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AMPHOTERICIN B - ERGOSTEROL INTERACTIONS; A SPIN LABEL STUDY

by

Peter E. Laks

B.Sc., Simon Fraser University, 1976

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Chemistry

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ABSTRACT

The interaction between the polyene macrolide antibiotic, amphotericin b, and ergosterol in EYL multilayers was investigated using spin-labelled lecithins as probes. Dicetyl phosphate and sterol oxidation products were used to increase the solubility of ergosterol past its' limit in pure EYL (~15 mole percent). At relatively low concentrations of sterol ($\leq$ 15 mole percent) and amphotericin B/ergosterol molar ratios less than 0.6, a complex of one molecule of each of the two components predominated. At higher concentrations of ergosterol, a complex of two ergosterol molecules and one amphotericin B was observed when there was insufficient antibiotic present to form a 1:1 complex. When the ratio of amphotericin B to ergosterol is greater than 0.5 at the higher concentrations, a larger 1:1 complex (or complexes) was formed. The complexation of ergosterol with amphotericin B at higher sterol concentrations was accompanied by a spectral change that can be interpreted as showing the bound ergosterol occupying a position closer to the membrane surface than the free sterol.

Acknowledgements

The author wishes to express his gratitude to Dr. I.C.P. Smith for his invaluable instruction, Dr. A.C. Oehlschlager for his advice throughout this project and Mandy Laks for her patience.

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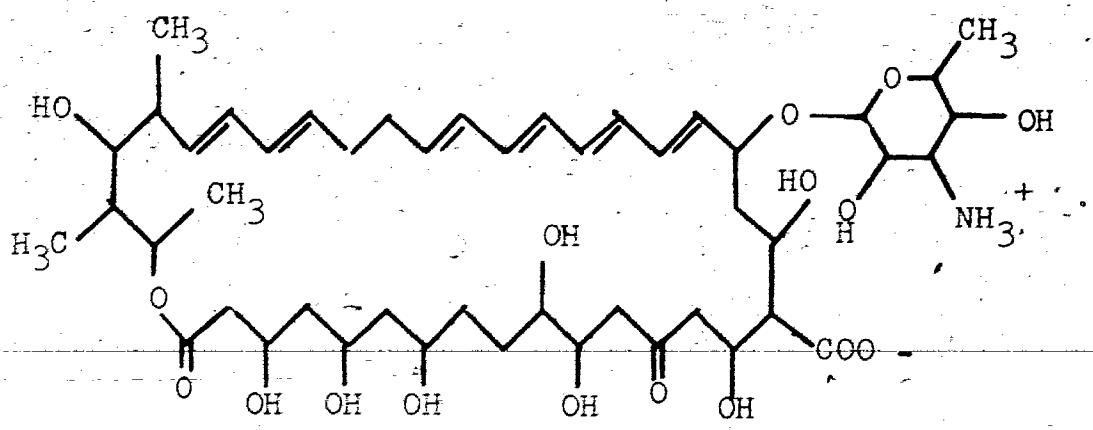
INTRODUCTION

I Polyene Macrolide Antibiotics

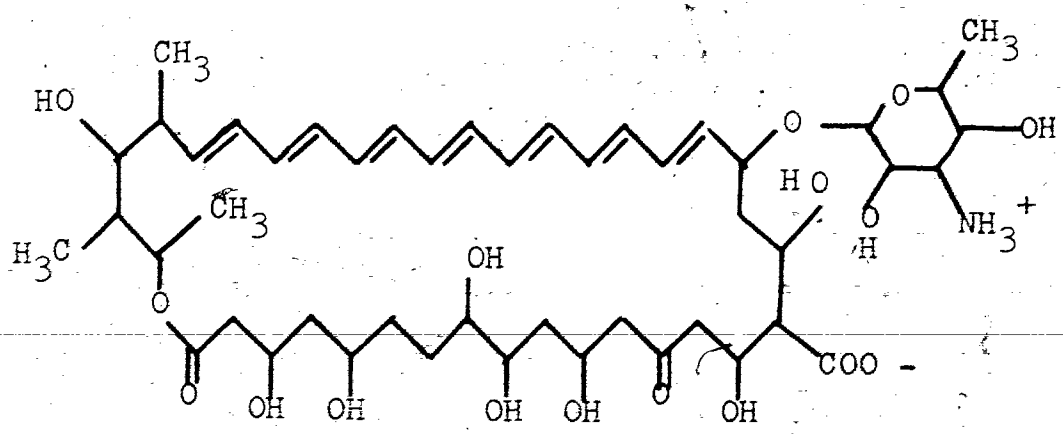
The polyene macrolide antibiotics are a group of antifungal agents produced by the actinomycetes. Although numerous polyene macrolides have been described in the literature (1), most work on the mode of action of these antifungals has been done with amphotericin B, nystatin, and filipin (Figure 1). Of these, amphotericin B, the polyene used in this study, is the only antibiotic whose tertiary structure and absolute configuration have been reported (2,3 Figure 2). There are several important features in the amphotericin B structure. The long conjugated polyene segment forms a rigid backbone holding the macrocycle in a rectangle-like shape. Most of the hydroxyl groups on the opposite side of the rectangle are axial and disposed to the same side of the ring plane. This structure gives the compound an obvious polarization about its long axis with one side being hydrophilic and the other side hydrophobic.

As antibiotics, the macrolides are highly effective in inhibiting the growth of a large number of yeasts and other fungi. They are commonly used in humans for control of topical and gastrointestinal infections by these microorganisms (4). However, bacteria are totally immune to these antibiotics (5). Subsequent work has indicated that a crucial step in the cytotoxic action of the polyene

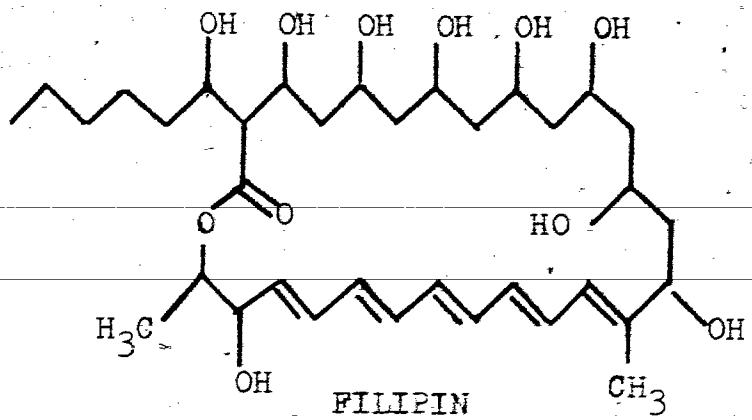
FIGURE 1



NYSTATIN



AMPHOTERICIN B



FILIPIN

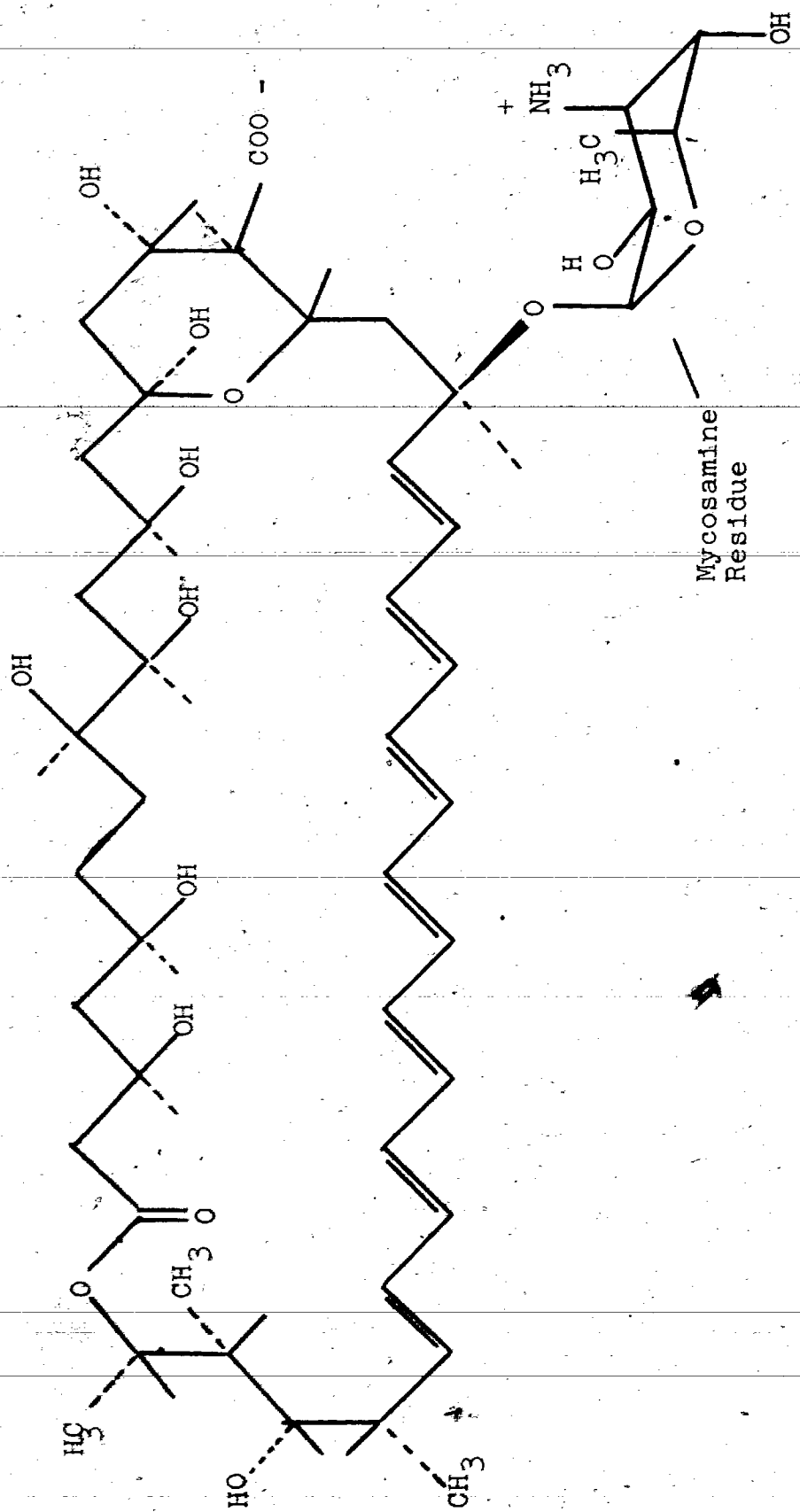
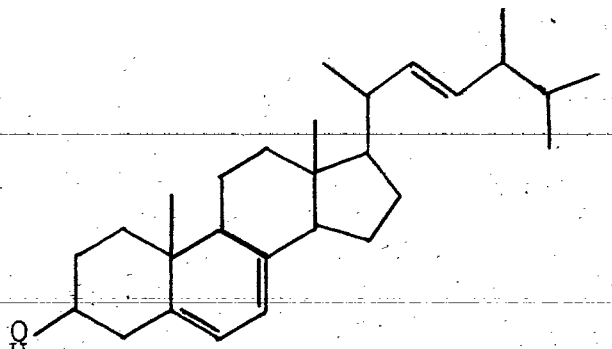

