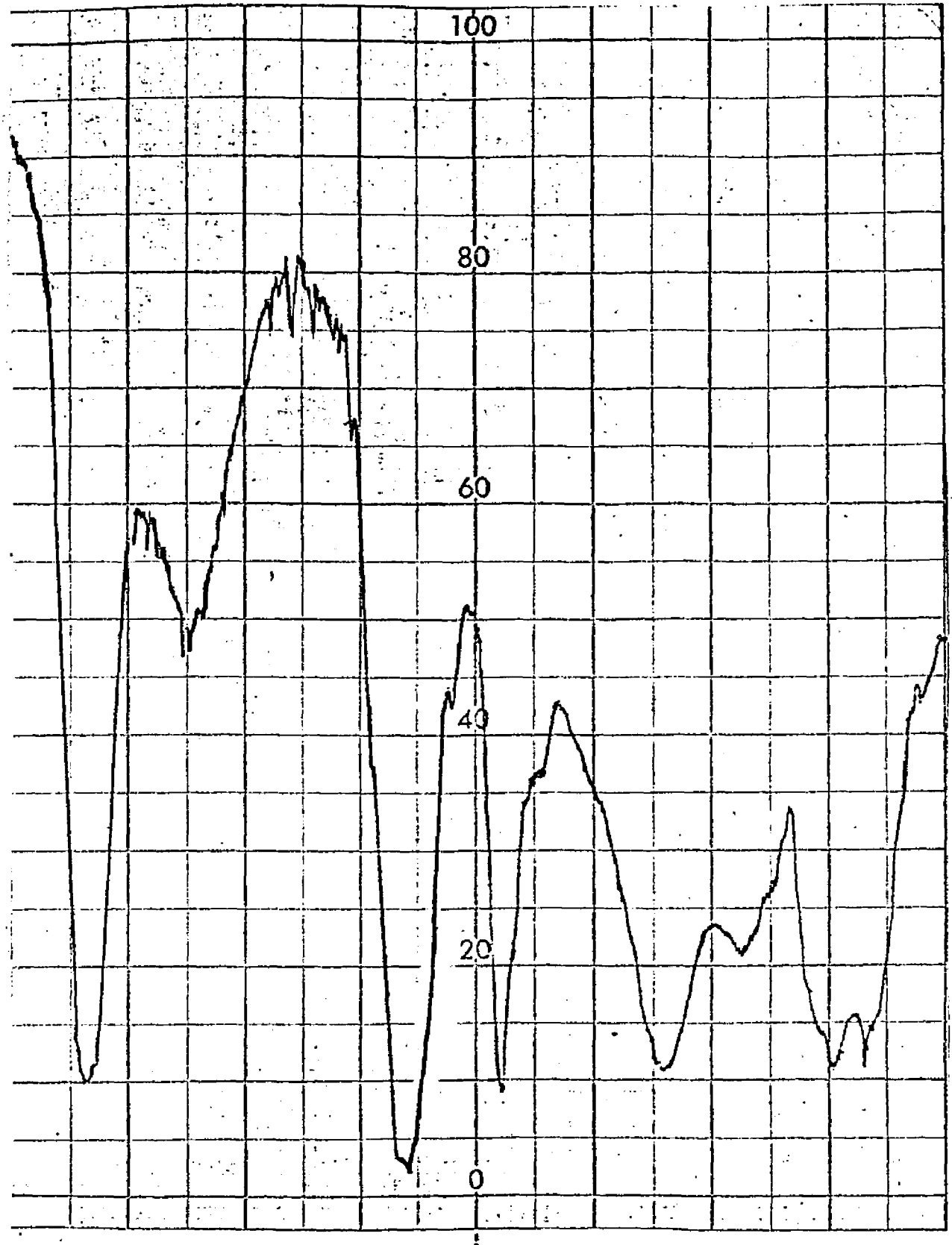

a)
WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
12
15
20
% Transmittance
100
80
60
40
20
0
3500
3000
2500
2000
1500
1000
500
FREQUENCY (CM-1)
b)
WAVELENGTH (MICRONS)
3
4
5
6
7
8
9
10
12
15
20
% Transmittance
100
80
60
40
20
0
3500
3000
2500
2000
1500
1000
500
FREQUENCY (CM-1)

Figure 19

The infrared spectra (1800 - 1100 cm^{-1}) of the 1/1 PC/PS vesicles after treatment with Ca^{+2} and Ca^{+2}/GlyGly.

A



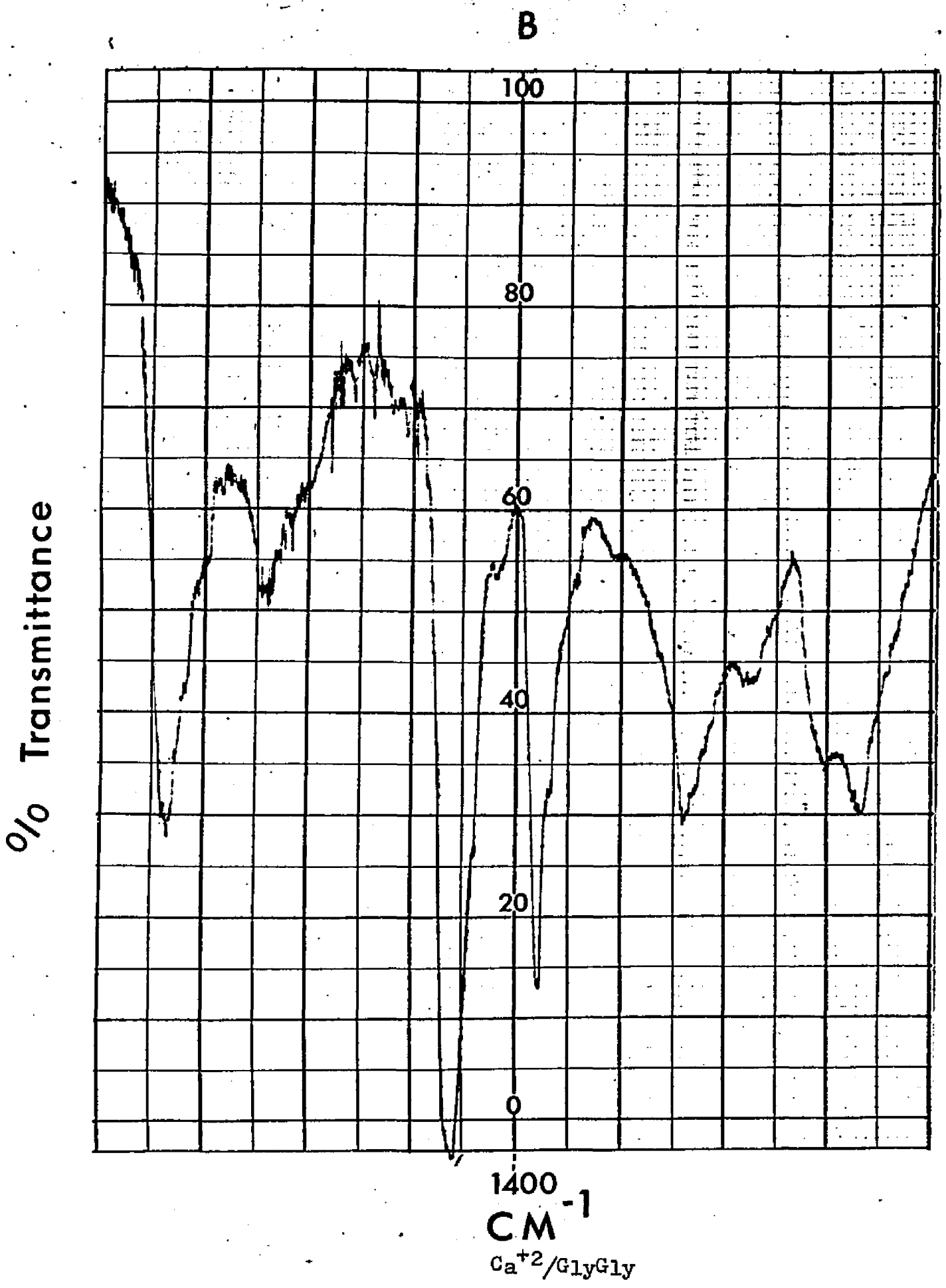
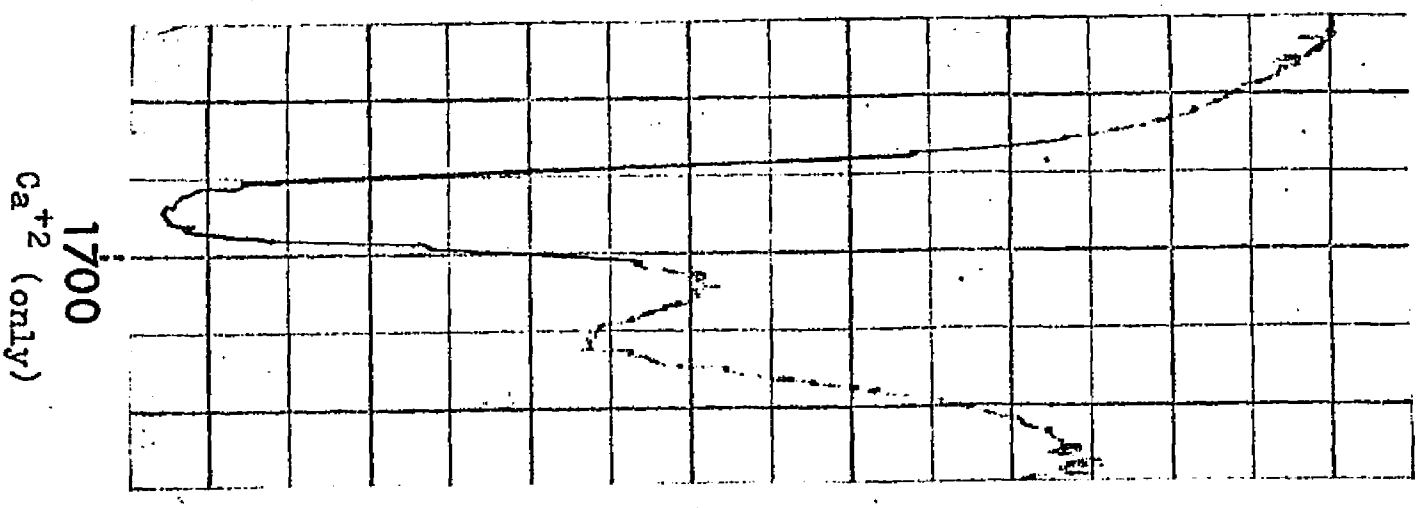

B
100
80
60
40
20
0
% Transmittance
1400
CM-1
Ca+2/GlyGly

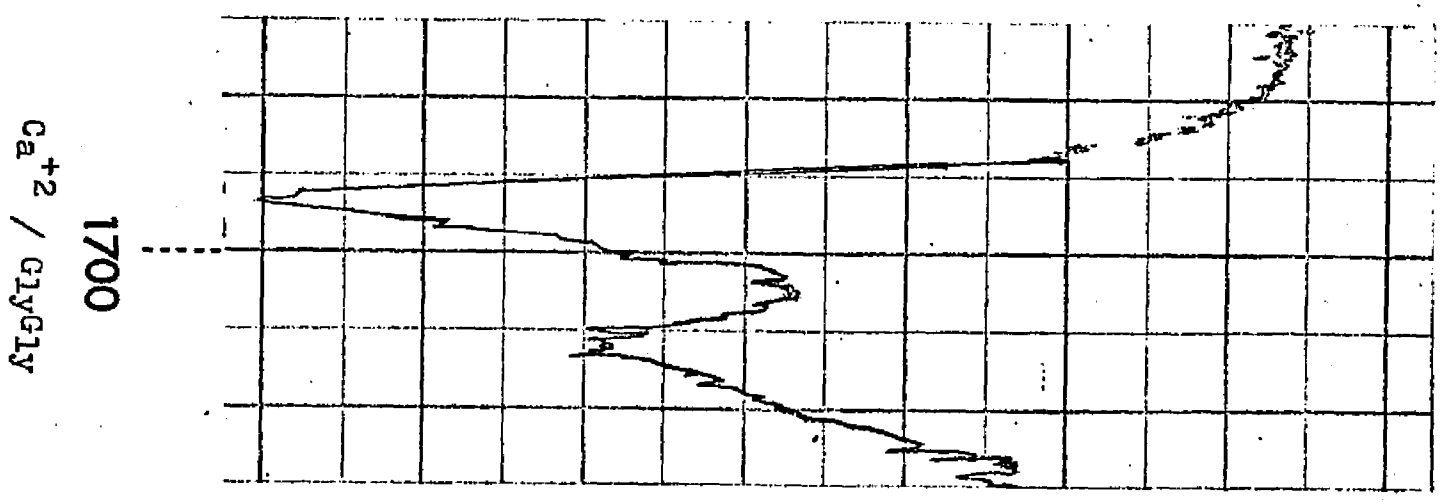
Figure 20

The infrared spectra (1800 - 1600 cm^{-1}) of the 1/9 mixed lipid PC/PS vesicles after treatment with Ca^{+2} and Ca^{+2}/GlyGly.

% Transmittance



% Transmittance



the sample size needed would have meant a dilute system or a system which would have precipitated the liposomes. Nevertheless, evidence was present for mixed ligand formation and this was the major emphasis of the preliminary mixed liposome experiments.

The next set of experiments involved the effect of both Ca^{+2} and Ca^{+2}/GlyGly on the properties of the liposome itself particularly to the release of solute from the liposome. The author chose glucose and inorganic phosphate to study the effect of efflux or permeability with complexation. Both phosphate[103] and glucose[103-4] have been studied on similar liposome systems but no data has been presented for the efflux of these materials from complexed vesicles. The following are the results of these experiments.

