NMR OBSERVATIONS OF SIMULTANEOUS HYDROPHILIC AND HYDROPHOBIC PROCESSES OF MIXED LABELLED LIPOSOMES

George J. Vella

A Thesis

in

The Department

of

Chemistry

Presented in Partial Fulfillment of the Requirements
for the degree of Master of Science at
Concordia University
Montreal, Quebec, Canada

September, 1976

ABSTRACT

NMR OBSERVATIONS OF SIMULTANEOUS HYDROPHILIC AND HYDROPHOBIC PROCESSES OF MIXED LABELLED LIPOSOMES

GEORGE J. VELLA

The silicon-containing fatty acid ester, methyl-13,13-dimethyl-13-silaheptadecanoate was synthesized and incorporated by sonication into lecithin vesicles. Nuclear magnetic resonance (NMR) line width measurements of the Si-methyl resonance and the N-methyl resonance of the lecithin enabled the observation of mixed liposomes undergoing a dynamic process. Some of the processes which were monitored involved lipid-electrolyte and lipid-hydrocarbon interactions. These interactions set the criteria for observing a cooperative lipid-ionophoric polypeptide effect. The latter process was observed with and without an electrolyte medium in order to elucidate any lipid architectural changes occurring during ion transport.

The observations, in this laboratory, concerning incorporation and function of the ionophore Gramicidin S are in variance with previously published results, stating that the effect is primarily an electrostatic interaction. Our results depict a hydrophobic as well as an electrostatic interaction which seems to suggest a dynamic incorporation process is taking place as opposed to the previously alleged surface binding only.

The silated fatty acid was biosynthetically incorporated into the membrane of the micro-organism Mycoplasma laidlawii. However, a sufficiently resolved NMR spectrum of the membrane fragments was not obtainable.

THIS THESIS IS

DEDICATED TO

MY PARENTS

JOSEPH AND MARY VELLA

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I wish to express my deep appreciation to Dr. Thomas Nogrady who proposed and directed the research reported in this Thesis. I am most grateful for his conscientiousness and concern as a research director as well as his stimulating and infinite source of ideas when problems arose.

The interest and suggestions by the members who served on my research committee, Dr. Jack Kornblatt and Professor Lawrence Colebrook, is also greatly appreciated.

I am indebted to Drs. Judith Kornblatt and Oswald Tee, for their most helpful discussions during the course of this research, and to Dr. Thomas Adley for obtaining and aiding with the interpretation of the mass spectral data.

I am especially appreciative of the friendship, encouragement and very helpful late night discussions with Dr. Alan Fraser whose concern helped me surmount many difficulties encountered.

Thanks are also due to Salem Daniel, Bruce Johnson and Rajender Sipehia for their uplifting spirits.

Finally, I would like to thank my loving wife, Ms. Lynn Watters, for her support, patience and understanding and especially for maintaining my sanity during times of anxiety.

CHAPTER I. INTRODUCTION

In the early 1900's, biological investigators believed that the sole purpose of a membrane was to contain a cell and its contents. Thus, research, at that time, was directed to elucidate the structure and composition of a biological membrane. For a number of years, the predominant idea was a bimolecular lipid leaflet arranged so that the hydrophobic tails opposed each other end to end on the inside, and the polar heads, which constituted a basic backbone structure, were on the outside[1]. It was not until the 1930's that protein layers were associated with this bimolecular leaflet to form a lipoprotein complex. This model for the structure of biological membranes, was published by Danielli and Davson[2] and forms the basic concept of membrane organization to the present day. The Danielli-Davson model proposed that the protein is in globular form and associated with the polar head group of the lipid leaflet. In 1957, Robertson[3] modified the Danielli-Davson model so that the protein layer was in extended β form; allowances were also made for asymmetric surfaces. This model was known as the unit membrane hypothesis. This hypothesis was universally accepted for a number of years until electron microscopy and X-ray diffraction data of various cell tissues revealed subunits in the plane of the membrane. The advent of these new observations led to the search for a more suitable membrane model so as to define more precisely biological membrane ultrastructure.

Up to this point, membrane function did not enter into the discussion of structure. Only within the past decade, have investigators been relating structure and function of a biological membrane as a dual intrinsic property. Thus the more recent hypotheses of biological membrane ultrastructure not only had to comply with spectroscopic evidence, but also had to contain some functional element besides a cytoplasmic envelope.

Currently, the most generally accepted hypothesis is the Singer-Nicolson mosaic model[4]. This model consists of helical and random coil proteins inside and outside of a bimolecular polar lipid leaflet. The proteins which penetrate the leaflet are partly made up of hydrophobic amino acid residues which may serve as a permeability gate in membrane transport mechanisms. A more detailed review of various hypotheses for membrane ultrastructure was published by R.W. Hendler[5] as well as E.D. Korn[6] and M.S. Bretscher[7].

It must be borne in mind, that these hypotheses are of a general nature and structure-function relationships in membranes vary throughout, thus making for instance the membrane of a nerve cell different from that of a rough endoplasmic reticulum. Because these structures differ in function, they also differ in membrane components giving rise to a new structure-function relationship. These hypotheses are - as always - subject to modification as membranologists discover more subtle intricacies related to membrane function which may shed new light on membrane structure. From the general hypotheses, the trend to

classify membranes into their site of origin, which is synergetically governed by their function and composition, seems to be justified.

Biological membrane phenomena can best be related to natural living membranes. Unfortunately, instrumental limitations (specifically spectral studies) are not entirely suitable to working with natural membranes. This problem had been circumvented by the use of membrane model systems made up of known membrane components.

In dilute aqueous suspensions, most membrane lipids spontaneously form closed vesicles, which, under ultrasonic irradiation, give rise to the formation of single walled lipid vesicles. Electron microscopy and X-ray diffraction data yield convincing evidence that these vesicles consist of lipid bilayers. These liposomes are far from being natural membranes, thus extrapolation of phenomena observed to living membranes must be done with extreme discretion.

Liposomes have been used extensively as working models for membranes. Not only are spectral studies possible, but they exhibit membrane properties which are principally due to membrane lipids. These liposomes provide a good understanding of the physicochemical properties of lipids in the presence of water. Biophysical studies of liposomes have revealed phenomena which are still unclear with respect to their biological implications, but it is possible that some structural transitions, which occur in these lipid-water models, may indeed be involved in natural membranes. Direct evidence for this is not yet available.

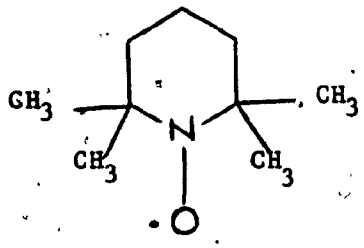
The most striking similarity between liposomes and intact membranes is the order-disorder transitions of the membrane lipids. Although these transitions are not totally understood, it is postulated that segments of hydrocarbon chains undergo the order-disorder transition under physiological conditions, inducing a local segregation of lipid molecules which may alter lipid-protein interactions thereby exerting a regulatory effect by the membrane[8].

Sonicated vesicles have been employed to establish correlations between structure and spectroscopic signals from various methods. Spin[9], fluorescence[10] and NMR[11] labels have been incorporated into these liposomes in order to display the different type of structures. Most of these studies have been conducted when the liposomes were in a static state with no external stimuli inducing any perturbation of the bilayer structure.

Only within the last few years have investigators been observing liposomes as a dynamic membrane model system so as to correlate any structural transition as a function of protein[12,13], peptide[14,15], antibiotic[16,17], or electrolyte[18,19] interaction.

Numerous approaches have been employed in order to unequivocally observe these interactions. Nuclear magnetic resonance and electron spin resonance have been successfully applied to these simple lipid systems. Unfortunately, natural membranes are far too complex to be studied by these methods since biological membranes are highly heterogeneous. Thus, even when a spectrum is obtained, the information is of little significance

since magnetic resonance studies reveal only averaged properties of lipids. NMR and ESR techniques become useful when specifically labelled lipids are incorporated into the membrane of an organism or in simple lipid vesicles. There has been a variety of NMR and ESR labels used in lipid systems. A review published by A.G. Lee et al.[20] outlines the various applications of NMR (and some ESR) to biological membranes. The most utilized ESR label used in liposomes and biological membranes are the TEMPO[21] spin labels such as 2,2,6,6, tetramethylpiperidine-1-oxyl. In conjunction with deuterium



TEMPO (II)

labelling of fatty acid chains[22], order parameters for various lipid systems have been calculated, and the data correlate very well between the two techniques. Other NMR probes used are ^{1}H, ^{31}P & ^{13}C techniques and to a lesser extent ^{19}F NMR.

Restricting ourselves to proton magnetic resonance studies of sonicated vesicles, the data obtained from ^{1}H spectra are generally more difficult to interpret than ^{13}C data. Although the PMR spectrum of lipid vesicles has been thoroughly studied[23,24] it is difficult to observe dynamic processes in these systems without the use of a probe.

A probe must be chosen with some discretion since an overlap of resonances may render a particular phenomenon unnoticed. Secondly, the size of probe must be kept in mind since extraneous local perturbation may lead to misinterpretation of data.

A hydrophobic probe developed and utilized in this laboratory, was a fatty acid chain containing a dimethyl silyl moiety, which would meet the criterion outlined above. The dimethyl silyl resonance appears in the high-field region of the PMR spectrum not affecting any other signals, thus, serving as a useful measuring devise for any processes taking place in the hydrophobic lipoid region of the lipid vesicles. A survey of the literature indicated that only one such probe was used and was reported by Green and Salton[25]. The probe used by these authors was a detergent molecule, sodium 2,2 dimethyl-2-silapentane-5-sulfonate (DMSP). The probe was used to study the hydrophobic properties of Micrococcus Lysodeikticus membranes. These authors also reported that their probe gave no evidence of an interaction with lipid fraction preparations.

The present investigation utilizes the reporter molecule 13,13-dimethyl-13-silaheptadecanoic acid in the form of its methyl ester. The synthesis of this silicon-containing fatty acid was first reported by Bunnel and Shirley[26], and was slightly modified in this laboratory to improve product yields.

The silated fatty acid ester was codispersed and cosonicated

in lipid vesicles composed of commercial egg yolk lecithin in D_2O. A high resolution PMR spectrum of lecithin vesicles is obtained, containing a strong absorption signal attributed to the protons of the methyl groups on the choline moiety of the polar head region.

Hence incorporating our hydrophobic reporter molecule into the lipid vesicles, our system affords a method whereby any structural changes in polar and apolar regions of the lipid bilayer can be explored simultaneously, as the $N\text{-}CH_3$ resonance of lecithin serves as an indicator of the polar, hydrophilic environment. Therefore, any structural change induced by external stimuli on the liposomes can be monitored by the resonance signals of the polar and apolar reporter molecules. Change in the spectral lines can be measured as a function of relaxation rates or mobility. Relaxation rates of the choline moiety have been measured on a qualitative and quantitative basis but difficulty is encountered when these parameters were sought for the fatty acid chains since the spectra of the side chains are not suited for this type of evaluation. Thus, the need for a hydrophobic probe is apparent for the purpose of this study.

The present investigation shows that independent and simultaneous observation of lipid vesicles can be conducted by monitoring the $Si\text{-}CH_3$ and $N\text{-}CH_3$ absorption lines while the liposomes are undergoing a dynamic process. In mixed vesicles containing n-dodecane, the hydrocarbon interacts only with the hydrophobic reporter molecule while the polar moiety remains unchanged. Conversely vesicles in an aqueous NaCl

environment shows perturbation of the polar head reporter molecule with no interaction with the silyl moiety. In the presence of divalent cations such as Ca^{+2}, a more noticeable effect takes place at the polar head region with a concomitant slight perturbation of the hydrophobic probe. This type of interaction indicates a stability phenomenon[27] being translated from the polar head moiety to the fatty acid chains as the zwitterionic group binds with Ca^{+2} ions, causing a generalized stabilization of the liposomes.

The incorporation and activity of the cyclopentadecapeptide, Gramicidin S, was also explored with our system. The antibiotic is known to possess ionophoric properties and our observations also seem to suggest ion transport. Unequivocal proof could not, however, be obtained in these experiments since our method scrutinizes only the liposome and not the peptide.

As previously mentioned, liposomal studies are investigating primarily biophysical behaviour of membrane lipids. In search for a more natural biological membrane, the hydrophobic probe was successfully incorporated into the membrane of Mycoplasma laidlawii in hope of attaining membrane fragments of this micro-organism. Unfortunately, a well resolved spectrum of the membrane fragments was not obtainable even at elevated temperatures. However, lipid extraction and isolation revealed that our hydrophobic probe was indeed incorporated biosynthetically, since the NMR spectrum of the lipid fraction indicated a sharp high-field resonance attributed to the $Si-CH_3$ moiety of our probe.

From this investigation, we propose a novel membrane model system whereby a further application of proton magnetic resonance can be utilized to study biological membranes and related systems.

CHAPTER II. RESULTS AND DISCUSSION

1. SYNTHESES OF LABELLED LIPIDS

1.1 INTRODUCTION

The main objective of this project, from a synthetic point of view, was to synthesise a fatty acid containing a dimethyl silyl moiety ($-Si(CH_3)_2-$) approximately midway on the chain. The fatty acid in mind was an analogue of stearic acid (18 carbons long and fully saturated - 18:0). The silyl moiety would serve as a reporter molecule or label in the hydrophobic region of our membrane model system.

Emphasis must be placed on the ultimate goal, the membrane model, and the synthetic endeavours were undertaken solely to achieve a plausible and novel membrane model system. Consequently many of the reaction by-products were not isolated nor characterised.

Organosilicon chemistry exposed us to many classical and non-classical reactions. A review of the literature indicated two possible difficulties which we might have encountered during the synthesis:

1) The formation of tetra-alkylorganosilicon compounds is rather difficult using conventional methods and reaction conditions.

2) The unfavourable side reactions, such as polymerizations and telomerizations, decrease the yield of expected products quite substantially.

The latter factor was another reason why the side products were not isolated, stressing once again that obtaining the labelled fatty acid was a prerequisite for the model system and not a new synthetic route for the synthesis of tetra-alkylorganosilicon compounds.

Having obtained the silicon-containing fatty acid, various attempts were carried out to incorporate this labelled acid as an integral part of a lecithin molecule. (Lecithin being a necessary component of the membrane model). Hence a labelled lecithin would be at our disposal. These attempts were successful only in very small yields, which proved to be inefficient for the utilization of this labelled lecithin for the experiments which were in mind.

The synthetic routes for the labelled lecithin ranged from partial lecithin synthesis (starting from the $CdCl_2$ complex of glycerolphosphatidylcholine), and a biosynthetic approach, utilizing the fatty acid auxotrophe organism, Mycoplasma laidlawii Strain B.

All these attempts were only partially successful since low yields (5 ÷ 15%) of the labelled lecithin were obtained from the partial syntheses. Incorporation of the labelled fatty acid in the

membrane of the bacteria also proved to be successful since the dimethyl silyl resonance was clearly visible in the NMR spectrum of the extracted lipids. Classification of the lipids, however, was not carried out.

Since the labelled lecithin was not conveniently obtainable, the membrane model system had to be modified so that the same technique (the same label) could still be employed in the experiments. The modified system was comprised of unlabelled egg yolk lecithin and the ester of the labelled fatty acid codispersed and cosonicated in heavy water.

1.2 SYNTHESIS OF SILICON-CONTAINING FATTY ACID

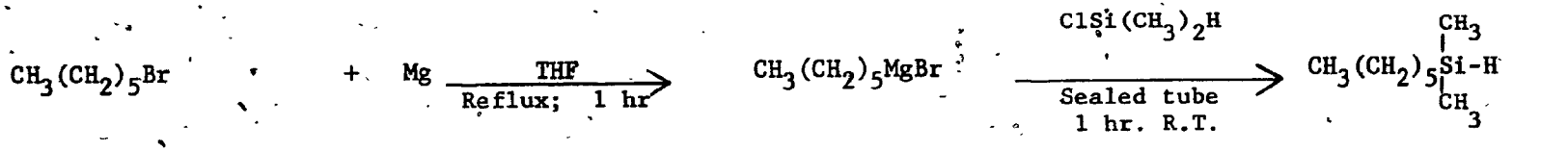
Our original aim was to place the dimethyl silyl moiety ($-Si(CH_3)_2-$) in the twelfth position from the carboxylic acid group in a chain eighteen atoms long, so as to observe any perturbation in the lipoid region. Due to synthetic difficulties as outlined in the Experimental Chapter, some classical chemical reactions did not proceed as smoothly as anticipated. Therefore, the silicon-containing fatty acid was modified to the extent that the long chain was reduced to thirteen atoms long, with the silyl moiety on the twelfth position. This approach was not successful. The next approach was to synthesize a silyl ether, thus alleviating any steric hindrance which may have been inherent in forming a Si-C bond of a tetra-alkylsilane. Silicon-oxygen bonds are easier to form and the chain conceivably could be made

as long as desired. Having had some success with this approach, a survey of the literature revealed the Bunnell and Shirley[26] synthesis of silicon-containing fatty acids with only a minor difference from the original labelled fatty acid we had in mind. The silyl moiety was in the thirteen position of a seventeen atom long chain. This synthesis was reproduced with a few minor modifications which increased the percent yield of all steps in this approach.

1.2.1 SCHEME I - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILA-STEARIC ACID

According to Scheme I, the first synthetic approach designed to obtain a "labelled" fatty acid, was to construct the chain around the dimethyl silyl moiety. Thus, dimethylhexylsilane (III) was synthesized in good yields by the action of n-hexyl Grignard reagent on dimethylchlorosilane. The coupling of (III) with the methyl ester of undecenoic acid (V) would result in an eighteen atom long silicon-containing fatty acid. Speier and coworkers[28] have shown that silanes add over a double bond in the presence of Group VIII metal catalysts, such as Platinum or Ruthenium[29]. This reaction is known as "hydrosilation". Speier has employed the catalyst chloroplatinic acid ($H_2PtCl_6.6H_2O$) in isopropanol, for these reactions and has shown that concentrations of up to 0.1 molar are most effective. Having attempted this hydrosilation reaction on a small scale, using the silane (III) and the unsaturated ester (V) with chloroplatinic acid in isopropanol, the NMR spectrum of the crude product still contained the resonances due to unsaturation. The reaction did not

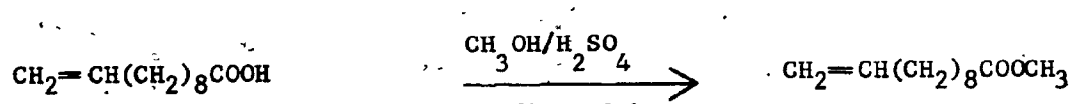
SCHEME I - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID



1 - Bromohexane (I)

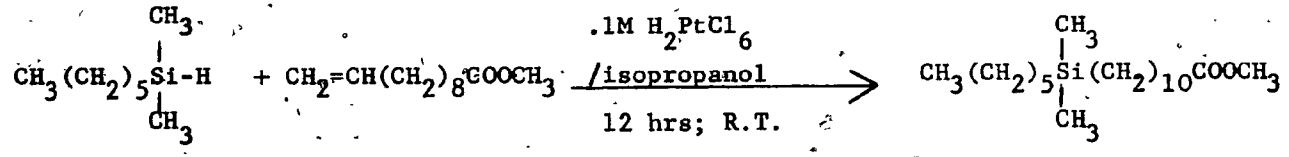
n-Hexylmagnesium bromide (II)

Dimethylhexylsilane (III)



10 - Undecenoic acid (IV)

methyl-10-undecenoate (V)



(III) (V) (N.R.)

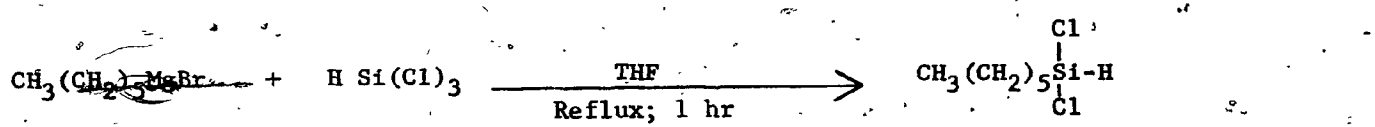
methyl-12,12-dimethyl-12-silyl stearate (VI)

proceed to any great extent since the crude product was essentially unreacted starting material. This hydrosilation reaction was carried out several times using chloroplatinic acid, then palladium-on-charcoal, and even benzoyl peroxide (since these reactions are also known to proceed via a free radical mechanism). These catalysts proved to be ineffective.

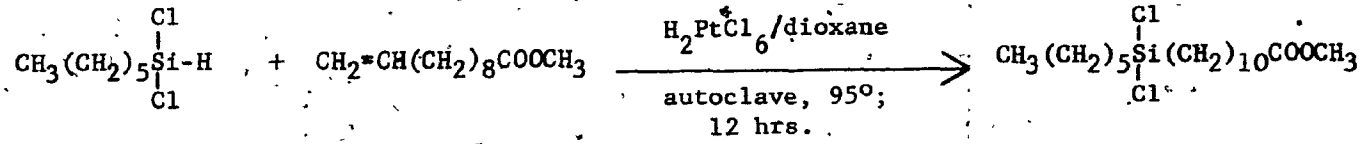
1.2.2 SCHEME II - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID

Petrov et al[30] reported that hydrosilation reactions are most effective when the silane contains a halogen i.e. $RSiHCl_2$ or R_2SiHCl. Therefore, the synthetic route had to be modified in order to carry out the hydrosilation reaction with a silane containing a halogen. Scheme II involves a reaction between n-hexyl Grignard reagent and trichlorosilane (VII) to yield the corresponding dichlorohexylsilane (VIII). The product, (VIII), was used in a crude state since the halogens may have hydrolyzed during purification. The hydrosilation reaction was carried out by reacting (VIII) with the unsaturated ester (V) to yield the expected product (IX) which was not isolated. Methyl-12,12-dichloro-12-silastearate (IX) was then reacted with a large excess of methyl magnesium iodide (X) to yield methyl-12,12-dimethyl-12-silastearate (VI), but upon attempted isolation of the product by distillation, no fraction yielded the corresponding fatty acid ester. Multiple splitting was observed in the silyl region of the NMR spectrum as well as concomitant reduction of the methyl ester resonance, probably due to carbinol formation by attack of excess Grignard reagent on the carboxyl group. Scheme II proved to be a fruitless attempt since the reaction by-

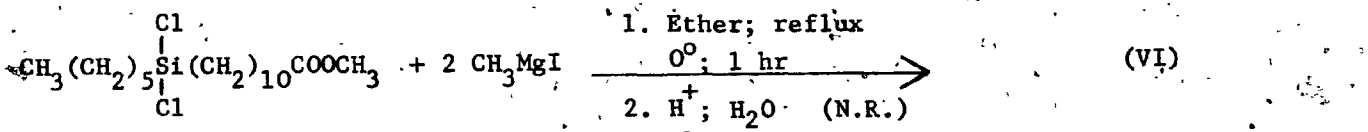
SCHEME II - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID



(II) Trichlorosilane (VII) Dichlorohexylsilane (VIII)



(VIII) (V) methyl-12,12-dichloro-12-silastearate (IX)



(IX) methyl magnesium iodide (X) (VI)

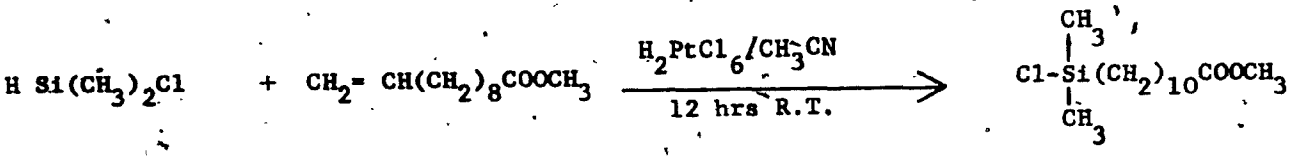
products were carried from the previous reaction thereby increasing the probability of undesired products.

Scheme II was attempted once more using a t-butyl ester instead of the methyl ester, in order to make the carboxyl group more sterically hindered. Upon distillation, the contents of the flask polymerized into a gel during heating. This approach was abandoned.

1.2.3 SCHEME III - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID

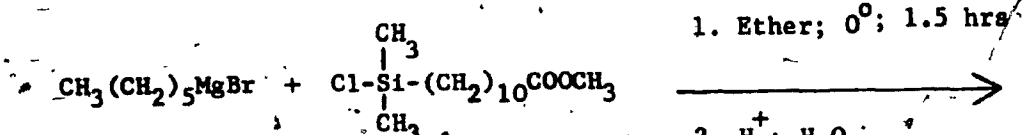
Another synthetic route was attempted, and this new approach was simply a variation of Schemes I and II. Therefore, according to Scheme III, the hydrosilation was first carried out on the methyl ester (VI) with dimethylchlorosilane to yield methyl-11-dimethyl-chloro-sila-undecanoate (XI), in the presence of H_2PtCl_6 in acetonitrile. Acetonitrile was used as a mediating solvent since hydrosilation reactions are known to also be catalyzed by base[30]. The hydrosilation product XI, was obtained in good yields and was purified as described in the Experimental Chapter. The halogen of product XI, could now be utilized as a reactive site for the n-hexyl Grignard reagent (II) to yield the desired product (VI). After the reaction between XI and II was carried out, the distillate was collected which crystallized upon cooling and accounted for 53% of theoretical yield. NMR of this product indicated no terminal CH_3 resonance. Mass spectroscopy exhibited a molecular ion at 530 m/e which was too high for the desired product. The product was thought to be a siloxane formed during the reaction and may have been formed as follows:

SCHEME III - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID



Dimethylchlorosilane (V)

methyl-12-chloro-12,12-dimethyl -12-silaundecanoate (XI)

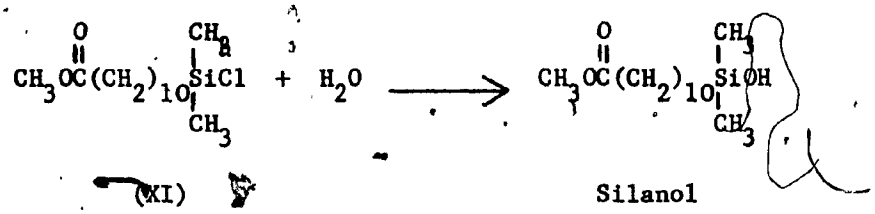


(II) (XI) (N.R.) (VI)

The final reaction involving the Grignard reagent (II) was carried out at low temperature so that carbinol formation would be kept at a minimum. But upon workup, a large fraction of n-hexane was collected indicating that the Grignard reagent did not react to any great extent at lower temperature. The hexane was probably formed during workup when the Grignard reagent is destroyed by ice/water and this reaction can be expressed by the following equation:

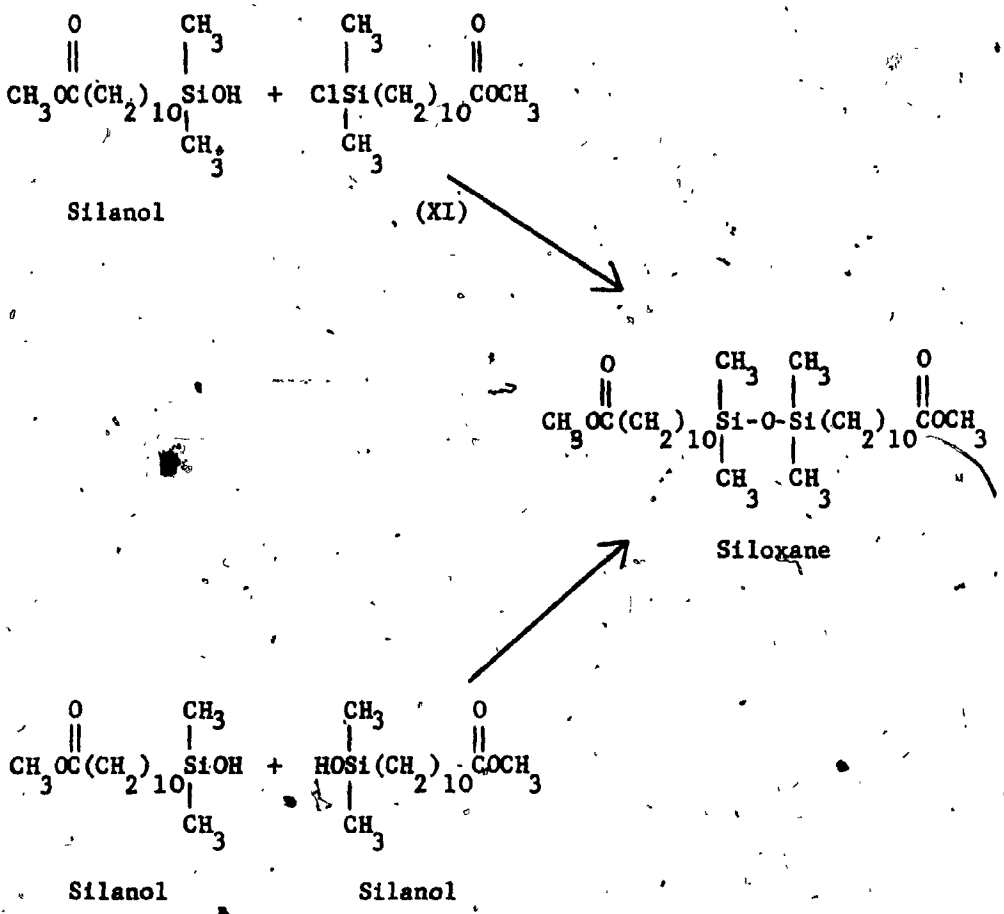
$$\underset{\text{(II)}}{C_6H_{13}MgBr} + H_2O \longrightarrow \underset{\text{n-Hexane}}{C_6H_{14}} + Mg(OH)Br$$

This reaction accounts for the n-hexane formation. But if the Grignard reagent did not react, the chlorosilane (XI) would still be present in the reaction mixture. Thus the chlorosilane in contact with ice/water would hydrolyse to the corresponding silanol as follows:



(XI) Silanol

The silanol being a very reactive species, could have easily reacted with another molecule of XI or another silanol molecule to form the more stable siloxane, as shown below:



Silanol (XI)

Siloxane

Silanol Silanol

The siloxane being a symetrical molecule, exhibited a 1:2 proton ratio for OCH_3 and $Si(CH_3)_2$ resonances, on the NMR spectrum of this product. Furthermore, no terminal-CH_3 resonance was observed and the methylene signal integrated for 20.3 H which is much lower than the integration count for the expected product (VI). The mass spectrum confirmed the siloxane formation yielding a molecular ion at 530 m/e, the molecular weight of the siloxane.