OH
+ NH3
H3C
H
O
O
O
Mycosamine
Residue
COO -
OH
OH
OH
OH
OH
OH
O
O
CH3
O
H3C
HO
CH3

FIGURE 2

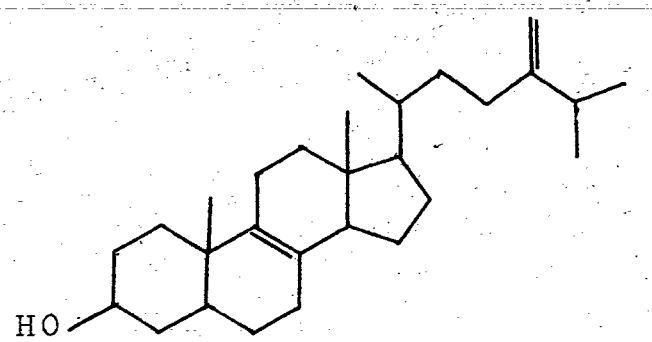
macrolide antibiotics involves some kind of interaction with sterol contained in the outer membranes of sensitive cells, probably forming a macrolide-sterol complex. Thus, the sterol - free bacteria are not affected. The membrane - antibiotic interaction causes a breakdown in the selective transport properties of the membrane, resulting in loss of cytoplasmic constituents and cell death. As an example of the in yivo evidence that supports this theory, Kinsky (6) has shown that the bacterium Mycoplasma laidlawii grown in the presence of sterols was sensitive to filipin, while the same microorganism grown without sterols was not sensitive. This bacterium normally has no sterol in its cell membrane but will absorb it from the medium if a sterol is present. Polyene antibiotics are also able to lower the DC resistance or rupture cholesterol-containing black film membranes. Cholesterol-free membranes are unaffected (7,8).

Sterol-containing organisms can also be resistant under certain circumstances. Antibiotic resistance can be conferred on normally sensitive organisms by growth on selective media. Insensitivity could result from a change in the amount or nature of the sterols available for complexation in the cell membrane. This has been investigated in antibiotic-sensitive and antibiotic-resistant strains of the yeast, Saccharomyces cerevisiae (9). The sensitive strain was shown to contain ergosterol as the predominant sterol

component, while a more resistant strain contained 96% fecosterol. Since the total amount of sterol in both strains was approximately the same, it was concluded that not only is sterol necessary for the antibiotic to be active, the right kind of sterol is necessary.



Ergosterol



Fecosterol

Information on the nature of the sterol - antibiotic complex has been obtained by a variety of methods. Studies using differential scanning calorimetry (10), permeability studies (11) and membrane binding methods (12) have shown that sterols and macrolide antibiotics do form nonisolatable complexes in which the ratio of antibiotic to sterol is a small number on the order of one. Spectroscopic and permeability studies have shown that the antibiotic - sterol

interaction is primarily hydrophobic in character (10). When increasing amounts of cholesterol are added to an aqueous - ethanol solution of amphotericin B, the antibiotic is solubilized with an accompanying change in the polyene UV chromophore. With this antibiotic, the reaction is complete when a ratio of 2:1, cholesterol/antibiotic, is reached. This infers that amphotericin B has two equal binding sites. Similar work with filipin and nystatin has shown they also have two binding sites, but of different affinities (13).

A large amount of information about the nature of the active antibiotic complex can be obtained by measuring the conductance of thin lipid membranes under appropriate conditions. Nystatin and amphotericin B can increase the resistance of sterol - containing films from a value of $10^{-8} \Omega\ cm^{-2}$ for the unmodified membrane to $10^{-2} \Omega\ cm^{-2}$ or higher when present in only micromolar amounts. The relevant characteristics of this process are listed below (14, 15, 8):

1) A sterol must be a component of the lipid membrane for the antibiotic to be effective.
2) There is a much greater increase in permeability when antibiotic is present on both sides of the membrane.
3) The conductance is proportional to a large power of the antibiotic concentration. The power can vary from 4 to 12 depending on the nature of the

antibiotic, type of phospholipid and other factors.

4) The antibiotic - treated membranes are primarily anion selective and discriminate among them on the basis of their Stokes - Einstein radius.

5) The conductance of the membrane decreases approximately 10^4 times for a 10° C temperature increase.

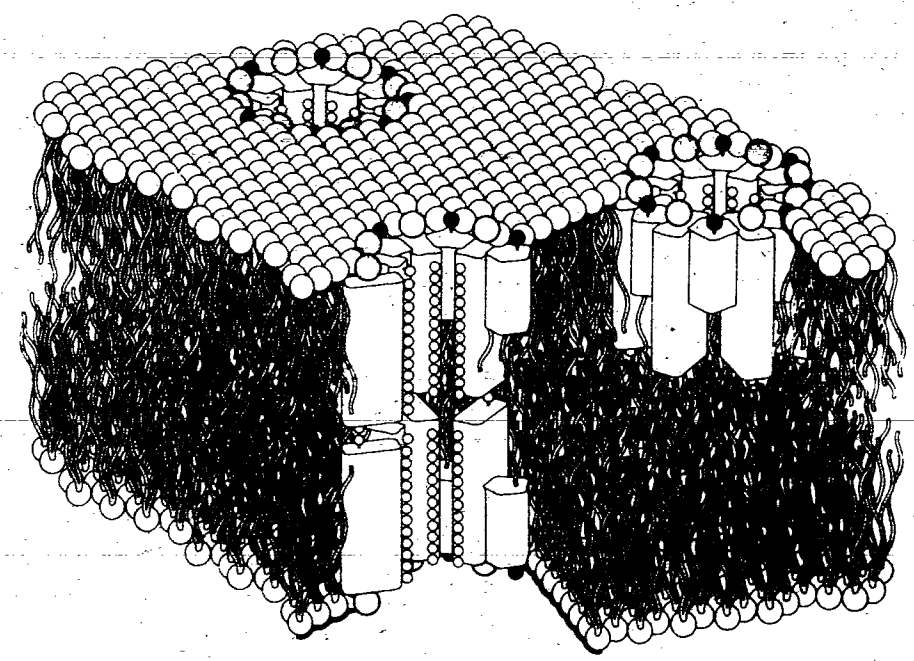
6) If the antibiotic is removed from the aqueous solutions on the sides of the conducting membrane, it reverts to its unmodified state.

The general picture which emerges from this data is of several antibiotic molecules interacting with sterol on each side of the membrane to form a conducting pathway. The extreme temperature dependence and reversibility of the conduction phenomenon implies a fast dynamic equilibrium between the complex and free sterol and antibiotic. As well as anions, the treated membrane is also permeable to water and non - electrolytes up to the size of glucose (Stokes - Einstein radius - 4Å) which is essentially impermeable (16). The pathways seem to be actual aqueous channels extending from one side of the membrane to the other. If this is true, it should be possible to observe discrete current jumps that can be attributed to a single ion moving across one pore. Such events have been observed in other antibiotics such as alamethicin (17) and monazomycin (18). Similar behaviour has been reported for amphotericin B and nystatin at 5% or less cholesterol/ox brain lipid membranes (19).

At higher concentrations of sterol, discrete jumps are not observed (20).

Mechanisms for the antibiotic action of the polyenes have been suggested by various groups (8, 21, 22, 23, 24). Of these, the proposal by De Kruijff and Demel (21) is the most detailed and perhaps the most consistent with available data. These authors used space - filling models of antibiotics and cholesterol to construct models for complexes that could act as a pore. The type of complex they propose appears to be a refinement of the earlier model of Finkelstein (8). The amphotericin B (or nystatin) - cholesterol complex is visualized as a circular aggregate forming aqueous channels that traverse the membrane (Figure 3). The channels consist of two half - pores, each a circular complex of 8 antibiotic molecules alternating with 8 cholesterol units. The antibiotic molecular structure fits in well with a complex of this type. The hydrophobic polyene side of the molecule is oriented to the outside where it can interact by Van der Waal's forces to both the phenanthrene part of the sterol and the phospholipid of the membrane. The hydroxyl groups on the other side of the antibiotic "rectangle" are oriented inwards forming a polar environment within the pore. Hydrogen bonding by the hydroxyl on the sterol to polar groups on the antibiotic gives additional strength to the complex. The charged mycosamine residue on the macrolide sits on the surface anchoring the complex to the polar region

Figure 3



of the membrane. This model is consistent with most of the experimental observations above. For example, the concept of the half pore requires antibiotic to be present on both sides of the membrane and the sterol/antibiotic ratio is 1:1.

II The Problem

The purpose of this research is to clarify the nature of the sterol - antibiotic complex by observing membrane perturbations induced by its presence in phospholipid bilayer systems using nitroxide spin - labelled lecithins as probes. These labelled molecules were constructed such that the spin label is situated in a specific region of the bilayer. By studying the perturbation in esr line shape which occurs in these labelled bilayer preparations upon addition of the antibiotic, one can deduce changes in label mobility. It was envisioned that this could allow location of the antibiotic - sterol complex within the bilayer.