The appropriate liposome (9/1, 3/1, etc.) lipids were sonicated in approximately 5.0 ml of the appropriate 1.0 M solution of either glucose or potassium phosphate. After sonication, the mixture was ultrafiltered to 1 ml volume and washed with 10 ml glass distilled/deionized H_2O. This procedure was followed until the supernatant did not render a positive colorimetric test for either glucose or phosphate. The filtrates of both preparations were then analyzed to determine the approximate amount of glucose and phosphate which was encapsulated in the respective vesicles. The values are shown in Table XI. The mixture was then

Table XI

Amount of Solute Entrapped
in the Mixed Liposome
Permeability Experiments

Concentration	Native (Mg/dl)	Ca^{+2} (Mg/dl)	Ca^{+2}/GlyGly (Mg/dl)
Glucose			
9/1	950	949	956
3/1	906	901	903
1/1	868	860	861
1/3	811	809	814
1/9	741	730	736
Phosphate			
9/1	18.9	18.9	18.8
3/1	18.0	17.9	18.0
1/1	17.3	17.2	17.2
1/3	16.9	16.9	16.9
1/9	16.2	16.1	16.0

diluted to 20 ml by addition of 2.0 ml pH 7.0 buffer and 8.0 ml 0.1 M KCl solution. Three separate reactions were run at each of the five mixed lipid vesicle concentrations. One was allowed to remain as it was - native - the second treated with an amount of $CaCl_2$ which was equimolar to the PS present and the third was treated with GlyGly and $CaCl_2$ again equimolar with PS. (The mixed ligand complex will not form if pH is 7.6 or greater.) The mixtures were kept at a constant temperature of 26°C by a Haake water bath and the appropriate aliquots were taken from each solution every hour for analysis. The condensed results from the glucose release Experiments are shown on Table XII, while the results of the phosphate release Experiments are shown on Table XIII. The amounts reported for the native liposome release are consistent with data previously reported. The Ca^{++} complex and the mixed ligand complexed liposome showed a greater tendency to release both glucose and phosphate. Figure 21 shows a graphic representation of the 1/1 mixture glucose release and is representative of the entire sequence of experiments. Figure 22 demonstrates the effect of the mixed ligand moiety on the vesicle as compared to the other concentrations. Figures 23 and 24 do the same for the phosphate release experiments. If the two separate experiments are compared, one can see the trend of greater release of the two quite different solutes with mixed ligand formation and with decreasing PS

Table XII

Percent Glucose Release
from Mixed PC/PS Phospholipid Vesicles
as a Function of State and Time (pH 7.0)

9/1 Time	Native	Ca^{+2}	Ca/GlyGly
1	0	0	0
2	0	6.9	6.9
3	2.3	19.1	39.7
4	3.5	23.7	49.3
5	3.5	29.5	54.8
6	4.5	35.9	67.0
7	5.2	39.9	90.8
8	5.8	41.1	97.7
9	5.8	42.8	97.7
10	6.4	45.2	97.7
11	6.4	45.2	97.7
2 Days	6.9	45.2	97.7

3/1			
1	0	0	0
2	0	6.7	17.0
3	0	11.6	32.3
4	1.8	14.6	36.5
5	4.2	17.7	39.6
6	6.7	20.7	42.6
7	9.7	23.8	47.5
8	10.3	27.4	47.5
9	10.3	30.5	47.5
10	10.3	30.5	47.5
11	10.3	30.5	47.5
2 Days	10.3	30.5	47.5

1/1			
1	0	0	0
2	0	6.4	11.5
3	1.3	11.5	18.5
4	3.2	14.7	18.5
5	4.4	18.5	19.8
6	5.5	21.7	25.5
7	6.7	24.3	37.0
8	8.9	24.3	38.9
9	9.5	25.6	38.9
10	8.9	26.2	38.9
11	9.5	26.2	38.9
2 Days	10.1	27.3	38.9

Table XIIa

Percent Glucose Release
from Mixed PC/PS Phospholipid Vesicles
as a Function of State and Time (pH 7.0)

1/3

Time	Native	Ca^{+2}	Ca/GlyGly
1	0	0	0
2	0	3.4	6.8
3	0	6.8	14.2
4	0.7	9.5	22.3
5	1.4	12.2	29.1
6	2.7	13.6	41.2
7	3.4	17.0	47.9
8	4.7	19.7	47.9
9	6.8	23.1	47.9
10	7.5	25.1	47.9
11	7.5	26.5	47.9
2 Days	9.5	34.6	47.9

1/9

1	0	0	0
2	0	0	6.7
3	2.2	2.3	13.4
4	4.4	6.8	17.9
5	6.7	6.8	23.1
6	10.2	10.5	28.4
7	11.1	12.0	29.9
8	13.3	14.3	29.9
9	17.1	17.3	29.9
10	16.3	18.8	29.9
11	17.1	20.3	29.9
2 Days	17.8	28.6	29.9

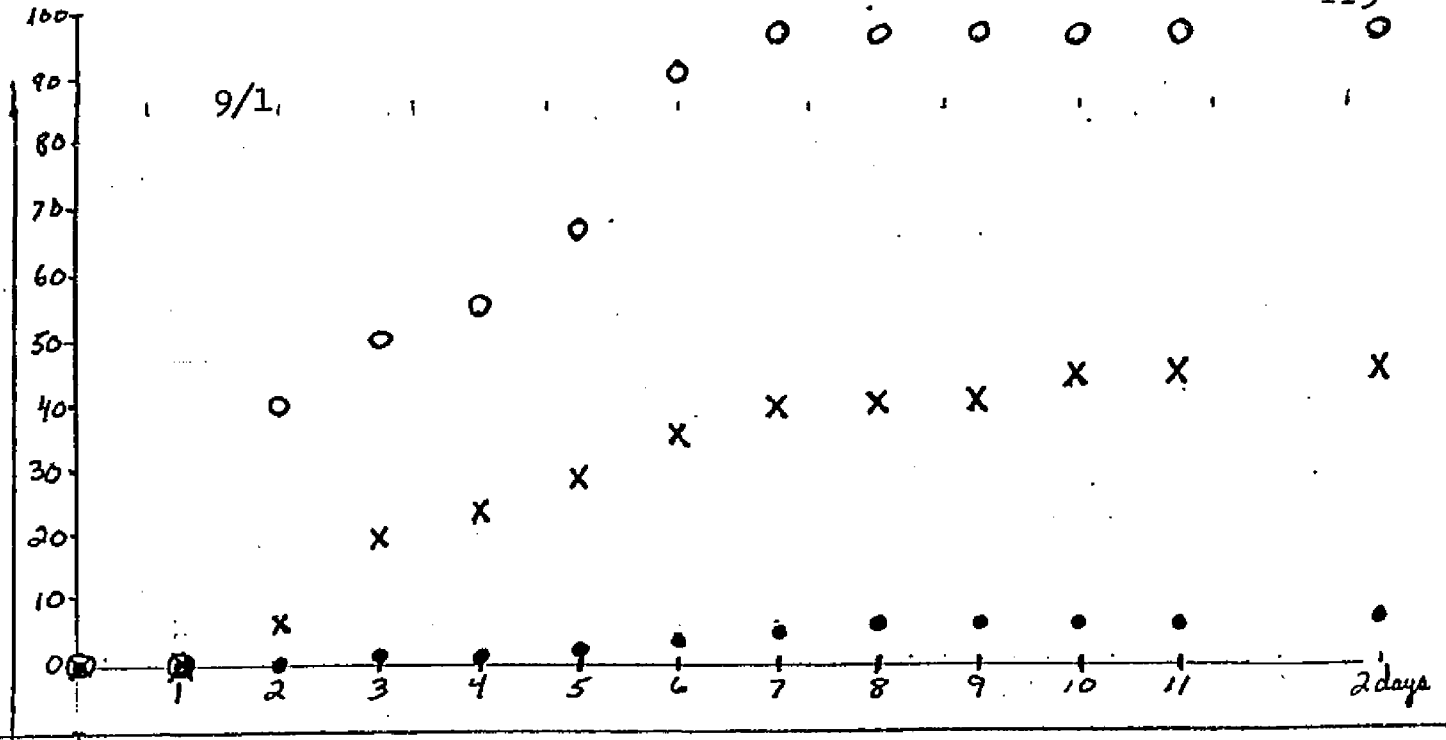
Figure 21

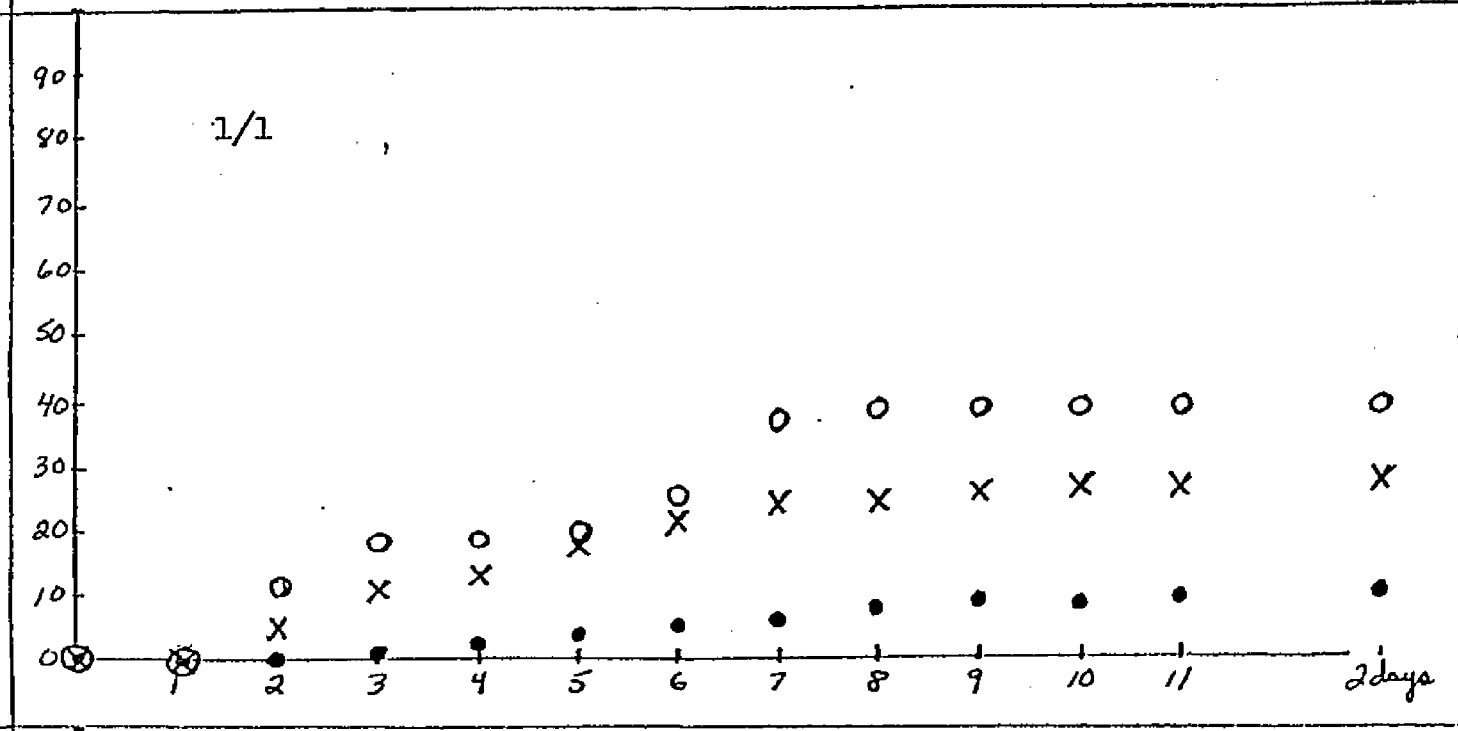
Graphic illustration of the glucose release experiments on the 9/1, 1/1 and 1/9 PC/PS vesicles as a function of time.

(The ordinate is % total glucose released and the abcissa is time measured in hours).

Legend:

- ● - Native Vesicle
- X - Ca^{+2} Complexed Vesicle
- o - Ca^{+2}/GlyGly Complexed Vesicle


9/1
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
2days


1/1
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
2days

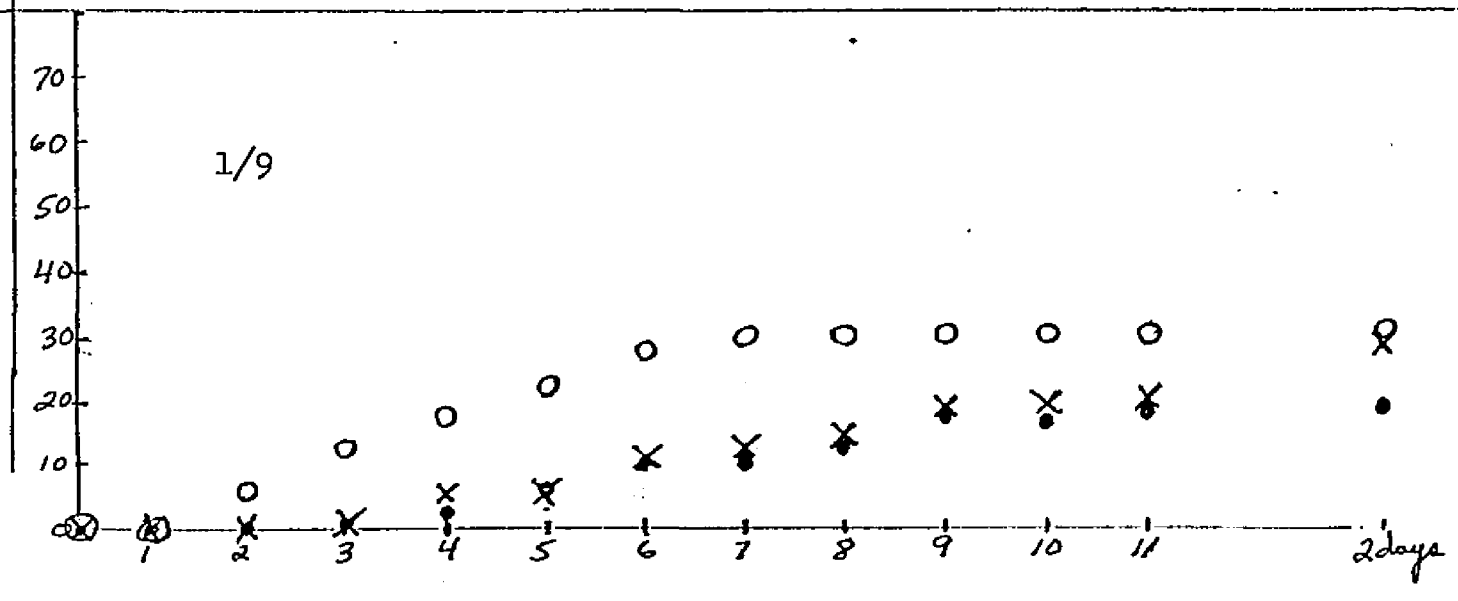

1/9
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
11
2days

Figure 22

The effect of mixed ligand complexation on glucose permeability in variable composition vesicles (PC/PS).