The problems encountered in Scheme III were twofold:

(1) Two reactive sites were present, which may be attacked by the Grignard reagent at. $0^{o}C$ (carbinol formation).

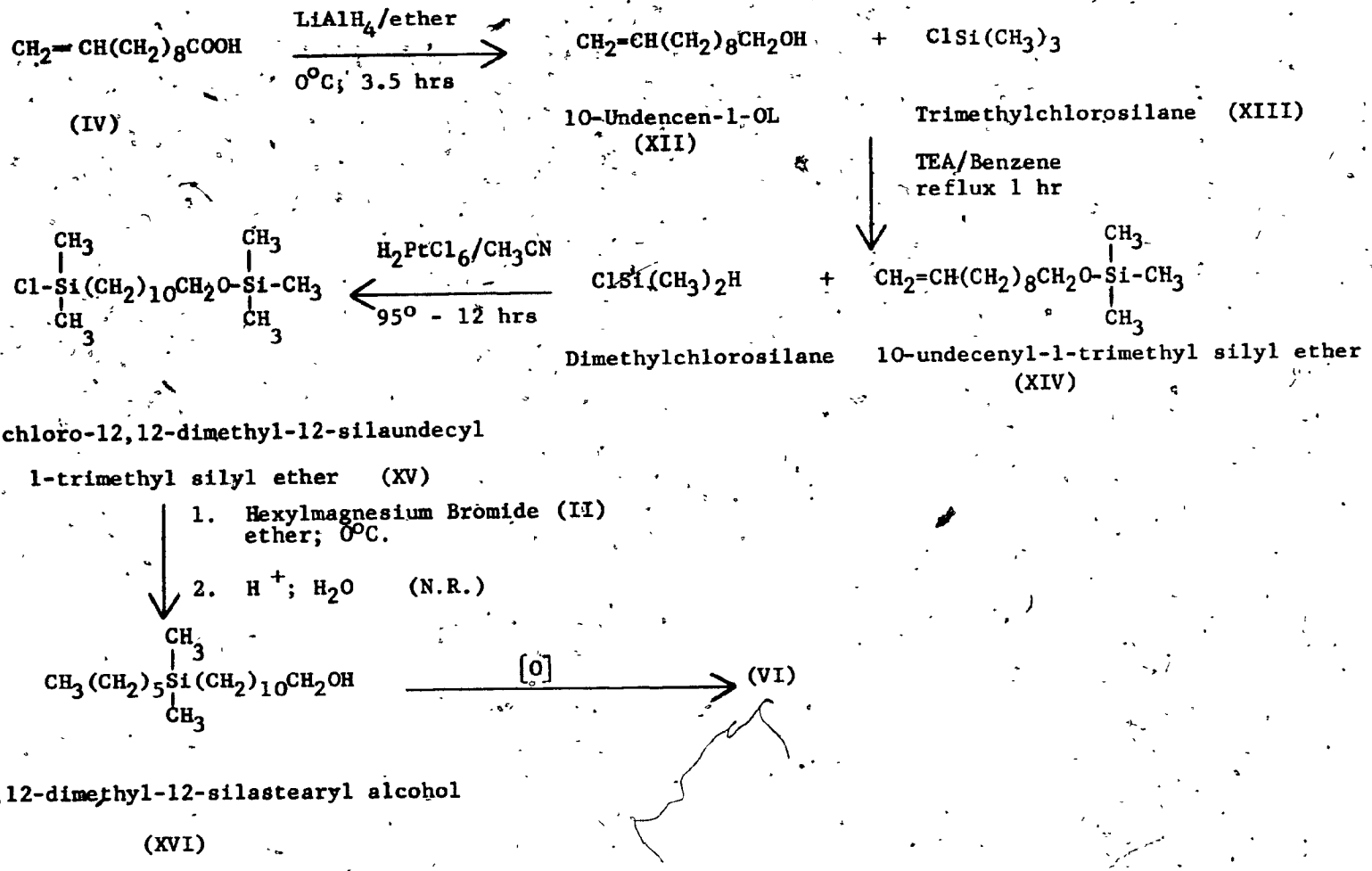
(2) At lower temperature, the Grignard reagent would react sluggishly which resulted in the siloxane formation with the unreacted starting material upon workup.

1.2.4 SCHEME IV - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID

As stated above, two competitive reactive sites were present in XI. By reducing the carboxyl group to the corresponding alcohol, which may then be protected, this particular problem may be alleviated. Furthermore, reaction conditions could probably be a little more vigorous so as to drive the reaction to completion. Once the competitive reaction was carried out, the protected alcohol could be converted to the free hydroxyl which could subsequently be oxidized to the acid; thus, obtaining the desired product (VI). The synthetic approach in Scheme IV outlines the intended route.

The acid (IV) was reduced to the alcohol (XII) by the action of lithium aluminum hydride. This reaction was carried out several times to obtain only moderate yields. Since the unsaturated alcohol was commercially available, the synthesis was commenced from this point.

SCHEME IV - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID



The active hydrogen of the alcohol was replaced by a trimethyl silyl moiety by reacting (XII) with trimethylchlorosilane in benzene and triethylamine as acid binder. The silyl ether is a versatile protecting group and can be easily cleaved off by the addition of water, dilute acid or base, to furnish the alcohol once again. The protected alcohol (XIV) was then subjected to the hydrosilation reaction with dimethylchlorosilane in the presence of H_2PtCl_6 in acetonitrile. The product (XV), 12-chloro-12, 12-dimethyl-12-silaundecyl-1-trimethyl silyl ether, was allowed to react with n-hexyl magnesium bromide (II). After workup, the reaction mixture was distilled and the first fraction collected ($36\text{-}39^{\circ}$/0.8 mm) was identified as n-hexyl alcohol implying that the Grignard reagent had decomposed. Further distillation yielded a product that contained no terminal-CH_3 resonance on the NMR spectrum, and a multiplet in the silyl region, probably due to a polymeric product formed during reaction.

To rationalize the decomposition of the n-hexyl magnesium bromide, one must keep in mind the limitations of the Grignard reagent. Grignard reagents are known to decompose in the presence of any compound containing a hydrogen atom bound to an electronegative atom such as oxygen. Presumably no active hydrogen was present in compound (XV), but the presence of a Si-O bond may have been responsible for the decomposition. Silicon is more electropositive than carbon or even hydrogen (electronegativities : Si=1.8; C=2.5; H=2.1). Conceivably, the Si-O bond may render the silicon atom acidic enough to decompose the Grignard reagent.

Therefore, this approach was shelved while a modified scheme was attempted.

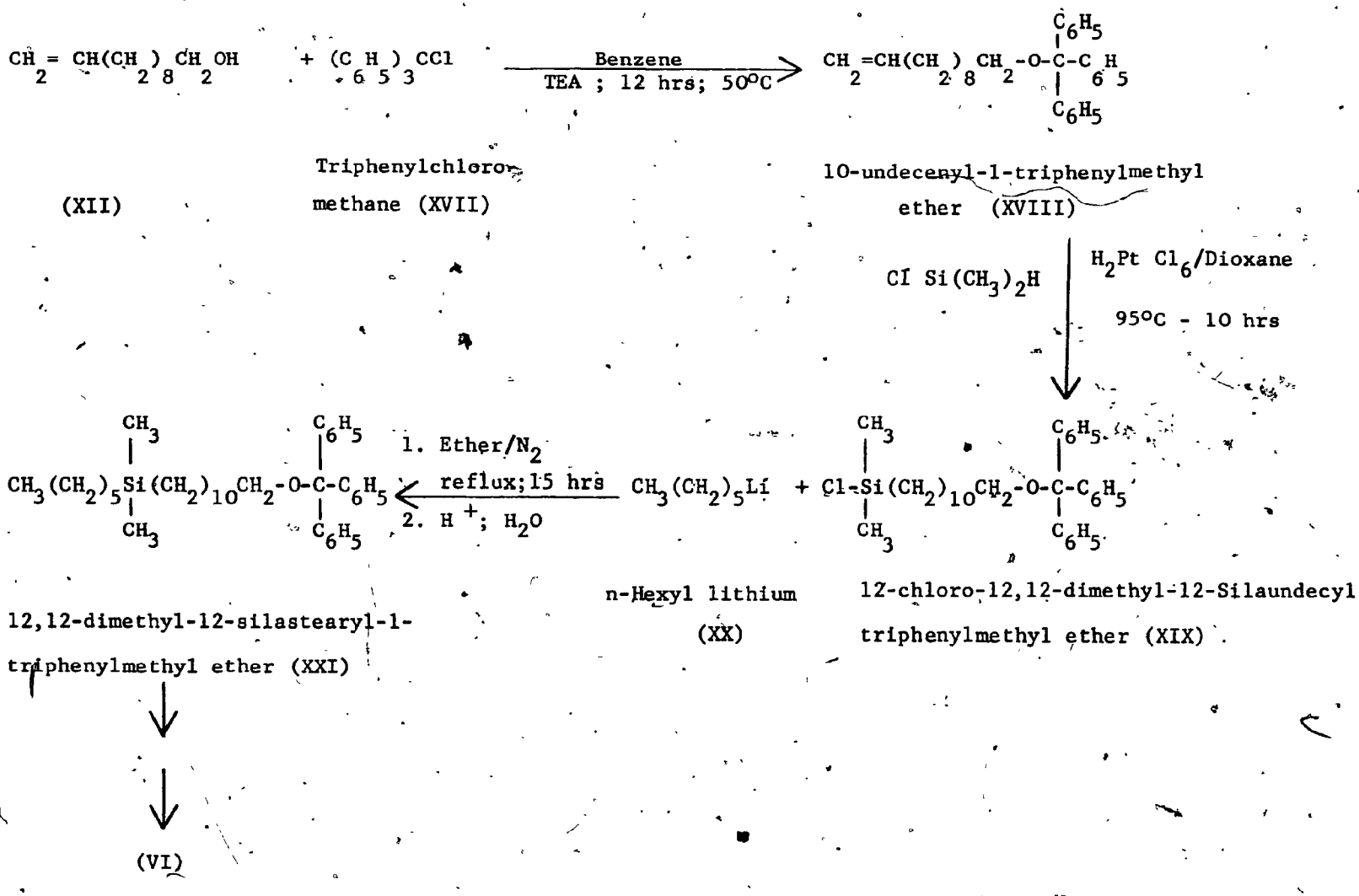
1.2.5 SCHEME IV A - MODIFIED APPROACH

Since the silyl protecting group was not as effective as expected, an alternate reaction scheme was devised. The first modification was the use of the trityl protecting group i.e. triphenylmethyl ether. The trityl group can be easily cleaved off by shaking with aqueous HBr at room temperature to restore the free alcohol. The second modification was the use of an organolithium reagent since these compounds are slightly more reactive than the Grignard reagents.

Therefore, 10-undecen-1-ol (XII) was reacted with trityl chloride in benzene and in the presence of an acid binder. The protected alcohol was used as a crude since the NMR spectrum of this crude product was satisfactory, and since decomposition may have resulted if distilled. The hydrosilation reaction was carried out by stirring the protected alcohol with dimethylchlorosilane in the presence of H_2PtCl_6 catalyst for 10 hrs at 95°C. The resultant crude product was analyzed by NMR and no unsaturation resonances were observed implying that the reaction had proceeded.

This reaction was carried out again under different reaction conditions (5 days at room temperature) and upon workup, a white crystalline precipitate was isolated. These crystals were identified as tritane, or triphenylmethane, (by mixed melting point and NMR) and constituted an 88.6% yield. This observation indicated that the protecting group had been cleaved. A possible reaction which may have resulted in

SCHEME IV A - MODIFIED APPROACH



tritane formation can be rationalized as follows:

The reaction mixture for hydrosilations requires chloroplatinic acid as a catalyst, whereby the platinum in this compound is reduced to metallic platinum. Prolonged exposure of the trityl ether to metallic platinum may have caused a hydrogenolysis resulting in tritane formation.

NMR of this hydrosilation product yielded a silicon-containing compound with virtually no trityl resonance (integration 1.4 H cf. Chapter III, Section 1.5.2).

The reaction with hexyl lithium (XX) was carried out with the hydrosilation products obtained by short exposures to the chloroplatinic acid catalyst, but upon workup, the crude product was unsatisfactory thus it was not purified. NMR spectrum exhibited a broad silyl resonance and a small trityl signal. Therefore, this approach was abandoned due to reasons outlined in the last two reactions.

Up to this point, Schemes I - IV demonstrated the difficulties encountered in attempting to synthesize a tetra-alkylsilane, containing two long chains. Therefore, the final product was modified and instead of methyl-12,12-dimethyl-12-silastearate (VI), the synthesis of short chained silicon-containing fatty acids was attempted.

1.2.6 SCHEME V - ATTEMPTED SYNTHESIS OF SHORT CHAINED SILICON-CONTAINING FATTY ACIDS

Scheme V outlines the synthesis of three possible short chained fatty acids containing the dimethyl silyl moiety in the twelveth position.

OPTION 1

Having obtained methyl-12-chloro-12,12-dimethyl-12-sila-undecanoate (XI) in good yields and in a fairly pure state, a reaction with methyl magnesium iodide was carried out utilizing the halogen on the silyl moiety as the reactive site. This would result in a compound containing a terminal trimethyl silyl moiety which would exhibit a stronger signal in the NMR spectrum.

The product, methyl-12,12-dimethyl-12-silatridecanoate (XXII), obtained by distillation from the crude reaction mixture contained a doublet in the silyl region of the NMR spectrum. This is certainly not satisfactory since only one resonance is desired for the silyl moiety.

The problem of having two reactive sites (as discussed in Scheme III) was inherent in the synthetic route for product (XXII). The Grignard reaction was carried out at lower temperature ($-18^{o}C$) since methyl magnesium iodide is considerably more reactive than the n-hexyl Grignard reagent. Nonetheless some carbinol formation was evident thus this approach was not pursued.

SCHEME V - ATTEMPTED SYNTHESIS OF SHORT-CHAINED SILICON-CONTAINING FATTY ACIDS

3 OPTIONS:

(1). CH_3MgI + $Cl\text{-}Si(CH_3)_2(CH_2)_{10}COOCH_3$ (XI) — 1. Ether; -18°C; 2 hrs; 2. H^+; H_2O → $CH_3\text{-}Si(CH_3)_2(CH_2)_{10}COOCH_3$

(X)

methyl-12,12-dimethyl-12-silatridecanoate (XXII)

(2). $CH_2=CH(CH_2)_9\text{-}O\text{-}Si(CH_3)_2\text{-}CH_3$ (XIV) + $ClSi(CH_3)_2H$ (DMCS) — H_2PtCl_6/Dioxane; 95°; 8 hrs → $CH_3\text{-}Si(CH_3)_2\text{-}O(CH_2)_{10}CH(CH_3)\text{-}Si(CH_3)_2\text{-}Cl$ (XV)

XV — 1. CH_3MgI, ether, 1.5 hrs; 2. H^+; H_2O → $HOCH_2(CH_2)_9CH_2Si(CH_3)_2\text{-}CH_3$

12,12-dimethyl-12-silatridecan-1-OL XXIII

$HOCH_2(CH_2)_9CH_2Si(CH_3)_2\text{-}CH_3$ — [O] → $HOOC(CH_2)_{10}Si(CH_3)_2\text{-}CH_3$

12,12 dimethyl-12-silatridecanoic acid XXIV

(3) $CH_3OOC(CH_2)_8CH=CH_2$ (V) + $H\,Si(CH_3)_2CH_2CH_3$ (Dimethylethylsilane XXV) — $h\nu$ or peroxide (N.R.) → $CH_3OOC(CH_2)_{10}Si(CH_3)_2\text{-}CH_2CH_3$

methyl-12,12-dimethyl-12-silatetradecanoate

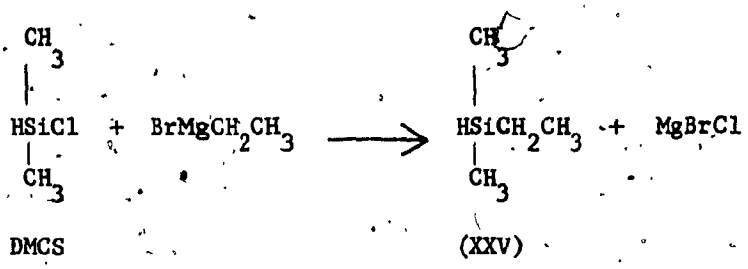
OPTION 2

A hydrosilation reaction was carried out with the organosilicon alkoxide (XIV) which was obtained conveniently from the unsaturated alcohol and dimethylchlorosilane. The resultant crude product, (XV), was reacted with methyl Grignard reagent - again an attempt to utilize the higher reactivity of the methyl magnesium iodide as opposed to that of the n-hexyl magnesium bromide. Upon workup, the crude product 12,12-dimethyl-12-silatridecan-1-ol (XXIII) was analyzed by NMR and a triplet and singlet were observed in the silyl region implying that attack by the Grignard reagent may have taken place at the ether oxygen once again (cf Scheme IV). This approach was abandoned.

OPTION 3

Hydrosilation reactions are known to take place with silanes which do not contain halogens but these reactions are catalyzed either by UV irradiation[31,32], or by the use of peroxides[33]. Not being equipped for any photochemical reaction, the latter catalyst was used in the form of benzoyl peroxide. Anti-Markovnikov additions over double bonds are usually catalyzed by benzoyl peroxide.

Starting from materials at hand, dimethylethylsilane (XXV) was synthesized by the action of ethyl magnesium bromide and dimethylchlorosilane, as follows:



DMCS (XXV)

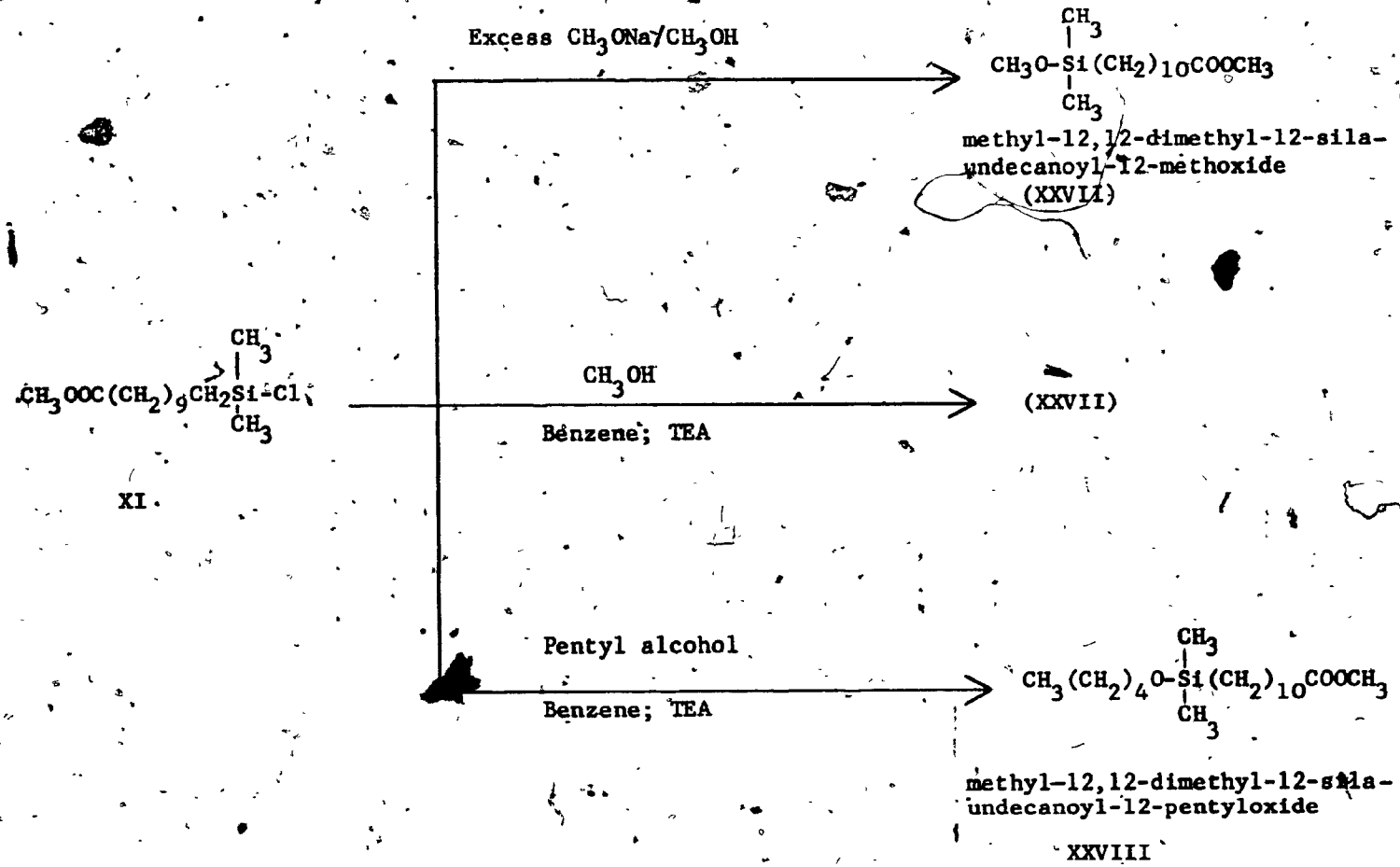
The reaction proceeded smoothly but the high volatility of dimethylethyl-silane (XXV) resulted in low yields (21%) due to loss by distillation. The silane (XXV) was confirmed by NMR and IR[34].

The reaction conditions for the hydrosilation reaction catalyzed by peroxides were described by G.M. Gadsby[33] who reported a 96% yield in the synthesis of triphenyl-silylundecanoic acid by reacting triphenylsilane with undecenoic acid in the presence of benzoyl peroxide catalyst. These conditions were reproduced when methyl-10-undecenoate (V) and dimethylethyl-silane were mixed together in the presence of recrystallized benzoyl peroxide. Upon workup, unreacted methyl-10-undecenoate was obtained. Repetition of this reaction under various conditions yielded the same result. Gadsby utilized a triarylsilane, which may be a more reactive species than the trialkylsilane due to the inductive effect of the phenyl moieties. This synthetic route was fruitless and thus not pursued.

1.2.7 SCHEME VI - ATTEMPTED SYNTHESIS OF SHORT CHAINED SILICON-CONTAINING FATTY ACIDS

The short chained fatty acid approach was not as efficient as was hoped, substantiating the difficulty of forming tetra-alkylsilanes. This difficulty may be circumvented by making an additional modification in the

SCHEME VI - ATTEMPTED SYNTHESIS OF SHORT-CHAINED SILICON-CONTAINING FATTY ACIDS



final product. This modification would facilitate the synthesis and the silyl moiety would still be present at the twelveth position.

Instead of synthesizing the tetra-alkylsilane, an attempt to synthesize a trialkyl silyl ether (organosilicon alkoxide) was undertaken. Thus the final product would contain a silicon-oxygen bond which may alleviate any steric effects that were encountered in forming the silicon-carbon bond.

The starting material for the silyl alkoxide was compound (XI) thus utilizing the halogen as a reactive site in a Williamson-type ether synthesis. The first attempt then was to react (XI) with sodium methoxide in methanol to obtain compound (XXVII), methyl-12,12-dimethyl-12-sila-undecanoyl-12-methoxide. The product was analyzed by NMR and two $-OCH_3$ resonances were observed at 3.43 and 3.20 ppm. Therefore, a reaction took place but did not proceed to completion since only 33% of the product (as calculated by integration) was evident.

The next approach was to react (XI) with methanol in benzene in the presence of triethylamine as an acid binder. Upon workup, the crude product proved to be the correct compound (XXVII) verified by NMR. The amine salt was isolated quantitatively indicating the reaction had proceeded to completion.

In order to obtain the free acid, the methyl ester of product (XXVII) must be hydrolyzed. An acid-catalyzed hydrolysis was carried out and upon

isolation of the crude product, NMR spectrum indicated that both the methyl ester and the methoxyl moiety had been hydrolysed. Only a very small resonance for the methoxyl group was evident at 3.5 ppm but the integration count was 0.25 H (cf Chapter III Section 1.7.3).

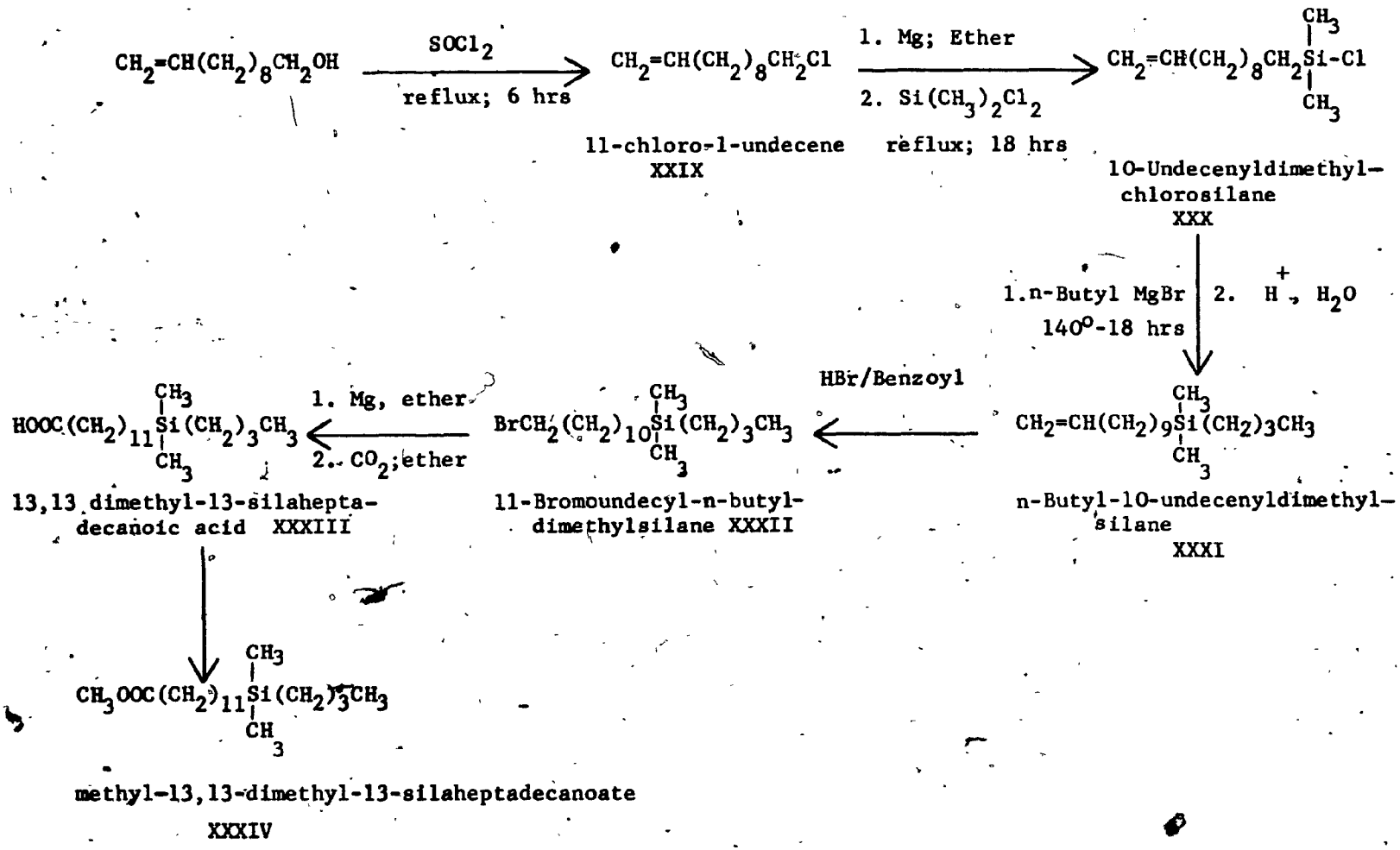
Since hydrolysis of the alkoxide is governed by the length of chain, the next approach was to synthesize a pentyloxy silane in order to prevent rapid hydrolysis of the alkoxide. Therefore, the pentyloxy silyl ether (XXVIII) was synthesized in the same fashion as described above. The crude product exhibited a correct NMR spectrum. The hydrolysis was carried out in the same manner and analysis of the product indicated that the methyl ester had hydrolysed with only slight hydrolysis of the pentyloxy silane. The silyl resonance indicated that some hydrolysis had taken place since a singlet (non hydrolysed product) and a doublet (polymeric hydrolysis product) was evident.