The compounds used in this work were amphotericin B and ergosterol. The latter was chosen because it is the major sterol found in fungi and yeast membranes which are the organisms against which these antibiotics are normally directed. It has been observed that ergosterol - containing thin membranes are much more sensitive to amphotericin B and nystatin than are cholesterol - containing membranes (25). This could be taken to mean that the ergosterol - antibiotic complex is the stronger one and so probably easier to observe

in these experiments. Amphotericin B was used because a large proportion of the research was done on the mechanism of the polyene macrolide anti-microbial action with this compound.

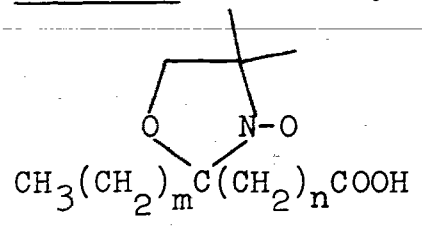
III The Spin - Label Technique

To use esr as a means of getting information about a system, an unpaired electron must be present. This requirement was met by attaching a paramagnetic moiety (a spin - label) to a compound compatible with the system. In this way, the perturbation of the unlabelled preparation is minimized. Usually a compound indigenous to the system is used as the carrier for the label. A variety of labelled compounds that have been used in membrane experiments of this type are shown in Figure 4. In this study PC(2)-5-PASL, PC(2)-8-PASL, PC(2)-12-SASL and PL-HL were used. These spin - labelled compounds are all derivatives of lecithin and so would be expected to have a similar position and orientation in the bilayer as an unlabelled lecithin molecule. With this set of probes we hoped to be able to monitor the bilayer fluidity at a variety of points in the membrane.

(A) The ESR Experiment

An unpaired electron can be viewed as a spinning orbiting charge. In an applied external magnetic field, $\vec{H}$, it will have two possible energy levels corresponding to components of the electronic magnetic moment aligned parallel or antiparallel to $\vec{H}$. These states are represented by spin

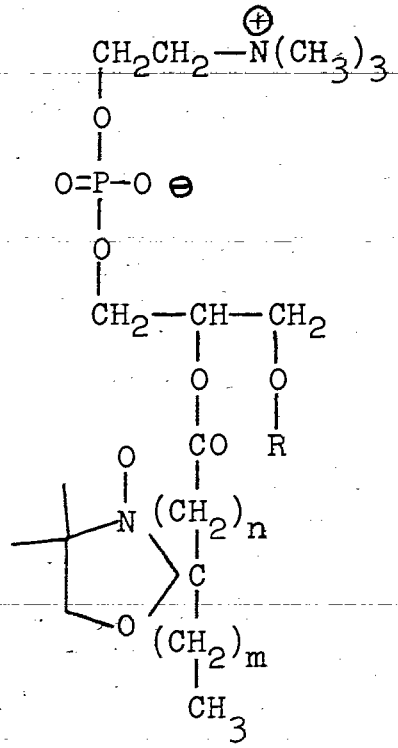
FIGURE 4 - Commonly Used Spin Labels



5-PASL - (m,n) = (10,3)

8-PASL - (m,n) = (7,6)

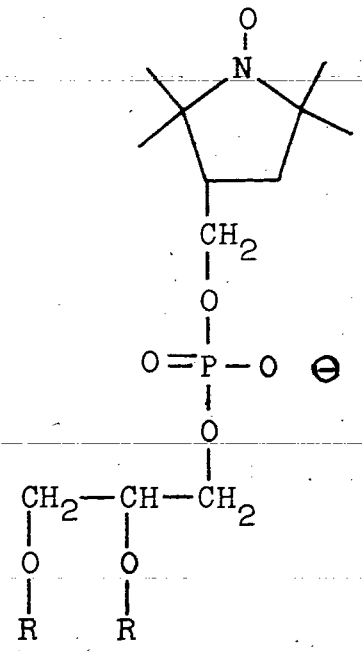
12-SASL - (m,n) = (5,10)



PC(2)-5-PASL - (m,n) = (10,3)

PC(2)-8-PASL - (m,n) = (7,6)

PC(2)-12-SASL - (m,n) = (5,10)



PL-HL

R - Fatty Acid Residue

PC(2)-X-FASL - Phosphatidyl Choline-X-Fatty Acid Spin Label

PL-HL - Phospholipid Head-Label

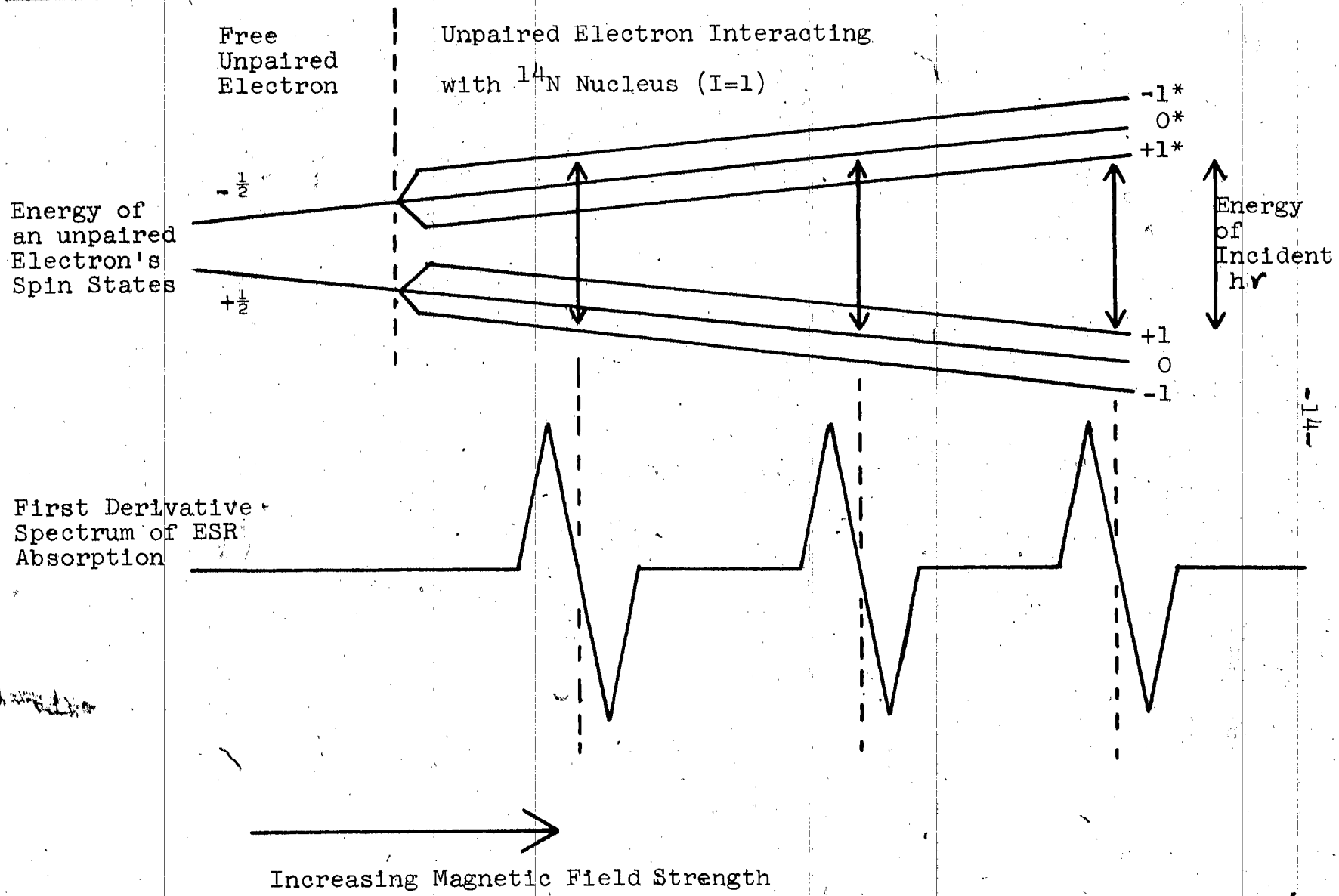
quantum numbers, $M_s = -\frac{1}{2}$ or $+\frac{1}{2}$. In the esr experiment, transition from the lower to higher state is induced by application of excitation energy to the sample in the form of radiation, $h\nu$, at right angles to H. Absorption will occur if the energy difference between the states corresponding to $M_s = -\frac{1}{2}$ and $M_s = +\frac{1}{2}$, E, is equal to the energy put in by $h\nu$. That is:

$$E = h\nu = gBH$$

where B is the Bohr magneton, a fundamental property of the electron, H is the external magnetic field strength and g is a constant, characteristic of the electron. The g-value characterizes the response of the isolated unpaired electron spin state to $\vec{H}$. Although g is a constant (= 2.00232), the experimentally observed g can vary because of local magnetic fields set up by the external H. These internal fields vary with the nature of the molecule containing the unpaired electron and its chemical and physical environment. Therefore, the observed g is characteristic of a certain spin - labelled molecule under specified conditions.