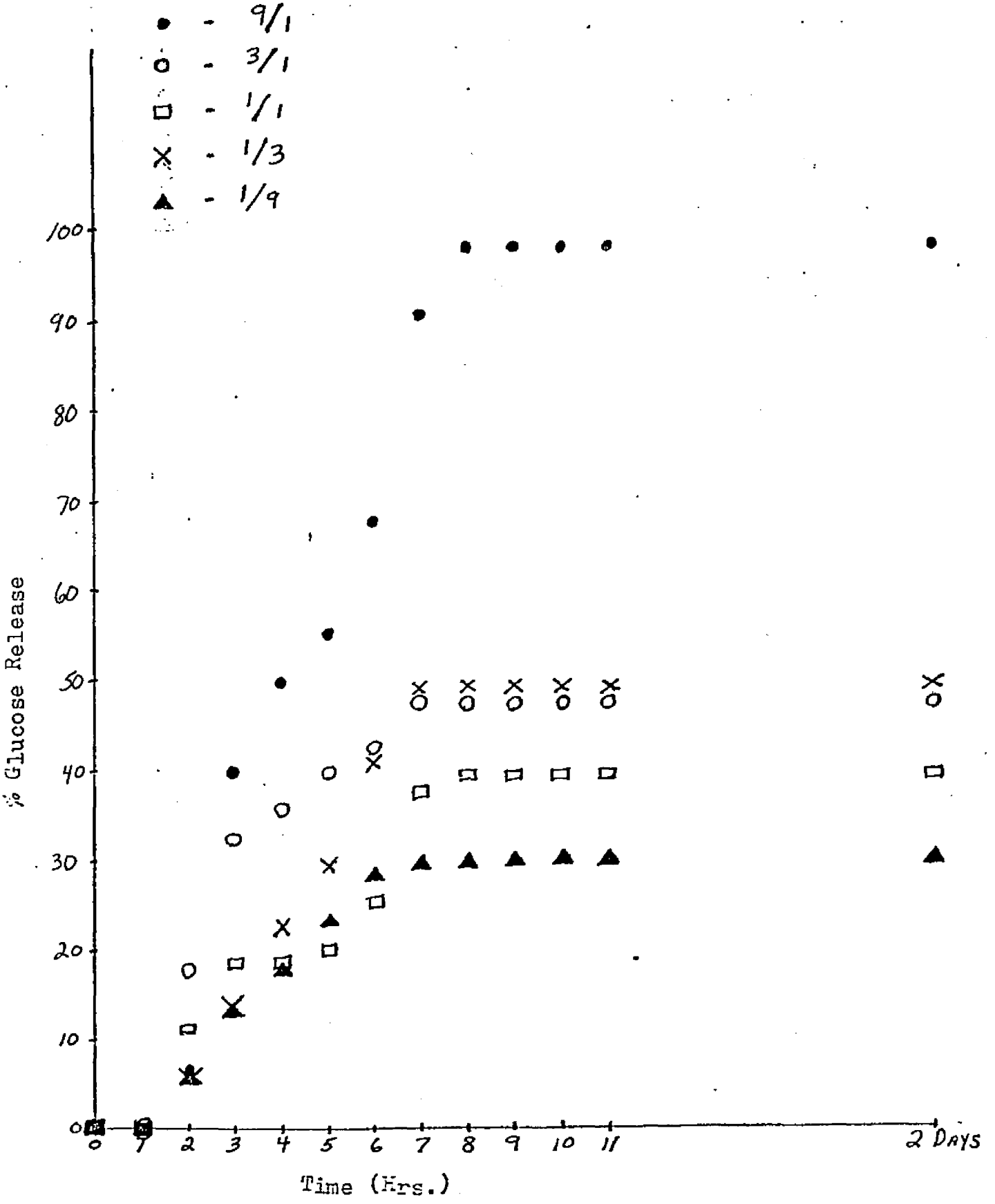

● - 9/1
o - 3/1
□ - 1/1
× - 1/3
▲ - 1/9
% Glucose Release
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
2 Days
Time (Hrs.)

Table XIII

Percent Inorganic Phosphate Release
from Mixed PC/PS Phospholipid Vesicles
as a Function of State and Time (pH 7.0)

9/1 Time	Native	Ca^{+2}	Ca/GlyGly	3/1 Native	Ca^{+2}	Ca/GlyGly
1	0	18.4	8.7	0	4.0	19.3
2	0	41.1	18.6	1.1	16.0	36.3
3	5.4	70.2	27.1	7.9	23.3	52.2
4	14.0	86.4	52.1	10.2	36.5	73.7
5	25.9	87.5	53.2	15.9	38.8	83.9
6	31.3	87.5	53.2	23.8	38.8	83.9
7	33.4	87.5	53.2	27.2	38.8	85.1
2 Days	48.6	89.6	87.9	38.6	85.5	85.1

1/1				1/3		
1	0	5.9	11.9	0	12.1	11.5
2	1.2	15.4	16.6	6.0	25.4	33.8
3	4.7	20.2	22.0	15.7	36.2	55.0
4	10.6	30.9	35.6	23.0	47.7	77.3
5	15.3	39.1	47.4	36.2	48.3	77.3
6	20.1	42.7	48.7	37.5	49.5	78.5
7	24.8	42.7	48.7	41.1	49.5	77.3
2 Days	54.3	60.5	59.3	70.1	77.3	78.5

1/9 Time	Native	Ca^{+2}	Ca/GlyGly
1	0	8.9	12.8
2	3.8	17.8	28.1
3	11.4	48.2	48.5
4	18.9	58.3	71.4
5	27.7	73.5	82.9
6	37.8	72.2	82.9
7	44.1	73.5	82.9
2 Days	76.9	86.2	86.8

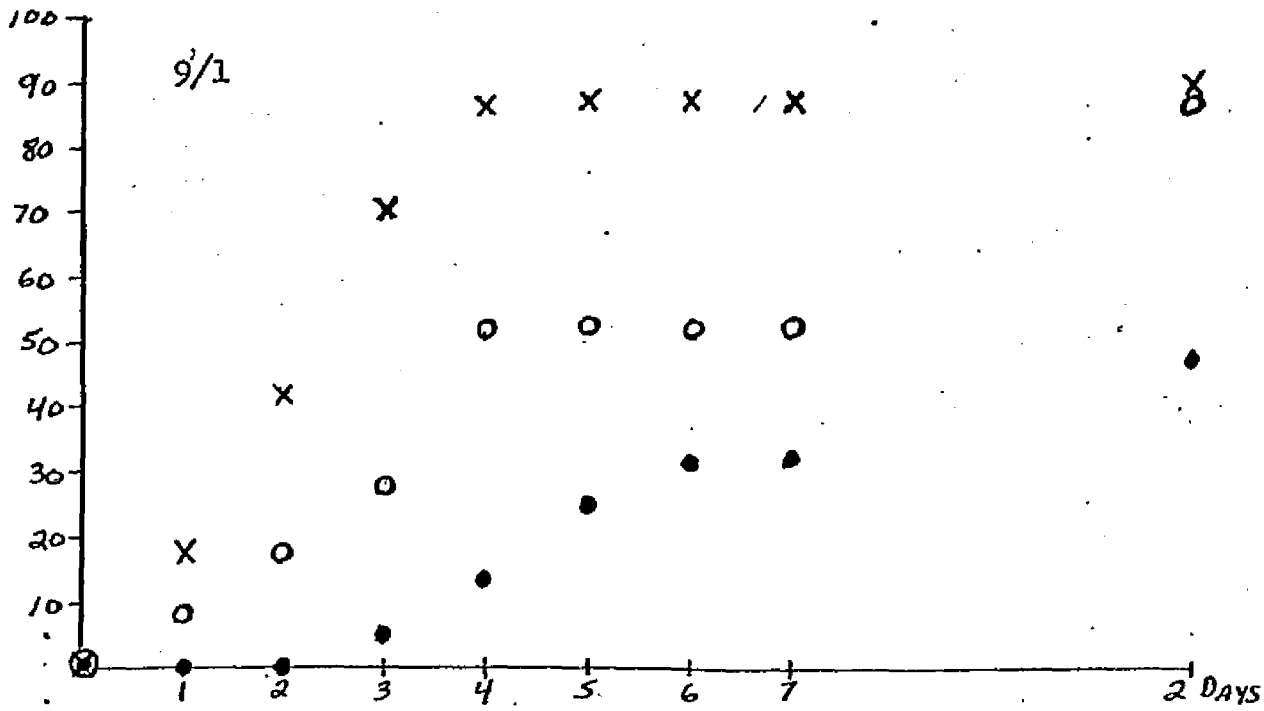
Figure 23

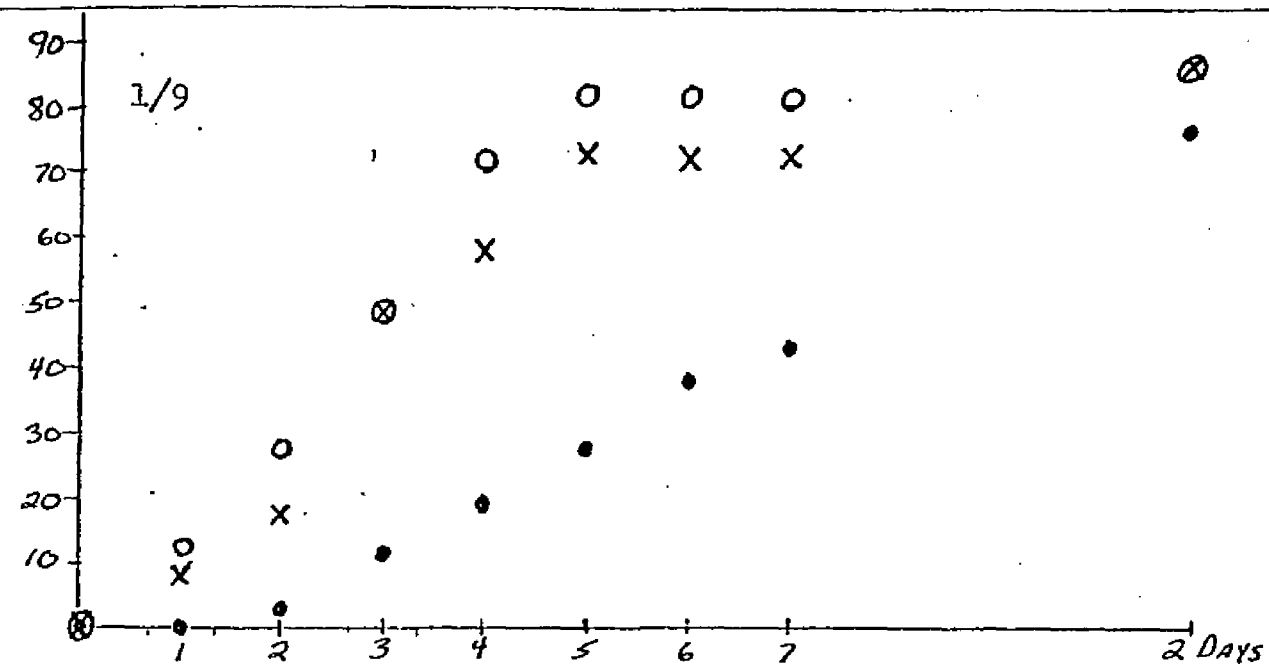
Graphic illustration of the phosphate release experiments on the 9/1, 1/9, 1/1, PC/PS vesicles as a function of time.

(The ordinate is % total phosphate released and the abcissa is time measured in hours).

Legend:

- ● - Native vesicle
- x - Ca^{+2} complexed vesicle
- o - Ca^{+2}/GlyGly complexed vesicle


9/1
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
2 DAYS


1/9
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
2 DAYS

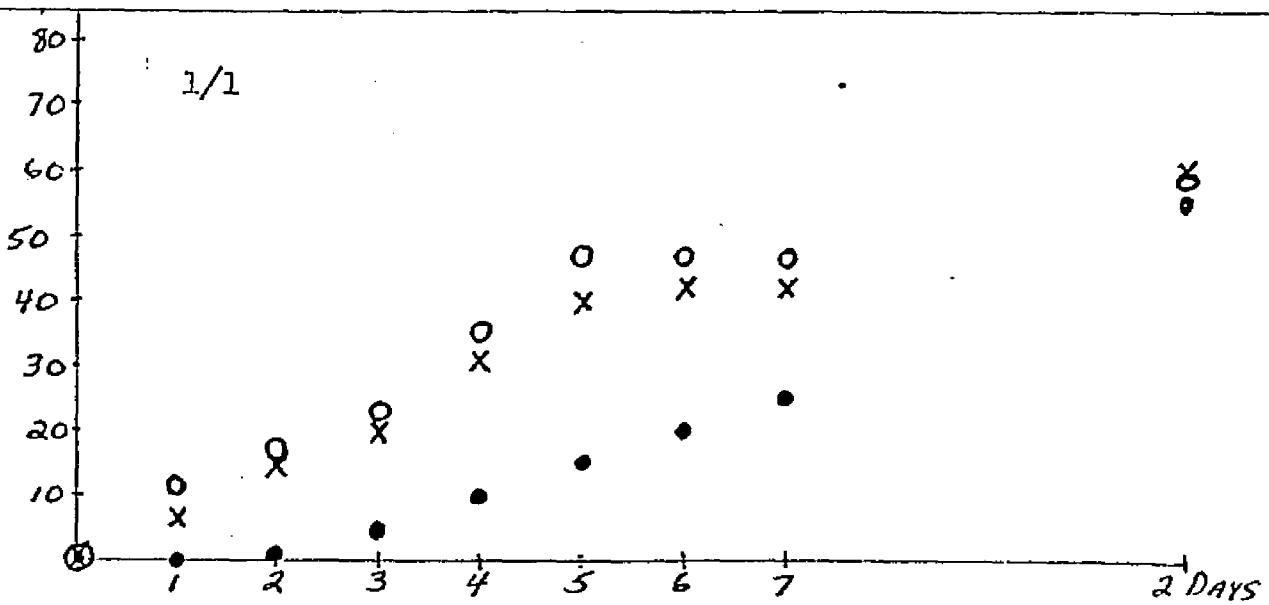
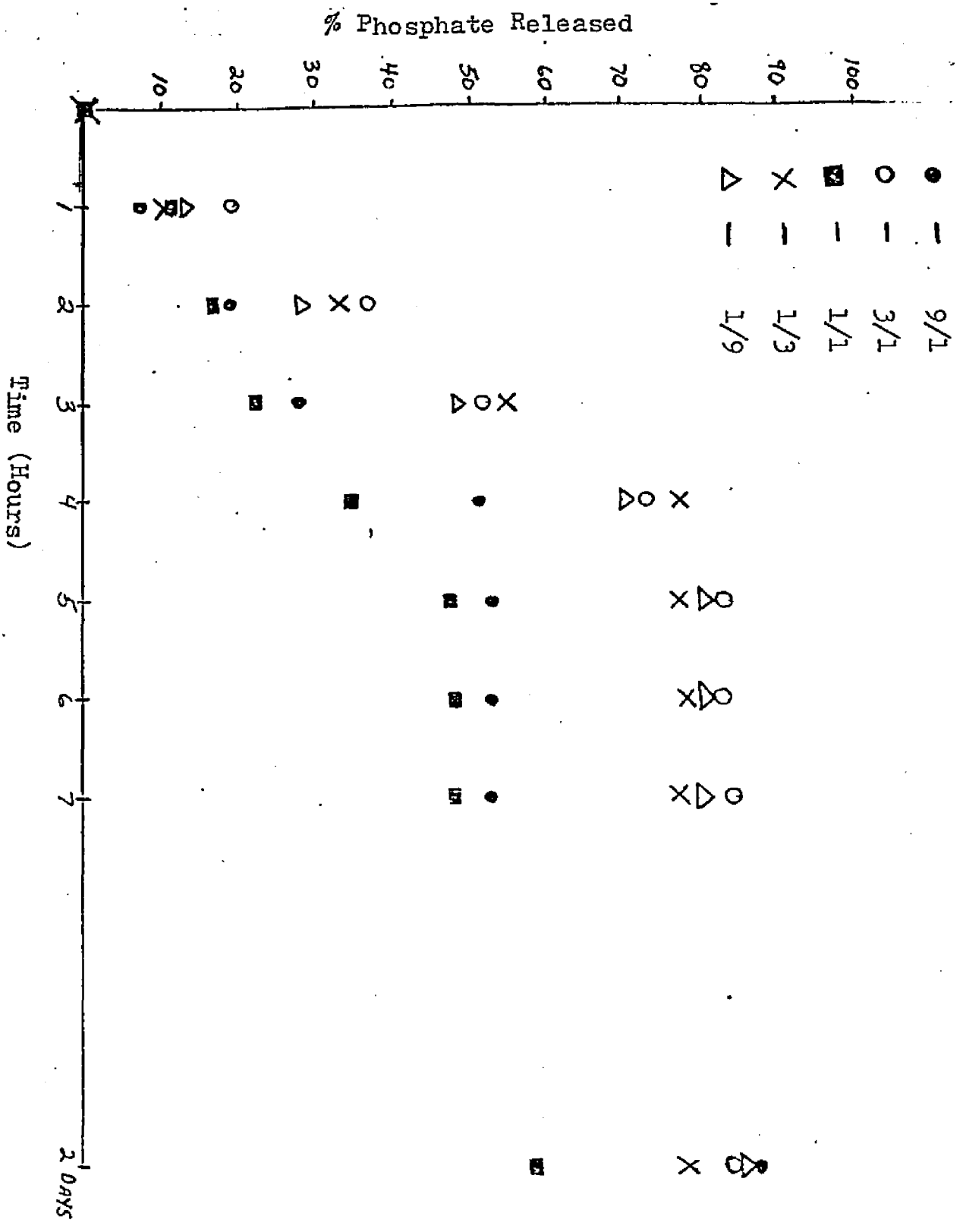

1/1
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
2 DAYS

Figure 24

The effect of mixed ligand complexation on the phosphate permeability of variable composition liposomes as a function of time.