Scheme VI was encouraging since the reactions were proceeding as expected with moderate yields. This approach was curtailed since a further investigation of the literature discovered a synthetic route for the synthesis of silicon-containing fatty acids.

1.2.8 SCHEME VII - SYNTHESIS OF 13,13-DIMETHYL-13-SILAHEPTADECANOIC ACID[26]

Bunnel and Shirley[26] describe a synthesis of a silated fatty acid where the dimethyl silyl moiety is placed on position thirteen of a seventeen atom long chain. The synthetic route utilizes classical

SCHEME VII - SYNTHESIS OF 13,13-DIMETHYL-13-SILAHEPTADECANOIC ACID



reactions since the hydrosilation reaction, using H_2PtCl_6, was not known at the time of publication. The difficulty in obtaining a tetra-alkylsilane was overcome by using only one functional group at a time, thus eliminating the possibility of competitive reactions. Secondly, the reaction conditions are much more vigorous thus forcing the reaction to take place and increasing product yield. The authors report that all yields in the six step synthesis were above 50%. Furthermore, the authors report several synthetic approaches which were unsuccessful in producing the final product and in doing so describe a siloxane ($R-(CH_3)_2Si-O-Si(CH_3)_2-R$) formation which is similar to the one which was obtained and described in Scheme III. The reaction sequences which were attempted by Bunnel and Shirley were similar to the attempts which were carried out in this laboratory and they reported that these reactions were ineffective in obtaining the end product, in agreement with our experience.

Thus, employing their synthetic scheme (with minor modifications), we were able to improve product yields considerably. In the five-step synthesis we report yields above 62% in all steps.

Scheme VII outlines the reaction sequence described by Bunnel and Shirley and the modifications we employed will be discussed where applicable.

Since the unsaturated alcohol, 10-undecen-1-ol (XII) was commercially available, the synthesis was commenced from this point. The conversion of (XII) to the corresponding alkyl chloride was reported to have been

carried out with excess thionyl chloride, in chloroform by refluxing for 8 hrs to yield 78% of pure product. In our case, the alcohol (XII) was reacted with thionyl chloride (distilled from linseed oil) and the latter reagent was used as solvent, therefore, in a very large excess. Our reaction time was 6 hrs and we obtained 92% pure product (XXIX), after distillation. Workup and purification of 11-chloro-1-undecene (XXIX) was carried out as described.

The alkyl halide (XXIX) was used as the precursor for the formation of the corresponding Grignard reagent. Once formed in the usual fashion, the Grignard reagent was reacted with dimethyldichlorosilane thus providing the product, 10-undecenyldimethylchlorosilane (XXX) in a 72% yield.

The product (XXX) was exposed to an excess of n-butyl magnesium bromide whereby the Grignard reagent would attack the halide on the silicon atom and thus form the long unsaturated silicon-containing chain, n-butyl-10-undecenyldimethylsilane (XXXI). This product was obtained in 74.5% yield. This reaction produced the tetra-alkylsilane since only one functional group was present and the reaction conditions were more vigorous (140°C for 18 hrs) than those employed in previous reaction schemes (-18 to 0°C for 2 to 12 hrs).

The next step in the synthesis utilized the double bond of product (XXXI) as a functional group. Thus by the use of benzoyl peroxide, an anti-Markovnikov addition of hydrogen bromide was taking place over the double

bond. The reaction product is (XXXII), 11-bromoundecyl-n-butyldimethylsilane, a primary alkyl bromide. The second alteration was in the work-up, whereby the washing with 5% sodium hydroxide was replaced by 5% potassium hydroxide. This washing removed the benzoic acid, as its corresponding salt, formed in the reaction and since potassium benzoate is more water soluble than sodium benzoate, it was felt that this was a more efficient method for the removal of benzoic acid. The product (XXXII) was obtained in 62.5% yield.

The alkyl bromide (XXXII) formed, was now used as a precursor for the formation of the Grignard reagent. Due to the length of the alkyl group, the Grignard formation was rather difficult. The reaction did not start spontaneously upon heating nor did it commence when a crystal of iodine was added. The reaction was initiated by grinding the alkyl bromide with magnesium in ether in a test tube and then adding it to the bulk of the reaction mixture. The authors report that the reaction was initiated by the addition of the more reactive n-butyl bromide to the magnesium and once the reaction was underway, the 11-bromoundecyl-n-butyldimethylsilane was added.

Once the Grignard reagent was formed, it was added to a slurry of dry ice and ether, described in Chapter III, Section 1.8.5. The slurry was prepared, and the reaction was carried out in a glove bag under an atmosphere of dry nitrogen. Anhydrous conditions must be maintained during the reaction so as to minimize any decomposition of the Grignard reagent by moisture and thus optimize the reaction conditions The corresponding

acid (XXXIII), 13,13-dimethyl-13-silahexadecanoic acid was obtained in 66% yield.

The acid (XXXIII) was further purified by converting to its barium salt and decomposing it back to the free acid once again after repeated washing of the salt with acetone.

The acid was converted to its methyl ester (XXXIV) by the action of excess diazomethane in ether. The ester was obtained in 80.5% yield.

The acid chloride and the acid anhydride were also formed by conventional methods. The acid (XXXIII) was reacted with oxalyl chloride in order to obtain the acid chloride. Oxalyl chloride was preferred over thionyl chloride[35] since the reaction conditions are not as vigorous and only slight decomposition was evident. With thionyl chloride, the resultant reaction mixture undergoes a drastic colour change to dark brown upon refluxing.

The acid anhydride was prepared in the usual manner by reacting the acid chloride with free acid (XXXIII) in the presence of an acid binder, triethylamine or pyridine.

1.3. PARTIAL LECITHIN SYNTHESIS

Lecithin synthesis starting from glycerophosphorylcholine (G.P.C.) or its cadmium chloride complex is known as the partial lecithin synthesis. Although lecithin can be obtained by total synthesis, incorporation of expensive or labile fatty acids render the total synthesis undesirable due to the number of steps involved and the poor yields obtained. Thus these particular lecithins can usually be conveniently obtained by the partial lecithin synthesis.

G.P.C. or (G.P.C.) $CdCl_2$ complex have been prepared by the deacylation of egg-yolk lecithin which can be purchased commercially[36,37]. Therefore, using Chadha's method in this laboratory, egg-yolk lecithin was deacylated in ether by utilization of tetrabutylammoniumhydroxide in methanol. The G.P.C. is the only insoluble product formed and can be isolated and converted to the cadmium chloride complex quite easily in 55% yield. Since free G.P.C. is extremely hygroscopic, a convenient method of storing this compound is by forming the cadmium chloride complex. The (G.P.C.) $CdCl_2$ can be readily converted to free G.P.C. by passing a solution of the cadmium chloride adduct through a mixed bed of ion exchange resins to obtain quantitative yields.

There have been numerous lecithin syntheses described[38-41], where identical saturated or unsaturated fatty acids were reacylated to free G.P.C. or its $CdCl_2$ adduct to form the corresponding 1,2 diacyl sn-glycero-3-phosphorylcholines or lecithins. Therefore, having obtained

the silicon-containing fatty acid and the (G.P.C.) $CdCl_2$ complex, attempts to synthesise the "labelled" lecithin were undertaken.

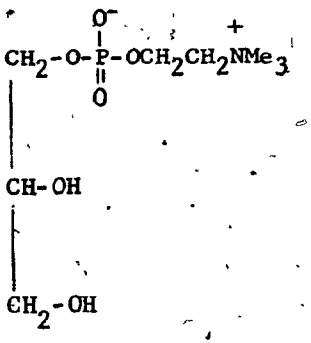
1.3.1 "LABELLED" LECITHIN SYNTHESIS BY E.C. ROBLES METHOD

Of all the partial lecithin syntheses described, the method reported by E. Cubero Robles was the most attractive since the product was reported to be obtained in 81.4% with no apparent side products formed. The reacylation is performed by heating a mixture of free G.P.C., fatty acid anhydride, and the potassium salt of the fatty acid in the molar ratio of 1:4:2 in vacuum for 48 hrs at 80°C. Therefore, free G.P.C. was mixed with "labelled" fatty acid potassium salt, (formed in situ by dissolving G.P.C., fatty acid and potassium hydroxide in methanol and the solvent evaporated under vacuum). The labelled anhydride was added and the reaction mixture heated in vacuo as described. Upon workup and analysis of all fractions obtained, no lecithin formation was evident. This method was repeated several times to yield the same results. It was checked by using stearic acid and 65% of crude lecithin was obtained.

1.3.2 "LABELLED" LECITHIN SYNTHESIS BY BAER-BUCHNEA METHOD

The Baer-Buchnea synthesis was the next method attempted in acquiring the "labelled" lecithin. This method is conducted by mixing G.P.C. in its $CdCl_2$ complex, and fatty acid chloride in 7-10 fold molar ratio excess, in the presence of pyridine. The acylation is carried out dry ethanol-free chloroform with glass beads for 30 minutes at 0°C, followed by an additional 2 hrs at room temperature. The lecithins, usually purified

ATTEMPTED PARTIAL LECITHIN SYNTHESIS - E.C. ROBLES METHOD[40]



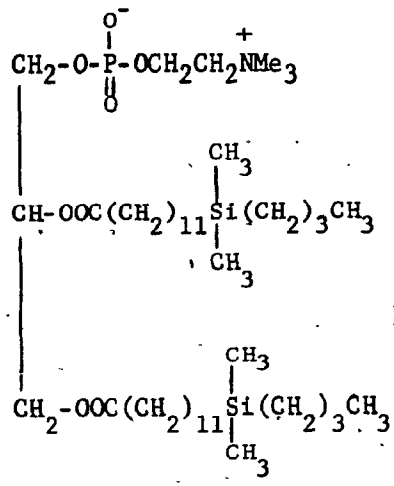
Glycerophosphorylcholine (GPC)

+ 2 $CH_3(CH_2)_3Si(CH_3)_2(CH_2)_{11}COOH$

(XXXIII)

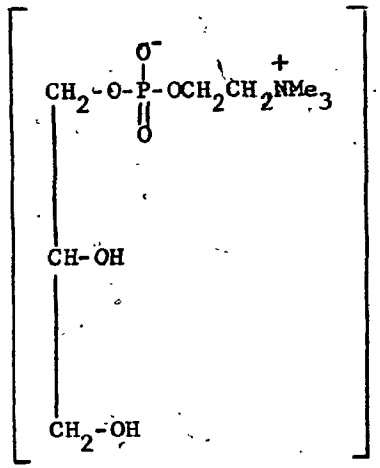
+ 4 (13,13-dimethyl-13-silaheptadecanoic anhydride)

$\xrightarrow[48\ hrs]{80^\circ C,\ in\ vacuo}$



Di-(13,13-dimethyl-13-silaheptadecanoyl) lecithin

ATTEMPTED PARTIAL LECITHIN SYNTHESIS - BAER-BUCHNEA METHOD



GPC - Cadmium Chloride adduct

$CdCl_2$

+ 10 $CH_3(CH_2)_3Si(CH_3)_2(CH_2)_{11}COCl$

13,13-dimethyl-13-sila-heptadecanoyl chloride

Pyridine/Glass beads → Di-(13,13-dimethyl-13-sila-heptadecanoyl) lecithin

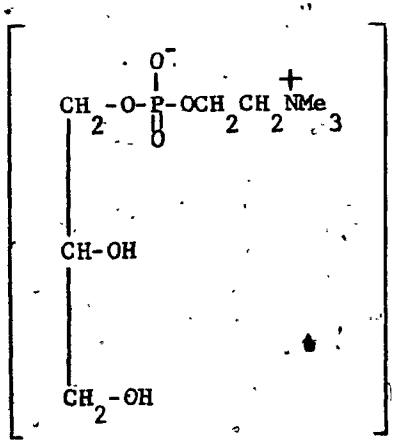
1. 30 min at 0°C
2. 2 hrs at R.T.

by elution through a mixed bed of ion exchange resins followed by elution through a silicic column are reported to range from 50-55% with the additional drawback that by-products are also formed. These by-products were indentified as cyclic lysolecithins, i.e. acyl-chlorodeoxy-glycero-phosphoryl-cholines[42]. Therefore, the reactants, (G.P.C.)$CdCl_2$, 13,13-dimethyl-13-silaheptadecanoyl chloride, and anhydrous pyridine were mixed together in dry ethanol-free chloroform for 30 minutes at 0°C followed by vigorous stirring at room temperature for an additional 2 hrs. Upon work-up, only 10.4% of crude labelled lecithin was formed. This method was attempted several times to yield between 5-15% yield of crude lecithin. This method proved to be inefficient since large amounts of the "labelled" lecithin were required. Therefore, another synthesis was attempted.

1.3.3 "LABELLED" LECITHIN SYNTHESIS BY KATE'S METHOD

Kates et al[41] reported a partial lecithin synthesis by reacting $(G.P.C.)_2(CdCl_2)_3$ with a mixed anhydride, formed in situ by mixing the free fatty acid with trifluoroacetic anhydride, in the presence of triethyl-amine. The reaction mixture was stirred for 48 hrs at room temperature under anhydrous condtions. The mixture was worked up and product purified by forming the methyl ester of the carboxylic acid, upon addition of methanol and, 0.01M HCl solution to free the cadmium chloride complex (no ion exchange is necessary). The solution was diluted with chloroform and eluted successively through a silicic column with chlorpform, chloroform-methanol 9:1 (V/V), 1:1 (V/V), and 1:4 (V/V) mixtures. The lecithin was observed in the 1:1 (V/V) eluant and the authors report a 24% yield.

ATTEMPTED PARTIAL LECITHIN SYNTHESIS - KATE'S METHOD



GPC $CdCl_2$

$CdCl_2$

+ 160 $CH_3(CH_2)_3Si(CH_3)_2(CH_2)_{11}COOH$

(XXXIII)

+ Trifluoroacetic anhydride

Triethylamine

48 hrs at R.T.

Di-(13,13-dimethyl-13-silaheptadecanoyl) lecithin

II - 41 A

This methodology was adapted to our system utilizing the cadmium chloride adduct of G.P.C., 13,13,dimethyl-13-silaheptadecanoic acid (XXXIII), trifluoroacetic anhydride, and anhydrous triethylamine. The reactants were mixed together and stirred at room temperature for 48 hrs. The workup and isolation was conducted in the same manner as described. The reaction mixture was passed through a silicic column and the eluates were monitored by NMR. Fraction I eluted by chloroform alone, exhibited a typical "labelled" fatty acid NMR spectrum, including a small resonance from the $-OCH_3$ of the esters formed. Fraction II eluted by chloroform-methanol 9:1 (V/V), contained primarily labelled fatty acid with a very small $N(CH_3)_3$ resonance which appeared as a doublet. A multiplet was also observed in the phenyl region but this cannot be accounted for. Fraction III eluted by chloroform-methanol 1:1 (V/V), contained a larger $N(CH_3)_3$ doublet, with a corresponding larger multiplet at 7.4 ppm. Both of these resonances integrated for the same number of protons (8.4 H). The methylene resonance integrated for 84 protons instead of 44 protons implying that some labelled fatty acid is still present in this third fraction. This fraction yielded only 7% of the crude lecithin. Fraction IV eluted by chloroform-methanol 1:4 (V/V) yielded a similar spectrum as Fraction III. The last two fractions were dark green in colour.

The method was repeated with ordinary stearic acid, in the same molar ratio, only to obtain a smaller choline resonance found in Fraction II.

In summary, the "labelled" lecithin was not conveniently obtainable from any of the partial lecithin syntheses attempted. The method described by Cubero Robles et al, has demonstrated its merits, since this procedure is the most convenient to prepare large quantities of saturated lecithins although the yields reported were found to be exaggerated[43]. Having reproduced their methodology with ordinary stearic acid, good yields of distearoyl phosphatidylcholine was obtained. Since the silicon-containing fatty acid is branched in the thirteenth position, steric effects may have hindered the reaction since no lecithin was formed at all. Hubbell and McConnell[44] acylated a fatty acid containing a nitroxide spin label via the Cubero Robles method but only obtained 20% yield. The dimethyl silyl moiety is not as large as the nitroxide spin label, but the length of the fatty acid chain also plays a role. Lecithin syntheses are generally carried out with C_{14} or C_{16} fatty acids and since yield of product decreases as chain length increases conceivably little or no lecithin may have formed when our "labelled" fatty acid was employed.

The Baer-Buchnea method produced the highest crude yield with our "labelled" fatty acid but, as mentioned previously, the formation of cyclic lysolecithin would probably reduce the yield of pure product quite considerably although no attempt to purify the crude product was undertaken.

Lastly, the method described by Kates et al seemed to show some promise, but a more detailed procedure for purification and

isolation of our "labelled" lecithin must be conducted in order to elute the product in one fraction. This method has yet to prove itself on larger scales.

The yields from all partial lecithin syntheses could probably be greatly improved if the acylation would be carried out in a homogeneous state. As far as we have seen, no attempts to find a suitable solvent to solubilize G.P.C. (or $CdCl_2$ adduct) and fatty acid (acid chloride or anhydride) have been carried out. Attempts in this laboratory were also unsuccessful. A homogeneous reaction would reduce reaction times and increase the efficiency of the reaction. Until such solvent is found, synthetic lecithins are obtained primarily by the methods described.

2. BIOSYNTHETIC INCORPORATION OF LABELLED FATTY ACID BY THE ORGANISM MYCOPLASMA LAIDLAWII STRAIN B

Mycoplasma laidlawii B organism was chosen since it is probably one of the most primitive micro-organisms known. These prokaryotic cells usually are dependant on an external fatty acid source and readily incorporate most fatty acids into their phospho - and glyco - lipids. This organism lacks a cell wall nor are internal membranes present, thus the bulk of the lipid is contained in the external cell membrane.

The organism was grown in a defatted medium supplemented with our "labelled" fatty acid as previously described (cf Chapter III - 1.10.1). The organism, which ordinarily grows in the coccoid form, assumed a filamentus morphology when the branched silicon-containing fatty acid was its only source of lipid. The cells also became extremely resistant to lysis by hypotonic shock or even sonication which substantiated an observation reported by Tourtellotte et al[45]. These gross differences were rationalized by these authors as changes in membrane lipid composition.

"Labelled" fatty acid incorporation was verified by lipid extraction by the method of Bligh and Dyer[46] and the isolated lipid was subjected to PMR analysis. The dimethyl silyl resonance was clearly evident at 0.12 ppm. No attempt was made to identify the lipids which contained the silated fatty acid.

The purpose for the incorporation of the fatty acid into the

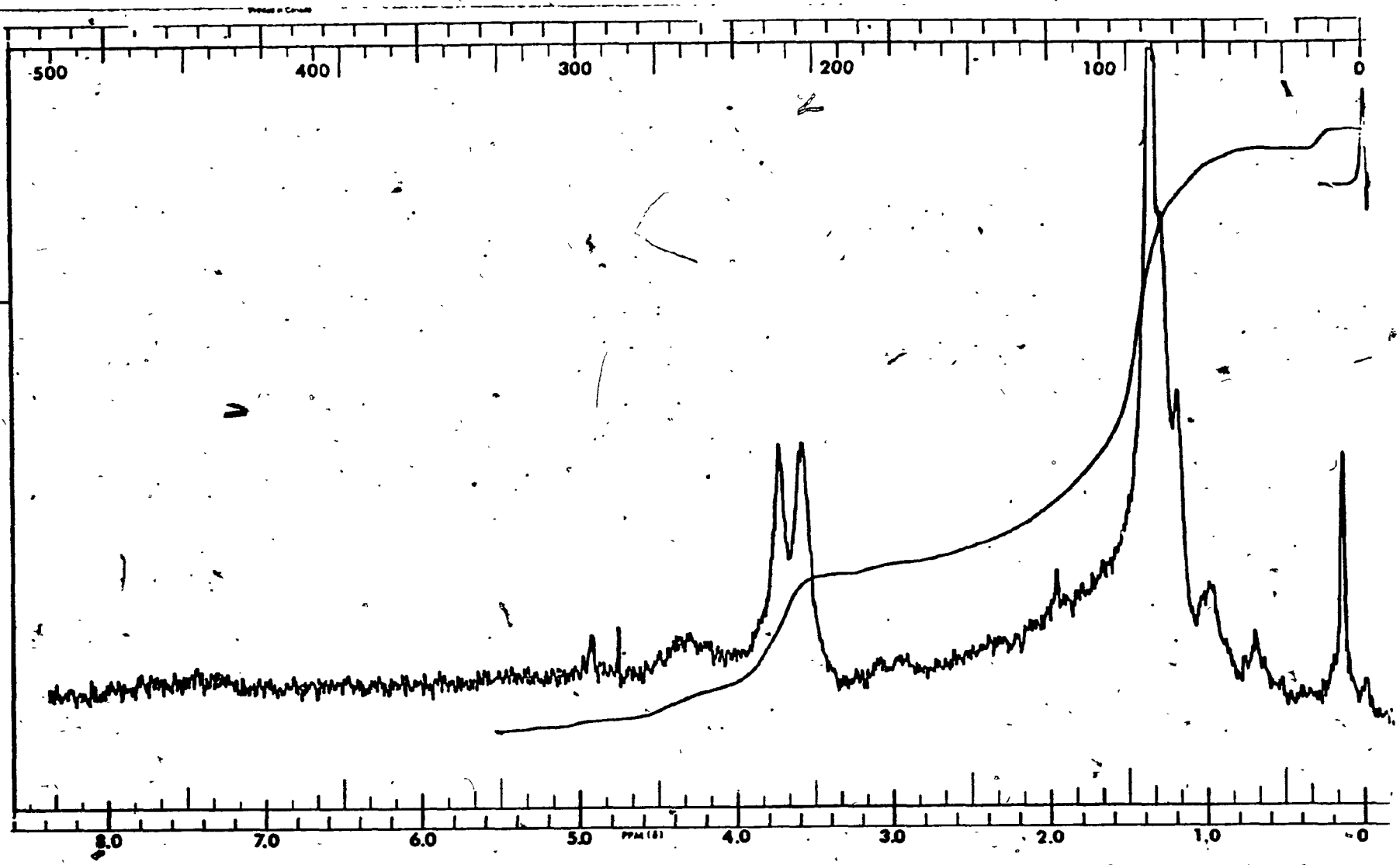
membrane of the micro-organisms was to ultimately obtain membrane fragments which would then be utilized as a natural membrane model system. These fragments would be monitored by NMR, and observation of the silyl reporter molecule in the hydrophobic region of the membrane may have elucidated some biological activity of cellular membranes which must be synergetically governed by molecular structure. For example, utilization of this natural membrane model system for the study of pharmacologically active agents, may have elucidated drug-membrane interaction, etc.

Unfortunately, this organism could not be utilized for two reasons:

(1) Due to the change in cell morphology by the incorporation of the branched "labelled" fatty acid, the cells could not be lysed to form membrane fragments or cell ghosts. Various methods were attempted but to no avail. The cells were finally disrupted by physical grinding with carborundum using a mortar and pestle.

(2) The membrane fragments, obtained by the grinding action, were isolated but no well resolved NMR spectrum could be observed even at elevated temperatures.

Since the mycoplasma membranes were not conducive to an improved natural model, all experiments with this organism were abandoned.



NMR spectrum of extracted lipids from the micro-organism Mycoplasma laidlawii grown in a medium containing the silicon-containing fatty acid (10 mg/l). Incorporation of the fatty acid is clearly visible. The spectrum was run with an external reference (TMS) in $CDCl_3$.

3. NMR STUDIES ON LABELLED LIPOSOMES

NMR relaxation time of a given molecule is a measure of its molecular motion. Spin-spin relaxation time (T_2 or transverse relaxation time) can be approximated by measuring the width at half-height ($\Delta\nu_{1/2}$) of the spectral line and using the following equation[47]:

$$T_2^{-1} = \pi \Delta \nu_{1/2} \qquad (I)$$

(where T_2^{-1} is the relaxation rate).

This relationship is only an approximation since all factors contributing to line broadening are not separated. In general, line broadening is not only due to non-secular (T_1 spin lattice relaxation time) and secular broadening (dipolar) but also due to diamagnetic susceptibility-dependent (magnetic anisotropy), dipole-dipole (in solids) and inhomogenate field-dependent broadening (instrumental artifact). Another broadening mechanism is the chemical shift effect (overlapping of spectral lines) which generally is of little importance.

Equation I, encompasses all these factors for a Lorentzian shaped absorption line, and thus it is apparent that T_2 is less well defined than T_1 (spin lattice relaxation time) since the latter corresponds to only one process whereas the former corresponds to several processes (including T_1). Therefore, Equation (I) can be written in the following manner:

$$T_2^{-1} = \pi \Delta \nu_{\frac{1}{2}} = 2\,T_1^{-1} + T_{2m}^{-1} + T_{2d}^{-1} \qquad (2)$$

where T_1^{-1} is spin lattice relaxation rate, T_{2m}^{-1} is the magnetic anisotropic effect and T_{2d}^{-1} is the dipole broadening of low frequency motions (e.g. solids). It is assumed here that the contributions from these line-broadening mechanisms mentioned, are small and can be included in the observed relaxation rate from equation I. Secondly, it is understood that without pulse NMR methods and data, one cannot rule out these contributions which lead to further line broadening. Therefore, we have chosen to report all data in terms of line widths ($\Delta\nu_{\frac{1}{2}}$) where applicable, implying that broadening of an absorption line is indicative of restricted mobility whereas the narrowing of the resonance is indicative of increased molecular motion.

In the membrane model system proposed, there are two absorption lines which can be monitored conveniently. Since the vesicular preparation is made up of egg yolk lecithin and the hydrophobic probe, methyl-13,13-dimethyl-13-silaheptadecanoate, codispersed in the former lipid, the two resonances, $-\overset{(+)}{N}(Me)_3$ (from lecithin) and $-Si(CH_3)_2$ (from the fatty acid ester) can be used as reporter molecules which would indicate any change in the degree of order or molecular mobility in the hydrophilic and hydrophobic regions respectively, of the bilayer system. Although precise values for T_2 cannot be obtained by the method employed, no natural hydrophobic resonance can be monitored conveniently (unless ^{13}C or ESR is used), thus the silicon-containing fatty acid ester becomes a valid probe

to be used in ^{1}H-NMR spectroscopy of lipid systems.

3.1 PROOF OF INCORPORATION AND CALIBRATION OF MEMBRANE MODEL

Incorporation of the silated fatty acid into a lecithin vesicle is, of course a prerequisite for its use as a hydrophobic probe. Fig. 1 shows the effect of lecithin/labelled fatty acid ester (LEC/LFAE) molar ratio on line width. An arbitrary amount of LFAE was kept constant throughout and increasing amounts of lecithin was added. The upper limiting ratio, 6.0, indicated formation of larger "onion-like" vesicles which separated during centrifugation. This is possibly due to increased vesicular size and compression of fatty acid chains since a broadening of both resonances were observed with increasing lecithin concentration.

The lower limiting ratio indicates a slight broadening of both resonances: (4.94 Hz for $-\overset{+}{N}(CH_3)_3$ and 3.47 Hz for the $-Si(CH_3)_2$ from their natural line widths (2.20 Hz for $-\overset{+}{N}(CH_3)_3$ and 1.50 Hz for the $-Si(CH_3)_2$). The broadening of the N-methyl absorption line is probably due to compression of the polar head group region thus immobilizing the molecule somewhat as a large amount of LFAE is being incorporated into the lipid.

The silyl resonance is broadened due to the new hydrophobic environment which restricts its natural mobility.