In the presence of magnetic nuclei such as ^{1}H, ^{14}N and ^{31}P, the unpaired electron spin can couple to the nuclear spin, splitting the electronic energy levels. Figure 5 illustrates what happens with ^{14}N. This nucleus has a nuclear spin quantum number, I, of 1. Therefore, there are $2I + 1 = 3$ energy levels possible with respect to the external magnetic field. Each of the two electronic energy levels is split

FIGURE 5

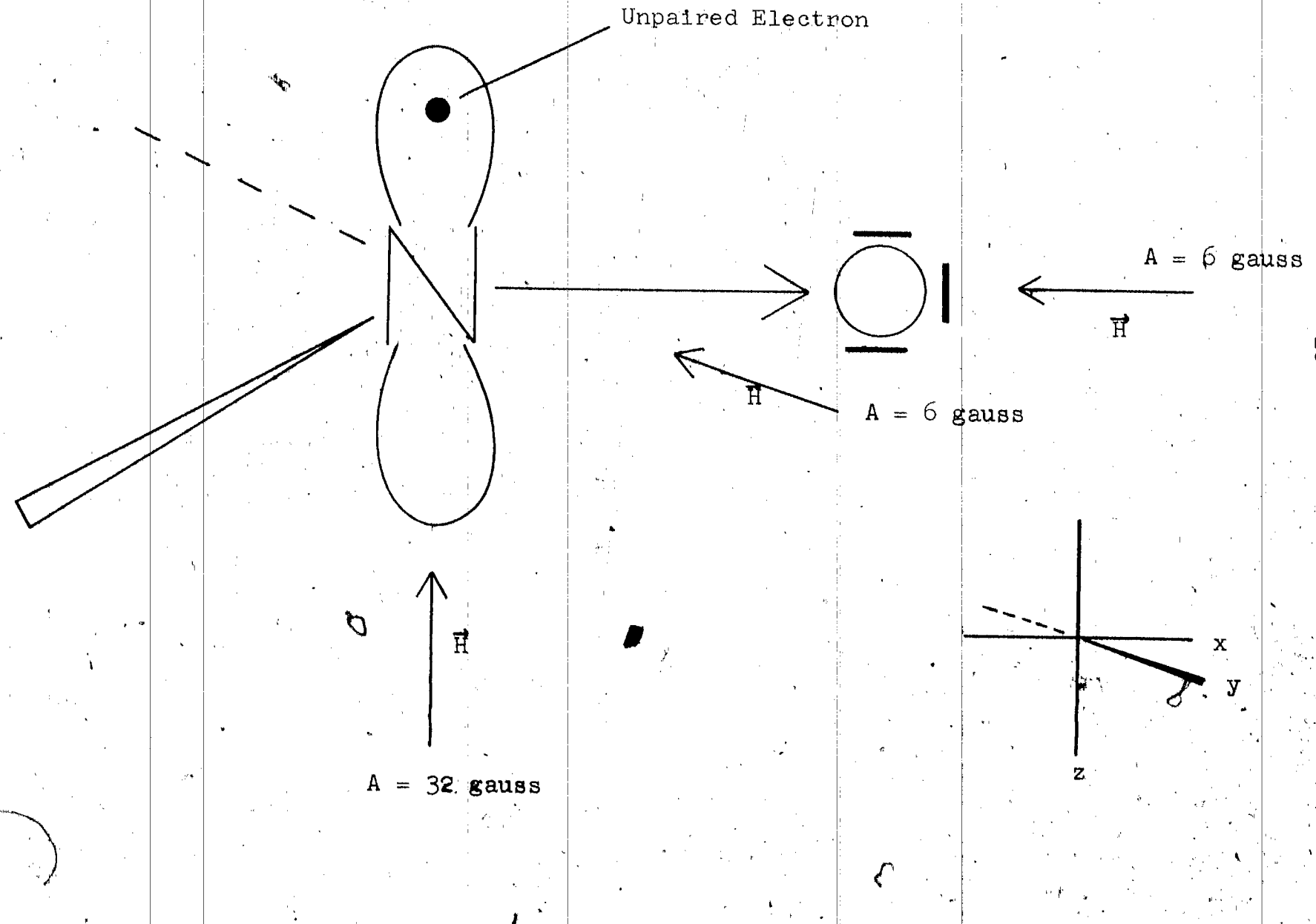

Free
Unpaired
Electron
Unpaired Electron Interacting
with ^{14}N Nucleus (I=1)
-1*
0*
+1*
$-\frac{1}{2}$
Energy of
an unpaired
Electron's
Spin States
$+\frac{1}{2}$
Energy
of
Incident
hν
+1
0
-1
First Derivative
Spectrum of ESR
Absorption
Increasing Magnetic Field Strength

into three sublevels, each characterized by a nuclear spin quantum number $M_1 = +1, 0, -1$. Transitions are allowed only between states with the same nuclear spin quantum number, which in this case are $+1 \longrightarrow +1^*$, $-1 \longrightarrow -1^*$ and $0 \rightarrow 0^*$. These are represented by the arrows in the diagram. Each transition is a change in the spin state of an electron coupled to a nucleus in one particular spin state. There is no change in the nuclear spin. Absorptions are observed by irradiating with a constant $h\nu$ and scanning $\vec{H}$. In this case three absorption peaks are recorded. The separation between the signals is called the hyperfine splitting, A. $\vec{H}$ is modulated at 10^2 Hz resulting in the first derivative of the absorption being taken as the esr spectrum. Integration will give the normal absorption spectrum and double integration, the relative areas.

The magnitude of the hyperfine splitting depends on the chemical and physical environment of the spin - labelled moiety and the angle of $\vec{H}$ to the orbital containing the unpaired electron. For a nitroxide, the chemical group containing the unpaired electron used in this work, A varies from 32 gauss when $\vec{H}$ is parallel to the nitrogen π orbital containing the unpaired electron, to 6 gauss when the field is at right angles to the orbital (see Figure 6).

In a low viscosity solution where the radical-containing molecules are tumbling rapidly with respect to the frequency corresponding to the energy difference between

FIGURE 6



the various hyperfine states, the hyperfine splittings average:

$$\frac{A_{xx} + A_{yy} + A_{zz}}{3} = 14.66 \text{ gauss}$$

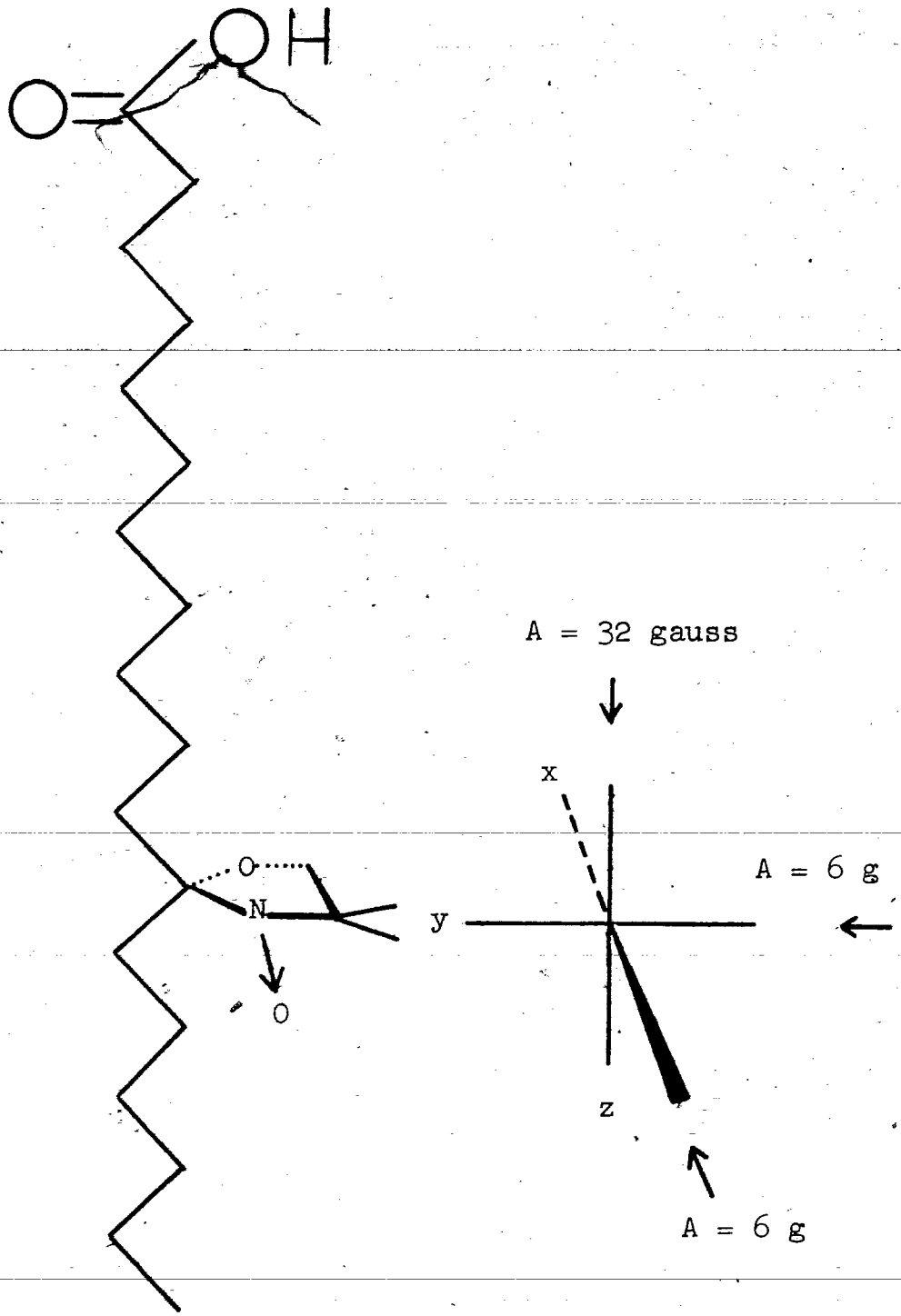
If the tumbling is too slow to average out as above, the spectra will show a non-averaged A, somewhere between 32 and 6 gauss. The magnitude of A can also be used as an estimate of the label environment polarity. A can vary from 17 G in polar solvents to 15 G in hydrocarbons.