% Phosphate Released
10
20
30
40
50
60
70
80
90
100
Time (Hours)
1
2
3
4
5
6
7
2 Days
9/1
3/1
1/1
1/3
1/9

concentration in the liposome. The results shown are reproducible if the preparatory procedures are followed exactly. Discussion of this work will be in the next section, Discussion III.

DISCUSSION

I. The Role of Ca^{+2} in Mixed Ligand Formation and Stability of Biological Systems

Ca^{+2} was found as early as 1894 to be necessary for the transmission of excitation from nerve to muscle[105] and for transmission of impulses across the nerve synapse.[106] From these early experiments to present, intra and extracellular Ca^{+2} has been implicated in numerous physiological functions such as lipoprotein formation,[44,46,107] cation transmembrane transport, oxidative metabolism and respiration in the mitochondria[108-9] and neural processes.[113] Each of these diverse systems involved acidic phospholipids such as PS, to act as an ion exchange medium or chelation site for the cellular surface. Thus Ca^{+2} is believed to play a leading role in the multiple physiological functions conducted by the cell and is virtually necessary for the very existance of each cell. On the other hand, the acidic phospholipids, e.g., PS, which has been discussed in the Introduction, comprise only from 10 - 20% of the total phospholipid composition of every cell membrane. However, it is this low percentage phospholipid content which seems to interconnect both the physiological functions of the membrane with the indispensible role of Ca^{+2}.

The cation binding tendencies of the acidic lipid, phosphatidyl-L-serine has been the topic of numerous reports [32,45,48,61,62,64,65] and the major facts are the following:

1. The PS carboxyl group is always accessible to Ca^{+2} or other metal binding. The phosphate group position is dependent on the liposome structure. [120]

2. Ca^{+2} absorption is primarily on the carboxyl group.

3. The amount of Ca^{+2} bound is not affected by a pH change in the range of pH 5-8 (see Figure 25).

4. The isoelectric point of PS is pH 1.2 at which point the phosphate is ionized and the free amine of the seryl group is protonated. The carboxyl pKa_1 is 4.0 and the pKa_2, the deprotonization of the seryl amine is 7.5.

5. PS shows the following metal affinity: Ca > Ba > Mg. [63]

6. Ca^{+2} may be displaced from a PS monolayer or bilayer by K^+ or Na^+.

7. Ca^{+2} binding constant to the carboxyl - 2.9×10^6 [64] (ranges from 1.17×10^4 [55] -- 1.4×10^7, [114] 10^7 [115]).

8. PS - Ca^{+2} complex from pH 5-8 is in a 1:1 ratio (dilute suspensions). [66]

To this information the following can be added from this work: 1) Addition of an amino acid, di/tripeptide or a protein to the PS/Ca^{+2} complex (either 1:1 or 2:1) will not form a mixed ligand complex. 2) Addition of an amino acid, etc. to the PS/Ca^{+2} complex in a mixed lipid (PC/PS) vesicle in a dilute solution will lead to the formation of a mixed ligand complex. 3) Addition of a Ca^{+2}/amino acid, etc. complex[116] to PS will produce a mixed ligand complex with the stabilities given in the Experiments and Results section. 4) Mixed ligand formation will occur whether one utilizes sonicated pure PS vesicles, mixed lipid PS liposomes, or multilamellar vesicles. 5) In addition to Ca^{+2}, only Mg^{+2} and to a slight extent Mn^{+2}, Sr^{+2}, Al^{+3} and Fe^{+3} will form a mixed ligand complex. 6) There is both a pH and time dependence for Mg^{+2} binding (as the tricomplex) in a competitive reaction. 7) Evidence favors chelation through the ionized carboxyl group of the respective species exclusively. 8) The more geometrically simple amino acids; alanine, glycine, sarcosine, valine, etc. show a greater tendency towards mixed ligand formation than the more complex amino acids. This trend is also seen in di and tripeptides, note that GlyGly is the best complexing ligand. 9) Protein will form the mixed ligand complexes only through their available ionized carboxyl groups. 10) Mg^{+2}, K^{+}, Na^{+} and the entire series of cations

used in the experiments of this thesis will not displace Ca^{+2} from a mixed ligand complex.

With this collection of facts one must proceed to a logical explanation of mixed ligand formation coupled with the pH dependence, the time dependence, the metal and ligand specificity. If one approaches this problem from a purely analytical chemistry viewpoint and examines the stability constants of various Ca^{+2} and Mg^{+2} complexes (Table 14), one notes the lack of a general trend favoring either Ca^{+2} carboxylate binding over Mg^{+2} carboxylate bonding or the opposite case of Mg^{+2} being favored over Ca^{+2}. However, it does appear that the Mg^{+2} and Ca^{+2} complex stabilities depend on the structure and the adjacent environment of the specific complex. This phenomenon, as yet unexplained, is true and is exemplified by the metal EDTA complexation reaction. In addition to the above stabilities, it has been reported that the PS-Mg^{+2} complex (log K = 4.34) is more stable than the PS-Ca^{+2} complex (log K = 4.03). However, this work has found that under the aqueous conditions studied, Ca^{+2} not only has a greater affinity for PS but also forms a more stable complex whether it be the mixed ligand or the dilipid species. Therefore, the greater carboxyl affinity for the Ca^{+2} species must play an important role in mixed ligand formation but does not explain why the various other

Table XIV

Various Carboxylate Stability Constants for Ca^{+2} and Mg^{+2}

Ligand	M^{+2}	Temp	Medium	Log of Equil. Const. $K(10^x)$	Method Analyzed
Acetic Acid	Ca^{+2}	25	ionic st	K_1 = 3.0	Glass electrode
	Mg^{+2}	18	$\longrightarrow$ 0	K_1^1 = 3.43	Conductivity
Malonic Acid	Ca^{+2}	25	.2 KCl	K_1 = 1.46	H^+ electrode
	Mg^{+2}	25	.2 KCl	K_1^1 = 1.91	H^+ electrode
Tartaric Acid	Ca^{+2}	25	.2 KCl	K_1 = 1.80	H^+ electrode
	Mg^{+2}	25	.2 KCl	K_1^1 = 1.36	H^+ electrode
Citric Acid	Ca^{+2}	25	$\longrightarrow$ 0	$K(Ca^{+2}+H_2l-2$ $CaH_2l)$= 3.29	Glass electrode
	Mg^{+2}	25	.09 KCl	K_1 = 1.60	Polarograph
Glycine (Acid)	Ca^{+2}	25	$\longrightarrow$ 0	K_1= 1.35 1.43	Solubility
	Mg^{+2}	25	$\longrightarrow$ 0	K_1^1 = 3.44	Glass electrode
Alanine (Acid)	Ca	25	$\longrightarrow$ 0	K_1 = 1.24	Glass electrode
	Mg	25	$\longrightarrow$ 0	K_1^1 = 1.96	Glass electrode
Aspartic Acid	Ca	25	.1 KCl	K_1 = 1.60	Glass electrode
	Mg	25	.1 KCl	K_1^1 = 2.43	Glass electrode
Glutamic Acid	Ca	25	.1 KCl	K_1 = 1.43	Glass electrode
	Mg	25	.1 KCl	K_1^1 = 1.9	Glass electrode
O,Phospho-Serine	Ca	25	.15 KCl	K_1 = 2.3	Glass electrode
	Mg	25	.15 KCl	K_1^1 = 2.4	Glass electrode
Glycyl-Glycine	Ca	25	$\longrightarrow$ 0	K_1 = 1.24	Solubility
	Mg	25	$\longrightarrow$ 0	K_1^1 = 1.06	Glass electrode
EDTA	Ca	25	.1($NaClO_4$)	K_1 = 10.7	Hg Amalgam elec.
	Mg	25	.1($NaClO_4$)	K_1^1 = 8.9	Hg Amalgam elec.
ATP	Ca	20	.1 KCl	K_1 = 3.60	Glass electrode
	Mg	20	.1 KCl	K_1^1 = 4.00	Glass electrode

Note: 1) $\longrightarrow$ 0 = extrapolated to zero

metals used in the subsequent investigations produced only the dilipid species even when reacted in a directly competitive manner (see Table V).

One may also look at the physical properties of various ions in an attempt to correlate why the metals act as they do. Table XV shows such an abbreviated listing of various ions. One can only speculate that the Ca^{+2} ion is of the right geometrical size for such complexations while the other metals are too large, have greater coordination numbers or possess greater nitrogen affinities than Ca^{+2}. These are all good tentative explanations for the metal role and the specificity in mixed ligand formation, for none is definitively known at this time.

The ligand specificity also poses such a problem. If one returns to Tables I and II, one will find a wide disparaging range of mixed ligand formation percentages. Of particular note, is that of aspartic and glutamic acids. At pH 7.0, 40% of the complexation reaction of aspartic acid PS and Ca was that of the mixed ligand species as compared to 1% for the glutamic acid reaction. At pH 7.3, the ratios were 51% to 10%. Structurally the only difference between the two acids is an added methylene group in the glutamic acid.

Aspartic Acid ($HOOC-CH_2-CHNH_2COOH$; Glutamic Acid $HOOC-CH_2-CH_2-CHNH_2COOH$)

Table XV

Some Physical Properties of
Hydrated and Unhydrated Ions (155)

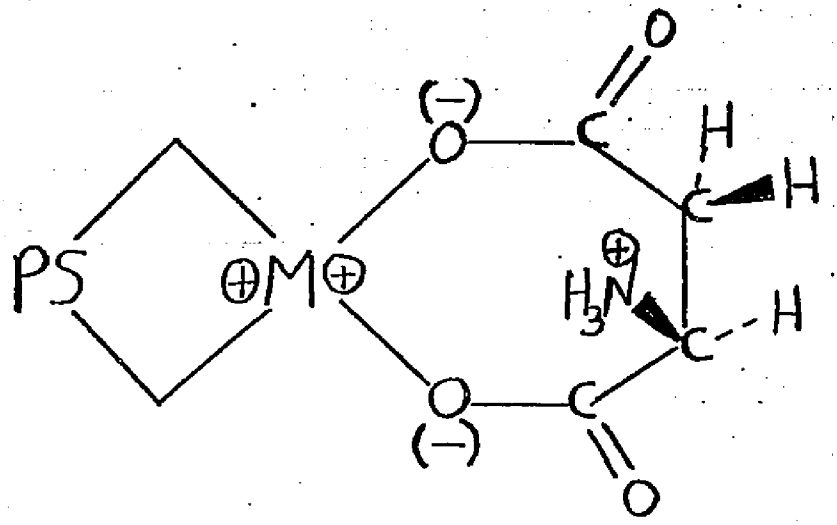
Ion	Crystal Ionic Radius A^o	Polarizability $\alpha_o \times 10^{-24} cm^3$	Hydration Energy K cal/g ion	Effective Radius A^o	Coordination Number
K^+	1.33	0.87	92	2.2	8
Na^+	0.95	0.21	116	3.4	6
Ca^{++}	0.99	0.531	410	4.5	6,8
Mg^{++}	0.65	0.012	495	5.9	4,6
Ba^{++}	1.35	1.69	346	3.7	6
Sr^{++}	1.13	1.42	370	3.7	8
Zn^{++}	0.74	0.114	528	4.5	4,6
Cu^{++}	0.96	0.67	536	4.5	4,6

This fact in itself may add an insight to mixed ligand formation, that of molecular geometry. Figure 26 shows the possible bonding scheme of both aspartic and glutamic acid with PS and Ca^{+2}. It is worth noting that the aspartic acid can form a more stable ring size with less electrostatic interactions than glutamic acid. (Both aspartic and glutamic acids are only bound to one PS even though the possibility exists for dual binding. The chelation point is assumed to be the amino carboxyl group). Therefore, the stability of the ring(s) or more accurately, the molecular geometry, of the mixed ligand complex may enhance the overall stability of these complexes and by so doing, render a plausible explanation as to the amino acid, di/tripeptide selectivity shown by the various ligands used in this work. But the stability of the mixed ligand complex would also depend on the length, steric bulk and functional groups of the side chains of the respective amino acids or peptides which is being complexed and not only on the ring size. If this factor does have a major role, both steric and electrostatic repulsions may hinder formation of such a mixed ligand complex and by so doing engender a natural selection mode for specific chelation of various metabolities or protein species.

In addition to these steric interactions one must also consider the net ionic charges of both reacting species. As noted above, mixed ligand formation may be

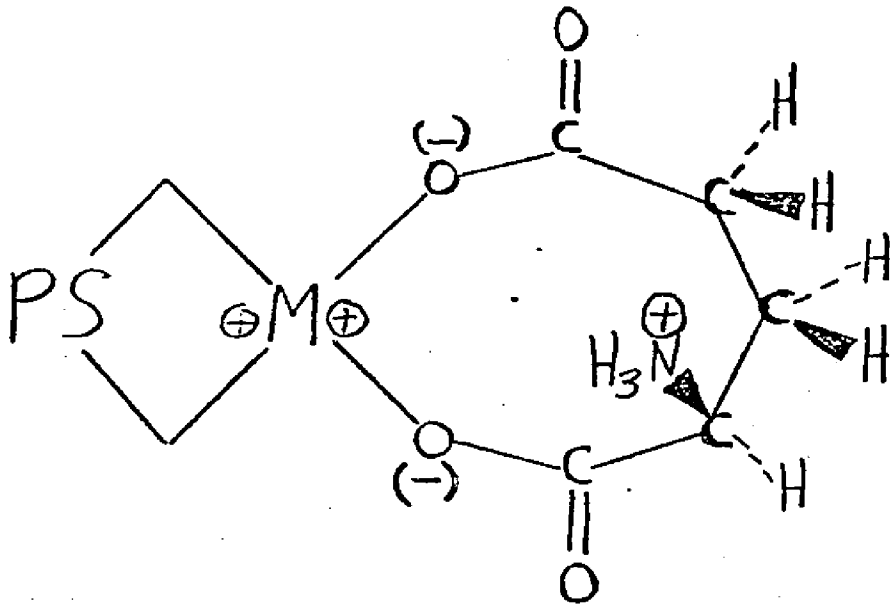
Figure 25

A graphic depiction of the possible structure of the aspartic and glutamic acid mixed ligand complex.