The molar ratio effect on line width was observed by maintaining the lecithin concentration constant throughout and increasing the LFAE concentra-

TABLE I - MOLAR RATIO LECITHIN/LABELLED FATTY ACID (CONSTANT)

Sample No.	LEC in mM (gm)	LFAE in mM (gm)	Molar Ratio LEC/LFAE	$\Delta\nu_{1/2}$ in Hz $N(CH_3)_3$	$\Delta\nu_{1/2}$ in Hz $Si(CH_3)_2$
1	50 (0.0404)	100 (0.0328)	0.5	4.94 ± 0.02	3.47 ± 0.14
2	100 (0.0807)	100 (0.0328)	1.0	5.31 ± 0.13	3.75 ± 0.31
3	200 (0.1614)	100 (0.0328)	2.0	5.49 ± 0.03	4.03 ± 0.26
4	300 (0.2421)	100 (0.0328)	3.0	5.69 ± 0.14	4.76 ± 0.19
5	400 (0.3228)	100 (0.0328)	4.0	6.41 ± 0.15	5.31 ± 0.13
6	500 (0.4035)	100 (0.0328)	5.0	6.59 ± 0.46	5.68 ± 0.27
7	600 (0.4842)	100 (0.0328)	6.0	7.61 ± 0.43	6.59 ± 0.06

TABLE I : Line width measurements of NCH_3 and $SiCH_3$ resonances contained in mixed vesicles. Labelled fatty acid ester (LFAE) concentration was kept constant at 100 mM while lecithin (LEC) concentration was varied so as to obtain a range of molar ratios (LEC/LFAE) from 0.5 to 6.0. Vesicles were prepared in 2 ml D_2O.

FIG. 1

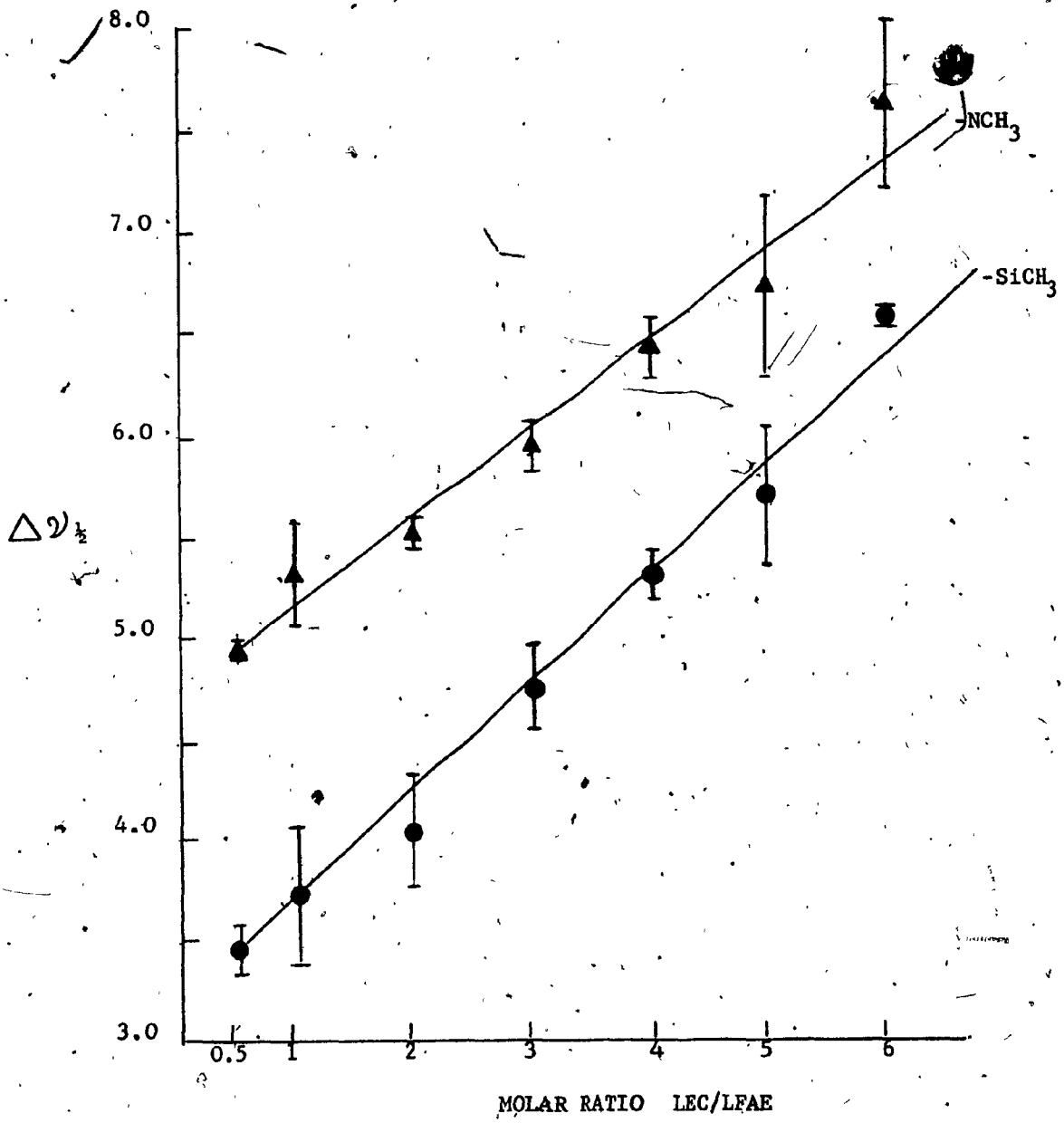


Fig. 1: Line width at half-height of the lecithin N-CH_3 resonance and the labelled fatty acid ester, methyl - 13,13-dimethyl-13-silaheptadecanoic acid, $SiCH_3$ resonance in mixed vesicles prepared by sonication in D_2O at 0^oC as a function of molar ratios. LFAE was kept constant at 100 mM and LEC concentration was varied.

tion in each sample. Figure 2 exhibits this relationship. The $+N(CH_3)_3$ resonance of the lecithin remains essentially unchanged as the labelled fatty acid concentration is increased. The silyl reporter moiety exhibits an increase in line width as the reporter molecule is incorporated into the lipid vesicle. Increased incorporation restricted molecular mobility in the lipoid region of the bilayer.

The lack of effect on the N-methyl signal is indicative that the silyl probe causes little perturbation of the lipid bilayer.

To substantiate the fact of the incorporation of LFAE into the liposomes, the absolute concentration of a standard 3:1 LEC/LFAE molar was varied, maintaining this molar ratio constant. As can be seen in Figure 3 , the line widths exhibit no change within experimental error with change in absolute concentration. At the lowest concentrations (molar ratio 50/16.6 LEC/LFAE) the signal to noise ratio was poor thus making measurements less precise,which may account for the scattering of points. But on the whole no change could be observed (see addendum).

It may be argued that the molecular distribution of the LFAE would not remain constant if larger "onion-like" vesicles were removed by centrifugation. To further substantiate LFAE incorporation and proof of statistical molecular distribution of the silated fatty acid, standard integration curves were obtained by monitoring the N-methyl and Si-methyl resonances, in carbon tetrachloride. Liposome preparations were then measured under identical conditions and their integral

TABLE II - MOLAR RATIO LECITHIN/LABELLED FATTY ACID

Sample No.	LEC in mM (gm)	LFAE in mM (gm)	Molar Ratio LEC/LFAE	$\Delta\nu_{1/2}$ in Hz $N(CH_3)_3$	$\Delta\nu_{1/2}$ in Hz $Si(CH_3)_2$
1	150 (0.1211)	300 (0.0984)	0.5	4.24 ± 0.03	2.12 ± 0.02
2	150 (0.1211)	150 (0.0492)	1.0	4.23 ± 0.04	2.38 ± 0.22
3	150 (0.1211)	75 (0.0247)	2.0	4.34 ± 0.05	2.98 ± 0.09
4	150 (0.1211)	50 (0.0164)	3.0	4.20 ± 0.02	3.08 ± 0.10
5	150 (0.1211)	37.5 (0.0124)	4.0	4.33 ± 0.02	3.79 ± 0.11
6	150 (0.1211)	30 (0.0098)	5.0	4.26 ± 0.02	3.36 ± 0.02
7	150 (0.1211)	25 (0.0082)	6.0	4.26 ± 0.02	3.46 ± 0.04

TABLE II : Line width measurements of NCH_3 and $SiCH_3$ resonances contained in mixed vesicles. Lecithin (LEC) concentration was kept constant at 150 mM while the labelled fatty acid ester (LFAE) was varied to obtain a molar ratio (LEC/LFAE) ranging from 0.5 to 6.0. Vesicles were prepared in 1 ml of D_2O.

FIG. 2

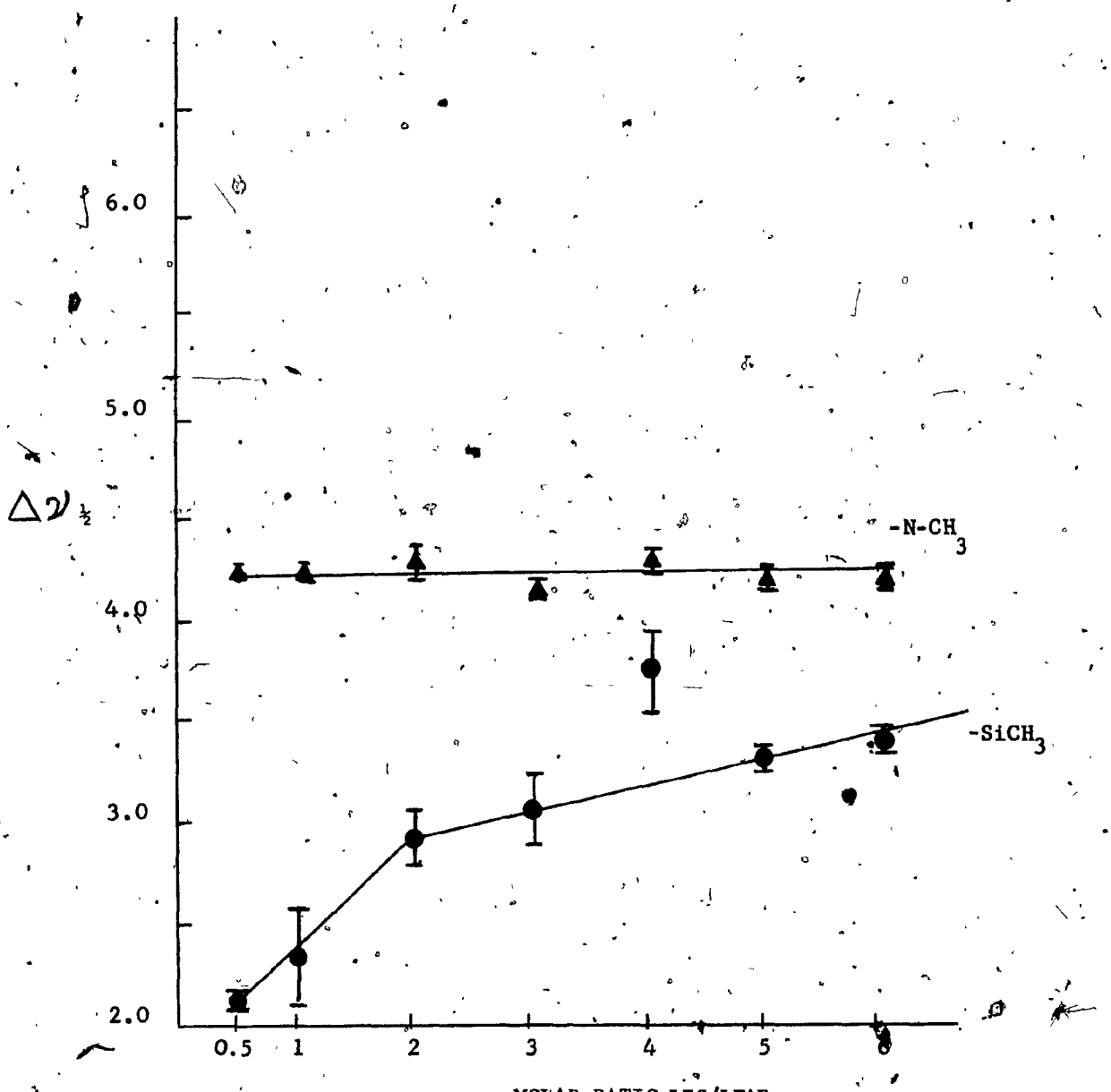


Fig. 2: Width of half-height of N-CH_3 resonance from the lecithin, concentration kept constant at 150 mM, and the $SiCH_3$ resonance from the labelled fatty acid ester - concentration varied, codispersed and cosonicated to form mixed vesicles in D_2O at 0°C as a function of molar ratios.

TABLE III - MOLAR RATIO LECITHIN/LABELLED FATTY ACID

3:1 CONSTANT; ABSOLUTE CONCENTRATION VARIED

Sample No.	LEC in mM (gm)	LFAE in mM (gm)	Absolute Molar Ratio LEC/LFAE	$\Delta\nu_{\frac{1}{2}}$ in Hz $N(CH_3)_3$	$Si(CH_3)_2$
1	50 (0.0404)	16.67 (0.0055)	3:1	4.09 ± 0.01	3.33 ± 0.14
2	100 (0.0807)	33.3 (0.0109)	3:1	4.19 ± 0.08	3.07 ± 0.13
3	150 (0.01211)	50 (0.0164)	3:1	4.10 ± 0.02	3.07 ± 0.03
4	200 (0.1614)	66.6 (0.0218)	3:1	4.10 ± 0.01	3.33 ± 0.23
5	300 (0.2421)	100 (0.0328)	3:1	4.08 ± 0.04	2.81 ± 0.16

TABLE III : Line width measurements of NCH_3 and $SiCH_3$ resonances contained in mixed vesicles. Molar Ratio (LEC/LFAE) was kept constant at 3:1 and the absolute concentration was varied ranging from 50 mM to 300 mM based on lecithin concentration.

FIG. 3

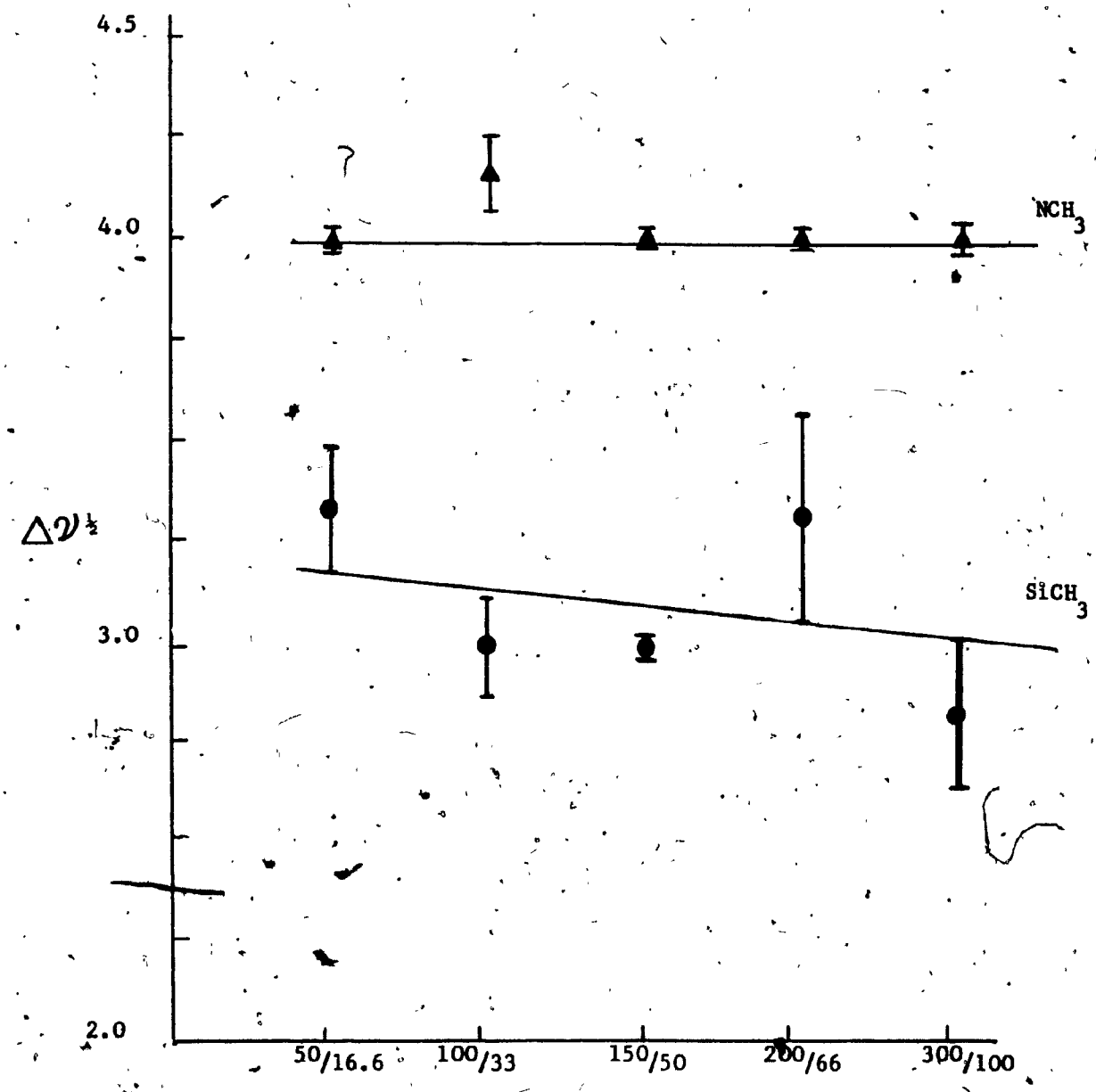


Fig. 3 : Effect of change in absolute concentration maintaining a molar ratio LEC/LFAE (3:1) constant (see addendum).

compared to the standard curves to yield 85.9% of the proton count for $-N^{+}(CH_3)_3$ and 83.0% for the $-Si(CH_3)_2$. Expressing these results in terms of molar ratio, 3.1:1 LEC/LFAE was observed.

Since all the liposomes were prepared in the same fashion, these results proved not only incorporation but also confirmed that a 3:1 molar ratio was maintained throughout. Figures 4 and 5 show the standard curves of the two resonances.

3.2 PERTURBATION OF REPORTER MOLECULES

In order to test the reporter properties of our hydrophilic and hydrophobic probes, various reagents were used to perturb these molecules. The polar head group probe $N^{+}(CH_3)_3$ being hydrophilic can be perturbed by the interaction of electrolytes on the zwitterionic head group. These cations, being impermeable to a lipid bilayer in the absence of any active transport, can only interact with the external polar head group, not affecting the hydrophobic probe by molecular or electronic interaction.

The silyl reporter molecule, being buried in the internal hydrophobic region of the bilayer, can be perturbed by incorporating an apolar organic reagent such as n-dodecane. The hydrocarbon reagent is insoluble in aqueous medium thus would preferentially be codispersed in the hydrophobic lipid region of the bilayer.

TABLE IV - STANDARD CURVE FOR-$N(CH_3)_3$

Sample No.	LEC in mM (gm)	Integration in mm
1	25 (0.0202)	3.0
2	50 (0.0404)	6.5
3	100 (0.0807)	11.0
4	150 (0.1211)	13.0
5	200 (0.1614)	14.0

TABLE IV - Standard curve (Fig.4) was obtained from the integration of the NCH_3 resonance from various concentrations of egg yolk lecithin in 1.0 ml of CCl_4. Vesicular preparation containing 150 mM of lecithin and 50 mM of LFAE indicate 85.9% proton count measured from the standard curve.

TABLE V - STANDARD CURVE FOR-$Si(CH_3)_2$

Sample No.	LFAE in mM (gm)	Integration in mm
1	10 (0.0033)	7.5
2	25 (0.0082)	16.0
3	40 (0.0131)	22.5
4	50 (0.0164)	30.5
5	75 (0.0246)	41.0

TABLE V : Standard curve (Fig.5) was obtained from the integration of the $SiCH_3$ resonances from various LFAE concentrations in 1.0 ml CCl_4. Vesicular preparation in 1 ml D_2O containing 150 mM of LEC and 50 mM of LFAE indicated an 83.0% proton count as measured from the standard curve.

FIG. 4

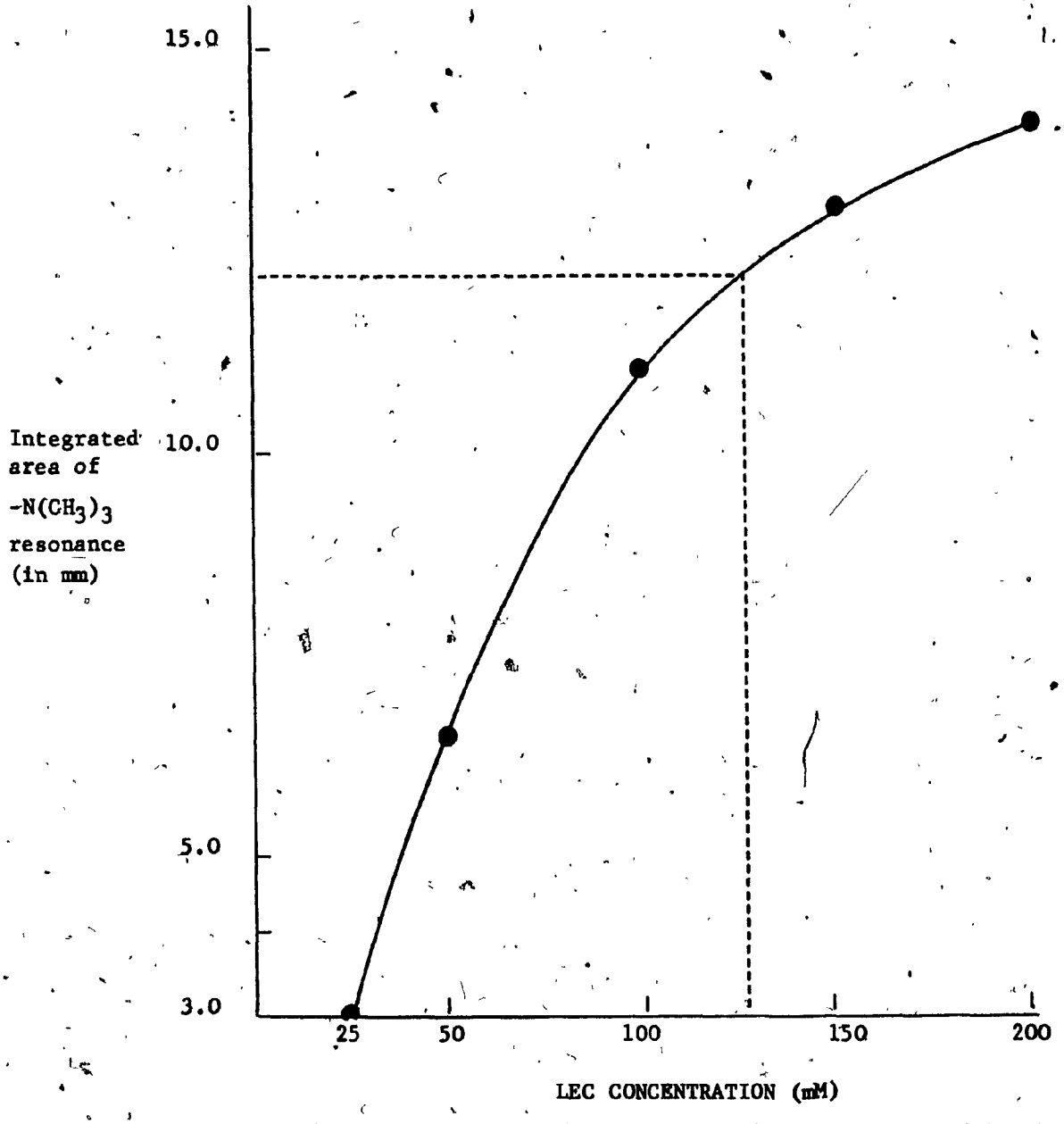


Fig. 4 : Standard curve for the N-methyl moiety obtained by the integral measurement of LEC dissolved in 1.0 ml of CCl_4. Integral of N-methyl resonance in liposomes, prepared by sonicating 50 mM of LFAE and 150 mM LEC, and centrifuging at 20,000 g., indicates a 128.75 mM concentration of LEC is codispersed in the D_2O supernatants as indicated by the dotted line.

FIG. 5

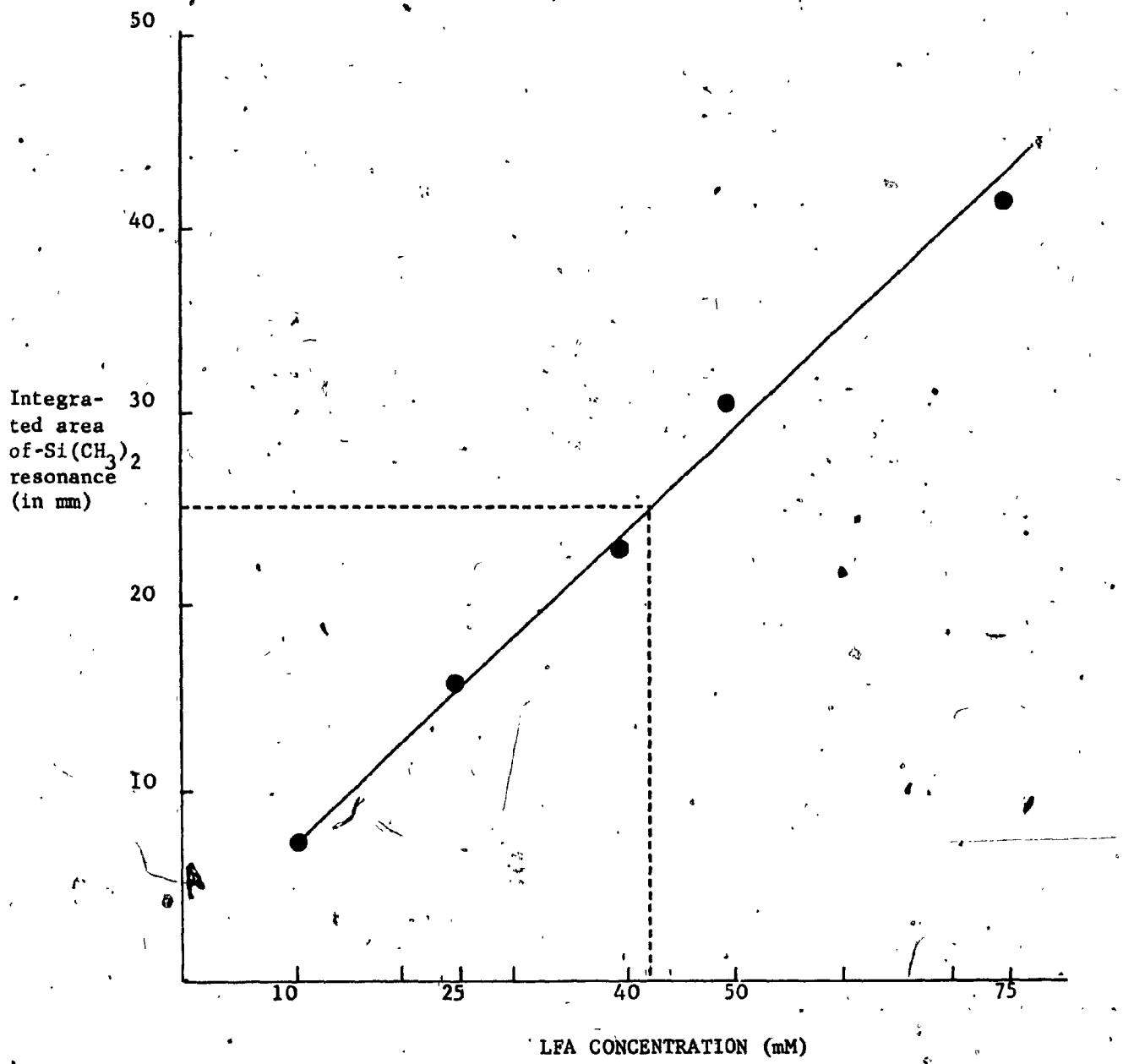


Fig. 5 : Standard curve for the dimethyl silyl moiety obtained by the the integral measurement of LFAE dissolved in 1.0 ml of CCl_4. Integral of silyl resonance in liposomes prepared by sonicating 50 mM of LFAE and 150 mM of LEC, and centrifuging at 20,000 g., indicates a 41.5 mM concentration of LFAE is codispersed in the D_2O supernatant as indicated by the dotted line.

3.2.1 EFFECT OF NaCl ON LIPOSOMES

Figure 6 shows the effect of increasing NaCl concentration on the line widths of both N-methyl and Si-methyl resonances. The line width of the hydrophilic reporter molecule increases as the salt concentration increases, reflecting some interaction between the zwitterionic polar head group and the electrolyte. The Si-methyl curve shows no change with increasing salt concentration below 0.75M. Above this concentration rapid precipitation induced by a "salting out effect" became evident with a marked increase in line width of the silyl resonance. Therefore, perturbation of the N-methyl groups by the increased ionic strength impedes the mobility of the probe which results in line widening. The electrolyte being impervious to the lipid bilayer shows no perturbation of the hydrophobic probe, hence reflecting no influence in the apolar region of the micelles.