As seen in the diagram, the g-value is also dependent on orientation. It may vary from 2.002 when H is parallel to the nitrogen orbital to 2.004 when H is at right angles. Again, if the tumbling motion is rapid, g is averaged in the same way as A:

$$\frac{g_{xx} + g_{yy} + g_{zz}}{3} = 2.003$$

The anisotropy of the hyperfine splitting and g-value components can be utilized to estimate the relative motional freedom of similar nitroxide-containing moieties in oriented bilayer systems. The situation encountered in most cases is a combination of rapid and slow anisotropic motions in two directions. In a fatty-acid spin-labelled lecithin molecule, the type of label used in this experiment, the Z axis of the nitroxide is parallel to the fatty acid residue's long axis (Figure 7). Rotation about the long axis would have no effect on the observed hyperfine splitting since the pairs A_{xx}, A_{yy} and g_{xx}, g_{yy} are already equal. End for end rotation

FIGURE 7

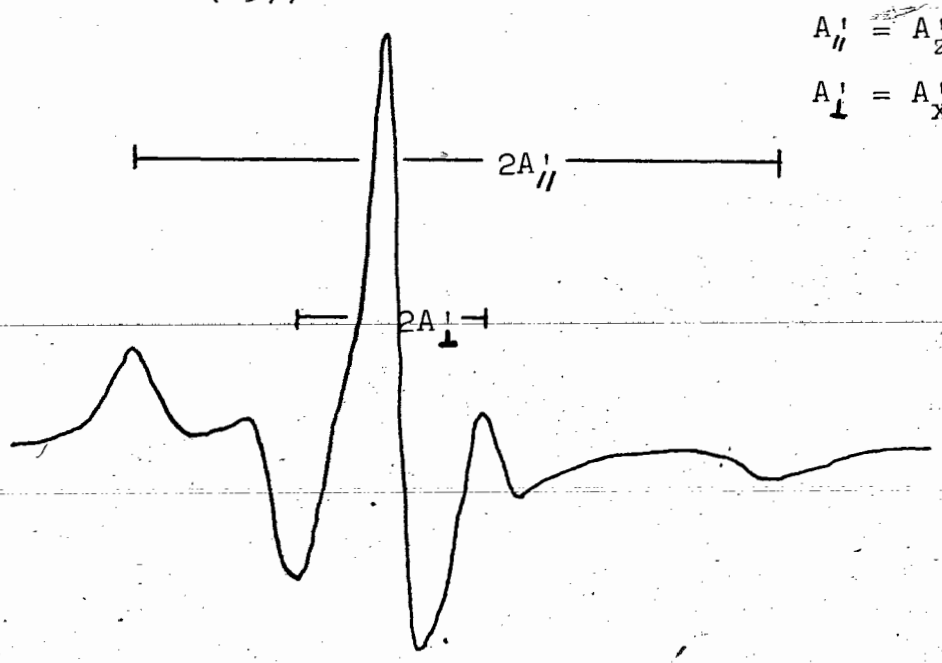


would be, of course, very slow and can be disregarded. Rapid "wagging" type motions of the fatty acid residue tails due to gauche-trans interconversions and general chain flexibility will tend to decrease A'_{zz} (the observed hyperfine splitting) and increase $A'_{xx} = A'_{yy}$. As the amplitude of this motion increases, so will the deviation of observed hyperfine splittings from the theoretical 32 and 6 gauss values. This effect is readily seen in Figure 8 which compares the splittings of two labelled lecithins. The spectra are taken of the labels in lecithin liposomes. In both cases, $2A'_{zz}$ is less than 64 gauss and $2A'_{xx}$ ($= 2A'_{yy}$) is larger than 12 gauss. However, for the 5-labelled compound, $2A'_{zz}$ is considerably larger than that for the 12-labelled compound, indicating that in the latter case there is much greater motion of the label.

(B) Interpretation of Results

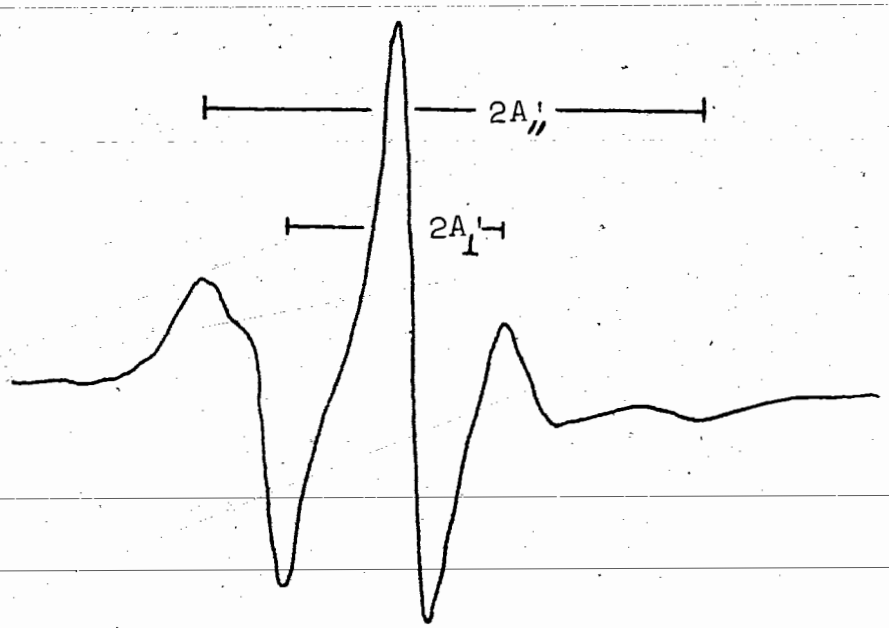
The spectra of fatty acid spin probes are usually interpreted in terms of the model formulated by Hubbell and McConnell (26). This model views the phospholipid fatty acyl chains contained in the membrane from two aspects; first, as a flexible chain of methylene groups rapidly undergoing gauche-trans conformational exchange about the C-C single bonds with the probability of motion increasing in proportion to the distance from the rigidly held polar region of the molecule. According to this model, the terminal methyl group would have a greater mobility than the methylene adjacent to the ester linkage. Second, the whole chain is considered as a "rigid

Figure 8 - Labels in Phosphotidyl Choline - Cholesterol Liposomes (2:1) (Adapted from Schreier-Muccillo and Smith (29))



PC(2)-5-SASL

10 gauss



PC(2)-12-SASL

stick" precessing in a cone about the axis normal to the bilayer surface. These motions can be related to a measured order parameter by the following equation:

$$\text{Log } S = n\text{Log } P_t + \text{Log } S_o$$

where n is the number of methylene groups between the nitroxide group and the ester linkage, P_t is the probability of the trans conformation, S_o is a "rigid stick" order parameter and S is the experimentally determined order parameter given by:

$$S = \frac{A'_{//} - A'_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})}$$

$A'_{//}$ and $A'_{\perp}$ are the observed hyperfine splittings when H is oriented parallel and perpendicular, respectively, to the labelled fatty acid chain long axis. A_{zz}, A_{xx} and A_{yy} are defined above. For the fatty acid-type label, $A_{xx} = A_{yy}$. S is simply the ratio of the observed hyperfine anisotropy to the theoretical maximum that would be obtained in the perfectly aligned rigid case. Thus, S = 1 in a perfectly ordered bilayer and S = 0 in a completely disordered system. In cases where $1 > S > 0$, the order parameter can be expressed in terms of $\bar{\gamma}$, the angle between the nitroxide $2P_\pi$ orbital and the normal to the plane of the bilayer.

$$S = \frac{(3\langle \cos^2 \bar{\gamma} \rangle - 1)}{2}$$

The angular brackets represent a time averaging over the entire molecular motion.

The major prerequisite for this type of analysis is that anisotropic motion must take place and it must be rapid on the esr time scale (approximately 10^{-8}s). A is also dependent on the polarity of the spin label environment and could vary by as much as 10% for a stearic acid spin label in water as compared to the same label in n-decane. The more polar solvent would cause the larger splitting. In cases where an accurate value for the order parameter is required, S is corrected by another term a', which accounts for solvent polarity. In this study, absolute numbers were not required so the order parameters determined were uncorrected.

Analysis of labelled membrane systems using the order parameter formalism has been described in a number of cases (28, 29, 31-34). In some experiments using cholestane spin label (CSL) or fatty acid-type labels, a simple three line spectrum is not observed. Figure 9 shows the spectrum obtained with CSL in multilayers of bovine brain lipid at low ionic strength. An extra line is observed 19 gauss downfield from the centre of the spectrum. This type of spectrum is obtained when there is a static distribution of CSL or fatty acid long axis orientations with retention of rapid motion about the long axes (29, 35). In other words, not all the bilayer fragments in the multilayer preparation (described below) are oriented parallel to each other. The more asymmetry

in the orientations, the more intense the downfield peak becomes with a proportional decrease in the intensity of the adjacent signal, D (Figure 9). The ratio of the peaks D/E has been used as an empirical order parameter (29, 36, 37). The greater the ratio, the narrower the distribution of label axes. In the case of a very large distribution, peak D may disappear entirely.

IV Model Membranes

Natural membranes are very complex in terms of composition and structure. The number of variables that would have to be controlled or taken into account when studying membrane phenomena has made the use of model systems a necessity. Using models, the complexity of a system can be increased in a controlled manner.

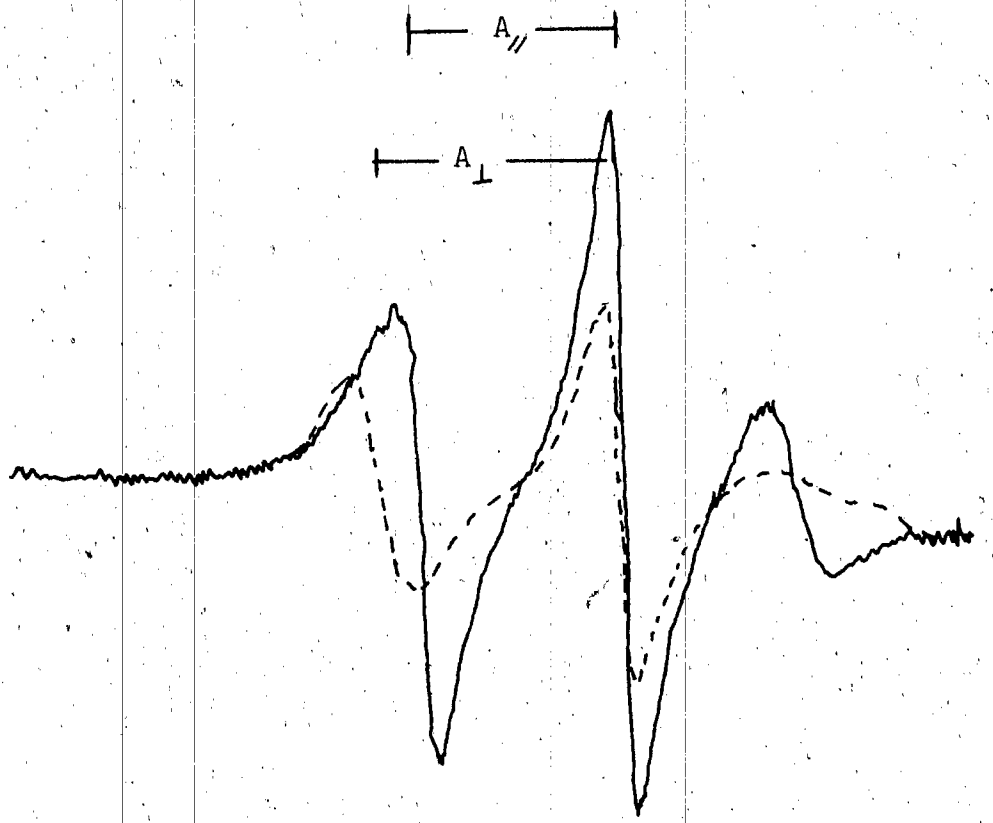
(A) Black Lipid Membranes

Black lipid membranes have been referred to previously in the antibiotic conductance experiments. The membrane is a single bilayer formed in a small hole connecting two aqueous solutions (38, 39). Due to its mechanical fragility, systems like these have not been studied by esr.