Aspartic Acid Complex
Ring Size-7

Normal Ring Stabilities: 6 > 7 > 8,5 > 9 > 4 > 3



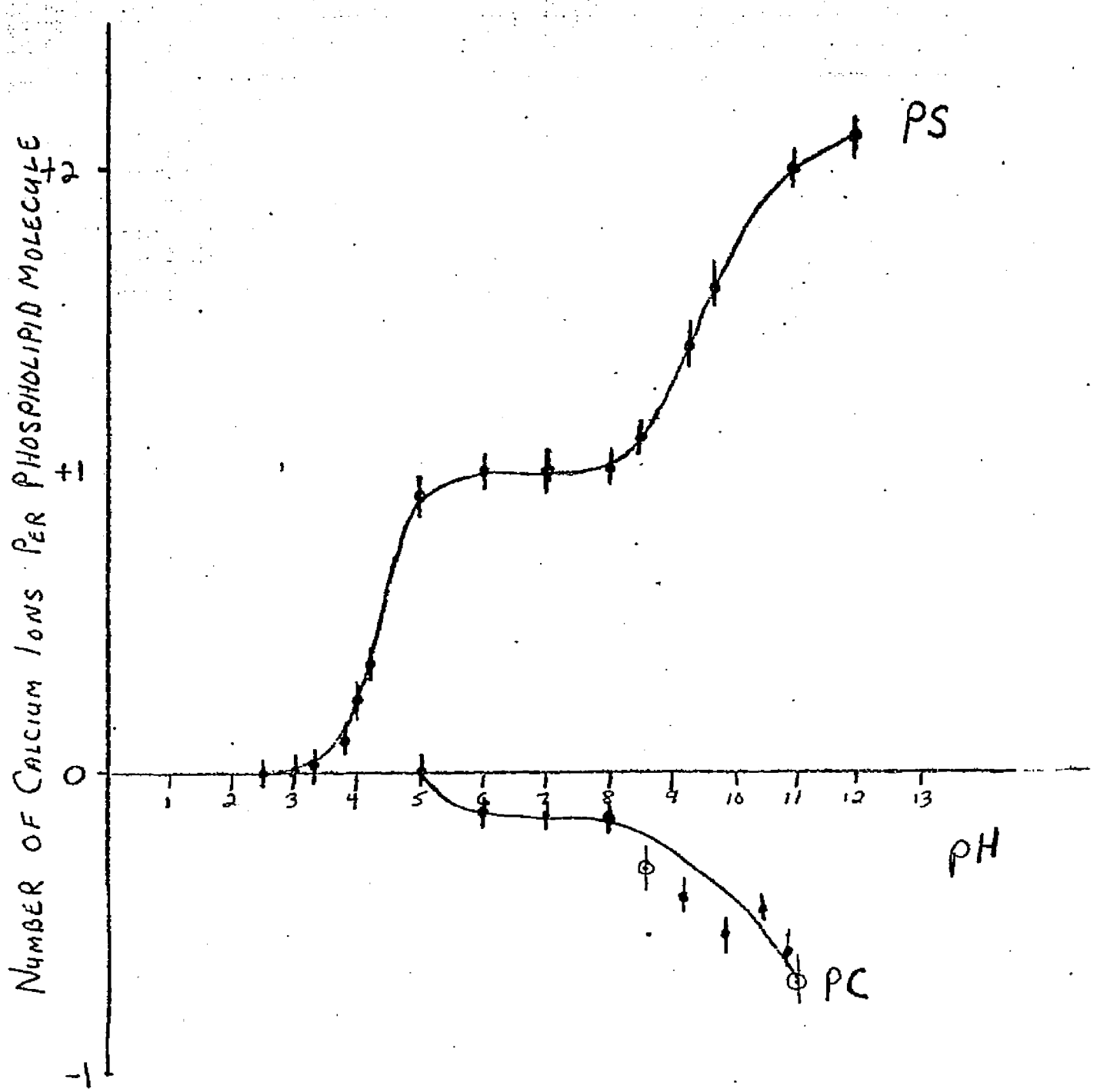
Glutamic Acid Complex
Ring Size-8

hindered by an ionized functional group which is actually positionally displaced from the chelation site but which does interfere in the coordination sphere. This interaction seems to be evident from the amino acid data displayed. However, the charge of the lipid is also important. It has been noted by many workers that PC (lecithin) having a net zero charge in the pH range studied, will not bind divalent metals to a great degree (Figure 26). PS, on the other hand, does have a net negative charge and binds very strongly. Therefore, it is the presence of this net charge or more specifically from the ionized carboxyl which facilitates mixed ligand formation.

The pH dependence of mixed ligand formation can be interpreted by coupling this phenomenon to two major factors. The first is the effect on the metals themselves. As the pH is increased, the sphere of hydration surrounding the ion is stripped off thus presumably activating the specific metal species toward a specific ligand. This phenomena is a well-known inorganic property, and can readily be observed through the binding tendencies of various metals to carboxylates such as acetates, tartarates, oxalates and EDTA at various pH values. The second major factor is in itself two-fold and will involve a time dependence as well. This is the effect of the pH on the lipid and peptide species. Generally, in

Figure 26

Ca^{+2} ion absorption per phospholipid molecule as a function of pH. [51]


NUMBER OF CALCIUM IONS PER PHOSPHOLIPID MOLECULE
+2
+1
0
-1
1
2
3
4
5
6
7
8
9
10
11
12
13
pH
PS
PC

the pH range used in these experiments both the phospholipid and the peptide species were fully ionized. However, as has been shown in Figure 26, there is pH effect on Ca^{+2} binding to PS as well as pH effects on the surface potential and the surface pressure of the PS vesicles, as demonstrated by Figure 27. Nevertheless, it is not only the above general pH effects on the lipids and peptide ligands themselves, but also the sensitive pH balance of the multiple equilibria system which these mixed ligand formation reactions entail. Graphically, Figure 28 displays the equilibria among the ionic groups of PS with Ca^{+2} and H^{+}. As one can see, the pH of the system becomes a deciding factor in this representation, however, this figure does not include the effect of Ca^{+2} and H^{+} on the amino acid, peptide or protein species and does not include the subsequent interaction which leads to mixed ligand formation. Totaled these comprise a formidable multiple equilibria system, which as yet has to be deciphered.

We must extend the pH considerations to include the possible denaturation of proteins or simply an unraveling effect experienced by most proteins when placed in various pH environments. This phenomena may well be important in the overall mixed ligand complexation reaction, since experiments carried out by this author show that both ovalbumin and bovine albumin will not form

Figure 27

Effect of pH on the physiochemical properties of phospholipids. Surface potential (ψ), surface pressure (π), Ca^{+2} binding ratio (Ca/P).[65]

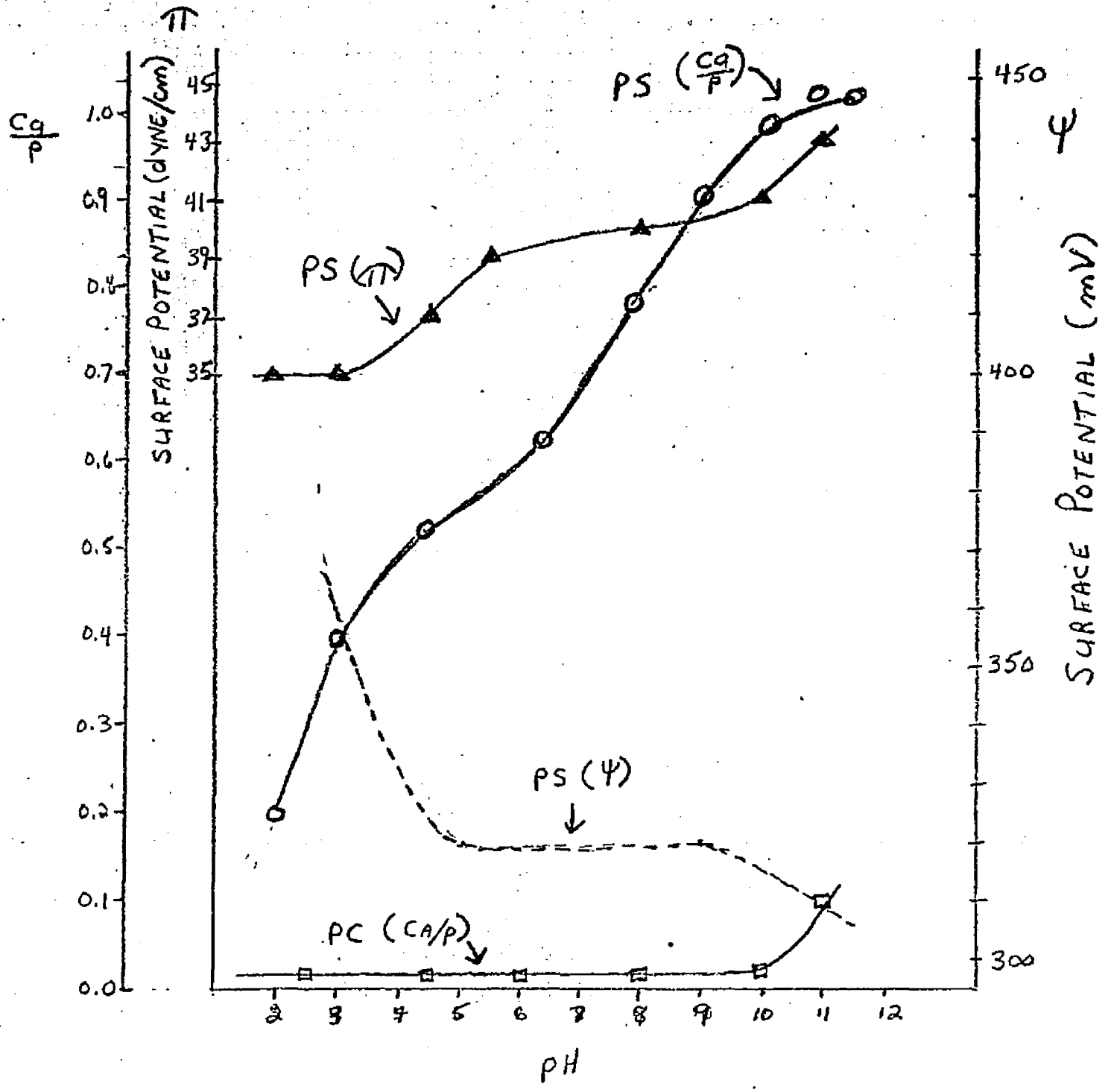
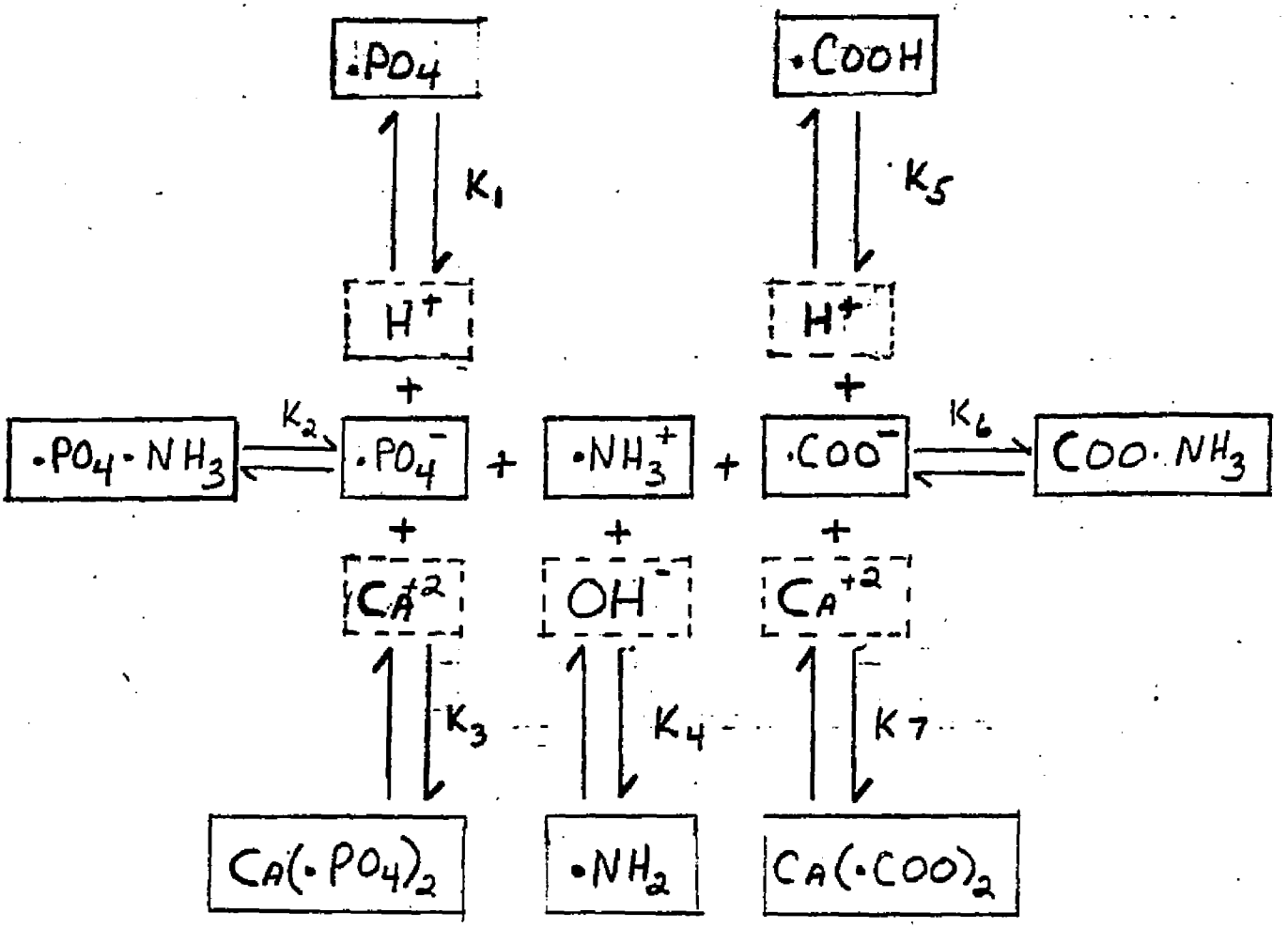

$\frac{C_A}{P}$
SURFACE POTENTIAL (dyne/cm)
Π
SURFACE POTENTIAL (mV)
Ψ
PS ($\frac{C_A}{P}$)
PS (Π)
PS (Ψ)
PC (C_A/P)
pH
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
45
43
41
39
32
35
450
400
350
300
2
3
4
5
6
7
8
9
10
11
12

Figure 28

Equilibria among the ionizable groups of acidic phospholipids, Ca^{+2} and H^{+}.[65]


·PO4
·COOH
K1
K5
H+
H+
·PO4·NH3
K2
·PO4−
·NH3+
·COO−
K6
COO·NH3
Ca+2
OH−
Ca+2
K3
K4
K7
Ca(·PO4)2
·NH2
Ca(·COO)2

a mixed ligand complex at pH values lower than 6.5. Phosvitin also shows the identical effect but will form a mixed ligand complex at pH 6.0, while both hemin and insulin - chain-a did not complex at all at any pH (5-9).

It is a general biological trend in proteins that as the pH is raised, the proteins begin to swell or unravel. This unravelling may or may not expose ionized functional groups to its aqueous environment. This denaturation process is experienced by the proteins in these experiments but show varying effects. The data reveals that the proteins,all possessing at least one ionized carboxyl side chain, will not form a mixed ligand complex below a certain pH level, e.g., 6.0. This may be due to either the inaccessibility of the group within the protein structure or the lack of a formal charge on that group. However, as the pH is raised, three of the five proteins bind PS in a mixed ligand complex indicating that for phosvitin, egg and bovine albumin the carboxyl sites are now exposed and ionized. The lack of complexation in the hemin and insulin - chain-a cases seems to indicate the inaccessibility of the carboxylate groups. This topic will again be examined in part II of this discussion.