3.2.2 EFFECT OF $CaCl_2$ ON LIPOSOMES

The role of calcium ions in membrane chemistry and biochemistry is still not totally understood, thus in testing our membrane model system, we were interested whether $CaCl_2$ would yield similar results obtained with the NaCl experiment.

There have been numerous contradictory reports in the literature on the mode of binding of cations with phospholipids[48]. Hauser et al. have outlined these reports and tried to clarify the controversy by using two different techniques: surface chemical and NMR methods. They concluded

TABLE VI - EFFECT OF NaCl

Sample - No	Molar Ratio LEC/LFAE	NaCl final Conc M	$\Delta \nu_{1/2}$ in Hz $N(CH_3)_3$	$Si(CH_3)_2$
1	3:1	0.01	6.04 ± 0.35	4.86 ± 0.1
2	3:1	0.04	6.32 ± 0.63	4.95 ± 0.03
3	3:1	0.1	6.20 ± 0.27	4.93 ± 0.02
4	3:1	0.25	6.78 ± 0.13	4.96 ± 0.02
5	3:1	0.5	6.74 ± 0.33	4.93 ± 0.01
6	3:1	0.75	7.05 ± 0.13	4.95 ± 0.01
7	3:1	1.0	7.47 ± 0.26	6.00 ± 0.04

TABLE VI : Effect of NaCl environment on the line widths of $N(CH_3)_3$ and $Si(CH_3)_3$ resonances of mixed lipid vesicles containing a 3:1 molar ratio (LEC/LFAE) based on 150 mM of LEC

TABLE VII - EFFECT OF $CaCl_2$

Sample No	Molar Ratio LEC/LFAE	$CaCl_2$ final Conc M	$\Delta \nu_{1/2}$ in Hz $N(CH_3)_3$	$Si(CH_3)_2$
1	3:1	0.01	6.51 ± 0.45	4.95 ± 0.20
2	3:1	0.02	7.53 ± 0.12	5.51 ± 0.77
3	3:1	0.03	7.52 ± 0.25	5.78 ± 0.22
4	3:1	0.04	7.63 ± 0.47	5.79 ± 0.40
5	3:1	0.05	7.99 ± 0.38	5.96 ± 0.33

TABLE VII : Effect of Ca^{+2} on the line width of $N(CH_3)_3$ and $Si(CH_3)_3$ resonances of mixed lipid vesicles containing a 3:1 molar ratio (LEC/LFAE) based on 150 mM of LEC.

FIG. 6

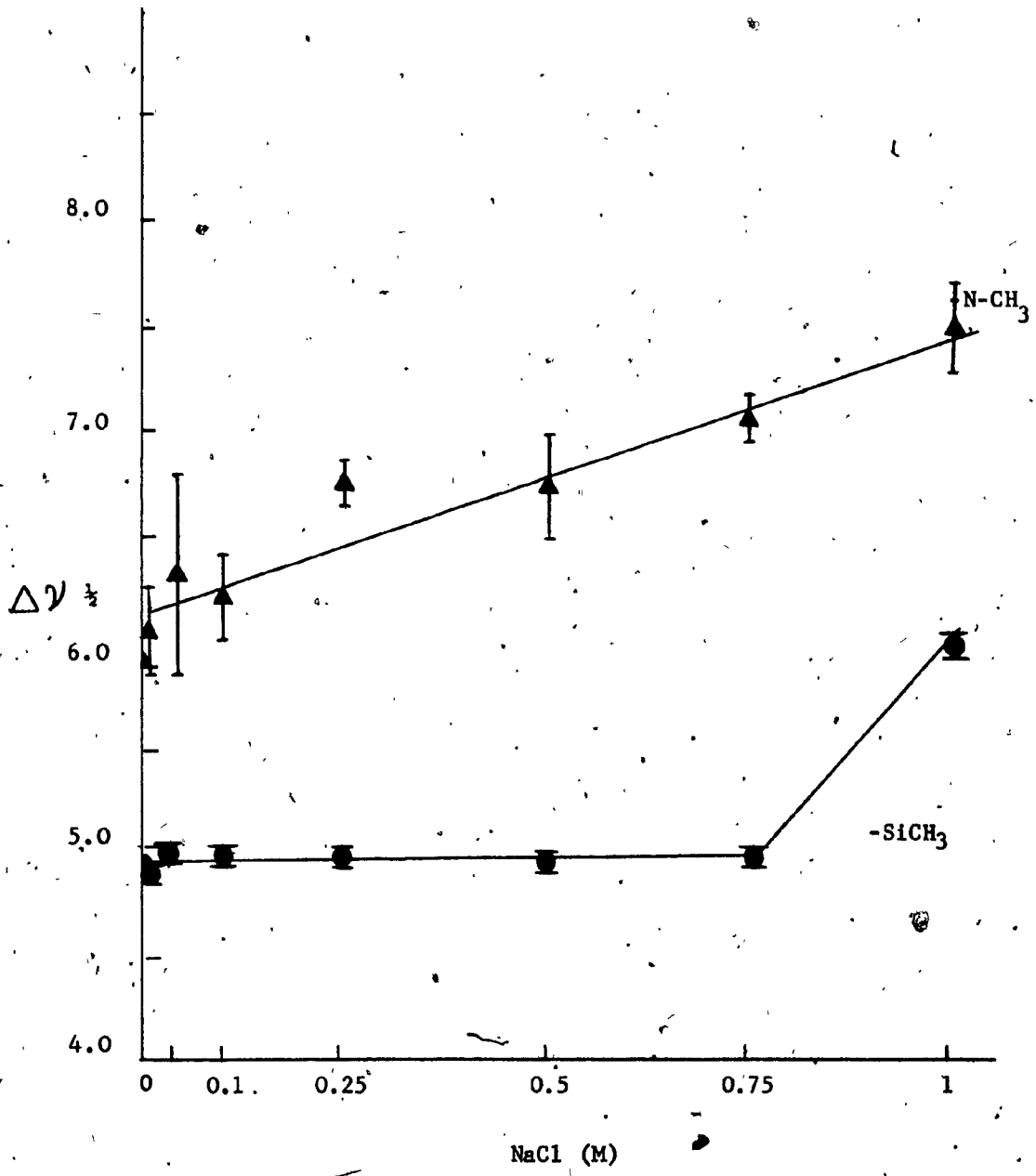


Fig.6: Effect of NaCl concentration on N-CH$_3$ and Si-CH$_3$ line width of a 3:1 LEC/LFAE mixed vesicle (150 mM lecithin).

FIG. 7

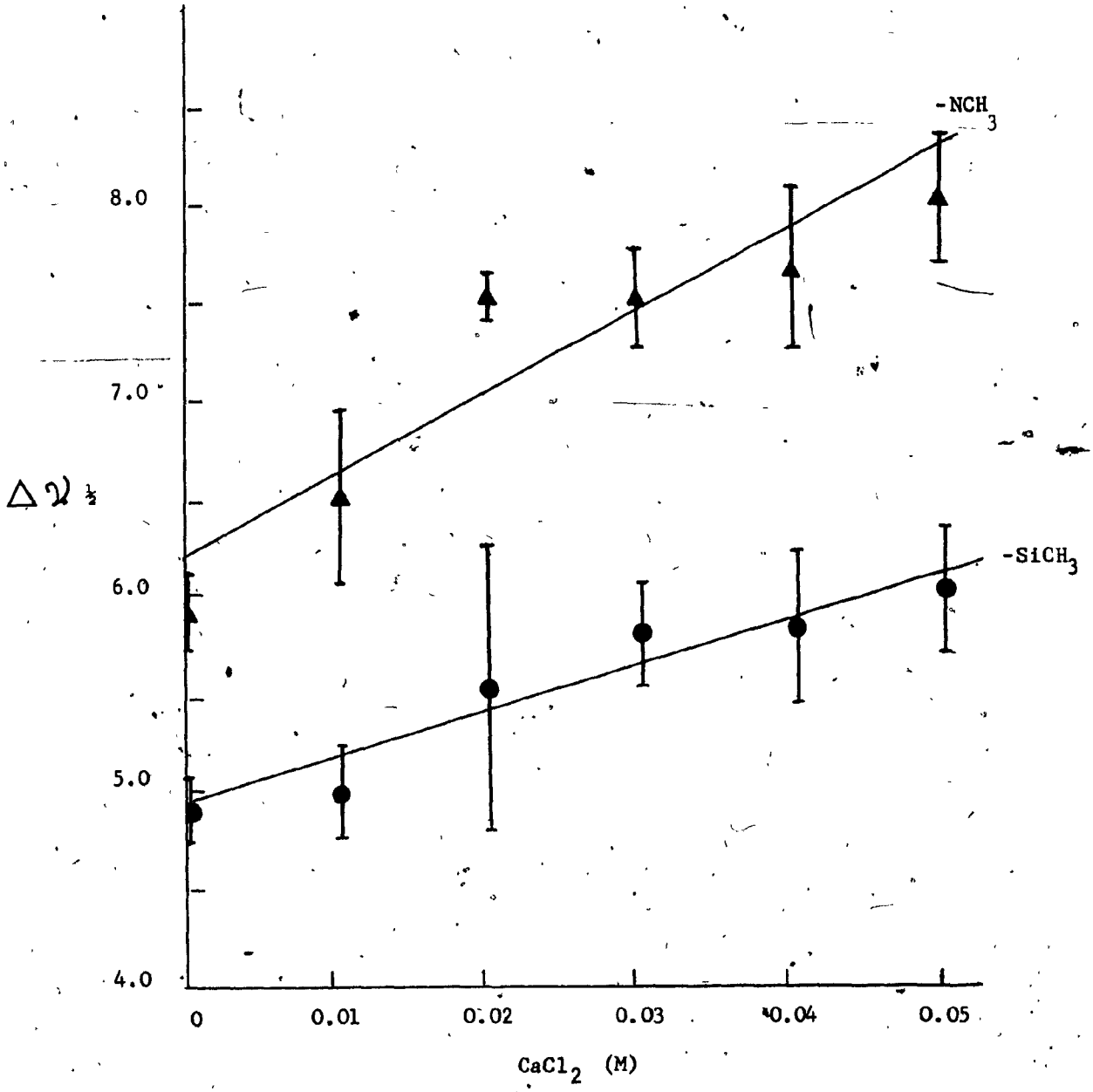


Fig. 7 : Effect of $CaCl_2$ concentration on the N-CH_3 and SiCH_3 line width of a 3:1 LEC/LFAE mixed vesicle (150 mM lecithin).

that no effect was observed on the ^{1}H, ^{13}C and ^{31}P NMR spectrum of lipid vesicles with Ca^{+2} concentrations of 0.1 to 0.2M. A very weak interaction was reported with surface chemical techniques when high Ca^{+2} concentrations were used. These observations support findings by Rojas and Tobias[49] and Hauser and Dawson[50], using surface radiography reported no Ca^{+2} interaction with pure egg lecithin at Ca^{+2} concentrations of 0.1 mM and 0.1 uM respectively. Dervichian[51] utilized potentiometric titrations and found no binding between Ca^{+2} and egg lecithin in the pH range 2 to 8.

On the other hand, Kimizuka et al.[52,53] utilized $^{45}Ca^{+2}$ and reported binding with monolayers and multilayers of egg, soybean and dipalmitoyl lecithin. White and coworkers[54] observed the Ca^{+2} distribution in a two phase system using synthetic lecithin and reported that the calcium ion was associated with the phospholipid. Shah and Schulmann[55,56] conducted surface potential measurements and concluded that Ca^{+2} in a 10 mM concentration, interacts with both saturated and unsaturated lecithins. Trauble[57] reported that Ca^{+2} binds to phospholipids which were monitored by the fluorescence of 1-anilino-8-naphthalenesulfonic acid incorporated into dipalmitoyl lecithin bilayers. Furthermore, Trauble reports that Ca^{+2} binds with a stability constant $K=10^{3}\ M^{-1}$. Faced with this dichotomy of reports, our observation from the interaction of $CaCl_2$ with our membrane model system favors a positive result as seen in Figure 7. The N-methyl curve acquires a positive slope as the ionic strength increases, indicating restricted mobility of the polar head group. The line widening implies an interaction between the lecithin and the electrolyte. The effect appears to be consider-

ably stronger than in the case with monovalent cation since a steeper slope is achieved at much lower concentrations. Above the Ca^{+2} concentration of 0.05M, (50 mM), the upper limit, aggregation and precipitation ensued yielding extensive broadening due to the formation of solid particulates in the sample tube. These points were omitted for this reason.

The hydrophobic probe responded with a slight departure from linearity. The lipid bilayer is impervious to Ca^{+2} ions in the absence of any active transport, yet the effect of restricted mobility is observed in the hydrophobic region of the bilayer. This change in the silyl reporter moiety imparts that the fatty acid chains experience an increased rigidity or stability. This stability supports the observation by Trauble[57] although no stability constant was calculated from our data. Furthermore, this observation substantiates the findings reported by Papahadjopoulos[58] where cationic effects were monitored by changes in the phase transitions of the phospholipid. Papahadjopoulos observed the absence of a phase transition between 0-70°C when Ca^{+2} (1×10^{-3} M) was added to phosphatidyl serine. Furthermore, X-ray evidence indicated that PS-Ca^{+2} bilayers are in a crystalline state at 24°C. No significant effect was observed when pure phosphatidyl choline was studied in the presence of mono-and divalent cations and he concluded that chain packing is affected by the lipid components, temperature, ionic composition and is particularly dependent on the chemical nature of the head group. Thus using mixed micelles, in the presence of Ca^{+2}, PS and PC, a 17 degree increase in the phase transition was observed.

The lecithin used in our micellar preparation was commercial grade, implying that a mixture of phospholipids is present. Since Ca^{+2}

has a stabilizing effect on bilayers which result in a shift to higher transition temperature, in mixed micelles, the changes in the line width of the hydrophobic probe implies a similar behaviour in commercial egg yolk lecithin, although no differential scanning calorimetry was conducted.

3.2.3 EFFECT OF DODECANE ON LIPOSOMES

The effect of the apolar hydrocarbon chain, dodecane, [redacted]he line widths of the hydrophilic and hydrophobic probes can be seen on Figure VIII. The perturbation of the silyl reporter molecule by the n-dodecane resulted in a negative slope as n-dodecane concentration increases. The line narrowing is indicative of an increased randomness in the hydrophobic interior as increasing n-dodecane tends to "solubilize" the acyl fatty acid chains, which renders them more mobile. The liposome disruption does not appear to be evident since line width of the N-methyl remains essentially unchanged. This observation also implies that no perturbation of the polar head group is taking place, as the hydrophobic interior achieves a more disordered state.

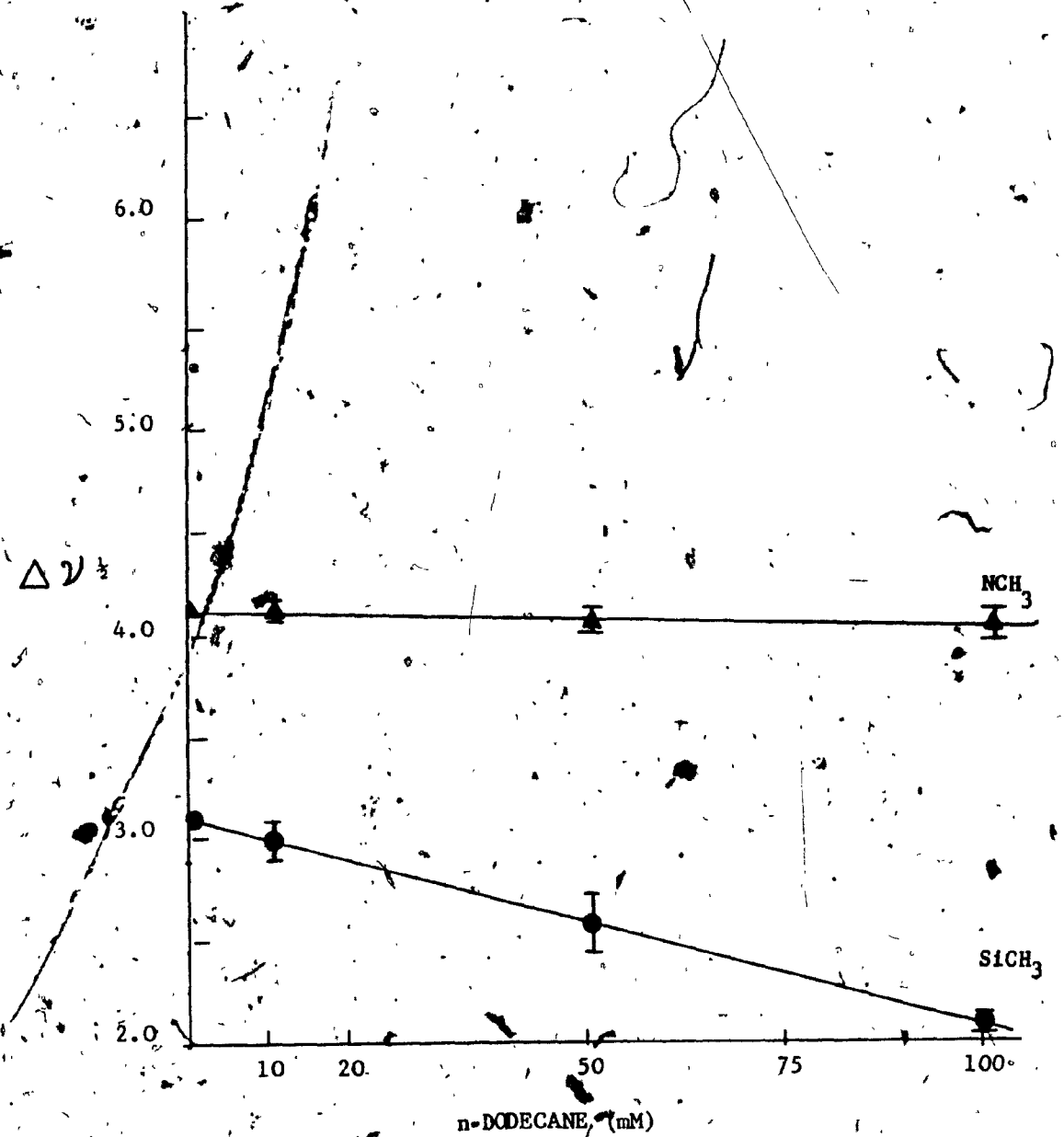
These experiments confirm the usefulness of the reporter molecule in the hydrophilic and hydrophobic regions of a phospholipid bilayer. These probes, which simultaneously exhibit various interactions or conformational changes, which may be induced by other reagents, offer a definite advantage as structure-function relationships in phospholipid bilayers or membranes are only starting to be investigated.

TABLE VIII - EFFECT OF DODECANE

Sample No	Molar Ratio LEC/LFAE	Dodecane Conc mM	$\Delta\nu_{1/2}$ in Hz $N(CH_3)_3$	$Si(CH_3)_2$
1	3:1	0	4.10 ± 0.02	3.10 ± 0.10
2	3:1	10	4.09 ± 0.01	3.00 ± 0.13
3	3:1	50	4.10 ± 0.02	2.60 ± 0.35
4	3:1	100	4.09 ± 0.03	2.10 ± 0.06

TABLE VII : Effect of the incorporation of n-dodecane into mixed lipid vesicles on the line widths of NCH_3 and $SiCH_3$ resonances The vesicles were prepared in 1.0 ml of D_2O based on 150 mM of LEC

FIG. 8



Fig, 8: Effect of n-dodecane on the line widths of the N-CH_3 and $SiCH_3$ resonances in mixed vesicles containing a 3:1 molar ratio of LEC/LFAE, based on 150 mM of lecithin. Dodecane was cosonicated with the lipid to form the mixed micelles.

Unfortunately, these results may not necessarily be extrapolated to natural membranes. A major component of natural membranes is the lipoprotein. Although proteins are not conducive to high resolution NMR, by monitoring our system in the presence of a polypeptide may result in the acquisition of structural information for the phospholipid-protein interaction.

3.3 EFFECT OF GRAMICIDIN S WITH AND WITHOUT NaCl

3.3.1 INCORPORATION OF GRAMICIDIN S INTO THE MEMBRANE MODEL

Gramicidin S is a cyclic pentadecapeptide antibiotic and its incorporation into a lipid bilayer is being investigated in this laboratory. Chapman et al.[59] and Finer et al.[60] have studied the interaction of this antibiotic with phospholipid bilayers. Some of the methods employed by these investigations are DSC, ESR, NMR, UV, IR, ORD and ultracentrifugation. Chapman concludes that Gramicidin S interacts with only the polar head group of phospholipid bilayers when the antibiotic is heated with lecithin in a 1:1 or 1:2 ratio. Furthermore, the antibiotic shifts the transition temperature to lower temperature (37°C) when equimolar ratios of lipid and Gramicidin S are used.

Chapman also reported that no broadening of the hydrocarbon NMR signal was observed when Gramicidin S was mixed with egg yolk lecithin, although $N^{+}(CH_3)_3$ signal is slightly reduced at high antibiotic concentration. Chapman, therefore, implies that the antibiotic interacts only with the polar head group rather than the hydrocarbon chain, thus no incorpora-

tion was evident and consequently no ion transport could be expected.

When Gramicidin S was mixed with dipalmitoyl lecithin, no N-methyl resonance or methylene signal was observed at room temperature. Chapman attributes this observation as the restricted mobility due to the gel state since room temperature is below the transition temperature of dipalmitoyl lecithin. At 55°C, these signlas become evident.

Finer et al. observed line broadening of the fatty acid chain signal only when Gramicidin S and the phospholipid are mixed in the presence of phosphate buffer. Finer also reported that further addition of the antibiotic to unsonicated egg yolk lecithin produced a high resolution NMR spectrum at a molar ratio of 1:1.3, but the bilayer structure is also destroyed. Thus the structure of this mixture is not known.

It seems that both these investigators used the dihydrochloride salt of the antibiotic which is scarcely soluble in water. Finer et al. also report that Gramicidin S is able to solubilize lecithin dispersed in water without sonication.

Figure 9 shows the results obtained in this laboratory when Gramicidin S free base is cosonicated with egg yolk lecithin. As may be seen, the Gramicidin S concentrations used are much lower than those employed by other investigators, and the low concentrations were chosen because these antibiotics show biological action at comparably low concentrations, and range from 50 μg - 250 μg/mole of lipid.

Both reporter moieties of the liposome behave in a parallel fashion as increasing amount of antibiotic is added, with an observed minimum (i.e. line narrowing) occurring at 100 μg/mole of lipid (9.925 μM) of antibiotic. Thus an initial narrowing of both line widths may be indicative of more spatial mobility caused by an expansion of vesicle size, as the Gramicidin S is solubulized by the phospholipid. This line narrowing reaches the minimum, perhaps the optimum vesicle size, and as more antibiotic is incorporated, a tighter packing of the lipid chain also occurs as is evident from the positive slopes on the graph of Figure 9. Above 250 μg/mole of lipid, aggregation and precipitation occurred.

Therefore, an effect of the antibiotic on the liposome was observed on both reporter moieties at much lower Gramicidin S concentration than that used by Chapman and coworkers. Furthermore, we observed a marked effect on the hydrophobic interior of the bilayer. The minimum observed at 100 μg/mole of lipid, as mentioned above, happens to occur at the same concentration of Gramicidin A as used by B.E. Cohen[61] in his studies of liposome permeability to non-electrolytes. The significance of this particular concentration is not readily understood, yet may be indicative of a cooperative effect whereby only a small amount of antibiotic is necessary for its function. This type of cooperative effect has been observed with other antibiotics such as alamethicin[62], where each antibiotic molecule induces a new form of aggregate comprised of 600 lipid molecules per molecule of alamethicin. Thus, with our system, 9.925 μM of antibiotic is equivalent to 13,400 lipid molecules per molecule of antibiotic - a 22.3 fold increased effect than that with alamethicin - supporting even more so the cooperative action of Gramicidin S.

TABLE IX - EFFECT OF GRAMICIDIN S

Sample No	Molar Ratio LEC/LFAE	Gramicidin µg/mole of lipid	$\Delta\nu_{1/2}$ in Hz $N(CH_3)_3$	$Si(CH_3)_2$
1	3:1	50	4.94 ± 0.07	4.38 ± 0.09
2	3:1	100(9.925µM)	4.83 ± 0.12	3.63 ± 0.13
3	3:1	150	4.96 ± 0.12	4.40 ± 0.08
4	3:1	200	4.88 ± 0.13	4.34 ± 0.30
5	3:1	250	5.48 ± 0.11	4.38 ± 0.57
6	3:1	500	5.92 ± 0.17	5.64 ± 0.25

TABLE IX : Effect of the incorporation of Gramicidin S on the line widths of NCH_3 and $SiCH_3$ of mixed vesicles. Vesicular preparation in 1 ml D_2O contained 100 mM of lecithin.

TABLE X - EFFECT OF GRAMICIDIN S AND NaCl

Sample No	Molar Ratio LEC/LFAE/GRAMICIDIN (mM)	NaCl final conc M	$\Delta\nu_{1/2}$ in Hz $N(CH_3)_3$	$Si(CH_3)_2$
1	$100:33:9.925\times10^{-3}$	0	4.92 ± 0.12	3.55 ± 0.08
2	$100:33:9.925\times10^{-3}$	0.01	5.21 ± 0.05	3.56 ± 0.13
3	$100:33:9.925\times10^{-3}$	0.04	5.51 ± 0.04	3.86 ± 0.06
4	$100:33:9.925\times10^{-3}$	0.10	6.56 ± 0.14	4.92 ± 0.05
5	$100:33:9.925\times10^{-3}$	0.15	7.13 ± 0.20	5.48 ± 0.15
6	$100:33:9.925\times10^{-3}$	0.20	7.19 ± 0.16	6.63 ± 0.26

TABLE X : Effect of NaCl on the line widths of NCH_3 and $SiCH_3$ of liposomes containing 100 mM of lecithin, 33 mM of LFAE and 9.925 µM of Gramicidin S.

FIG. 9

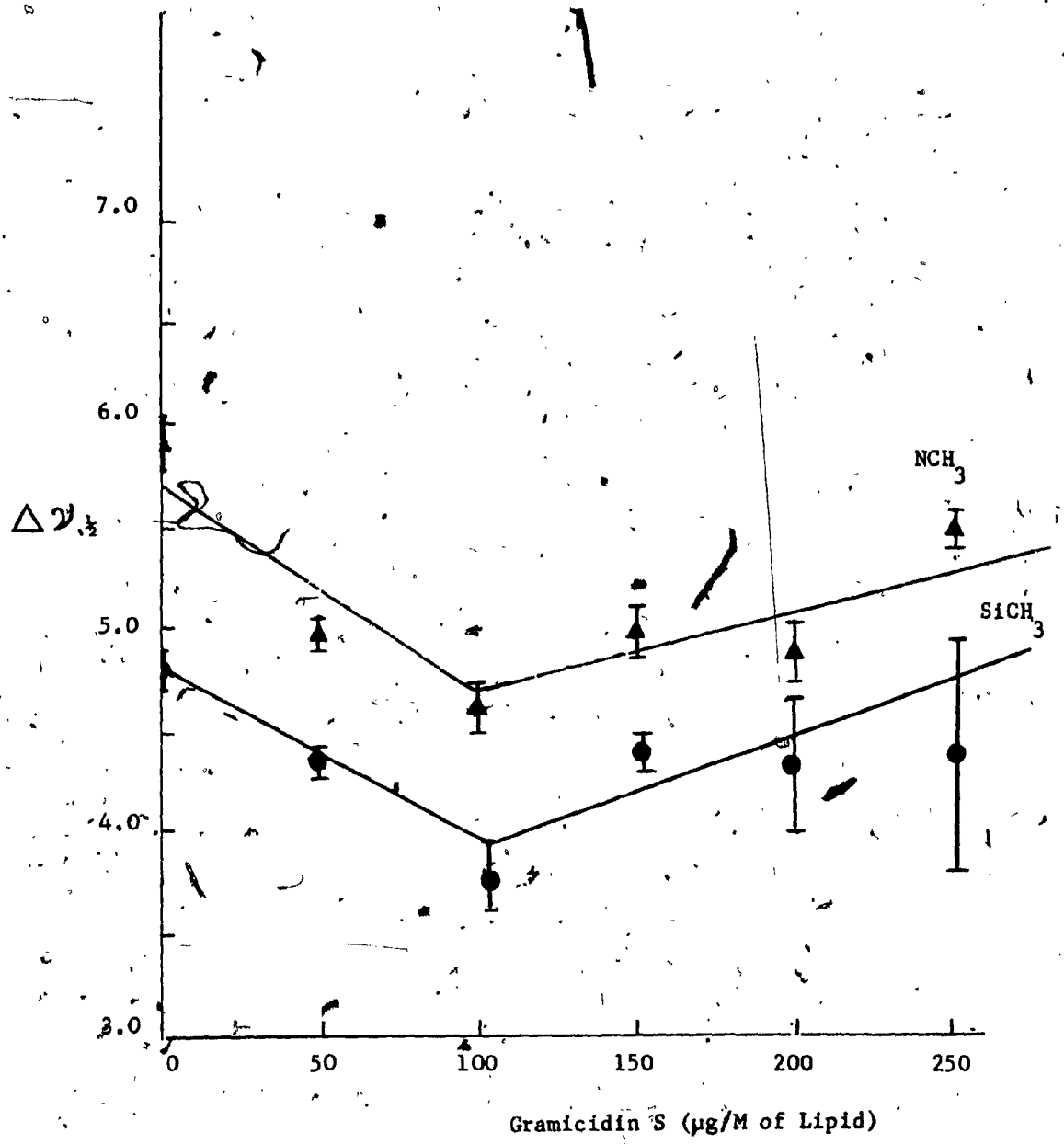


Fig. 9: Effect of Gramicidin S on the line widths of NCH_3 and $SiCH_3$ resonances in mixed vesicles. Gramicidin and lipid were cosonicated in D_2O at 0°. Vesicles molar ratio was 3:1 based on 100 mM of lecithin.