(B) Liposomes

Liposomes are multilamellar vesicles composed of concentric spheres of phospholipid bilayers separated by a water layer. They are made by sonication of phospholipid dispersions in water, resulting in aqueous suspensions of the

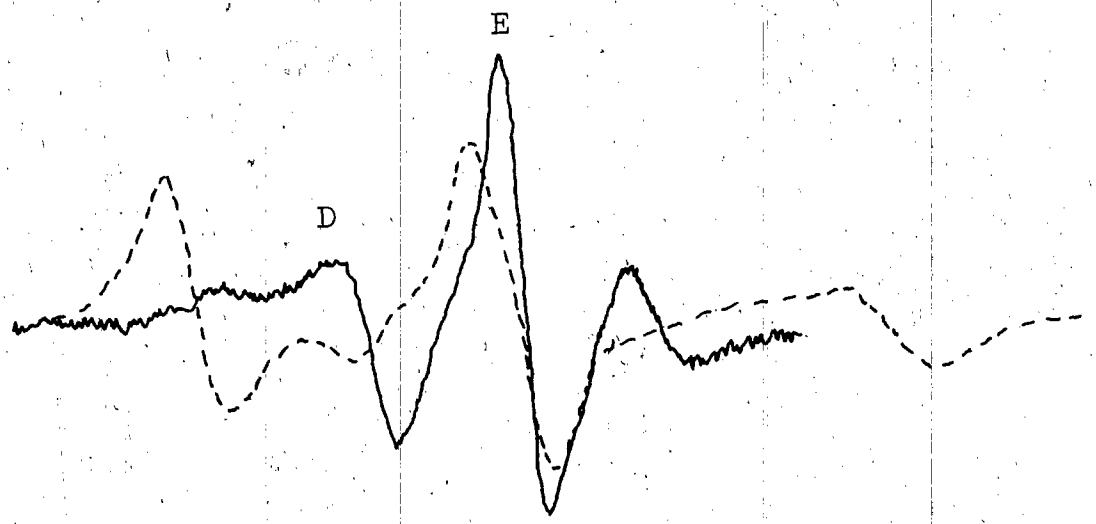
Figure 9 - PC(2)-8-SASL in 10% Ergosterol-EYL Multilayers



— H parallel to multilayers

- - - H perpendicular to multilayers

Figure 9 (con't) - An Example of a Distorted Spectrum PC(2)-5-SASL in EYL multilayers Containing 5% Amphotericin B

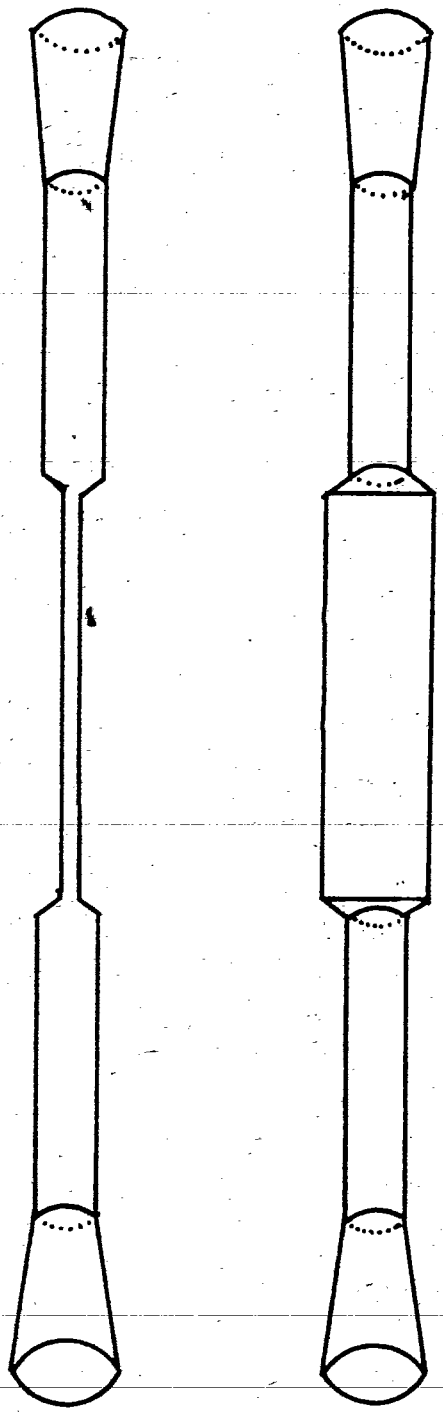


liposomes. Due to their relative ease of preparation and versatility, liposomes have been studied by a variety of physical techniques. However, this system does have complications when used for a spin label experiment. The packing of the lecithin fatty acid residues varies with the size of the liposome making the esr spectrum of a probe sensitive to the degree of sonication (40). Also, the motions of the inside and outside lecithin acyl chains are different (41). Due to these factors and the symmetry of the system, the esr spectra of spin-labels in liposomes tend to be complex and interpretation is sometimes ambiguous (29).

(C) Multilayers

Multilayers are parallel sheets of bilayers deposited on a flat surface. This model system is commonly formed by two methods. Evaporation of a chloroform-lipid solution on a glass surface such as a microscope slide cover slip will result in well ordered multilayers after solvent removal in vacuum and hydration (27, 42). After mounting in a suitable holder, $A'_{//}$ and $A'_{\perp}$ can be measured directly by rotating the holder within the microwave cavity of the esr machine. In the second method, the multilayers are formed on the inside of a flat esr cell (Figure 10) by bubbling wet nitrogen through a chloroform solution of lipid in the cell itself (27). The orientation with respect to H can be changed in the same way to obtain $A'_{//}$ and $A'_{\perp}$.

FIGURE 10



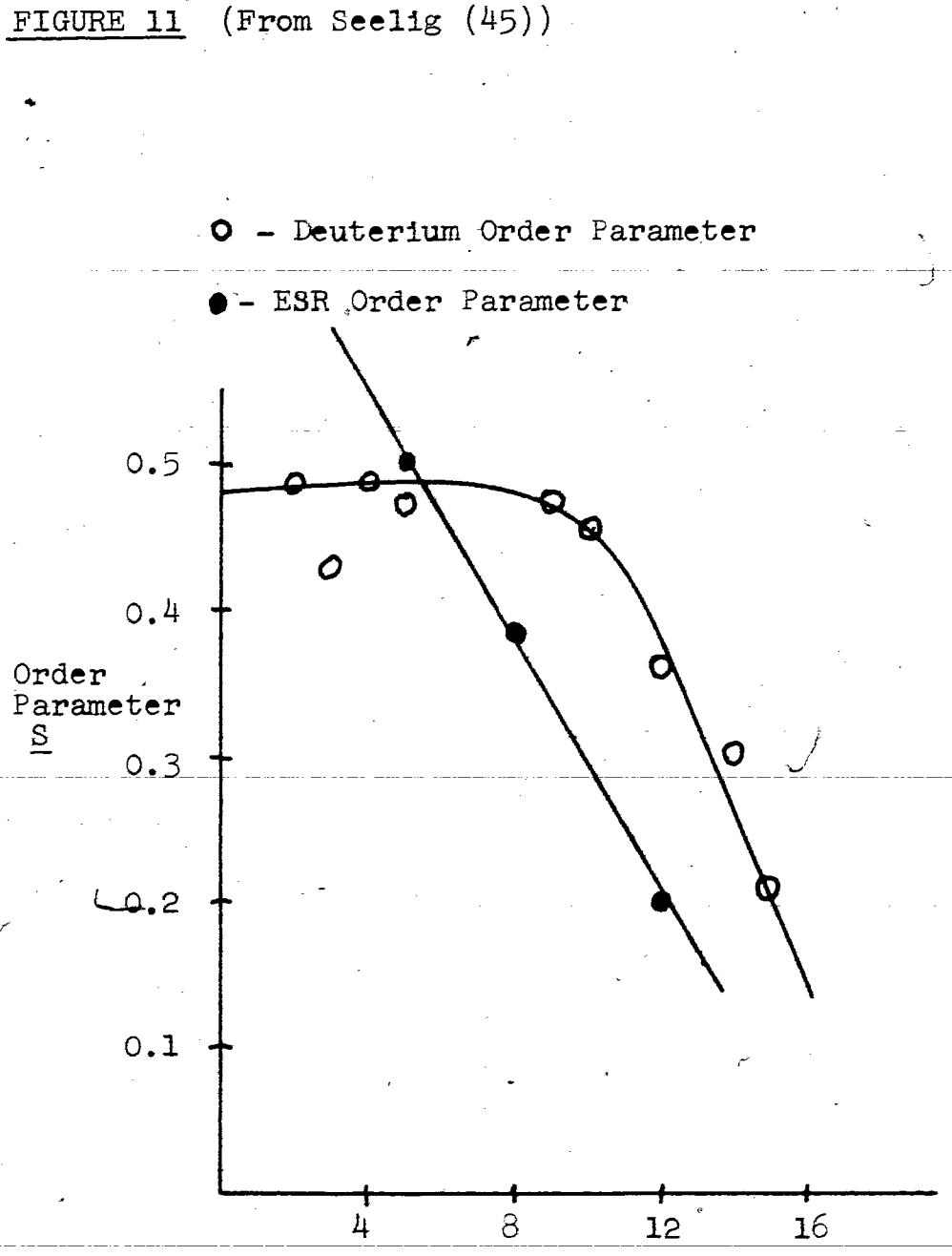
McConnell has demonstrated that spin-labelled phospholipids migrate within the bilayer at a rate measurable by ESR. The movement is slow on the ESR time scale (26). The parallelity of the bilayer fragments that make up a multilayer preparation has been confirmed by X-ray diffraction(12).