Another consideration of pH dependence is the effect it shows on the mixed ligand complex itself. As noted in the experiments and results, the Ca^{+2} complex

showed acidic stability, while dissociating in an alkaline medium (pH 7.6). The Mg^{+2} mixed ligand complex was base stable (in fact, it only fully complexed at approximately pH 7.8), and dissociated in acidic medium (pH 6.5). Both complexes could be re-established by careful adjusting of the respective pH's, but only the Ca^{+2} mixed ligand complex will reform completely. The Mg^{+2} complex shows an increase in the dilipid species as previously noted. This tendency may be due to a kinetic vs. thermodynamically controlled situation connected with the multiple equilibria system. Yet it is surprising and more or less unique that only the Ca^{+2} mixed ligand system does not see such a kinetic effect except in the direct Mg^{++}/Ca competition reaction. It is even more unique that only the Ca^{+2} mixed ligand complex will form a "membrane" structure on standing in its supernatant.*

So why does a mixed ligand complex form? Is it only a pH dependent phenomena? If so, why do Ba^{+2}, Pb^{+2} and Ni^{+2} not form such a mixed ligand complex in the pH range examined. Could it be that Ba^{+2} has a greater affinity for PS as opposed to the peptide ligand? Why then does Ca^{+2} have a greater affinity for PS than Ba^{+2} and yet form a mixed ligand complex? Why?

* This effect was not seen with Egg or Bovine Albumin or with a mixture of dilipid/tricomplex material. If the dilipid species is removed, the membrane will form.

As one can see, there is no simple explanation for mixed ligand complexation. It appears that all of the factors previously discussed from the pH effect on ligand ionization and the subsequent effects on the multiple equilibria system to the overall geometric structure of the complex itself play crucial roles in the formation and stability of the mixed ligand species. Factors such as the effective radius of the metal ion, the overall charge of both the lipid and peptide, the size, length and composition of the peptide side chains, the accessibility of carboxyl groups, the stability of the chelated complex itself and the coordination number of the metal ion all contribute to the formation of a system seeking its lowest free energy. Graphically, the complexation observations are as follows:

$$\text{PS} + \text{Peptide} + M^{+2} \xrightarrow{(\text{pH } 5\text{-}8)} \begin{cases} \rightarrow \text{PS-}M^{+2} + \text{Peptide-}M^{+2} + M^{+2}(\text{PS})_2 \\ \rightarrow \text{PS-M-Peptide} \\ \rightarrow \text{PS-M-Peptide} + M^{+2}(\text{PS})_2 + \text{Peptide-}M^{+2} \end{cases}$$

Experimentally, Tables I, II and III reveal the physical binding tendencies of various systems with a number of metal species. The only trends that can be found are that Ca^{+2} is the predominant mixed ligand complexing metal and that the mixed ligand complexes seem to form more readily with the geometrically simple amino acids and peptides. Yet, these trends cannot explain why methionine

will not form a mixed ligand complex while bovine albumin will. It appears from the results obtained from this thesis that one cannot predict or rule out the possible formation of a mixed ligand complex from "trends" alone. Each case necessitates individual examination to ascertain the likelihood of mixed ligand complexation. However complicated, the fact remains that mixed ligand complexes do exist.

One can only speculate at this time that it is a compilation of the various effects (pH, molecular geometry, electrostatic interactions, binding affinities and the numerous equilibria) which lead to the formation of and accounts for the stability of the mixed ligand complex displaying both metal and peptide ligand specificity.

II. Specific Lipid/Protein Interactions

There have been a few investigations into the formation of a protein/lipid complex mediated through the use of divalent cations.[43-47,119] A number of investigators have noted the necessity or presence of Ca^{+2} in such a molecular complex.[30,39,41,84,107,121-23] Two authors, J.G. Fullington and H.S. Hendrickson have done extensive work on the interaction of phospholipid-metal complexes with various proteins. They have found that dilute suspensions of the acidic phospholipid-metal complexed liposomes can interact with various protein species (water soluble proteins of wheat flour, bovine serum albumin, histone (calf thymus), egg albumin, poly-L-lysine and poly-L-aspartic acid) to form a lipid/protein complex. Their analysis using starch gel, electrophoresis have observed these complexes and have also indicated that a metal ion is necessary for such complex formation. However, these authors believe that the association of the lipid and protein is due the proper orientation of groups within a protein chelating into an available coordination position on the metal ion already bound to a phospholipid. Their work will be contrasted to this thesis to show the differences and similarities of the two diverse investigations and the comparison itself will add an insight to such metal induced lipid/protein interactions.

Throughout the above mentioned investigations there was little concerted effort to characterize such complexes as to the number of lipid and metal molecules bound to the protein or to the site of such chelation on both the protein and the lipid. In the experiments performed in this thesis, these issues were investigated and have been reported in the results.

Generally, it can be stated that the anionic proteins: phosvitin, ovalbumin and bovine albumin will form mixed ligand complexes at pH 7.0 with the following ratios:

Phosvitin: 29-31 moles PS/mole PV

Bovine Albumin: 6.9-7.1 moles PS/mole B.A.

Ovalbumin: 4.1-4.5 moles PS/mole E.A.

In the mass balance of these complexes, there appears to be approximately an equimolar amount of Ca^{+2} (to PS) bound in these systems. The sites of chelation are assumed to be that of free carboxyl groups based on the accumulated experimental evidence in this dissertation. This evidence includes:

1) PC (lecithin) a lipid without a free carboxyl group will not form a mixed ligand complex.

2) Both inorganic phosphate and ionized ortho-phosphoserine will not form a mixed ligand complex through the phosphate oxygen.*

* This statement does not imply that such an interaction cannot exist, it only states that such an interaction itself does not lead to a stable complex.

3) Infrared analysis of the mixed ligand species has shown that the primary point of chelation is on the carboxyl and not at the phosphate.

4) Reaction of PS at a pH where only the phosphate is ionized will not form a mixed ligand complex but does form the dilipid complex.

It is interesting to note that although Hendrickson and Fullington did not try to ascertain the exact point of chelation they did note that both methylation and acetylation of the protein led to an increase and decrease respectively in binding to that protein. They also noted that the amount of phospholipid bound to the protein decreased with increasing pH; that the binding seemed to follow no set pattern and that their reaction product could be separated into a "weakly" bound and "tightly" bound complex. In contrast to this thesis, it is obvious that there are some distinct differences in observations. Disregarding the fact that most of their experiments were performed in a biphasic solution and at a higher pH range, this dissertation noted that there was an increase in lipid binding as the pH increased. The lipid bound was found to be specific and reached a maximum limit. It appears that the complex which Hendrickson and Fullington have observed is only partly that of the mixed ligand complex as proposed in this thesis. Their "weakly" and "tightly" bound complexes seem to be the dilipid-metal complex and the mixed ligand

complex since they were separated by a $CHCl_3/ETOH/H_2O$ system which has been shown to solublize the dilipid complex. Therefore, it appears that their complex is primarily a lipid/protein species in which there is a large amount of hydrophobic interaction of the lipid fatty acid chains coupled with a coordination of available sites on the protein. This model is consistent with their other observations of: 1) reducing the net positive charge (acetylation) with the increase in lipid metal binding; and 2) decreasing the net negative charge (methylation) with the subsequent decrease in lipid binding (but not elimination). In sharp contrast to the above work is the fact that from this work the removal of the net negative charge of a peptide specie led to no mixed ligand complexation. It appears that the complexes of both investigations are for the most part different lipid/protein species.

An important finding in the recognition of mixed ligand complexes and the reaction site itself is the actual stoichiometry of the phosvitin complex. For it is with this protein that the exact number of ionized accessible carboxyl groups are known to be 31. The results of these complexation experiments show a range of lipid complexation of 29 - 31/mole PV but no evidence for higher binding at pH (7.0). However, at the same pH there are an additional 136 ionized phosphate groups

which can bind an average of 127 Ca^{+2}/molecule of PV. Therefore, the possibility exists for a greater number of lipids to be bound to PV but no such case was found. It is possible from a steric point of view that only 29 - 31 molecules of lipid can bind to the protein but this seems to be to coincidental with the number of ionized carboxyl groups of PV. The absence of a low lipid/protein ratio complexes also indicates that there must be a determining factor other than the total number of accessible ionized group influencing mixed ligand formation. The total reaction itself was found to be not as spontaneous as was the other mixed ligand formation reactions, taking approximately 3 hours to fully precipitate. This observation may well be due to the fact that the numerous ionized phosphates act as an ion exchange medium preventing the immediate formation of the dilipid species while forming the mixed ligand species (with a presumably high formation constant) very slowly with the available carboxyl groups.

The remaining two proteins, ovalbumin and bovine albumin have also displayed the tendency to presumably form a mixed ligand complex. This assumption is based on the experimental evidence as follows: 1) Both proteins when mixed with PS (without Ca^{+2}) form a precipitate (presumably through hydrophobic and electrostatic interactions) which is soluble in the $CHCl_3$/MEOH (ETOH)/ H_2O system. The protein-Ca^{+2}-PS system for each protein

is insoluble in this solvent. 2) The protein-Ca^{+2} complex (without PS) when treated with EDTA will produce a metal free protein. Treatment of either protein - Ca^{++} - PS system has no effect. 3) The infrared spectra of the precipitates (Nujol Mull) although quantitatively inconclusive does display the Ca^{+2}-PS carboxyl binding frequency (1645-1640 cm^{-1}). 4) The chemical analysis of the precipitates:

Egg Albumin (Moles)		PS (Moles)	Ca^{+2}
a)	1	4.3	4.9
b)	1	4.5	5.0
c)	1	4.1	4.6
Bovine Albumin			
a)	1	6.9	6.9
b)	1	7.1	7.1

is consistent with a mixed ligand species. 5) Both bovine and egg albumin were found to form a complex in the Hendrickson, Fullington experiments.

It should be noted that the above data in (4) have two apparent inconsistent features. The first is the non-intergral number of PS(Ca^{+2}) moles bound. This can be explained by the fact that the true molecular weight of the albumins is not accurately known. The presence of impurities (other proteins or mannose) may lead to such numbers in the subsequent calculations. The second disparity is the amount of Ca^{+2} bound in the

Egg Albumin results. Although internally consistent, an added 0.5 mole factor above the PS level, is indicated. This finding can be justified by the fact that the Albumin has many possible binding sites (141 carboxyl side chains) which may be accessible to Ca^{+2} binding but not to mixed ligand formation (steric or electrostatic interactions). The fact that the excess amount of Ca^{+2} is consistent throughout seems to indicate that this is the case. However, it is of interest to compare the Ca^{+2} bound results of the three proteins discussed so far. All three proteins have anionically charged sites in excess of the amount complexed by the metal and lipid in a mixed ligand complex. Yet only the Egg Albumin resulted in an increased amount of Ca^{+2} being bound. This fact indicates that on the egg albumin itself there must be anionic sites which are accessible to Ca^{+2} after mixed ligand formation. It is possible that for both phosvitin and bovine albumin the formation of the mixed ligand complex prevents the further chelation of Ca^{+2} or more simply that the sites are no longer available for chelation either by being sterically blocked or by participating in a coordinate bonding scheme. Nevertheless, the stoichiometry for the phosvitin egg and bovine albumin mixed ligand complex has been calculated, the importance of which is still to be determined.

It has been previously mentioned that Hendrickson and Fullington have investigated lipid/protein interactions utilizing a mixed solvent system.[44] They would dissolve a lipid and protein in a biphasic solution of $CHCl_3$/MEOH/H_2O. Addition of a salt (Ca, Mg, or Ni) led to a precipitate formation at the interface of the aqueous and organic layers. They analyzed these products by starch gel electrophoresis and found both weakly and strongly bound metal/lipid/protein complexes. Their complex product for this reaction has been discussed previously. However, what will be discussed is the results of similar reactions carried out in this thesis.

Either poly glycine, alanine or hydroxy proline was added equimolar to PS in a biphasic $H_2O/CHCl_3$, system. The mixture was agitated and an equimolar amount of $CaCl_2$ was added (pH of mixture approximately 7.0). A precipitate formed at the interface of the H_2O and lipid rich $CHCl_3$ phase. This same experiment was carried out with decanoic, lauric, oleic and stearic acid as well as ovalbumin, hemin and insulin - chain-a. In each case a precipitate was formed at the interface. The subsequent analysis showed the following:

1) Adding MEOH or ETOH to the $CHCl_3$ phase "dissolved" completely the precipitates from the hemin and insulin experiments.

2) The alcohol addition reduced or "partially dissolved" the amount of precipitate in all the poly peptide experiments substantially. (Indicates dilipid complex was present).

3) Acidifying the biphasic system had varying effects but every precipitate did dissolve to a lesser (polyaminoacid) or greater degree (fatty acids).

4) EDTA treatment "dissolved" all the precipitates from the fatty acid experiments and had no effect on the others.

Specifically, these results show that hemin and insulin chain-a do not form a mixed ligand complex as was also seen in the aqueous experiments.

This fact in itself is interesting from a biochemical point of view because it indicates that certain proteins will not undergo such chelation reactions. Whether it be the fact that the binding sites of the protein are inaccessible or that the formation of the mixed ligand complex is sterically or electrostatically impaired by the protein is not drastically important. What is important is the knowledge that certain proteins, peptides and amino acids have the tendency to form a mixed ligand complex with PS under physiological conditions while other proteins, peptides and amino acids will not (see Tables I and II). Therefore, it seems probable that

such mixed ligand formation at a membrane surface may play a pivotal role in many membrane surface or trans-membrane processes, especially since the formation is ligand specific, structurally stable and pH controlled. (An interesting parallel to this idea is the role of metals in enzyme catalyzed reactions).[148]

The poly-amino acid experiments show that a large part of the precipitate formed is the dilipid complex which may then interact hydrophobically with the acid system.[44,149-152] This fact is consistent with the Hendrickson/Fullington experiment since treatment of the interface precipitate with a $CHCl_3/MeOH/H_2O$ solution does dissolve the precipitate substantially. The remaining precipitate may well be a mixed ligand complex but the analytical and spectral data of that precipitate is inconclusive.

Ovalbumin forms a mixed ligand complex in these biphasic experiments but it was not totally characterized in this set of experiments. The infrared spectrum of this interface precipitate was identical with that of the aqueous egg albumin mixed ligand complex. It was therefore assumed that the initial precipitate formed in this experiment was the mixed ligand complex together with a large amount of the dilipid complex.

The fatty acids all form what appears to be a very weak mixed ligand complex (low formation constant).

In summation the chemical and infrared spectral analysis was inconclusive for the majority of reactants examined but it did show that: 1) the stoichiometry (qualitatively) was much greater than 1:1 for the poly amino acids; 2) there was a significant and equal increase in the CH and $COO^{(-)}$ infrared absorbtion bands for the fatty acid complexes; 3) the ovalbumin spectrum was identical to that of the aqueous preparation; 4) the precipitate in the hemin and insulin experiments was the Ca^{+2} dilipid species.