FIG. 10

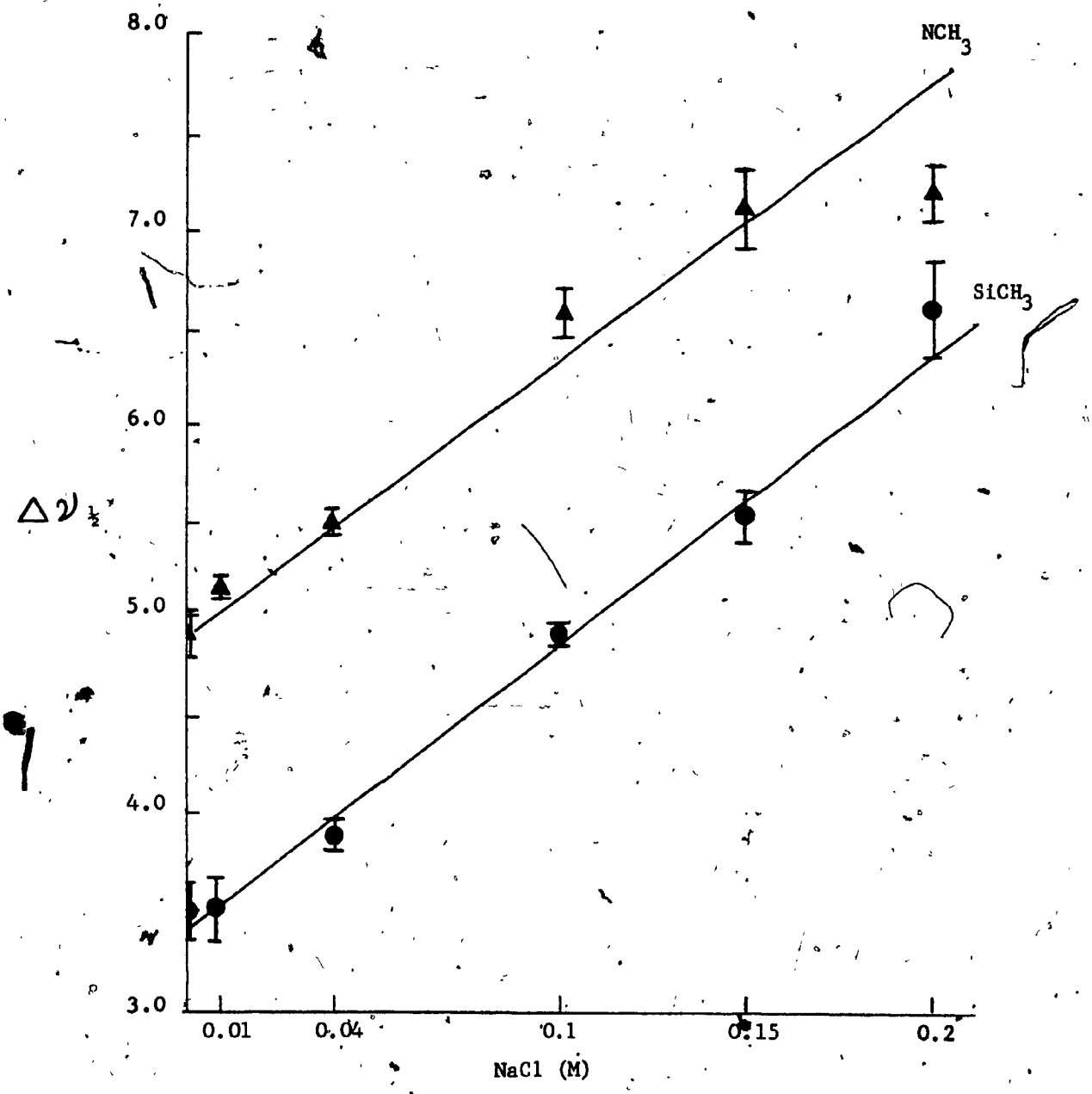


Fig. 10 : Effect of NaCl on the line widths of NCH_3 and $SiCH_3$ resonances of mixed vesicles containing LEC/LFAE, in a 3:1 molar ratio (100 mM of lecithin), and 100 µg/mole of lipid (9.925 µM) of Gramicidin S.

The next approach was to observe this lipid-polypeptide complex in the presence of cations such as Na^+.

3.3.2 EFFECT OF LIPID-GRAMICIDIN S COMPLEX IN THE PRESENCE OF NaCl

Our standard molar ratio of LEC/LFAE was maintained at 3:1, based on 100 mM of LEC, with the added feature that 100 µg/mole (9.925 µM) of Gramicidin S was now incorporated into the system. The object of this experiment was to elucidate any changes in line widths of the reporter molecules in this new lipo-peptide system which may be brought about by the presence of NaCl.

The effect of the electrolyte on the lipid-antibiotic system can be seen on Figure 10. If compared to the NaCl interaction in the absence of the antibiotic, (Figure 6), the changes in line widths vary quite drastically. Firstly, the interaction exhibited on Figure 10 occurs at much lower electrolyte concentration. Almost a four-fold increase of the effect has taken place by the introduction of the cyclic antibiotic, where maximum interaction occurs at 250 mM of sodium chloride concentration as compared to the 1.0M in Figure 6. Above 250 mM, precipitation and breakdown of the lipid system was apparent.

Secondly, comparing the N-methyl curves of both graphs, a steeper slope is observed in the system containing the antibiotic. This result is most likely comprised of not only electrolye-head group interaction but also cation-antibiotic interaction. The latter interactions may, perhaps, be separated by subtracting the effect shown on Figure 6

(effect of NaCl on liposomes without antibiotic) from that shown on Figure 10 (effect of NaCl on liposomes in the presence of the antibiotic). This electrolyte-Gramicidin S interaction may be brought about by conformational changes in the antibiotic hydrophilic sites or changes in conformation of the peptide if indeed the cation is being transported through the hydrophilic channel. Differentiation of these two processes cannot be achieved with the system at hand although an effect is evident.

Thirdly, the most drastic difference between the two graphs is the change in the hydrophobic probe, $-Si(CH_3)_2$. Figure 6 shows absolutely no change in the line width except at very low electrolyte concentrations, (due to stability as discussed in the $CaCl_2$ experiment). But this effect is very small.

In the presence of the antibiotic, the hydrophobic reporter molecule exhibits a linear change of line width with increasing electrolyte concentration. This perturbation of the interior reporter moiety may be indicative of increased stability of the bilayer. It may reflect conformational changes taking place in the hydrophobic amino acids of the antibiotic as sodium ions are being transported. Such conformational changes would then result in the immobilization of the lipid phase. The condition that lipid bilayers are impermeable to ions still holds true unless the antibiotic creates an ion leak. The changes observed on the behaviour of the hydrophobic probe, therefore, imply that the antibiotic is incorporated into the lipid bilayer and also suggests ion binding if not transport

(since transport in the strict sense depends on the existence of an ion gradient, whereas an equilibrium will be reached quickly in our system).

The two systems behave differently due to the incorporation of the cyclic antibiotic, Gramicidin S. A general "freezing" of the liposomes can be ruled out, even though Chapman et al. report that Gramicidin S in solution forms a gel in the presence of salts (such as KCl). In our experiment, however, no gel formation was evident when sodium chloride was added to the solution, therefore, we assume that our explanation of the experimental findings describes specific phenomena and not a general effect.

Our results do not directly answer the question of how the antibiotic functions but we believe the nature of the interaction, between lipid and antibiotic, appears to be hydrophobic as well as electrostatic. We also believe that a cooperative effect similar to that seen with alamethicin may be present with Gramicidin S, since only (100 μg/mole of lipid) was required to observe an effect in both regions of the bilayer. It also appears likely that the antibiotic is incorporated into the lipid bilayer, in variance with the results of the Chapman group.

CHAPTER III EXPERIMENTAL

1. SYNTHESIS OF SILICON-CONTAINING FATTY ACID

1.1 SCHEME I - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILA-STEARIC ACID

1.1.1 PREPARATION OF n-HEXYL-MAGNESIUM BROMIDE (II)

Bromohexane, I, (24.8 g - 0.15 mole) was added to magnesium turnings (5.4 g - 0.225 gram-atom) in 50 ml of THF (dried over $LiAlH_4$). A crystal of I_2 was necessary to initiate the reaction. Once the initial reaction had subsided, the reaction mixture was refluxed for 1 hour when finely divided magnesium was evident since the solution turned a grey-black colour. After that time, the reaction mixture was cooled and filtered rapidly to recover the unreacted magnesium (1.9 g - 0.079 gram-atom - 99% formation of Grignard reagent). The filtrate, containing the n-hexyl magnesium bormide, II, was reacted immediately with dimethyl-chlorosilane (DMCS) (PCR Incorporated, Gainsville, Florida).

1.1.2 PREPARATION OF DIMETHYLHEXYLSILANE (III)

Dimethylchlorosilane (14.2 g - 0.15 mole) was dissolved in 20 ml of dried THF and placed in a dropping funnel. This solution was added to the Grignard reagent (II), with cooling at $0^{\circ}C$ over 15 minutes. Slight warming of the flask was evident as well as the formation of the magnesium salts, indicating that a reaction was taking place. The reaction mixture was allowed to warm to room temperature and then

gradually warmed to refluxing temperature and maintained for 1 hr. The magnesium salts appeared to be slightly soluble in THF at refluxing temperature.

The reaction mixture was then cooled, filtered and washed with the same solvent, to recover 77% of the magnesium salts. The solvent and excess dimethylchlorosilane of the filtrate was evaporated on a rotary evaporator and a partly crystalline slurry remained. The residue was taken up in 20 ml of ether, washed twice with 15 ml of 10% HCl, and washed thrice with 15 ml of distilled H_2O. The ethereal phase was dried over anhydrous Na_2SO_4 and the ether evaporated to yield a yellow liquid (68.5% crude yield).

The crude silane, III, was distilled and the main fraction was collected at 146-154°/760 yielding 5.04 g (46.5%) of III. A yellow residue remained in the distillation flask.

NMR (III): $Si(CH_3)_2$ - doublet at 0.16 ppm*; 6.0 H
Si-H - heptet at 3.8 ppm; 1.0 H
$(CH_2)_4$ - singlet at 1.16 ppm; 8.5 H

* all chemical shifts are given in δ values at 60 MHz.

1.1.3 PREPARATION OF METHYL-10-UNDECENOATE (V)

10-undecenoic acid (IV) (12.3 g - 0.067 mole) was dissolved in 75 ml of absolute methanol. To this mixture, 1 ml of H_2SO_4 was added and the reaction mixture was refluxed for 3 hours.

Twenty-five ml of H_2O was added to the reaction mixture and the ester was extracted with 20 ml of ether. The ethereal phase was washed twice with 15 ml of 8% $NaHCO_3$ solution, then thrice with 10 ml of H_2O. The ether layer was dried over anhydrous Na_2SO_4 and solvent evaporated yielding a viscous yellow liquid. The crude ester was distilled under vacuum and the main fraction was collected at 79-81°/0.7 mm which yielded 92% of the ester, V.

NMR (V) : $-(CH_2)-_6$ - singlet at 1.16 ppm; 12.0 H

$-OCH_3$ - singlet at 3.22 ppm; 3.0 H

$C\underline{H}_2=CH$ - multiplet at 3.78 ppm; 2.0 H

$CH_2=C\underline{H}$ - multiplet at 4.45 ppm; 1.0 H

$=CH-C\underline{H}_2$ - multiplet at } 2.0 ppm; 4.1 H

CH_2-COO - multiplet at }

IR (V) : $(CH_2)-CH$ - at 3.45 μ (S) and 3.52 μ (M)

C-COO - at 8.00 (W); 8.30 (M); 8.51 (M)

C = O - at 5.90 μ (S)

C = C - at 6.20 μ (W)

$CH_2=CH$ - at 7.05 μ (S)

1.1.4 PREPARATION OF METHYL-12,12-DIMETHYL-12-SILASTEARATE (VI)

Methyl-10-undecenoate (V) (2.0 g - 0.01 mole) and dimethylhexylsilane (III) (1.5 g - 0.01 mole) were mixed together with 0.5 ml of 0.1M chloroplatinic acid in isopropanol and sealed in a tube. This was left standing overnight at room temperature.

The sealed tube was opened and the reaction mixture had acquired a dark brown coloration. The isopropanol was evaporated on a rotary evaporator to yield the crude product (VI).

NMR (VI) crude : $-C\underline{H}_2=C\underline{H}$ - multiplet at 3.78 ppm; 2.3 H

1.2 SCHEME II - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID

1.2.1 PREPARATION OF n-HEXYL MAGNESIUM BROMIDE (II)

n-Hexyl Grignard reagent was prepared as previously described in Section 1.1.1.

1.2.2 PREPARATION OF DICHLOROHEXYLSILANE (VIII)

Trichlorosilane (VII) was dissolved in dried THF and an equimolar amount of n-hexyl Grignard reagent (II) was added to the solution with cooling and stirring at 0°C. The reaction mixture was refluxed for 1 hr whereupon the magnesium salts started to precipitate. The reaction mixture was cooled, filtered rapidly and washed with the same solvent. The solvent was removed on a flash evaporator yielding an oil and more magnesium salts. The residue was taken up in dry ether, filtered once more, and a clear solution was obtained. The ether was then removed on a flash evaporator to yield an oil. The product (VIII) was used without further purification and not isolated since the halogens present may have been easily hydrolysed.

1.2.3 PREPARATION OF METHYL-12,12-DICHLORO-12-SILASTEARATE (IX)

The crude product (VIII) was taken up in dry dioxane. Methyl-10-undecenoate (V) was added to this solution in the same solvent. The reaction mixture was placed in a stainless steel autoclave containing 1 ml of 0.1M H_2PtCl_6 which was dried azeotropically (using benzene and chloroform) dissolved in dioxane. The autoclave was sealed and left overnight at 95°C. The solvent was then removed by evaporation yielding the crude product IX (?) which was not isolated and used without further purification.

1.2.4 PREPARATION OF METHYL-12,12-DIMETHYL-12-SILASTEARATE (VI)

The crude product (IX) was dissolved in ether and allowed to react with the methyl magnesium iodide (X) prepared in a large excess in the usual manner. The addition was carried out at 0°C with stirring. The reaction proceeded with slight warming and magnesium salts were evident. The reaction mixture was maintained at 0°C for 1 hr, then warmed up to room temperature, and worked up by washing with dilute acid to decompose excess Grignard reagent. The ethereal phase was further washed with H_2O and dried over anhydrous Na_2SO_4. The solvent was removed on a flash evaporator yielding a dark brown oil. Upon distillation no fraction yielded the corresponding ester (VI) when analyzed by NMR.

1.3 SCHEME III - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID

1.3.1 PREPARATION OF METHYL-12-CHLORO-12,12-DIMETHYL-12-SILA UNDECANOATE (XI)

Methyl-10-undecenoate (V) (2 g - 0.01 mole) and 100% excess of dimethylchlorosilane (1.8 g - 0.02 mole) were dissolved in 20 ml of dry acetonitrile and placed in an autoclave containing 0.5 ml of 0.1M solution of the catalyst H_2PtCl_6 in acetonitrile.

The autoclave was sealed and maintained at 0°C for 2 hrs then allowed to warm to room temperature and left overnight. The resultant reaction mixture was evaporated under vacuum to remove excess dimethylchlorosilane as well as solvent. The crude product (XI) was then distilled under vacuum and a clear oil was collected at 130-132°/0.5 mm to obtain 76% yield.

NMR (XI) : $-Si(CH_3)_2$ - singlet at 0.35 ppm; 6.0 H
$-(CH_2)_{10}$ - singlet at 1.25 ppm; 20.8 H
$-OCH_3$ - singlet at 3.25 ppm; 3.0 H

no $CH_2=CH$ resonances evident.

MASS SPECTRUM : molecular ion at 292 m/e. (calculated 292 m/e.)

1.3.2 PREPARATION OF METHYL-12,12-DIMETHYL-12-SILASTEARATE (VI)

n-hexyl magnesium bromide (II) was prepared as previously described from (1.7 g - 0.009 mole) of n-hexyl bromide, (0.5 g -

0.02 gram-atom - 100% excess) of magnesium in 50 ml of ether. The resulting Grignard reagent was added to (2.63 gms - 0.009 mole) of methyl-12-chloro-12,12-dimethyl-12-silaundecanoate (XI) dissolved in 15 ml of sodium dried ether. The addition was carried out at $0^{o}C$ over 30 minutes and left stirring at that temperature for 1 hr. The reaction mixture was worked up in the usual manner; poured over ice/water, neutralized with 15 ml of 10% HCl, washed twice with 10 ml of 8% $NaHCO_3$, washed thrice with 10 ml of H_2O, dried over Na_2SO_4 and solvent removed on a rotary evaporator to yield the crude product.

The residue was fractionally distilled under vacuum and the main fraction was collected at $195-205^{o}/0.3$ mm to produce a 53% yield. Upon cooling the distillate crystallized. The precipitate was taken up in boiling petroleum ether and recrystallized. The melting point was recorded at $50-51.5^{o}$.

NMR (crude) ; - $Si(CH_3)_2$ - singlet at 0.2 ppm; 6.0 H
- $(CH_2)_n$ - singlet at 1.2 ppm; 34.0 H
- OCH_3 - singlet at 3.2 ppm; 2.2 H
- CH_3 (terminal) - distorted triplet at 0.9 ppm; 3.1 H

NMR (main fraction) (siloxane):- $Si(CH_3)_2$ - singlet at 0.2 ppm; 6.6 H
- $(CH_2)_n$ - singlet at 1.2 ppm; 20.3 H
- OCH_3 - singlet at 3.2 ppm; 3.0 H
no terminal CH_3 peak present

MASS SPECTRUM : molecular ion at 530 m/e. (which proved to be the siloxane).

1.4 SCHEME IV - ATTEMPTED SYNTHESIS OF 12,12-DIMETHYL-12-SILASTEARIC ACID

1.4.1 PREPARATION OF 10-UNDECEN-1-OL (XII)

$LiAlH_4$ (3 g - 0.08 mole - 33% excess) was suspended in sodium dried ether. 10-undecenoic acid (IV) (10 g - 0.05 mole) was dissolved in the same solvent and added dropwise to the former solution at 0°C with stirring, over a period of 30 minutes. The reaction vessel was maintained at 0° for 3 hrs. The resultant slurry was then hydrolyzed cautiously, with cooling, by the addition of 3 ml of H_2O/gm of $LiAlH_4$ used. The reaction mixture was left stirring until a totally white precipitate ($LiAlO_2$) was present. The lithium salts were filtered, and washed several times by resuspension in ether. The filtrate was washed with H_2O, dried over Na_2SO_4 (anhydrous), and solvent was removed on the flash evaporator. The resultant residue was distilled under vacuum to yield 52% (9.6 g) of the unsaturated alcohol (XII), B.p. 130-132°/16 mm.

NMR (XII) :	$-(CH_2)_8$	- singlet at 1.29 ppm;	16.0 H
	$-CH_2O$	- triplet at 3.40 ppm;	2.01 H
	-COH	- singlet at 4.35 ppm;	0.96 H
	$C\underline{H}_2$= CH	- multiplet at 4.90 ppm;	1.8 H
	CH_2= $C\underline{H}$	- multiplet at 5.65 ppm;	0.93 H

1.4.2 PREPARATION OF 10-UNDECENYL-1-TRIMETHYL SILYL ETHER (XIV)

10-undecen-1-ol (XII) (5.2 g - 0.035 mole) and triethylamine (3.3 g - 0.033 mole) were dissolved in 20 ml of dry benzene. Trimethylchlorosilane (XIII) (3.5 g - 0.033 mole) was placed in a dropping funnel

with 15 ml of the same solvent. The silane was added dropwise with stirring at 0°C and a precipitate (triethylamine hydrochloride) was evident immediately as well as slight heat evolution. The addition was completed within 15 minutes and the reaction mixture was warmed gently until refluxing commenced. The reaction mixture was refluxed for 1 hr, cooled and filtered to remove the salt formed. The hydrochloride salt was washed with benzene, dried and weighed to yield 4.5 g (100% recovery).

The filtrate was evaporated under vacuum and a 96% crude yield was obtained. The crude product was distilled under vacuum and the main fraction was collected at 84-88°/0.5 mm. The distilled product was 78% of theory with very little residue remaining in the distillation flask.

NMR (XIV) : - $Si(CH_3)_2$ - singlet at 0.16 ppm; 9.0 H
- $(CH_2)_7$ - singlet at 1.2 ppm; 14.3 H
- CH_2O - triplet at 3.45 ppm; 2.0 H
$C\underline{H}_2$= C$\underline{H}$ - multiplet at 4.85 ppm and 5.60 ppm; 3.0 H

no C-O$\underline{H}$ resonance evident.

1.4.3 PREPARATION OF 12-CHLORO-12,12-DIMETHYL-12-SILAUNDECYL-1-TRIMETHYL SILYL ETHER (XV)

The silyl ether (XIV) (9.8 g - 0.04 mole) was placed in the autoclave with 25% excess dimethylchlorosilane (5.0 g - 0.051 mole) and 1.5 ml of 0.1M H_2PtCl_6 in acetonitrile (dried). The reaction vessel was

sealed and heated for 12 hrs at 95°C. The reaction mixture was then evaporated to remove acetonitrile and excess silane and the residue distilled under vacuum to yield unreacted silyl ether at 88-90°/0.5 mm; a mixture of products at 92-120°/0.5 mm and a third fraction at 126-129°/0.5 mm; a 46% (6.21 g) yield.

NMR (3rd fraction) (XV) :
- $Si(CH_3)_3$ - singlet at 0.25 ppm; 7.4 H
- $ClSi(CH_3)_2$ - doublet at 0.15 ppm; 7.8 H
- $(CH_2)_{10}$ - singlet at 1.2 ppm; 21.4 H
- CH_2O - triplet at 3.40 ppm; 2.0 H

1.4.4 PREPARATION OF 12,12-DIMETHYL-12-SILASTEARYL ALCOHOL (XVI)

The product (XV) prepared in the previous section was used without further purification. Thus the product (XV) (6.21 g - 0.018 mole) was dissolved in 20 ml of sodium dried ether. To this solution was added n-hexyl magnesium bromide (II) (3.6 g - 0.019 mole - 10% excess based on XV) prepared in 35 ml of ether as previously described. The addition was carried out at 0°C with stirring and was complete in 30 minutes. The reaction mixture turned a grey-brown colour but no precipitate was evident. The reaction mixture was warmed to room temperature and 5 ml of THF was added to enhance precipitation of the magnesium salts. After 1 hr at room temperature, a slight precipitate was evident and was left stirring for 12 hrs.

The workup was carried out in the normal fashion; poured over ice/H_2O, washed with 15 ml of 10% HCl, washed twice with 10 ml of 8% $NaHCO_3$, washed three times with 10 ml portions of H_2O, dried over.

anhydrous sodium sulfate and solvent removed on a rotary evaporator. The residue was fractionally distilled under vacuum to yield a large fraction at 36-39°/0.8 mm and a minor fraction collected at 180-230°/0.3 mm.

NMR (38-39°/0.8 mm) : - $(CH_2)_n$ - singlet at 1.35 ppm; 13.10 H
- CH_2O - triplet at 3.50 ppm; 2.0 H
- OH - singlet at 4.90 ppm; 1.0 H
- $Si(CH_3)_n$ - multiplet at 0.15 ppm; 1.2 H
- CH_3 (terminal) - triplet at 0.85 ppm; 5.0 H

NMR (180-230°/0.3 mm): - $(CH_2)_n$ - singlet at 1.3 ppm; 34 H
- CH_2O - triplet at 3.75 ppm; 2.0 H
- $Si(CH_3)_2$ - doublet at 0.20 ppm; 11.0 H
no terminal CH_3 evident.

1.5 SCHEME IV A - MODIFIED APPROACH

1.5.1 PREPARATION OF 10-UNDECENYL-1-TRIPHENYLMETHYL ETHER (XVIII)

The unsaturated alcohol (XII) (5.0 g - 0.03 mole) was dissolved in 50 ml of dry benzene with triethylamine (3.25 g - 0.032 mole) as acid binder. Chlorotriphenylmethane (9.0 g - 0.05 mole - 66% excess) was dissolved in the same solvent and added to the former solution, dropwise with stirring. Upon addition the amine salt precipitated immediately and the reaction mixture was warmed to 50°C and maintained for 12 hrs. The amine salt was filtered, washed, dried and weighed to yield 83% recovery. The filtrate was evaporated under vacuum to remove solvent and unreacted amine. A dark brown oil remained as residue combined with small crystals (triphenyl carbinol). The residue was taken up in hexane and the crystals were filtered, washed and dried. Melting point of crude crystals was

recorded at 158-161°. (Triphenyl carbinol: m.p. 162°). The filtrate was evaporated and crude product was analysed by NMR. This product (XVIII) was not distilled for fear of decomposition.

NMR (XVIII) crude $-(CH_2)_7$ - singlet at 1.35 ppm; 16.6 H

$-OCH_2$ - triplet at 3.05 ppm; 2.0 H

$-C(C_6H_5)_3$ - multiplet at 7.22 ppm; 18.0 H

$CH_2=CH$ - multiplet at 4.89 & 5.75 ppm; 3.0 H

no hydroxyl resonance evident.

1.5.2 PREPARATION OF 12-CHLORO-12,12-DIMETHYL-12-SILAUNDECYL-1-TRIPHENYLMETHYL ETHER (XIX)

The crude trityl ether (XVIII) was placed in an autoclave containing 1.5 ml of 0.1M H_2PtCl_6 in dioxane and the chlorodimethylsilane (20 ml) was used as solvent. The reaction vessel was sealed and heated for 10 hrs at 95°C. The reaction mixture was then evaporated under vacuum to remove solvent (excess chlorodimethylsilane). NMR of this crude product indicated that hydrosilation took place since no unsaturation resonances were observed.

This reaction was repeated at the same molar ratios with the exception that the autoclave was left at room temperature for five days. Upon workup, the product contained a white crystalline precipitate. The solution was decanted and the crystals were taken up in hot methanol and recrystallized from the same solvent. The crystals were filtered, washed and weighed 5.3 g. The melting point of these crystals was 85-89°C.

(Triphenylmethane m.p, 94°). NMR of these crystals confirmed that triphenylmethane was formed. The tritane formation was 88.6% of theory.

NMR (tritane)	:-C-H	- singlet	at 5.42 ppm;	1.0 H
	$-C-(C_6H_5)_3$	- multiplet	at 7.20 ppm;	15.2 H
NMR (filtrate)	:$-Si(CH_3)_2$	- doublet	at 0.2 ppm;	8.42 H
	$-(CH_2)_{10}$	- singlet	at 1.22 ppm;	23.5 H
	$-CH_2O$	- triplet	at 3.30 ppm;	2.0 H
	$-C(C_6H_5)_3$	- multiplet	at 7.00 ppm;	1.4 H

1.5.3 PREPARATION OF n-HEXYL LITHIUM (XX)

Finely cut lithium metal (0.90 g - 0.13 gram-atom - 100% excess) was suspended in 50 ml of dry ether under N_2. n-Hexyl bromide (I) (10.8 g - 0.065 mole) was placed in a dropping funnel neat. A few drops were added to initiate the reaction and the remainder of the alkyl halide was dissolved in 25 ml of dry ether. The addition was carried out over 1 hr with cooling and stirring. Once completed, the reaction mixture was refluxed overnight under anhydrous conditions. The organolithium reagent could have been used immediately or stored in a sealed vessel under N_2 at 0°C.