Compared to liposomes, multilayers provide more accurate values for the hyperfine splittings and therefore, the order parameter. Another factor in this case is the large size of the sterol-antibiotic complex (according to DeKruijff's model, about 30 Å in diameter (21)). The complex may be deformed or not form at all in the relatively small liposomes (250 Å in diameter for single bilayer vesicles (43)). For these reasons, multilayers were used as the model membrane in these experiments.

V Validity of the Spin-Label Method

Recently there has been a controversy concerning the validity of the spin-label technique as compared to ^{13}C and ^{2}H-NMR of bilayers (44, 45). The argument centres about the flexibility gradient of bilayers. This gradient is the difference in motion one encounters moving down the chain of a phospholipid fatty acid residue in a bilayer. The spin-label order parameter decreases rapidly as the paramagnetic nitroxide moiety approaches the end of the fatty acid chain (and so the centre of the bilayer)(26,35,

FIGURE 11 (From Seelig (45))



46). The ^{2}H-NMR experiments show a slightly different picture (47, 48). The deuterium order parameters are constant in the outer two thirds of the bilayer and then drop approaching the centre (Figure 11). It has been argued by McConnell (49, 31) that the discrepancy in gradients is due to the difference in time scale of the two methods. ESR measures fluctuations with frequencies $\geq 10^8$ Hz while ^{2}H-NMR is slower, detecting movements with frequencies $\geq 10^5$ Hz. Therefore, motions with frequencies of 10^5-10^7 Hz are not included in the ESR order parameter but are detected by ^{2}H-NMR. The relationship between the order parameters S and S_D, respectively, should be (45):

$$S_D = 1/2(3\overline{\cos^2\gamma} - 1)S$$

where $\overline{\cos^2\gamma}$ is the average angular fluctuation in the 10^5-10^7 Hz range, γ was defined previously. From the equation, the time scale effect should have the effect of making S_D smaller than S. However, when order parameters were determined by both methods in identical systems, S_D was found to be larger than S (45, 48). This is the opposite of the prediction derived by time-scale arguments. The discrepancy has yet to be resolved.

^{2}H-NMR shows the physical state of an unperturbed system while spin labels show the response of the system to a small perturbation, the presence of the nitroxide chemical group. It appears that if spin label spectra

are interpreted with this in mind and especially if the data analysis is qualitative, as in this work, the use of the spin-label method can be justified. It is possible that in some cases, the presence of the bulky reporting group may be an advantage, the slightly perturbed system could be more sensitive to steric changes than the original system.

EXPERIMENTAL

I Preparation of Multilayers

Egg yolk phosphatidylcholine was prepared by the method of Pangborn (50) except for the use of 1:1 chloroform/methanol as the extraction solvent instead of ethanol. When stored for over two weeks, the EYL was kept at -20° C as a frozen, degassed benzene solution. For shorter periods and before use in a multilayer preparation, the lecithin was stored in chloroform under N_2 at the same temperature. Ergosterol (MCB) was purified by three recrystallizations from methanol/chloroform and stored as the solid at -20° C. A chloroform solution was prepared immediately before use. Amphotericin B (Calbiochem, Lot 300655, A Grade) was stored with the ergosterol in a desiccator at the above temperature, then dissolved in acidic methanol (1 drop conc. HCl/ 10 ml methanol) before use. The spin labels were stored in chloroform at -20° C and their purity monitored by TLC occasionally. When necessary, the labels were repurified by preparative TLC.

The oriented multilayers were prepared as follows: the solutions described above were either prepared or allowed to warm to room temperature, then appropriate aliquots were combined in a test-tube. 1.3×10^{-6} moles of EYL were used per preparation. The amounts of the other components needed were then determined from this amount; e.g., if a 10% ergo-

sterol preparation was required, 1.4×10^{-7} moles of the sterol was added. Spin-label concentration was kept at 1% or less. The combined solution was then transferred to a flat quartz esr cell (Willmad Glass Co.) and the solvent evaporated by bubbling with a stream of wet nitrogen. The lipid was deposited on the side of the cell by the bubbling action. Any remaining solvent was evaporated by evacuation for at least 8 hours. The multilayers were hydrated by exposure to 0.15 M NaH_2PO_4, pH 7, for 15 minutes. After draining, the esr spectrum of the preparation was recorded on a Varian E-4 spectrometer. When using the antibiotic, the multilayers were kept in the dark as much as possible during and after their preparation. Typical machine settings would be as follows:

Scan Range	- 100 G
Field Set	- 3360 G
Time Constant	- 1.0 s
Scan Time	- 16 min
Modulation Amplitude	- 1.0×10^{0} G
Receiver Gain	- 5×10^{3}
Microwave Power	- 20 mW
Microwave frequency	- 9.450 GHz

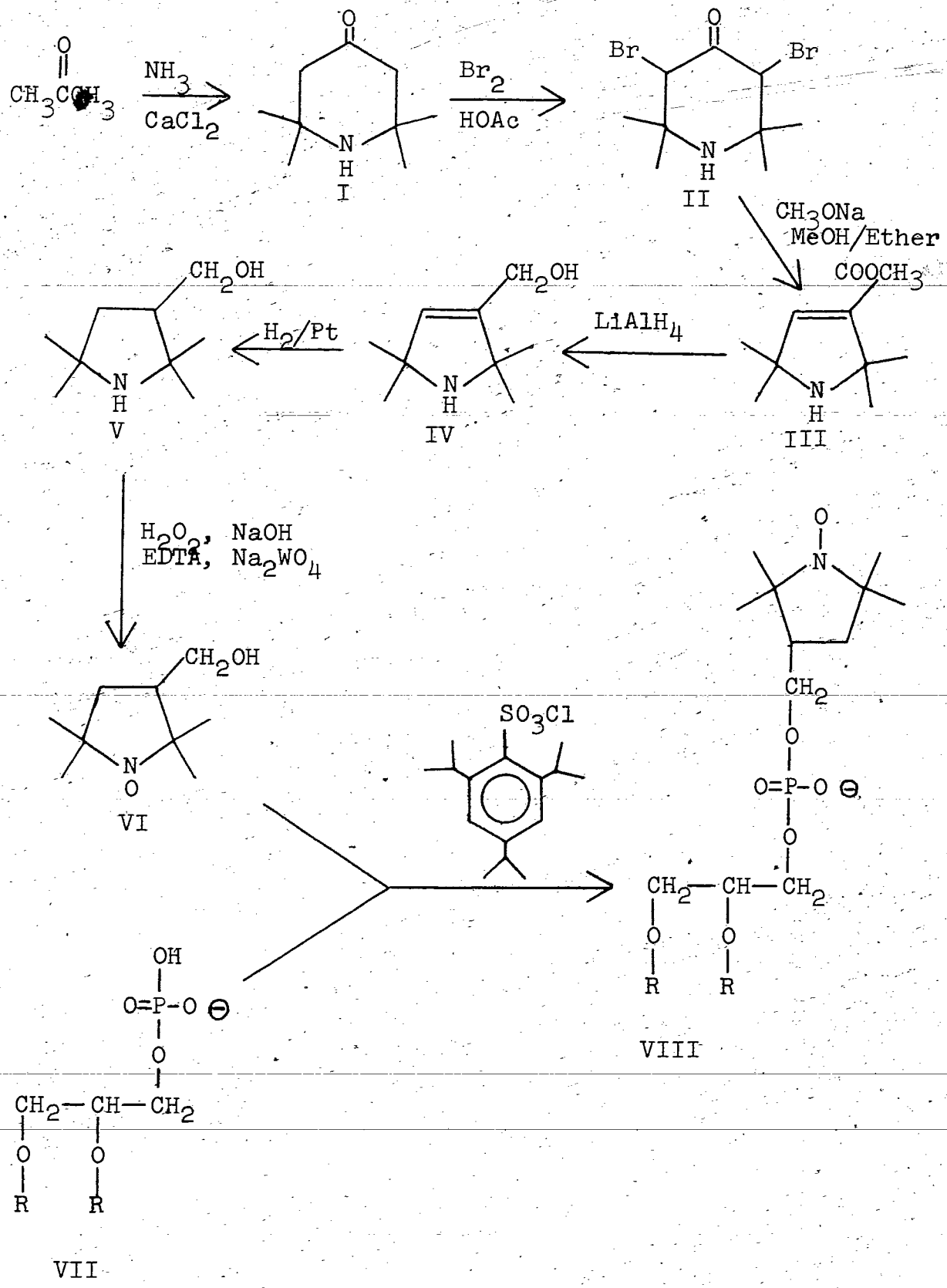
II Synthesis of Spin-Labelled Compounds

Mass spectra were taken on a Hitachi Perkin-Elmer RMU-6E spectrometer. 100 MHz NMR were recorded for deuterochloroform solutions on a Varian XL-100 spectrometer using TMS as an internal standard. Nitroxide-containing compounds were first reduced by the addition of phenylhydrazine to the sample in the NMR tube. Infra-red spectra were determined on a Perkin-Elmer spectrophotometer model 457. A Varian E-4 EPR spectrometer was used for esr spectra. Organic solvent solutions were first dried with magnesium sulphate before evaporation on a rotary evaporator (temperature less than 40° C).