The Hendrickson/Fullington experiment showed analagous results except that their findings showed that Ni^{+2}, Mg^{+2} and Ca^{+2} all formed these mixed phospholipid/protein complexes. Ca^{+2} demonstrated the weakest chelation or more simply the least stable complex. These authors believe that the metal reduces the surface potential of either the lipid or the protein. As a consequence, this effect increases the possibility of hydrophobic interactions and/or weak chelation as was previously discussed. It should be noted that their experiments were carried out at a pH of 8 or above. At this pH, it has been found that a precipitated mixed ligand Ca^{+2} complex is completely dissociated while a liposome mediated mixed ligand complex remains partially complexed. It is also known that a pH 8, the seryl amine group of PS is completely deprotonated. Therefore, there is no real

inconsistency in the data of this or the Hendirckson/Fullington experiments because at the higher pH values one would expect a greater utilization of the nitrogen in complex formation. Since both Ni^{+2} and Mg^{+2} are preferentially bound to nitrogen bases (Ca^{+2} to multidentate anions) in biological systems, it would explain why Hendrickson/Fullington found the stabilities of the complexes reported above. It also supports the contention that their complex is not the same complex as is investigated in this thesis. A graphic representation of their proposed metal complex is shown in Figure 29.

This author believes that such a structure may exist but that experimental evidence bears out the following facts:

1) Such a PS-Metal complex can only exist at pH's greater than 8.

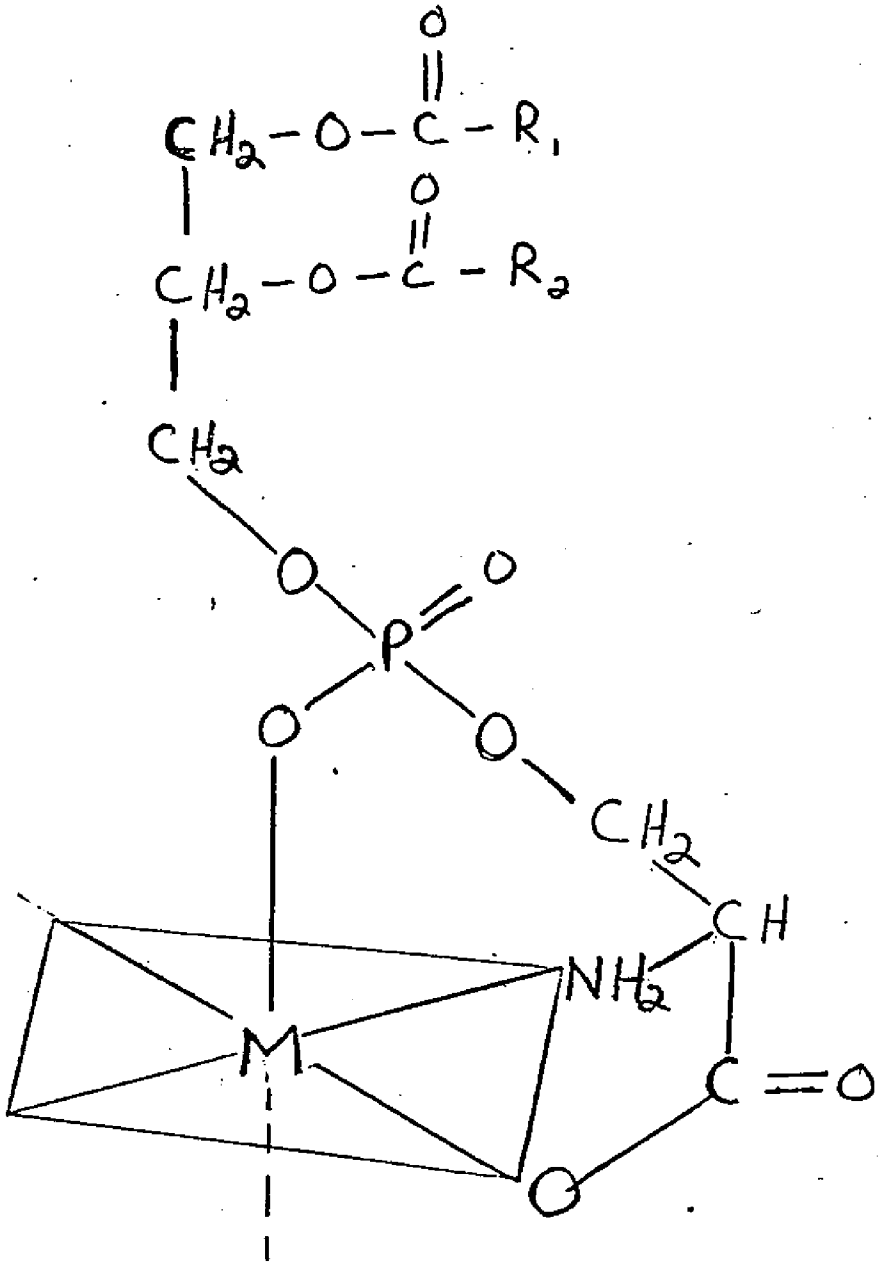
2) This 1:1 PS-Metal complex has only been detected in extremely dilute vesicle systems.

3) Treatment of the vesicle system in (2) does not produce mixed ligand formation in the pH range of $6.5 \longrightarrow 7.5$.

4) Preparation of a 1:1 PS-Metal complex in a mixed ligand vesicle under dilute conditions and subsequent treatment with a suitable peptide ligand will produce a mixed ligand complex which is bound through the respective carboxyl groups.

Figure 29

Possible structure for the phosphatidyl-serine metal complex in the pH range of 9 → 14.


O
CH2-O-C-R1
O
CH2-O-C-R2
CH2
O
P
O
O
O
CH2
CH
NH2
M
C=O
O

5) Treatment of PS as a multilamellar vesicle or a dispersion with Ca^{+2} produces a dilipid complex.

6) The dilipid complex will not form a mixed ligand complex if so reacted.

7) The dilipid complex may associate with a protein but the complex can easily be dissociated by treatment with a $CHCl_3/MeOH/H_2O$ solution.

8) A mixed ligand complex will not dissociate with the above solution.

9) An amino acid (peptide)/metal complex (as the mono substituted species,[116,137-9] i.e., $AA\text{-}Ca^{+}$) will form a mixed ligand complex when added to a PS suspension. (This, of course, is dependent on the metal and ligand employed).

10) At pH 8.0 the Ca^{+2} mixed ligand complex is at best only partially formed and is extremely labile.

The above facts from this thesis and the observations and results of the Hendrickson/Fullington experiments delineate between two separate and distinct types of lipid/protein complexes. They are:

1) Hendrickson/Fullington complex - a lipid/protein complex which is formed between a 1:1 metal/PS chelated complex and a protein. The primary modes of association are believed to be hydrophobic attractions and electrostatic coordination of the remaining available metal sites by the

appropriate functional groups on the protein. The metal/lipid chelate effectively reduces the net ionic charge of PS (at pH 8.0) to 0, thus facilitating the above suspected interactions.

2) Mixed Ligand Complex - A lipid/protein complex which is formed by the direct chelation of a protein to an acidic phospholipid. In such a complex, Ca^{+2} actually chelates or "bridges" the anionic carboxyl groups of PS with the respective peptide/amino acid or protein species.

Experimentally, it seems that the Hendrickson/Fullington complex is energetically favored at a pH greater than 8.0. The stability of this lipid/protein complex appears to be dependent upon the nitrogen affinity of the specific metal utilized and the amount of hydrophobic interactions involved.

The mixed ligand complex, on the other hand, is favored in the physiological pH range of 6.0 $\longrightarrow$ 7.6. The lipid/protein complex is specific in that it is the direct chelation of the respective carboxyl groups (verified through thorough chemical and spectral analysis of the complex). The overall stability of this complex, as discussed in Section I of the Discussion, depends on a variety of factors from the pH and molecular geometry of the complex to the specific peptide ligand and metal utilized.

The results do not imply that these lipid/protein interactions are present in a "living" biological membrane. However, their chemical presence and their overall pH and molecular stability warrants that they not be discounted in the overall scheme of biological and physiological lipid/protein interactions.

III. The Effect of Mixed Ligand Complexes on Pure and Mixed Lipid Vesicles

The use of model systems such as vesicles or liposomes is a convenient method of being able to simulate a biological membrane while circumventing the gross pitfalls of working with natural membranes. Mueller et al [125] first reported using a protein-free model membrane system, the so-called black lipid membrane in 1962. The merits of this powerful technique were soon realized and numerous experiments concerning the surface pressure[62] and potential [126,7] of these BLMs were undertaken on these mechanically supported membranes. Yet historically, liposomes, lyotropic smectic mesophases, have most likely been forming and reforming in an aqueous environment since the formation of lipid-like molecules in time. What this actually entails is the clustering of "lipids" at an air/water interface.[128] Simple principles such as aqueous hydration, electrostatics and surface free energy are now recognized as being the major stabilization of these membrane structures. Ironically, the use of liposomes or vesicles was patented in England by J.Y. Johnson.[129] A certain pharmaceutical company had discovered that certain drugs when mixed with lecithins can be injected into the body and released quite slowly! The preparation of this injection reads exactly like a standard vesicle preparation technique used today. As science progressed, lipid aqueous

suspensions became more in vogue and scientifically useful. Today liposomes which can actually be broken down to three distinct types of phospholipid assemblages, namely, multilamellar, microvesicular and macrovesicular, are utilized in various biological investigations. Briefly, these types differ in size, preparation and use. The multilamellar structure can be pictured as numerous bimolecular leaflets piled on top of each other with a thin aqueous stratum separating the "membranes". This system has been the virtual work horse for biological model investigation.[130-132] Such studies as cation permeability,[133] properties of ion carriers,[134] binding of basic proteins such as cytochrome C[135-6] and effects an antibiotics[137] all have been carried out on this system.* They are the easiest lipid system to prepare and one can incorporate or encapsulate any given solute such as glucose or inorganic phosphate through simple mechanical or manual agitation of the lipid/solute/water system.

The microvesicle is a structurally distinct version of the multilamellar system. The concentration of phospholipid to H_2O is very low and the solution is ultrasonicated to produce clear sols comprised of single shelled bilayer spherules with an average diameter of 160 A^{o}. These structures are ideal for studying the ion

* For more References, see "Methods in Membrane Biology, 1 (E.D. Korn, ed.) Plenum Press, N.Y. 1974.

exchange capabilities of pure acidic phospholipids. It has also been found that both PS and PC form microvesicles when sonicated.[99]

Macrovesicles are smectic mesophase systems prepared in a non-electrolyte medium which when prepared is the "in between" of the multilamellar and microvesicle systems. Since the presence of electrolytes disrupts this system, it is used only in very specific cases involving the study of non-polar aliphatic materials on these "membranes."

Throughout the course of this dissertation, both the multilamellar and microvesicle systems have been used and have produced identical results especially in the mixed ligand formation and metal competition studies. However, in the efflux studies it was found that on ultrafiltration the microvesicles seemed to congeal forming a multilamellar-like system. Subsequent dilutions (washing) and reconcentration had the same effect on the microvesicles so that at the final stage in which all solute was removed from the supernatant liquid and the volume adjusted both original preparations appeared to be similar to that of a multilamellar liposome. Analysis as to the average size of the liposome was not performed but was assumed to be approximately 200 A^{o}.

Using both the sonication and ethanol injection methods for microvesicle preparation (see Experimental

Techniques) the mixed phospholipid vesicles (PC/PS-9/1; 3/1;1/1;1/3;1/9) were prepared as clear sols and initially used to observe mixed ligand formation. The results of these experiments have already been reported and discussed in this thesis. Therefore, since mixed ligand complex formation did take place at the surface of the vesicle, it became of interest to investigate the overall effect of such complexation on the physical properties of the vesicle itself.

Numerous experiments have been performed on vesicle systems noting the effect of bi and multivalent metals on surface potential and surface pressure.[59,62,88,154] Recently, various proteins have been observed to increase the permeability of phospholipid membranes.[46,135-6] Although the reason for such increased permeability of various solutes is unknown, it has been correlated with the degree of penetration of the protein into the lipid membrane.[135] The decision was, therefore, made to investigate the effects of mixed ligand complexation on the glucose and phosphate permeability of variable composition PC/PS vesicles. Glucose and inorganic phosphate were selected because previous permeability studies had been performed on similar vesicle systems thereby giving a basis of comparison for this experiment.

After the initial preparation of the mixed lipid vesicles encapsulating the glucose and phosphate solute (see Procedural Techniques), the Ca^{+2} and the Ca^{+2}/GlyGly was

added to the respective reaction vessels. At this concentration approximately 1 x 10^{-3} M in lipid, the observation of PS dissociating out of the vesicle is not seen. The liposomes remain intact. Aliquots were then removed from the reaction vessels (at constant 26° C temperature) and analyzed as per the Procedural Techniques section. The results as shown in Tables XII and XIII and in Figures 21-24 can best be interpreted as follows: There is a definite trend in the glucose permeability experiments indicating that glucose release is enhanced with mixed ligand complexation especially in the case of the liposome containing the least amount of PS. Ironically, it is this 9/1 PC/PS vesicle which compositionally resembles a natural membrane. The Ca^{+2} complexation experiments also follow this trend. The difference in the two separate experiments is primarily the amount of glucose released.

The native liposome experiments in this series displays its own peculiar trend of solute efflux. Glucose release seems to be favored in the most negatively charged liposome, that of the 1/9 native mixture. This effective anionic surface charge trend, however, does not appear to be consistent as can be seen by the amount of glucose present in the supernatant 2 days after the initial complexation: 9/1 - 65.93 mg/dl; 3/1 - 93.41 mg/dl; 1/1 - 87.91 mg/dl; 1/3 - 76.92 mg/dl; 1/9 - 131.87 mg/dl. The same inconsistencies exist as can be readily seen in the tables for both the Ca^{+2} complexed species and the mixed ligand complexed species. Therefore, a number of factors

must be involved in controlling the efflux of glucose from the liposomes.

The major controlling factors of glucose permeability seem to be a combination of the net surface charge and the lipid composition of the vesicle. As the net natural surface charge (anionic) of the native liposomes increases (with PS concentration), glucose release increases. Experimentally as the net surface anionic charge is reduced with either Ca^{+2} or mixed ligand formation, glucose release is greatly enhanced. However, the least naturally charged liposome, the 9/1 - PC/PS; displays the greatest glucose permeability after complexation. If one were only considering the net surface charge of the native vesicles, the 9/1 liposome would have a slight negative charge diffused through the surface of the vesicle as opposed to the 1/9 liposome which has a large net anionic charge. After complexation with Ca^{+2} which was observed to be in a 1:1 stoichiometry, the liposome charge is no longer anionic, it is now cationic with the 9/1 vesicle acquiring the least overall positive charge. This charge reversal is also true for the Ca^{+2}/GlyGly mixed ligand complexation. It is now interesting to note that the native vesicle with the greatest negative charge and the complexed vesicles either Ca^{+2} or Ca^{+2}/GlyGly with the least positive charge displayed the greatest glucose permeability. Unfortunately, a simple correlation of the two effects cannot be made on the basis of net surface charge because if surface anionic

characteristics are necessary for enhanced glucose permeability why do the positively charged complexes show a greater permeability? If neutrality or cationic surface characteristics are necessary, why then does the pure PC liposome (neutral)[105] show the identical permeability as the greatest cationically charged liposome (1/9 complexed) while both are only a fraction of the permeability of the least positively charged vesicle (9/1-PC/PS - Ca^{+2} and Ca^{+2}/GlyGly)?