1.5.4 PREPARATION OF 12,12-DIMETHYL-12-SILASTEARYL-1-TRIPHENYLMETHYL ETHER (XXI)

The crude product (XIX) was dissolved in 50 ml of dry ether and the n-hexyl lithium reagent (XX) was placed in a dropping funnel and

added to the former solution under a N_2 blanket with stirring at 0°C over 15 minutes. No reaction was evident during addition and the reaction mixture was allowed to reach room temperature. The reaction vessel was then gently heated to refluxing temperature and maintained for 15 hrs. The reaction mixture contained a white precipitate (LiCl) after this time, and was worked up by pouring over ice/H_2O, then washed with 20 ml of 10% HCl, washed twice with 10 ml of 8% $NaHCO_3$ solution, washed three times with 15 ml portions of H_2O, dried over Na_2SO_4 (anhydrous) and ether removed on a flash evaporator. A yellow-orange oil remained as the crude product. NMR of this product was not satisfactory thus it was not purified.

NMR (XXI) crude :	$-Si(CH_3)_3$	- broad singlet	at 0.25 ppm; 7.6 H
	$-(CH_2)_{15}$	- singlet	at 1.25 ppm; 25.8 H
	$-CH_3$ (terminal)	- triplet	at 0.80 ppm; 6.15 H
	$-OCH_2$	- triplet	at 3.92 ppm; 2.24 H
	$-C(C_6H_5)_3$	- multiplet	at 7.15 ppm; 12.4 H

1.6 SCHEME V - ATTEMPTED SYNTHESIS OF SHORT CHAINED SILICON-CONTAINING FATTY ACIDS

OPTION 1:

1.6.1 PREPARATION OF METHYL-12,12-DIMETHYL-12-SILATRIDECANOATE (XXII)

Methyl-12-chloro-12,12-dimethyl-12-silaundecanoate (XI) (1.83 g - 0.006 mole) previously described, was dissolved in 15 ml of dry ether and cooled with stirring at -18°C. Methyl Grignard reagent prepared in the usual manner by reacting methyl iodide (0.98 g - 0.007 mole) with

magnesium turnings (0.16 g - 0.007 gram-atom) in 15 ml of ether (dried), was added to the first solution dropwise. The reaction mixture was left stirring until the bath temperature reached between -5 to 0°C, and was worked up at that time by pouring over ice/H_2O, then washed with 10 ml of 10% HCl, washed twice with 10 ml of 8% $NaHCO_3$ solution, washed thrice with 10 ml portions of H_2O, dried over anhydrous Na_2SO_4 and solvent removed on a rotary evaporator yielding a dark yellow oil which weighed 1.71 g (102% yield).

NMR (XXII) crude	:-$Si(CH_3)_3$	singlet at 0.00 ppm;	5.9 H
	-$(CH_2)_{10}$	singlet at 1.25 ppm;	18.8 H
	-OCH_3	singlet at 3.55 ppm;	2.4 H
NMR (XXII) 160-180°/0.1 mm	:-$Si(CH_3)_3$	doublet at 0.00 ppm;	8.7 H
	-$(CH_2)_{10}$	doublet at 1.20 ppm;	18.6 H
	-OCH_3	singlet at 3.55 ppm;	3.0 H

OPTION 2:

1.6.2 PREPARATION OF 12,12-DIMETHYL-12-SILATRIDECAN-1-OL (XXIII)

10-Undecenyl-1-trimethyl silyl ether (XIV), prepared as previously described from 10-undecenol (5 g - 0.029 mole), was used as the crude product and reacted with 20 ml of chlorodimethylsilane (used as solvent) in the presence of 1.5 ml of 0.1M H_2PtCl_6/dioxane solution. The reactants were placed in an autoclave, sealed and heated at 95°C for 8 hrs. The reaction mixture was evaporated to remove excess solvent, yielding the crude product (XV) which was used without further purification.

Product (XV) was taken up in 30 ml of ether and cooled with stirring at 0°C. To this solution, was added methyl magnesium iodide prepared in the usual manner from 4.17 g (0.029 mole) of methyl iodide and 0.7 g (0.029 gram-atom) of magnesium turnings in 25 ml of dry ether. The reaction was exothermic and 5 ml of THF was added to aid the precipitation of the magnesium salts. The reaction mixture was heated to refluxing temperature and maintained for 1½ hrs. No precipitate was evident after this time so reaction was left stirring at room temperature overnight. A white precipitate ensued, thus the reaction mixture was worked up in the usual manner. It was poured over ice/H_2O, washed with 15 ml of 10% HCl, washed with 20 ml of 10% $NaHCO_3$ solution, washed twice with 20 ml portions of H_2O, dried over sodium sulfate, and solvent was removed on a rotary evaporator to yield 8.5 g (119%) of crude product (XXIII).

NMR (XXIII) crude : - $Si(CH_3)_3$	- singlet at 0.01 ppm; - triplet at 0.2 ppm;	9.09	H
- $(CH_2)_{10}$	- singlet at 1.20 ppm;	20.0	H
- OH	- singlet at 4.6 ppm;	1.27	H
- CH_2O	- triplet at 3.90 ppm;	2.7	H

OPTION 3:

1,6.3 PREPARATION OF DIMETHYLETHYLSILANE (XXV)

Ethyl magnesium bromide was prepared in the usual manner from 11.6 g (0.106 mole) of ethyl bromide, 2.54 g (0.106 gram-atom) of magnesium and 50 ml of THF. The resultant Grignard reagent was added to chlorodimethylsilane (10 g - 0.106 mole) dissolved in 20 ml of dried

THF, at 0°C with stirring. A white precipitate was evident immediately, and the reaction mixture was left stirring for 2 hrs at 0°C, then left overnight at room temperature. The reaction mixture was worked up by addition of ice/water, to decompose unreacted Grignard reagent, washed three times with 15 ml of distilled water, and dried over anhydrous sodium sulfate. The organic phase was then fractionally distilled utilizing a 30 cm column packed with 4 mm glass beads, to give 1.96 g (21%) of dimethylethylsilane (XXV), b.p. 44-46°C. Literature b.p.: 45.7°C.

NMR (XXV): $-Si(CH_3)_2$ - doublet at 0.61 ppm; 6.0 H
$-Si(H)$ - multiplet at 3.49 ppm; 1.0 H
$-Si(CH_2CH_3)$ - multiplet from 0.00 ppm to 0.84 ppm; 5.8 H

IR (XXV) : $-SiH$ - 4.74 μ (2120 cm^{-1}) (S)
$-SiCH_3$ -11.6 μ (875 cm^{-1}) (S)
- 7.95 μ (1255 cm^{-1}) (M)
-11.8 μ (840 cm^{-1}) (S)
$-CH_2CH_3$ - 3.4 μ (2950 cm^{-1}) (S)
- 7.05 μ (1420 cm^{-1}) (M)
- 6.85 μ (1460 cm^{-1}) (M)

IR coincided perfectly with spectrum published by W. Westermark[34].

1.6.4 PREPARATION OF METHYL-12,12-DIMETHYL-12-SILATETRADECANOATE (XXVI)

Methyl-10-undecenoate (V) (1.0 g - 0.005 mole) dissolved in 15 ml of n-hexane dimethylethylsilane (XXV) (0.44 g - 0.005 mole) dissolved in 10 ml of the same solvent, and benzoyl peroxide (0.122 g - 0.0005 mole) - recrystallized from cold $CHCl_3$/MeOH (1:2 V/V) were sealed in an autoclave and heated to 95°C for 12 hrs. The reaction mixture was neutralized with 15 ml

of 10% KOH, then washed twice with 5 ml of 2% $Na_2S_2O_5$ (sodium meta bisulfite) solution to decompose unreacted benzoyl peroxide. The hexane phase was washed twice with 20 ml of H_2O, dried over Na_2SO_4 and solvent removed on a rotary evaporator yielding the crude product. NMR of crude indicated no reaction since no silyl resonance was evident.

This reaction was repeated at the same scale using 20 ml of benzene under the same conditions to yield no reaction again. Even at elevated temperature (130°C) for 48 hrs, and 250% excess of silane yielded predominantly starting material with a very small silyl signal.

1.7 SCHEME VI ATTEMPTED SYNTHESIS OF SHORT CHAINED SILICON-CONTAINING FATTY ACID

1.7.1 PREPARATION OF METHYL-12,12-DIMETHYL-12-SILAUNDECANOYL-12-METHOXIDE (XXVII)

Sodium methoxide was prepared by reacting clean sodium metal (0.5 g - 0.022 gram-atom) with 50 ml of absolute methenol. The resultant turbid methanolic solution was added dropwise to a solution of compound (XI) (2.0 g - 0.007 mole) dissolved in 10 ml of sodium dried ether, whereupon a white precipitate (NaCl) formed. The reaction mixture was heated to reflux and maintained for 2½ hrs, after which time the reaction vessel was cooled, and diluted with approximately 20 ml of ice/H_2O to decompose the excess methoxide. The ethereal phase was washed with 20 ml of 10% HCl, then twice with 10 ml of 10% $NaHCO_3$, and finally twice with 20 ml of H_2O, dried over anhydrous calcium chloride (which also binds traces of

alcohol) and the solvent was removed on a flash evaporator to yield a yellow oil, 1.80 gm (91%) crude yield.

NMR (XXVII) crude : - $Si(CH_3)_2$ - singlet at 0.09 ppm; 6.0 H
- $(CH_2)_8$ - singlet at 1.12 ppm; 16.9 H
- $COOCH_3$ - singlet at 3.43 ppm; 2.9 H
- OCH_3 - singlet at 3.20 ppm; 1.4 H
- OH - singlet (broad) at 4.28 ppm; 0.5 H

1.7,2 PREPARATION OF XXVII VIA AN ALTERNATE METHOD

Compound (XI) (1.0 g. - 0.0035 mole) was dissolved in 15 ml of dried benzene and was added to a solution of absolute methanol (0.25 g - 0.008 mole - 100% excess) dissolved in 15 ml of the same solvent containing triethylamine (0.34 g - 0.0035 mole). The amine salt precipitated immediately upon addition and an exothermic reaction ensued. The reaction mixture was left stirring at room temperature for 4 hrs after which time the amine salt was filtered, washed with the same solvent, and dried to yield a 92.5% recovery. The filtrate was evaporated on a flash evaporator to remove the excess methanol and the solvent, benzene, and yielded an oil with residual amine salt dissolved in methanol. The residue was taken up in 10 ml of ether and the precipitate was filtered, washed and dried. The total amine salt recovered was now 99%. The ether in the filtrate was removed by vacuum to yield 0.98 g (100%) of the crude product (XXVII).

NMR (XXVII) crude : - $Si(CH_3)_2$ - singlet at 0.09 ppm; 5.80 H
- $(CH_2)_8$ - singlet at 1.10 , 16.00 H
- $COOCH_3$ - singlet at 3.40 ppm; 2.98 H
- $SiOCH_3$ - singlet at 3.18 ppm; 2.68 H

no OH resonance.

1.7.3 HYDROLYSIS OF XXVII

The crude product XXVII (0.98 g - 0.0035 mole) was dissolved in 30 ml of methyl alcohol and 0.14 g (0.0035 mole) of NaOH dissolved in 10 ml of H_2O was added. The reaction mixture was refluxed for 1 hr and worked up by evaporating the solvent to approximately one-third its volume. The residue was diluted with 10 ml of H_2O, 25 ml of ether, and 10 ml of 10% HCl. The ethereal layer was separated and washed twice with 10 ml of H_2O, dried over $CaCl_2$ and solvent evaporated on a flash evaporator to yield 90% crude product.

NMR (hydrolysis product):

- $Si(CH_3)_2$	- singlet at 0.08 ppm;	5.5	H
	- doublet at 0.08 ppm;		
- $(CH_2)_8$	- singlet at 1.12 ppm;	16.0	H
- OCH_3	- singlet at 3.50 ppm;	0.25	H
- COOH	- singlet at 9.08 ppm;	1.3	H

1.7.4 PREPARATION OF METHYL-12,12-DIMETHYL-12-SILAUNDECANOYL-12-PENTYLOXIDE (XXVIII)

Methyl-12-chloro-12,12-dimethyl-12-silaundecanoate, (XI) (1.0 g - 0.003 mole) was dissolved in 20 ml of dried benzene and added with stirring to a solution containing amyl alcohol (0.92 g - 0.01 mole - 300% excess) and triethylamine (0.34 g - 0.003 mole) in 10 ml of the same solvent. Upon addition, the amine salt precipitated and an exothermic reaction was evident. The reaction mixture was left stirring at room temperature for 12 hrs. The amine salt formed was filtered, washed with the same solvent, dried and weighed giving a 74% recovery of the amine as its salt. The

filtrate was evaporated under vacuum to remove the excess amyl alcohol as well as the solvent, to yield an oil as a crude reaction product. The residue was dissolved in 20 ml of ether and washed several times with H_2O. The ethereal layer was separated, dried over $CaCl_2$, and the solvent was removed on a rotary evaporator to yield the crude product.

NMR (XXVIII) crude :	- $Si(CH_3)_2$	- singlet at 0.06 ppm;	6.07 H
	- $(CH_2)_{11}$	- singlet at 1.24 ppm;	21.06 H
	- CH_3 (terminal)	- triplet at 0.80 ppm;	3.57 H
	- $COOCH_3$	- singlet at 3.50 ppm;	superimposed total 4.84 H
	- OCH_2	- triplet at 3.45 ppm;	

26

1.8 SCHEME VII SYNTHESIS OF 13,13-DIMETHYL-13-SILAHEPTADECANOIC ACID

1.8.1 PREPARATION OF 11-CHLORO-1-UNDECENE (XXIX)

10-undecen-1-ol (10.0 g - 0.06 mole) was placed in the reaction vessel and thionyl chloride (35 ml - 57.33 g - 0.48 mole), distilled from linseed oil, was added dropwise with stirring over a period of 1 hr. As the addition took place, the reaction vessel was gently heated until refluxing occurred after approximately 30 minutes. The reaction mixture was refluxed for 6 hrs once the addition was completed, after which time the reaction mixture was cooled, the excess thionyl chloride was removed on a rotary evaporator, and the residue was diluted with 75 ml of ice/water, to destroy any residual thionyl chloride. 100 ml of ether was added and the ethereal layer was separated, washed with 10% NaOH (20 ml) washed twice with 20 ml portions of H_2O, dried over anhydrous sodium sulfate and solvent was removed under vacuum to yield a dark oil. The crude product

was distilled under vacuum and a main fraction was collected at 61-63°/0.25 mm (10.22 g) which was a 92% yield of the product (XXIX). This fraction exhibited a very strong Beilstein test indicating the presence of halogen.

NMR (XXIX) :	$-(CH_2)_7$	- singlet	at 1.10 ppm;	14.0 H
	$-CH-C\underline{H}_2$	- multiplet	at 1.80 ppm;	2.0 H
	$-CH_2-Cl$	- triplet	at 3.24 ppm;	2.07 H
	$C\underline{H}_2=CH$	- multiplet	at 4.72 ppm;	2.00 H
	$CH_2=C\underline{H}$	- multiplet	at 5.25 ppm;	1.03 H

This reaction was repeated at 50 and 100 g scales to obtain 92% and 90% yields respectively.

1.8.2 PREPARATION OF 10-UNDECENYLDIMETHYLCHLOROSILANE (XXX)

A few drops of the alkyl halide (XXIX)(5.0 g - 0.027 mole) were added neat to magnesium turnings (0.75 g - 0.03 gram-atom - 17% excess) in 10 ml of dried ether, to initiate the Grignard reagent formation. The remaining alkyl halide was dissolved in 20 ml of same solvent and added dropwise to the reaction mixture which was refluxing spontaneously. The addition was completed in 15 minutes and the reaction mixture was refluxed for 3 hrs until the reaction was complete. The unreacted magnesium was recovered to indicate 100% formation of the Grignard reagent.

The resultant Grignard reagent was added to dimethyldichlorosilane (15.0 g - 0.116 mole - 330% excess) dissolved in 50 ml of dried ether. No reaction was evident during addition and the reaction mixture was left to reflux for 18 hrs. A white precipitate (magnesium salts) was evident

15 minutes after refluxing had commenced. Most of the ether was removed on a flash evaporator and the resulting residue was filtered rapidly to remove the magnesium salts. The filtrate was distilled under vacuum to yield 4.59 g (72%) of the product, (XXX), collected at 84-85°/0.4 mm. This reaction was carried out several times at 10, 20 and 50 g scales to produce a 78%, 69%, 70.5% yield respectively.

NMR (XXX) : - $Si(CH_3)_2$ - singlet at 0.11 ppm; 6.0 H
- $(CH_2)_7$ - singlet at 1.08 ppm; 14.4 H
= CH-$C\underline{H}_2$ - multiplet at 1.75 ppm; 2.00 H
$C\underline{H}_2$=CH - multiplet at 4.69 ppm; 1.9 H
CH_2=$C\underline{H}$ - multiplet at 5.45 ppm; 1.0 H

1.8.3 PREPARATION OF n-BUTYL-10-UNDECENYLDIMETHYLSILANE (XXXI)

n-Butyl magnesium bromide was prepared, in a 100% excess, by reacting magnesium (0.90 g - 0.038 gram-atom) with n-butyl bromide (4.35 g - 0.032 mole), initiated by a crystal of I_2, in 50 ml of dried ether. The reaction mixture was decanted rapidly, so as to separate the excess magnesium turnings, and added to a solution of 10-undecenyldimethylchlorosilane (XXX) (3.87 g - 0.016 mole) dissolved in 20 ml of same solvent. No reaction was evident upon addition or during a subsequent reflux period. Diethyl ether was replaced by adding 50 ml of n-butyl ether and the solvent was distilled from the mixture until the temperature reached 100°C. As distillation was taking place, the appearance of the magnesium salts became evident. The mixture was left refluxing with stirring for 18 hrs. The resulting slurry was poured over an ice/H_2O mixture containing

sufficient dilute HCl to dissolve the magnesium salts. The two phases were separated and the ethereal layer was washed with H_2O several times and dried over anhydrous Na_2SO_4. The solvent was removed on a flash evaporator and the resulting residue was distilled under vacuum. The product (XXXI) was collected between 99-101°/0.3 mm to yield 2.13 g (50.7%) of the product. The reaction was repeated to yield 74.5% product at a 15 g scale and a 79% product at 35.4 g scale.

NMR (XXXI) : - $Si(CH_3)_2$ - singlet at 0.16 ppm; 5.98 H
- $(CH_2)_{10}$ - singlet at 1.20 ppm; 20.0 H
- CH_3(terminal)- triplet at 0.77 ppm; 3.16 H
- $CH\text{-}C\underline{H}_2$ - multiplet at 1.92 ppm; 2.37 H
$C\underline{H}_2$=CH - multiplet at 4.84 ppm; 2.14 H
CH_2=$C\underline{H}$ - multiplet at 5.55 ppm; 1.07 H

1.8.4 PREPARATION OF 11-BROMOUNDECYL-n-BUTYLDIMETHYLSILANE (XXXII)

n-Butyl-10-undecenyldimethylsilane (4.92 g - 0.018 mole) was dissolved in 15 ml of petroleum ether, with 0.1 g of benzoyl peroxide, recrystallized from cold $CHCl_3$/MEOH 1:2 V/V. Two drops of H_2O were added and the reaction vessel was cooled in an ice bath with stirring. Hydrogen bromide gas was bubbled in from a gas cylinder for 1½ hrs whereupon the solution became saturated and obtained a yellow coloration. The vessel was then stoppered and left standing at room temperature overnight. The resulting solution was washed with H_2O, then aqueous ferrous sulfate (to decompose any peroxide), then twice with 5% KOH (to form the potassium salt of benzoic acid), and finally three times with H_2O. The mixture was dried over anhydrous sodium sulfate and solvent evaporated

on a flash evaporator to yield the crude product. The residue was distilled under vacuum and the product (XXXII) was collected between 148-151°/0.5 mm yielding 3.98 g (62.5%) of 11-bromoundecyl-n-butyldimethylsilane (XXXII). Repetition of this reaction at 5.0 g and 37.58 g scales yielded 63% and 78% of the product.

A note worth mentioning when larger scales are used is, that in order to obtain respectable yields, bubbling of HBr should not be carried out on batches exceeding 20.0 g of starting material. After the addition of HBr, the batches may be combined and worked up together.

NMR (XXXII) : - $Si(CH_3)_2$ - singlet at 0.12 ppm; 5.95 H
- $(CH_2)_{11}$ - singlet at 1.20 ppm; 22.0 H
- CH_3 (terminal) - triplet at 0.82 ppm; 3.1 H
- CH_2Br - triplet at 3.22 ppm; 1.8 H

no unsaturation resonances evident.

1;8.5 PREPARATION OF 13,13-DIMETHYL-13-SILAHEPTADECANOIC ACID (XXXIII)

11-Bromoundecyl-n-butyldimethylsilane (XXXII) (5.0 g - 0.014 mole) was reacted with magnesium metal (1.0 g - 0.04 gram-atom - 190% excess) in 50 ml of ether. The Grignard formation was initiated in a test tube with grinding and once started, it was added to the remaining reaction mixture, and was refluxed for 3 hrs. The resultant mixture was decanted, so as to separate the Grignard reagent from the excess magnesium, and then added to a carbon dioxide/ether slurry in a glove bag under an atmosphere of dry N_2.

The slurry was prepared under N_2 by taking small pieces of solid CO_2 (in excess) and dipping each piece in an acetone bath (to remove any H_2O) and then in an ether bath (to remove any acetone). The pieces were ground up with a mortar and pestle and placed in a beaker. Anhydrous ether was added slowly with stirring until the slurry had formed.

The Grignard reagent was cooled and added very slowly with vigorous stirring to the slurry in the glove bag. After evaporation of the CO_2, the reaction mixture was cautiously hydrolyzed by the addition of H_2O and an excess of dilute HCl. The ethereal layer was separated, washed several times with H_2O, dried over anhydrous Na_2SO_4 and solvent was removed on a flash evaporator yielding the crude acid. The residue was distilled under vacuum and the product (XXXIII) was collected at 166-170°/0.5 mm as a viscous clear oil. The product obtained was 43% of theory.

Repetition of this reaction at a larger scale (27.68 g), yielded 66% of the product collected at 180-182°/0.75 mm.

A small amount of the acid was further purified by converting it to its barium salt, as described[26] to give the following mass spectral analysis:

MASS SPECTRUM (XXXIII) : molecular ion at 314 m/e (calculated 314 m/e)

NMR (XXXIII) : - $Si(CH_3)_2$ - singlet at 0.16 ppm; 6.0 H
$CH_3(CH_2)_{14}$ - multiplet at 0.04-2.1 ppm; 31.9 H
- $OOCCH_2$ - multiplet at 2.10 ppm; 2.04 H
- COOH - singlet at 8.91 ppm; 0.92 H
- $(CH_2)_{11}$ - singlet at 1.20 ppm; 21.7 H
- CH_3 (terminal) - triplet at 0.85 ppm; 3.1 H
- $Si(CH_2)_2$ - triplet at 0.38 ppm; 4.1 H

IR (XXXIII) : - C=O at 6.0 μ (S)
$Si(CH_3)$ at 8.15 μ (M)
$Si(CH_2)$ at 12.15 μ (W)
OH at 3.0 μ (S)

1.8.6 PREPARATION OF METHYL-13,13-DIMETHYL-13-SILAHEPTADECANOATE (XXXIV)

The silicon-containing fatty acid (XXXIII) (4.0 g -0.012 mole) was dissolved in 15 ml of anhydrous ether and cooled to 0°C. Diazomethane (1.5 g - 0.035 mole) prepared by the action of 50% KOH on nitroso-methyl-urea in ether, was carefully added to the former solution. Frothing and gas evolution (N_2), was evident immediately upon addition which required approximately 1 hr. The mixture retained the characteristic yellow colour and was left standing overnight at room temperature to decompose the excess diazomethane. The resultant mixture was dried over anhydrous Na_2SO_4 and solvent was removed on a flash evaporator to yield the crude ester. The residue was distilled under reduced pressure and the product was collected at 158-161°/0.9 mm. The ester obtained (3.33 g) was 80.5% of theory.

MASS SPECTRUM (XXXIV) : molecular ion at 328 m/e (calculated 328 m/e)

NMR (XXXIV) : - $Si(CH_3)_2$ - singlet at 0.16 ppm; 6.0 H
- $(CH_2)_{11}$ - singlet at 1.19 ppm; 21.4 H
- CH_3 (terminal) - triplet at 0.84 ppm; 3.15 H
- OCH_3 - singlet at 3.24 ppm; 2.8 H

1.8.7 PREPARATION OF 13,13-DIMETHYL-13-SILAHEPTADECANOYL CHLORIDE

The acid (XXXIII) (3.0 g - 0.01 mole) was dissolved in 25 ml of benzene and was added dropwise with stirring to a solution of oxalyl chloride (2.0 g - 0.015 mole - 50% excess) dissolved in 15 ml of same solvent. Gas evolution was evident immediately upon addition. Reaction mixture was left stirring at room temperature until no more gas evolution was evident, after which time the mixture was refluxed for 3 hrs to decompose excess oxalic acid. The mixture was cooled and solvent and excess oxalyl chloride were removed on a flash evaporator to yield the crude acid chloride which was used without further purification.

NMR : no carboxyl hydrogen evident
all resonances and integration remained the same as the carboxylic acid (XXXIII)

IR : ClC=O stretching at 5.55 u (1795 cm^{-1}) (S)
no OH stretching between 3.0 u and 3.32 u (3300-3000 cm^{-1})

1.8.8 PREPARATION OF 13,13-DIMETHYL-13-SILAHEPTADECANOIC ANHYDRIDE

The acid chloride (3.2 g - 0.01 mole) was dissolved in 20 ml of dry benzene containing 2.0 g (0.02 mole - 100% excess) of triethylamine. The acid chloride/triethylamine complex was formed as the reaction mixture was stirred for approximately 5 minutes. To this mixture, was

added a solution of the acid (XXXIII) (3.1 g -0.01 mole) dissolved in 15 ml of dry benzene, over a 10 minute period with stirring. The amine salt separated out during addition, and once complete, the mixture was left stirring at room temperature overnight. The amine salt was filtered, washed, dried and weighed to obtain a 57.5% recovery. The solvent and excess triethylamine were removed on a flash evaporator to yield a brown coloured crude product. The anhydride was used without further purification.

IR : -C=O at 5.6 μ (1807 cm^{-1}) (8)

1.9 PARTIAL LECITHIN SYNTHESIS

1.9.1 PREPARATION OF L-α-GLYCEROPHOSPHORYLCHOLINE - CADMIUM CHLORIDE [36]

Commercial egg yolk lecithin (25.0 g) was dissolved in 250 ml of anhydrous ether and filtered through cotton wool. Tetrabutylammonium-hydroxide (25% in methanol; 25 ml) was added to the filtrate and shaken for 2 minutes. After approximately 5 minutes, the solution became turbid and a brown solid started to precipitate. The reaction mixture was left standing for 2 hrs at room temperature and then decanted. The precipitate was washed with an additional 50 ml of anhydrous ether by resuspension. The solution was decanted after about 10 minutes and the residue was boiled with absolute alcohol (75 ml) in which it is partially soluble. Hyflo Super-cell (0.5 g) was added and the mixture was filtered hot. The filtrate was cooled and 125 ml of anhydrous ether was added whereupon a brown precipitate was formed which was allowed to settle and the supernatant

was decanted. 20 ml of boiling water was added to the precipitate as well as a solution of cadium chloride ($CdCl_2.2.5H_2O$, 4.0 g in 10 ml of H_2O). Norite (0.5 g) and hyflo (1.0 g) were added and the mixture was brought to boiling and filtered hot. Ethanol (approximately 125 ml) was added to the colourless filtrate until a persistent turbidity became evident. The solution was cooled to 0-5°C and allowed to stand overnight at that temperature. The L-α-Glycerophosphorylcholine cadmium chloride complex separated as a colorless precipitate. The product was filtered, and washed with absolute alcohol, benzene, and ether then dried in a vacuum oven, overnight at 60°C/10 mm. The product yield was 7.2 g (50.9%).

Analysis for Phosphorus : Cal'd - 7.03%; Found - 6.94%

NMR in D_2O : $-N(CH_3)_3$ - singlet at 3.26 ppm; 9.0 H
$CH_2CH\ CH_2$ - multiplet between 3.45-4.1 ppm;
CH_2CH_2-N - multiplet between 3.45-4.1 ppm; } 8.9 H

Free L-α-GPC was obtained by passing an aqueous solution of the cadmium chloride adduct through a mixed bed ion exchange resin Amberlite IRC-50 and IR-45. Free GPC is extremely hygroscopic.

1.9.2 ATTEMPTED SYNTHESIS OF Di-(13,13-DIMETHYL-13-SILAHEPTADECANOYL) LECITHIN[40]

GPC-$CdCl_2$ (0.58 g - 0.0013 mole) was dissolved in a minimum amount of H_2O and passed through a mixed bed of ion exchange resins to yield 0.36 g (92.5%) of free GPC after drying under vacuum.