Although there is no simple solution for the above question, the results of the glucose permeability experiments do add a further insight into membrane specificity. The lipid composition and the pH of the environment of a membrane controls the net surface charge of the species which then dictates the amount of complexation which will/can take place. Experimental evidence of various investigators have shown that on addition of divalent cations to mixed lipid vesicles the following phenomena occur: 1) for PS or mixed PS/PC vesicles with a high (> 50%) PS content - vesicle contract on salt additions; PC vesicles swell.[140] 2) Ca^{+2} binds to the PS molecules in the vesicle forming rigid aggregates (Mg^{+2} ineffective) thereby separating the PC into clusters.[68] 3) Addition of the proteins spectrin or albumin to the vesicle (before Ca^{+2} addition) increases the permeability of both pure and mixed PS/PC liposomes.[46] Subsequent Ca^{+2} addition would lead to

perturbations of the bilayer structure increasing permeability. 4) "Membrane" formation phenomena displayed by the mixed ligand complexes incorporates the ligand within the membrane or for this case the vesicle.

With this information and the results of the glucose permeability experiment, the data become more easily understood. On the addition of Ca^{+2} or Ca^{+2}/GlyGly to a liposome a complex is formed. Ca^{+2} alone will aggregate the PS into rigid clusters allowing the PC itself to cluster. The 9/1; PC/PS liposome for example would be a nearly homogeneous vesicle with small pockets of PS rigid clusters disrupting or perturbing the vesicle surface and the hydrophobic interior. The same is true for the Ca^{+2}/GlyGly systems except that the GlyGly is incorporated into the vesicle presumably interacting with the charged PC species quite near the vesicle surface. In any case, the positive charge of the GlyGly is somewhat displaced from the surface of the vesicle. This charge displacement may play an important role in the overall glucose permeability since both the Ca^{+2} and Ca^{+2}/GlyGly complexed systems have identical positive charges yet the Ca^{+2} complex which is positionally closer to the membrane surface displays a lower glucose permeability. As the concentration of PS increases with the analagous

increase in Ca^{+2} and Ca^{+2}/GlyGly, the complexed systems become more rigid reducing the fluidity of the PC portion of the vesicle while increasing the positive charge of the vesicle. Experimentally, this increased rigidity lowers the permeability of glucose from the vesicle systems. Therefore, it appears to be a combination of the reduction of surface charge and the remaining fluid content of the vesicle which controls glucose permeability in these experiments. Other possible explanations such as a differential in the amount of glucose entrapped in the original liposome preparation can be disregarded since the concentration of the glucose entrapped was calculated for each preparation and was above the levels indicated in the results. Reference to the Juliano, Kimelberg, Papahadjopoulos[46] observation, of increased permeability with protein and Ca^{+2} binding was explained in the following manner. Both spectrin and albumin are added (before Ca^{+2}) to the vesicles (or monolayer) forming a lipoprotein complex, presumably both hydrophobic and electrostatic in nature. These proteins are actually incorporated into the vesicle, "swelling" the size of the vesicle and thus affecting the hydrophobic core of the liposome. Subsequent Ca^{+2} addition, experimentally caused gross perturbations to the lipid structure thus increasing the permeability of the liposome to Na^{+} flux.

Their experiment and those described here are quite different. First, glucose permeability is being measured and second, glycylglycine, a dipeptide, is being directly chelated to the carboxyls of the vesicles. This peptide cannot interfere with the hydrophobic region of the vesicle and more probably remain on or near the surface of the vesicle interacting with the negative phosphate group of the PC species. It, therefore, indicates that mixed ligand complexation, the direct metal chelation of the carboxyl groups on a lipid and peptide species, can play an important role in selective membrane permeability without a direct interference into the hydrophobic core of that membrane.

The phosphate analysis may be approached in the same manner as that of the glucose permeability study. The results show that for the native liposomes, phosphate permeability is also enhanced by the presence of an anionic charge. In the Ca^{+2} and the Ca^{+2}/GlyGly complexed vesicles, however, a trend is not as obvious. Comparison of the amount of phosphate released after 2 days is virtually the same for both complexed species (Figure 23) but the rate of release and the amounts released in the first seven hours do not follow a pattern as was seen with the glucose experiment. The Ca^{+2} 9/1 complex vesicle, the liposome with the lowest positive charge, shows the greatest phosphate permeability while the

1/9 Ca^{+2} vesicle, the liposome with the largest positive charge, is second. The Ca^{+2}/GlyGly experiment is even more confusing with the 3/1, 1/3 and 1/9 complexed liposomes showing almost the identical amount of phosphate released (Figure 24).

These results indicate that the net surface charge is not directly involved in the permeability of ionized phosphate or at least plays a minor role. In the overall reaction, there seems to be little difference in the Ca^{+2} and Ca^{+2}/GlyGly complexation on the vesicle permeability to phosphate which supports the above statement. Therefore, there must be other factors or combination of such which control phosphate efflux.

One factor which should be considered is the solute itself. The ionized phosphate encapsulated in a lipid hydrophobic region would have quite a different effect on that environment than would glucose. The phosphate may itself disrupt the hydrophobic vesicle interior causing the permeability observed. This facet of vesicle perturbation may also explain why there is a much shorter induction period in the phosphate efflux experiments. The primary effect of the phosphate in the lipophilic region may well be a disruption of the natural fluidity of the vesicle thereby increasing the rigidity effect of Ca^{+2} and Ca^{+2}/GlyGly complexation and increasing the phosphate efflux.

Numerous other biophysical phenomena such as osmotic pressure, surface tension and the phosphate binding affinity may also be involved in the above experiments but have not been discussed. Obviously, no one effect can explain the results obtained.

It appears that various combinations of factors such as: 1) the swelling and contracting of vesicles by salt or mixed ligand formation, 2) the fluid nature of the vesicle, 3) the nature of the solute being studied, 4) the surface charge of the liposome and 5) the vesicle composition itself, all contribute to the observed permeability and affect the rates of release observed in these and other liposome experiments.

CONCLUSION

The importance of lipid/protein interactions cannot be overestimated. These interactions lead to the formation of lipid/protein complexes which are involved in a multitude of various physiological and biochemical processes. Specific lipid/protein complexes are the actual structural macromolecular components of all biological membranes. Other such complexes are involved in cell secretion,[145] active transport,[146] cell permeability, calcification of various human tumors, neural response and are an indispensible component of the energy transducing system in the mitochondria. Each of these lipoprotein complexes are functionally and structurally unique, yet the modes of lipid/protein interaction are still not fully understood.

This investigation examined one such possible mode of interaction, that of mixed ligand metal chelation. The work demonstrated that: 1) a mixed ligand complex can be formed and is stable under physiological conditions. 2) The complex is formed by the direct chelation of the respective ionized carboxyl groups of both a lipid and protein by a multivalent cation. No phosphate bonding was observed. 3) Only certain cations will form such a complex in the pH range of 6 - 8. They are: $Ca^{+2} \gg Mg^{+2} > Sr^{+2}, Mn^{+2}, Al^{+3}, Fe^{+3}$. 4) The mixed ligand complex is

ligand selective. 5) The mixed ligand complexes of Ca^{+2} and Mg^{+2} demonstrated a pH dependence which in a natural membrane could well be a mechanism for regulating cell permeability or transport. 6) The mixed ligand complex formed with proteins depended on the accessibility of carboxyl groups. 7) The stoichiometry of the mixed ligand complex of PS/Ca and either amino acid or peptide ligands was 1:1:1. 8) The stoichiometry of the protein mixed ligand complexes was as follows:

1. Phosvitin - 29-31 PS and Ca^{+2}/PV
2. Ovalbumin - 4.3 PS and 4.8 Ca^{+2}/Ovalbumin
3. Bovine Albumin - 7.0 PS and Ca^{+2}/Bovine Albumin

9) Mixed ligand complexes will be formed on mixed lipid vesicles. 10) The amount of mixed ligand formation on a vesicle is dictated by the amount of acidic phospholipid present. 11) Mixed ligand formation has an extensive effect on the vesicle permeability of glucose and phosphate.

It is not my intention to state that this mode of lipid/protein interaction exists under the biological and physiological conditions of the cell. It is contended, however, that mixed ligand chelation, often invoked and speculated in various investigations, is a viable possibility in protein/phospholipid interactions as has been demonstrated by the results of this thesis. Therefore, mixed ligand complexation, the direct metal

chelation of the respective ionized carboxylate groups, should not be discounted as a functional and structural force in biological membranes.

APPENDIX

I. Liposome Permeability Coefficients

It should be understood at the outset of this section that this thesis addendum does in no way modify, contradict, expand or contract the experimental observations or conclusions made previously in this dissertation. The purpose of this section is to qualitatively calculate the experimental vesicle permeability coefficients, noting the effect, if any, of metal or mixed ligand complexation on the vesicle permeation of glucose and inorganic phosphate. These coefficients can then be compared to literature values for the same solutes and vesicle systems thus possibly supplying an added insight into the membrane efflux phenomenon.

If one assumes that both glucose and inorganic phosphate permeation follow first order kinetics, the permeability coefficient, $k_{glucose}$ (k_g) or $k_{phosphate}$ (k_p) can be calculated from the following equation: [156]

$$- \ln (1 - \Delta N/\Delta N_{\infty}) = (A/V)\, k\, t$$

In this case ΔN and ΔN_{∞} are the concentrations of glucose or inorganic phosphate expressed as the % of the total solute efflux at time t and at equilibrium, respectively. A is the surface area and V the internal volume of the vesicle.

It is at this point that the qualitative nature of such a calculation becomes evident. One must now estimate both the surface area and the internal volume of these mixed lipid vesicles. Since there is no literature data available for such mixed lipid systems, both parameters were calculated from "known" values for the pure PC or PS systems. The following base information was obtained for these systems.

PC - surface area [59,157] = $1.9 \pm .2 \times 10^3$ cm^2/u mole lipid

- internal volume [156,158,159] = $1.6 \pm .1 \times 10^{-4}$ ml/u mole lipid
- volume/molecule = 62 to 71 A^{o2}

PS - surface area [89] = $2.3 \pm .1 \times 10^3$ cm^2/u mole lipid

- internal volume [89] = $2.0 \pm .1 \times 10^{-4}$ ml/u mole lipid
- volume/molecule = 70 A^{o2}

Utilizing this information, the mixed lipid vesicles systems were estimated as follows:

PC/PS	Surface Area ($\times 10^3$ cm^2/u mole L)	Internal Volume ($\times 10^{-4}$ ml/u mole L)	V/A ($\times 10^{-7}$ cm)
9/1	1.94 ± .19	1.64 ± .1	0.845
3/1	1.90 ± .18	1.70 ± .1	0.895
1/1	2.10 ± .15	1.80 ± .1	0.857
1/3	2.20 ± .13	1.90 ± .1	0.864
1/9	2.26 ± .10	1.96 ± .1	0.867

The Δ N and ΔN_∞ values were taken from Tables XII, XIIa and XIII. ΔN_∞ was considered that point in which the amount of

diffused solute remained the same over a period of time. ΔN was obtained by halving the equilibrium time and utilizing that respective solute value. For the cases in which no obvious equilibrium concentration was obtained, the last reading (before 2 days) was used as the ΔN_{∞} with ΔN corresponding to one-half of this time value. Table XVI shows the permeability coefficients calculated using the above assumptions. For both the glucose and phosphate experiments the permeability coefficients increased with complexation of either Ca^{+2} or the Ca^{+2}/GlyGly systems. However, no further correlation can be made from these numbers.

If a comparison is now made to permeability coefficients that have already been documented in the literature, an interesting observation is made. The below are permeability coefficients which have been reported.

PC - glucose permeability a) 2.5×10^{-10} cm/sec [160] (bilayer membrane)
b) 4.0×10^{-11} cm/sec [161] (unilamellar vesicles at 36°C)

PS - glucose permeability a) 41.3×10^{-12} cm/sec [161]

PS/50% cholesterol - glucose permeability 17.5×10^{-12} cm/sec [161]

Human red cell phospholipid - 6.2×10^{-8} cm/sec [104] (glucose)

Reconstituted erythrocyte membranes - 3.4×10^{-8} cm/sec [103] (inorganic phosphate)
(.5 mM $MgCl_2$) - 8.8×10^{-9} cm/sec

(No data exists for the mixed lipid vesicles systems used in this thesis.)

Table XVI

Vesicle permeability coefficients for the glucose and phosphate efflux experiments.

		k_g (cm/sec x 10^{-12})		
	PC/PS	Native	Ca^{+2}	Ca^{+2}/GlyGly
Glucose	9/1	3.718	4.378	4.121 x 10^{-12}
	3/1	1.2165	3.870	9.445
	1/1	2.971	5.084	3.837
	3/1	0.995	3.205	3.772
	1/9	2.595	2.163	5.545
		k_p (cm/sec x 10^{-12})		
Phosphate	9/1	1.477	4.95	5.585
	3/1	3.339	7.633	8.072
	1/1	2.101	5.098	4.787
	1/3	4.329	10.50	9.671
	1/9	2.865	8.854	7.043

The striking note is that the coefficients which were qualitatively calculated from the vesicle data reported in this thesis are well within the area of the accepted value for glucose permeability in the pure PS vesicle. Notwithstanding the primary assumptions which were previously made in order to calculate the permeability coefficients, there is only a 10-20 fold difference in the respective coefficients. There is no correlation with the reported pure PC permeability coefficients but for the 50% cholesterol/PS mixture there is only a 5-10 fold difference in the reported and calculated coefficient values.

It must be reiterated that the above calculated coefficients are at best described as qualitative. This permeability treatment suffers from a number of serious disadvantages. They are:

1. These results cannot be expressed as fluxes or true permeabilities because the actual surface area of the dispersion was not measured.

2. The size distribution of the vesicles was not measured but was assumed to be that of reported preparations.

3. Permeability coefficients are usually calculated by the flux of a solute into a membrane with subsequent extrapolation to a vesicle system. These calculations used solute efflux data from a vesicle species.

4. Large liposomes are susceptible to mechanical rupture.

5. The coefficient itself is dependent upon the time (t) chosen. One could, therefore chose a time close to the equilibrium point and thus obtain a greater numerical value for the permeability coefficient.

6. It was assumed that the vesicles were homogeneous as to the mixture (PC/PS) which was intended.

7. Although the exact amount of solute entrapped was known, the true internal volume or "trap" volume was not known.

8. There has been little experimental data on these mixed lipid vesicles systems which would lead to a beneficial comparison of the calculated values.

However, keeping the above pitfalls in mind, the calculated coefficients do fall into the correct range for the PS vesicle glucose permeability. Whether this be serendipitous or concrete experimental evidence is extremely hard to say. Yet complexation (metal or mixed ligand) does increase the value of the respective permeability coefficient for both the glucose and inorganic phosphate experiments. This phenomenon may be due to the effects discussed previously in this thesis. It is even more interesting to note that the coefficients for the two vastly different solutes are virtually the same. There is no

apparent answer to this observation and this author will not speculate on this point at this time.

In summation, the calculated permeability coefficients are consistent with the evidence compiled from the liposome experiments and can be used qualitatively to note the effect of complexation (non-hydrophobic core disrupting) on a vesicle or liposome system.

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