Free GPC (36.0 g - 0.0014 mole) was mixed with 13,13-dimethyl -13-silaheptadecanoic acid (0.950 g - 0.003 mole - 114.3% excess), and potassium hydroxide (0.183 g - 0.0033 mole - 10% excess) in 20 ml of absolute methanol. The solution was evaporated to dryness under reduced pressure on a rotary evaporator to obtain a white powder which was dried in a vacuum dessicator over P_2O_5 for 5 hrs. 13,13-dimethyl-13-silaheptadecanoic anhydride (3.70 g - 0.006 mole - 228% excess) was added to the powder as well as a few glass beads, to increase surface area, and the heterogeneous mixture was closed under vacuum and heated in an oil bath at 78-79°C for 48 hrs with stirring. The anhydride (in large excess) dissolved the powder to form a thick homogeneous mixture. The reaction mixture was cooled to room temperature and 25 ml of anhydrous ether was added and the solution filtered to separate a very small amount of precipitate. Robles[40] states that the lecithin is contained in this precipitate which can be obtained by dissolving this precipitate in boiling chloroform and letting the anhydride separate out. The lecithin would be in the filtrate. The small amount of precipitate we obtained was insoluble in boiling chloroform.

This procedure was repeated several times to give the same results. All fractions were analyzed and the bulk of the acid and anhydride were found in the ethereal fraction. The chloroform fraction yielded neither lecithin nor GPC.

1.9.3 ATTEMPTED SYNTHESIS OF DI-(13,13-DIMETHYL-13-SILAHEPTACANOYL) LECITHIN[39]

GPC-$CdCl_2$ (0.5 g - 0.001 mole) was placed in a reaction vessel with a few glass beads. The flask was cooled in an ice bath, and to the rapidly stirred mixture, a thin stream of freshly prepared 13,13-dimethyl-13-silaheptadecanoyl chloride (4.9 g - 0.015 mole) dissolved in 10 ml of ethanol - free chloroform[39], followed by a solution of anhydrous pyridine[64] (0.9 g - 0.011 mole) in 15 ml of same solvent, was added. After 30 minutes, the reaction vessel was warmed to room temperature and the stirring continued for 2 hrs. The reaction mixture was then poured through a Buchner funnel without filter, and the glass beads were washed several times with same solvent. The combined filtrates were centrifuged and the solvent was removed on a flash evaporator; the resultant residue was dried overnight under vacuum (1 mm) in a bath at 35°C.

The dried residue was suspended in 75 ml of anhydrous acetone[39] and centrifuged. The pellet obtained, was washed with an additional 25 ml of anhydrous acetone in the same manner, followed by a washing with 25 ml of anhydrous ether. The resultant pellet was dried under vacuum and last traces of cadmium chloride, and pyridine hydrochloride were removed by dissolving the pellet in 75 ml of chloroform, methanol, water (5:4:1 V/V) and passing this solution through a mixed bed of ion exchange resins. The bed was washed with 100 ml of same solvent mixture and the combined eluants were evaporated on a rotary evaporator which yielded a yellow oily film (0.10 g - 10.4%) - the crude lecithin.

NMR - $N(CH_3)_3$ very small singlet at 3.47 ppm;

TLC - ($CHCl_3/CH_3OH/H_2O$ - 65:25:4 V/V) of commercial egg yolk lecithin and crude product revealed one predominant spot, R_f=0.8. for the product which appears to be the labelled fatty acid.

This method of synthesizing the "labelled" lecithin was attempted several times only to yield between 5-15% crude product.

1.9.4 ATTEMPTED SYNTHESIS OF Di-(13,13-DIMETHYL-13-SILAHEPTADECANOYL) LECITHIN[41]

The cadmium chloride complex of GPC (0.5 g - 0.00047 mole)- based on $(GPC)CdCl_2$ was mixed with free 13,13-dimethyl-13-silaheptadecanoic acid (3.64 g - 0.012 mole), trifluoroacetic anhydride (4.71 g - 0.023 mole) and anhydrous triethylamine[65] (0.06 g - 0.0005 mole). The reaction mixture was stirred under anhydrous conditions for 48 hrs at room temperature. The mixture was heterogeneous and a light coloration ensued. Upon addition of reactants, slight warming of reaction vessel was evident.

Absolute methanol (1 ml) was added, to form the methyl ester of the excess acid, as well as chloroform (20 ml) and 1.5 ml of 0.01M HCl solution, to free the $CdCl_2$ (ion exchange not necessary). The resultant solution was centrifuged and the supernatant was decanted. The pellet was washed twice wi[illegible] ml of benzene and the combined supernatants were evapo-

rated to dryness on a flash evaporator. The residue was dissolved in 10 ml of $CHCl_3$ and passed through a 10 cm silica column. The column was eluted with 150 ml of chloroform (where most of the brown coloration was removed), then with 100 ml of 9:1 $CHCl_3$ / MeOH solution (to remove any free acid), then followed by 200 ml 1:1 $CHCl_3$ / MeOH (which elutes the lecithin formed), and finally eluted with 200 ml of 1:4 $CHCl_3$ / MeOH (which elutes any lysolecithin formed). All fractions were evaporated to dryness on a flash evaporator to obtain the following residues:

Fraction I - a dark yellow oil (fatty acid and methyl ester)

Fraction II - a dark yellow oil (predominantly fatty acid)

Fraction III - a dark green oil (lecithin ?) - 7.0%

Fraction IV - a dark green sticky mass (lysolecithin ?)

NMR (Fraction I) : typical labelled fatty acid spectrum containing the - OCH_3 resonance of the methyl ester (small) at 3.35 ppm.

NMR (Fraction II): typical labelled fatty acid spectrum containing a small choline resonance.

$-Si(CH_3)_4$	- singlet	at 0.2 oom;	12.0 H
$-(CH_2)_{22}$	- singlet	at 1.22 ppm;	69.1 H
$-N(CH_3)_3$	- doublet (?)	at 4.00 ppm;	4.57 H
(?)	- multiplet	at 7.40 ppm;	4.57 H

NMR (Fraction III) - 7%

$-Si(CH_3)_4$	- singlet	at 0.2 ppm;	12.00	H
$-(CH_2)_{22}$	- singlet	at 1.22 ppm;	84.0	H
$-N(CH_3)_3$	- doublet (?)	at 4.00 ppm;	8.4	H
(?)	- multiplet	at 7.40 ppm;	8.4	H

NMR (Fraction IV) : - identical spectrum as Fraction III.

The same experiment was repeated using stearic acid in the same molar ratio only to obtain an even smaller choline resonance found only in Fraction II.

1.10 INCORPORATION OF LABELLED FATTY ACID INTO BACTERIAL MEMBRANE:

1.10.1 ORGANISM AND GROWTH CONDITIONS:

Mycoplasma laidlawii strain B ATCC* No.14192 was grown in a basal medium described by Razin et al[66] with minor modifications. The medium consisted of 2% tryptose (Difco Laboratories), 0.5% NaCl, 0.5% Tris (hydroxy methyl) amino methane, 10% bacto PPLO serum fraction, 0.5% glucose and (1:2000 W/V) of thallium acetate[67] instead of penicillin G. Growth was carried out in 10 ml volume of medium in test tubes incubated statically at 37°C. A 10% inoculum every 48 hrs into fresh medium, maintained the organism thriving. Approximately 3 passages were required for normal growth, which was monitored by nephelometry (optical density at 420 nm).

* American Type Culture Collection

Once viability of the organism was confirmed, a 10% inoculum was added to a basal medium containing 2% tryptose which had been defatted by 12 hrs Soxhlet extraction with chloroform-methanol (2:1 V/V) followed by a 12 hr extraction with anhydrous ether, and supplemented with 10 mg/l of the sodium salt of the silicon-containing fatty acid. No PPLO plasma was added and the rest of the ingredients remained unchanged as well as the growth conditions. Approximately five passages were required for normal growth which was not as vigorous as growth in normal medium. Once viability was confirmed, the volume of the medium was scaled up 10-fold i.e. organism was grown in 100 ml volume of medium, contained in 250 ml Erlenmeyer flasks.

The organism was harvested 36 hrs after inoculation (12 hrs beyond the end of the logarithmic phase of growth) by centrifugation at 20,000 g for 20 minutes. The sedimented cells were washed twice with β-buffer[68] containing 0.156M NaCl, 0.05M Tris, 0.01M mercapto-ethanol in deionized water adjusted to pH 7.4 with HCl. The cells were centrifuged again to yield 0.04 to 0.07 g wet weight/100 ml culture.

1.10.2 LIPID EXTRACTION

The washed cell paste, from various batches, was pooled together and homogenized by hand in 20 ml of chloroform-methanol (1:2 V/V) mixture[46]. To the mixture was added 20 ml of chloroform and 20 ml of distilled water and further homogenized. The homogenate was then filtered on a Buchner funnel, the layers were allowed to

separate to clarity and the chloroform phase drawn off and evaporated to dryness on a rotary evaporator to yield 0.09 g of a sticky mass (the crude lipids).

NMR spectrum of the extracted lipid clearly indicated the dimethyl silyl resonance at 0.12 ppm. A doublet was observed at 3.61 ppm and is probably a glucose residue from phosphatidyl glucose[67], since mycoplasma bacterium contains very little phosphatidyl choline. The methylene resonance appeared as a large singlet at 1.35 ppm.

Lyophilization of the aqueous phase yielded the water/methanol soluble protein. No further classification of the lipid nor of the protein was carried out.

2. INVESTIGATIONS ON LABELLED LIPOSOMES

2.1 DESCRIPTION OF INSTRUMENTS

All nuclear magnetic resonance spectra were recorded on a Varian A60 spectrometer or a Varian T60 spectrometer equipped with a constant temperature probe (38°C) or a variable temperature probe. The latter, was interfaced with a PDP-11 computer which was utilized as a time averaging instrument. This interface was designed and built by Dr. E. Cerny and H. Lam[69] of the Electrical Engineering Department, Concordia University, Loyola Campus.

All NMR samples were run in CCl_4 (reagent grade) or D_2O (99.8% purity - Aldrich Chemical Co.) unless otherwise mentioned.

The IR instruments utilized for the identification of samples were: Beckman IR-8, Perkin Elmer 457, or a Perkin Elmer 225 spectrophotometer.

The mass spectra used to verify synthetic samples were taken on a Hitachi RMU-6 spectrometer.

The UV instrument employed for the nephelometric observation of the micro-organism Mycoplasma laidlawii was a Beckman DK-2A ratio recording spectrophotometer equipped with a tungsten lamp.

2.2 PREPARATION OF LIPOSOMES

Commercial egg yolk lecithin (242.1 mg) (Sigma Chemicals) and the methyl ester of the silicon-containing fatty acid, methyl-13,13-dimethyl-13-silaheptadecanoate, (16.4 mg), were dissolved in 2-3 ml of peroxide free ether and evaporated to dryness under vacuum. Deuterium oxide (2 ml) was added and the resultant mixture was sonicated at 0-2°C for 15 minutes or until optically clear, with a "Sonic 300" dismembrator (Artek Systems Corp.) at a 180-W output at 20,000 Hz. The sonicated mixture was centrifuged in the cold at 15,000 rpm for 30 minutes to remove any metal fragments and large multilamellar "onion-like" vesicles. The supernatant was used for all the experiments without further classification. All experiments were carried out in triplicate and data shown are mean values. The data were subjected to standard deviation calculations and curves were fitted by least squares method unless mentioned otherwise.

2.3 CALIBRATION OF MEMBRANE MODEL

2.3.1 OPTIMIZATION OF MOLAR RATIO : LECITHIN/LABELLED FATTY ACID ESTER

An arbitrary amount of the labelled fatty acid ester (LFAE), methyl-13,13-dimethyl-13-silaheptadecanoate (0.0328 g - 100 mM) was kept constant throughout seven samples. Increasing amounts of egg yolk lecithin (LEC) (50 mM to 600 mM) were added to these samples so that the molar ratio (LEC/LFAE) ranged from 0.5 to 6.0. Each of these samples were codispersed, cosonicated and centrifuged as described above. Plotting the width at half-

height ($\Delta\nu_{1/2}$) vs molar ratio (LEC/LFAE) shown on a Table I, the graph on Fig.1 was obtained.

Similarly the previous experiment was carried out once again but this time each sample contained a constant arbitrary amount of egg yolk lecithin (0.2421 g - 150 mM). The labelled fatty acid concentration was varied so as to achieve a final molar ratio ranging from 0.5 to 6.0. After codispersion, cosonication and centrifugation, the spectrum of each sample was recorded. The data are shown on Table II and the graph on Fig.2 was obtained by plotting $\Delta\nu_{1/2}$ vs molar ratio (LEC/LFAE).

2.3.2 EFFECT OF ABSOLUTE LIPID CONCENTRATION ON LIPOSOMES

Maintaining a 3:1 (LEC/LFAE) molar ratio constant, the absolute concentration was varied, ranging from 50 to 300 mM based on lecithin concentration. The data on Table III show the amounts of lecithin and labelled fatty acid ester and by plotting $\Delta\nu_{1/2}$ vs absolute molar ratio the graph on Fig. 3 was obtained.

2.3.3 PROOF OF STATISTICAL DISTRIBUTION OF LABELLED FATTY ACID IN LECITHIN VESICLES

A standard curve was obtained by monitoring the $\overset{+}{N}(CH_3)_3$ resonance by integration of various concentrations of egg yolk lecithin in 1.0 ml of CCl_4. Lecithin concentrations varied from 25 mM to 200 mM. A 0.5 ml aliquot was placed in an NMR tube and the spectrum was

recorded at 38°C. Table IV indicates the data obtained and Figure 4 depicts the standard curve for the $\overset{+}{N}(CH_3)_3$ resonance when integration is plotted against concentration of lecithin.

Similarly a standard curve for the labelled fatty acid ester was obtained by the integration of the $Si(CH_3)_2$ resonance. LFAE concentrations varied from 10 mM to 75 mM. Table V and Figure 5 show the data and the standard curve for the silyl resonance.

Liposome samples with LEC/LFAE (150/50 mM) were prepared as described and integration indicates that the supernatant contains 128.85 mM of lecithin and 41.5 mM of LFAE, which is 3.1:1 molar ratio, obtained from the standard curves.

2.4 MONO AND DIVALENT CATION INTERACTION WITH MEMBRANE MODEL

2.4.1 EFFECT OF NaCl

Liposomes were prepared as previously described containing lecithin and the labelled fatty acid ester in a 3:1 molar ratio. The liposomes in solution were divided into 0.5 ml aliquots in 10 ml Erlenmeyer flasks. Stock solutions containing various concentrations of NaCl were added to each of the aliquots, so that the final sodium chloride concentration ranged from 0.01M to 0.75M. To achieve a 1.0 molar salt solution, NaCl was added in solid form since a concentrated stock solution could not be prepared due to solubility.

The Erlenmeyer flasks were gently shaken by hand and the solutions were observed by NMR. Table VI shows the data and Fig. 6 indicates the graph obtained when $\Delta\nu_{\frac{1}{2}}$ in Hz is plotted against NaCl concentration.

2.4.2 EFFECT OF $CaCl_2$

Similarly, $CaCl_2$ was added in solution to preformed liposomes containing the same molar ratio but the $CaCl_2$ concentrations ranged from 0.01M to 0.1M where precipitation of the lipid was evident at the latter concentration. Table VII and Fig. 7 show data and graph obtained.

2.5 APOLAR REAGENT INTERACTION WITH MEMBRANE MODEL

2.5.1 EFFECT OF n-DODECANE

n-Dodecane was incorporated into the liposomes by adding the hydrocarbon to the lecithin/labelled fatty acid ester mixture prior to liposome formation. The three components were dissolved together in ether, evaporated to constant weight, cosonicated for 15 minutes or until optically clear, and then centrifuged in the same manner as described previously. The apolar reagent is assumed to be in the hydrophobic region of the vesicles and its effects are shown by the data on Table VIII. By plotting $\Delta\nu_{\frac{1}{2}}$ vs dodecane concentration, the effect can be seen graphically on Fig. 8.

2.6 POLYPEPTIDE ANTIBIOTIC INTERACTION WITH MEMBRANE MODEL

2.6.1 EFFECT OF GRAMICIDIN S

Various methanolic stock solutions were made up so as to deliver from 50 μg/mole of lipid to 500 μg/mole of lipid of Gramicidin S. These solutions were added to the lipid solution prior to liposome formation. The mixture was codispersed in dry ether and the ether and methanol were removed by evaporation to dryness. The resultant residue was then sonicated and centrifuged as previously described. The data on Table IX depict the effect observed and the graph on Fig. 9 was obtained when $\Delta\nu_{\frac{1}{2}}$ was plotted against Gramicidin S concentration.

2.6.2 EFFECT OF GRAMICIDIN S IN THE PRESENCE OF NaCl

Liposomes were prepared, as described in the previous section, containing 100 mM of lecithin, 33.3 mM of labelled fatty acid ester, and 100 μg/mole of lipid or 9.9 μM of Gramicidin S. Sodium chloride in various concentrated solutions was added to these liposomes and shaken by hand until well mixed. The final NaCl concentration ranged from 10 mM to 250 mM solutions. The effect of sodium chloride on liposomes containing the channel-forming antibiotic can be seen by the data on Table X. This effect can be visualized graphically by plotting $\Delta\nu_{\frac{1}{2}}$ vs NaCl concentration (Fig.10). A salting out of the lipo-peptide occurred at salt concentration higher than 250 mM.

CHAPTER IV. SUMMARY AND CONCLUSION

The silicon-containing fatty acid probe proved to be a useful and novel tool for the NMR spectroscopist investigating biological membranes and related systems. Incorporation of this hydrophobic probe into lecithin vesicles enabled simultaneous observation of both regions of a phospholipid bilayer during a dynamic process. Therefore, a summary of experiments conducted and interpretation of the observations is as follows:

(1) The synthesis of the silicon-containing fatty acid, 13,13-dimethyl-13-silaheptadecanoic acid, was achieved.

(2) The unsuccessful attempts to incorporate the labelled fatty acid as an integral part of a lecithin molecule led to a modified mixed lipid vesicle which comprised of a mixture of commercial egg yolk lecithin, with the methyl ester of the labelled fatty acid codispersed and cosonicated in D_2O as our membrane model system.

(3) Optimization of the molar ratio of the components in the vesicular system made the NMR study feasible. Calibration of the system and proof of statistical distribution of the labelled fatty acid in the lipid vesicles strengthened the reproducibility of such a model system.

(4) Selective and simultaneous observation of the hydrophilic reporter molecule $\overset{+}{N}(CH_3)_3$ (from the lecithin) and the hydrophobic probe $-Si(CH_3)_2$ (from the LFAE) of the liposomes in an aqueous sodium chloride solution indicated an electrolyte interaction with the zwitterionic polar

head group only. No perturbation of the silyl moiety was evident until high salt concentration (0.75M) were used, which also caused the lipid vesicles to aggregate and precipitate out of solution.

(5) In an aqueous calcium chloride environment, the $\overset{+}{N}(CH_3)_3$ reporter moiety exhibited a stronger effect than that caused by sodium chloride, at lower concentrations than that of the previous electrolyte. The silyl reporter moiety also showed an increase in line width, indicative of restricted mobility due to the stabilization of the lipid vesicle. This stabilization is believed to be a result of a cross-linking of polar head groups as the divalent cation binds with the zwitterionic moiety[19].

(6) The external stimulus provided by the incorporation of n-dodecane as a hydrophobic probe perturbator exhibited no influence to the hydrophilic reporter molecule line width. The mobility of the lipid region increased as the dodecane concentration increased as indicated by the line narrowing of the hydrophobic probe's signal. The introduction of the hydrocarbon, n-dodecane, caused a "solubilization" of the fatty acid side chains (and probe) rendering the lipoid region to acquire a more "fluid" state.

(7) Polypeptide-lipid interaction was observed with our membrane model system by introducing the cyclic pentadecapeptide antibiotic, Gramicidin S, into the liposomes by sonication. By simultaneously observing the interior hydrophobic probe and the exterior hydrophilic reporter molecule, a parallel behavior is exhibited; implying an inter-

action in both regions of the bilayer as the antibiotic is incorporated into the liposomes.

(8) The two reporter molecules were monitored as liposomes containing 9.9 μM of Gramicidin S were exposed to an aqueous sodium chloride environment. Both moieties exhibited line broadening as sodium chloride concentration increased indicative of structural changes taking place as the electrolyte interacts with the lipid-peptide complex. The antibiotic is known to possess ionophoric properties but ion transport could not be equivocally elucidated by our model system.

(9) The hydrophobic probe was successfully incorporated biosynthetically into the membrane of the micro-organism Mycoplasma laidlawii. Extraction and isolation of membrane lipids verified the biosynthetic incorporation. Membrane fragments, obtained with great difficulty as previously discussed, did not yield a high resolution NMR spectrum, however.

As it may be seen from this investigation, the utilization of the silyl reporter molecule in the NMR studies of liposomes, can be added to the large collection of methods useful in elucidating membrane phenomena.

ADDENDUM

The statistical treatment of the data obtained was conducted by calculating the standard deviation for each data point which was measured in triplicate. The standard deviation is portrayed on the graphs in the form of error bars and the points plotted are averaged data.

Figure 3 depicts a slight negative slope for the silyl resonance. We assume that this change in the ordinate (0.2Hz) over the five concentrations is sufficiently small that it may be considered to experience no change in line width as the absolute concentration is increased, although the molar ratio is maintained constant.

The instrumental accuracy is limited to $\pm$ 0.3Hz which further substantiates that the silyl line may be assumed to have a slope of zero. The points at 100/33 and 150/50 (LEC/LFAE) have the least error and depict no change in line width. These were the concentrations and ratios used throughout the project.

REFERENCES

1. E. Groter and F. Grendel, J. Expt. Med., 41, 439 (1925).
2. J.F. Danielli and H. Davson, J. Cell. Comp.Physiol., 5, 495 (1935).
3. J.D. Robertson, J.Biophys. Biochem. Cytol., 3, 1043 (1957).
4. S.J. Singer and G.L. Nicolson, Science, 175, 720 (1972).
5. R.W. Hendler, Physiol. Rev., 51, 66 (1971).
6. E.D. Korn, Science, 153, 1491 (1966).
7. M.S. Bretscher, Science, 181, 622 (1973).
8. T. Gulik-Krzywicki, Biochim. Biophys. Acta, 415, 1 (1975).
9. L.C.P. Smith, Chimia 25, 349 (1971).
10. V.G. Bieri and D.F.H. Wallach, Biochim. Biophys. Acta, 389, 413 (1975).
11. J. Seelig and W. Niederberger, J. Amer. Chem. Soc., 96, 2069 (1974).
12. G. Eytan, M.J. Matheson and E. Racker, Febs. Lett., 57, 121 (1975).
13. G.B. Warren, M.D. Houslay and J.C. Metcalfe, Nature, 255, 684 (1975).
14. M. Montal, J. Membr. Biol., 7, 245 (1972).
15. M.C. Goodall, Arch. Biochem. Biophys., 157, 514 (1973).
16. M. Hsu and S.I. Chan, Biochemistry, 12, 3872 (1973).
17. R.I. MacDonald, R.C. MacDonald, N.W. Cornell, Biochemistry, 13, 4018 (1974.
18. K. Jacobson and D. Papahadjopoulos, Biochemistry, 14, 152 (1975).
19. H. Trauble and H. Eibl, Proc. Nat. Acad. Sci. USA., 71, 214 (1974).
20. A.G. Lee, N.J.M. Birdsall, J.C. Metcalfe, "Methods In Membrane Biology" Vol.2, Ed. E.D. Korn, Plenum Press, New York, 1974. p.1.
21. W.L. Hubbell and H.M. McConnell, J. Amer. Chem. Soc., 93, 314 (1971).
22. J. Seelig and W. Niederberger, Biochemistry, 13, 1585 (1974).
23. A.F. Horowitz, W.J. Horsley and M.P. Klein, Proc. Nat. Acad. Sci. USA, 69, 590 (1972).

24. D. Chapman and A. Morrison, J. Biol. Chem., 241, 5044 (1966).

25. D.H. Green and M.R.J. Salton, Biochim. Biophys. Acta, 298, 664 (1973).

26. R.H. Bunnel and D.A. Shirley, J. Org. Chem., 17, 1545 (1952).

27. H. Trauble, Naturwiss. 58, 277 (1971).

28. J.L. Speier, J.A. Webster and G.H. Barnes, J. Amer. Chem. Soc., 79, 794 (1957).

29. J.F. Harrod and A.J. Chalk, J. Amer. Chem. Soc., 87, 1133 (1965).

30. A. Petrov, V.F. Mironov, V.A. Ponomrenko and E.A. Chernysev, "Synthesis of Organosilicon Monomers" Consultant Bureaus, London, 1964. p. 23.

31. R. Calas, Rev. Frac. Corps. Gras., 3, 5 (1956).

32. J.L. Speier, U.S. Pat. - 2,723,987, (1955).

33. G.N. Gadsby, Research (London), 3, 338 (1950).

34. H. Westermark, Acta Chem. Scand., 9, 947 (1955).

35. B.J. Howe and T.Malkin, J. Amer. Chem. Soc., 73, 2663 (1951).

36. J.S. Chadha, Chem. Phys. Lipids, 4, 104 (1970).

37. H. Brokerhoff and M. Yurkowski, Can. J. Biochem., 43, 1977 (1965).

38. N.H. Tattrie and C.S. McArthur, Can. J. Biochem. Physiol., 35, 1165 (1957).

39. E. Baer and D. Buchnea, Ibid., 37, 953 (1959).

40. E. Cubero Robles and Van Den Berg, Biochim. Biophys. Acta, 187, 520 (1969).

41. E.L. Pugh and M. Kates, J. Lipid Res., 16, 392 (1975).

42. R. Aneja and J.S. Chadha, Biochim. Biophys. Acta, 239, 84 (1971).

43. A.J. Slotboom, H.M. Varheiz and G.H. De Haas, Chem. Phys. Lipids, 11, 295 (1973).

44. W.L. Hurbell and H.M. McConnell, J. Amer. Chem. Soc., 93, 314 (1971).

45. M.E. Tourtellotte, D. Branton and A. Keith, Proc. Nat. Acad. Sci. USA, 66, 909 (1970).

46. E.G. Bligh and W.J. Dyer, Can. J. Biochem. Physiol., 37 37, 911 (1959).

47. A.F. Horwitz, "Membrane Molecular Biology" Ed. C.F. Fox and A. Keith, Sinauer Associated Inc., Publishers, Stamford, Conn., 1972. p. 164.

48. H. Hauser, M.C. Phillips, B.A. Levine and R.J.P. Williams, Eur. J. Biochem., 58, 133 (1975).

49. E. Rojas and J.M. Tobias, Biochim. Biophys. Acta, 94, 394 (1965).

50. H. Hauser and R.M.C. Dawson, Eur. J. Biochem., 1, 61 (1967).

51. D.G. Dervichian, Biochemical Problems of Lipids (ed. G.Popjak and E. Le Breton) p.p. 3-13, Butterworths, London, 1956.

52. H. Kimizuka and K. Koketsu, Nature, 196, 955 (1962).

53. H. Kimizuka, T.Nakahara, U.Uejo, A. Yamauchi, Biochim. Biophys. Acta, 137, 549 (1967).

54. M.S. White and N. Lakshminarayannaiah, Currents in Modern Biology, 3, 39 (1969).

55. D.O. Shah and J.H. Schulmann, J. Lipid Res., 6, 341 (1965).

56. D.O. Shah and J.H. Schulmann, J. Lipid Res., 8, 227 (1967).

57. H. Trauble, Naturwiss., 58, 277 (1971).

58. K. Jacobson and D. Papahadjopoulos, Biochemistry, 14, 152 (1975).

59. W. Pache, D. Chapman and R. Hillaby, Biochim. Biophys. Acta, 255, 358 (1972).

60. E.G. Finer, H. Hauser, D. Chapman, Chem. Phys. Lipids, 3, 386 (1969).

61. B.E. Cohen, J. Membr. Biol., 20, 235 (1975).

62. H. Hauser, E.G. Finer and D. Chapman, J. Mol. Biol., 53, 419 (1970).

63. L.F. Fieser and M. Fieser, "Reagents for Organic Synthesis" Vol. I, J. Wiley, New York, 1967. p.196.

64. Pyridine reagent was distilled over NaOH pellets under anhydrous conditions.

65. TEA reagent was dried over NaOH pellets and distilled under anhydrous conditions.

66. S. Razin, H.J. Morowitz and T.M. Terry, Proc. Nat. Acad. Sci. USA, 54, 219 (1965).

67. P.F. Smith and C.V. Henrikson, J. Lipid Res., 6, 106 (1965).

68. J.D. Pollack, S. Razin, M.E. Pollack and R.C. Clacerdon, Life Sci., 4, 973 (1965).

69. E. Cerny and H. Lam, Report No. EE - 73 - 103.