(A) Synthesis of the Phospholipid-Head label (PL-HL)

The synthesis of PL-HL (VIII, Figure 12) was according to the method of Aneja and Davies (51) except where noted. Acetone was first condensed with ammonia to form 2,2,6,6-tetramethylpiperid-4-one (I)(52). This was brominated to form the 3,5-dibromo derivative (II) by the method of Sandris and Ourisson (53). Treatment of II with sodium methoxide produced III via a Favorskii rearrangement. Reduction of III with lithium aluminium hydride followed by catalytic hydrogenation gave IV and then V. The oxidation of the secondary amine to a nitroxide was done with hydrogen peroxide catalysed by sodium tungstate and EDTA as described by Kornberg and McConnell (54). VI was linked to phosphatidic

FIGURE 12 - Synthesis of PL-HL



acid (derived from egg yolk lecithin (41, 54)) using 2, 4, 6,-tri-isopropyl-benzenesulphonyl chloride in pyridine to form the spin labelled phospholipid (PL-HL,VIII). The principal peaks in the mass spectra taken of the intermediate compounds corresponded to the literature.

(1) Preparation of I

Dry acetone (750 ml) and anhydrous $CaCl_2$ (189 g) were combined in a 1 litre round-bottom flask equipped with a mechanical stirrer, condensor and a gas addition tube. Ammonia gas bubbled through the suspension at 4 hr intervals with stirring over a 2 day period. The solution was refluxed for 1 hr, filtered, and the filtrate distilled until only constituents boiling above 75° C remained. The red viscous liquid which resulted was cooled to 3° C in a salt-ice bath and small portions of H_2O added until there was no further increase in temperature upon addition. The solution was cooled to 0° C, filtered and the solid recrystallized from ether saturated with water. 42.9 g of triacetonamine (I) was obtained.

M.S. - 155 (M^+), 140, 98, 84, 83, 58, 55, 42

(2) Bromination of I

A solution of Br_2 (51 g) in glacial acetic acid (70 ml) was added dropwise to 25 g of I dissolved in 100 ml of glacial acetic acid. The rate of addition was such that the temperature did not exceed 40° C. After the reaction was complete, the solution was allowed to cool while HBr gas was given off and the product precipitated. The solution was stirred for an additional 2 hrs, filtered and the precipitate washed successively with several 50 ml portions of glacial acetic acid followed by water, methanol and ether, then dried. The product obtained (II, 51.1 g) was used without further purification in the following step.

M.S. - 313 (M^+), 234, 232, 153, 83, 58

(3) Favorskii Rearrangement of II

To a suspension of II (27 g) in a solution of ether (340 ml) and methanol (35 ml) was added a sodium methoxide solution, prepared from 6 g of Na dissolved in 75 ml of methanol and 400 ml of ether, over a one hour period with magnetic stirring at 2° C. The mixture was stirred at this temperature for an additional 2 hrs, then warmed to 25° C and filtered. The filtrate was evaporated to give a yellow viscous liquid. The crude product was purified on a 120 g Unisel silica gel column eluted with 5% methanol in chloroform, to give 8.3 g of pure III.

M.S. - 168 ($M^+ - CH_3$), 167, 152, 136, 109, 108

(4) Reduction of III

A solution of dry ether (100 ml) containing 3.54 g of $LiAlH_4$ was refluxed for 1 hr. While the solution was still warm, 8.3 g of III was added dropwise with magnetic stirring at a rate that maintained a gentle reflux. After addition was complete, the solution was refluxed for an additional 1½ hr, cooled and stirred overnight. Excess $LiAlH_4$ was destroyed by addition 3 ml 15% NaOH and 10 ml H_2O. The solution was filtered and the filtrate extracted with water saturated with NaCl. The ether layer was evaporated and dried under vacuum to give 5.5g of IV.

M.S. - 155 (M^+), 140, 122, 110

(5) Reduction of IV

Hydrogenation of IV (5.5 g) was carried out in a Parr apparatus with 100 ml of methanol at pH 2 and 150 mg of prereduced PtO_2 catalyst for 11 hrs. The solution was filtered and the filtrate pH adjusted to 13 with 5N NaOH. The solution was then evaporated and extracted with NaCl-saturated water and ether. The ether layers were combined and evaporated to give 3.7 g of V. NMR showed no olefinic hydrogens in the product.

(6) Oxidation of V

A combination of 3.7 g V, 1.7 g EDTA, 0.95 g NaOH, 2 g $Na_2WO_4 \cdot 2\ H_2O$ and 25 ml 30% H_2O_2 in 100 ml of water was left for 24 hrs without stirring after which another 25 mls of 30% H_2O_2 was added. After a further 24 hrs the unreacted H_2O_2 was destroyed by vigorous magnetic stirring. The yellow solution was then extracted with four 75 ml portions of chloroform. The $CHCl_3$ layers were dried with $MgSO_4$ and evaporated to give a yellow residue. The crude VI was recrystallized from ether-hexane to give 3.0 g of the nitroxide.

M.S. - 172 (M^+), 127, 85, 71

(7) Preparation of VIII

The phosphatidic acid used in this step was prepared by the method of Kornberg and McConnell (41) from freshly prepared EYL. 190 mg of VI was combined with 425 mg of egg phosphatidic acid and the combination evaporated from dry pyridine and chloroform three times. The residue was dried under vacuum, over P_2O_5 for 2 days. The dry mixture was dissolved in 40 ml of dry pyridine with 360 mg of dry 2,4,6-tri-isopropyl-benzenesulphonyl chloride and left shaking for 9 hrs at 25° C. After filtration, 70 ml of H_2O was added to the filtrate with cooling, and allowed to stand for 12 hrs. The suspension was evaporated to dryness, then placed under vacuum over P_2O_5 for 12 hrs. The dry solid was triturated with hexanes, filtered and the filtrate evaporated

to give a yellow solid which was triturated, filtered and dried a second time giving 200 mg of an amorphous yellow solid. The crude reaction mixture was purified by preparative TLC on 0.75 mm silica gel plates using a 75 $CHCl_3$/25 methanol/4 conc NH_4OH solvent system to yield 25 mg of VIII. The IR and NMR spectra of VIII corresponded to those given in the literature (51).

(B) Synthesis of the Tail-labelled Phospholipids (PC(2)-5-PASL, PC(2)-8-PASL, PC(2)-12-SASL

The tail-labelled phospholipid precursors, 5-PASL, and 8-PASL, were synthesized by the method of Hubbell and McConnell (26,ACO). 12-SASL was synthesized from 12-hydroxystearic acid (IX,ICN Pharmaceuticals) by methylation to form the methyl ester (X,CH_2N_2), Jones oxidation to form the 12-keto acid (XI,55) and condensation with 2-amino-2-methylpropanol (56,Aldrich) to form the oxazolidine derivative(XII). Oxidation with m-chloroperbenzoic acid and hydrolysis of the ester gave the labelled acid (XIV,Figure 13,56). The acids were linked to lysolecithin (XVII,Sigma) to form the PC(2)-X-FASL compounds (XVIII,Figure 14,57).

PC(2)-X-FASL

Three 5 ml portions of benzene (dried over Na) were evaporated from 200 mg of the spin-labelled acid in the reaction flask, The residue was dissolved in 2.5 ml of dry benzene with 100 mg of N,N-carbonyldiimidazole (CDI,Aldrich)

FIGURE 13 - Synthesis of 12-SASL

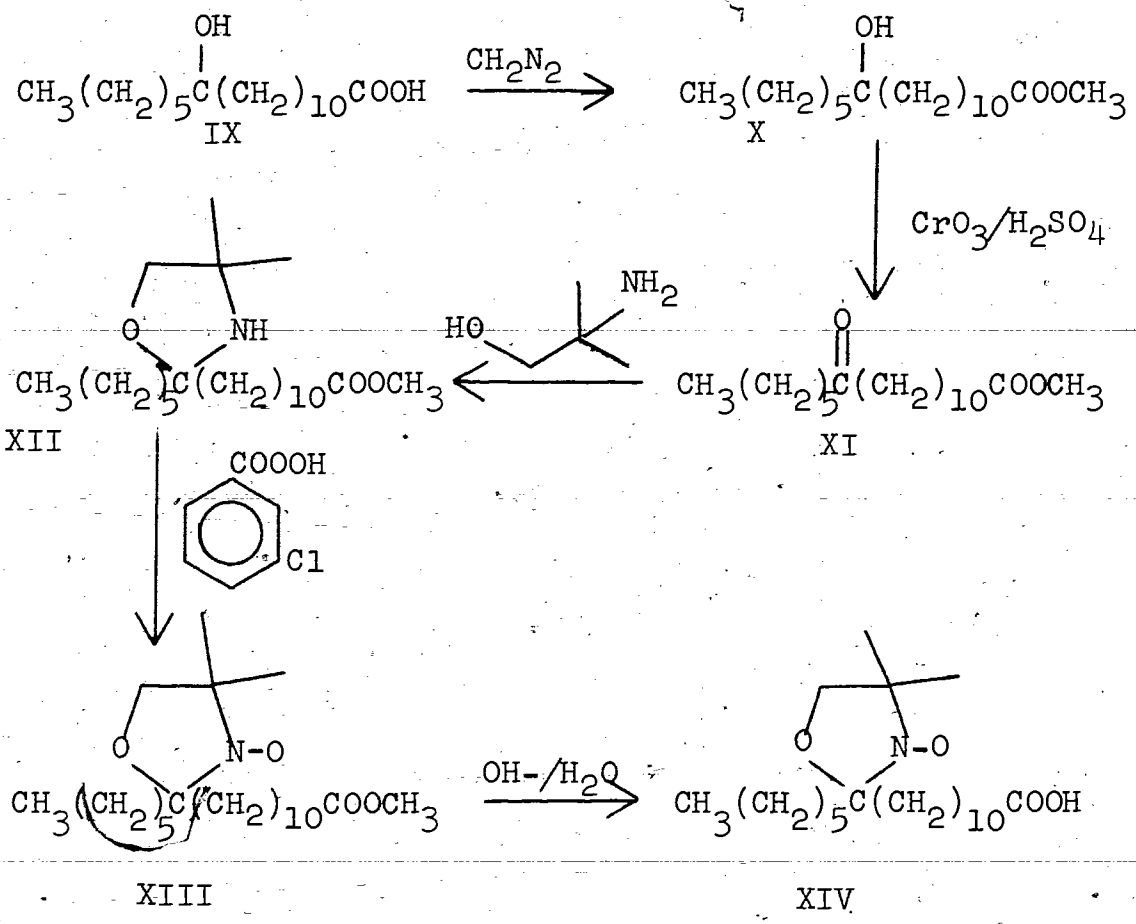
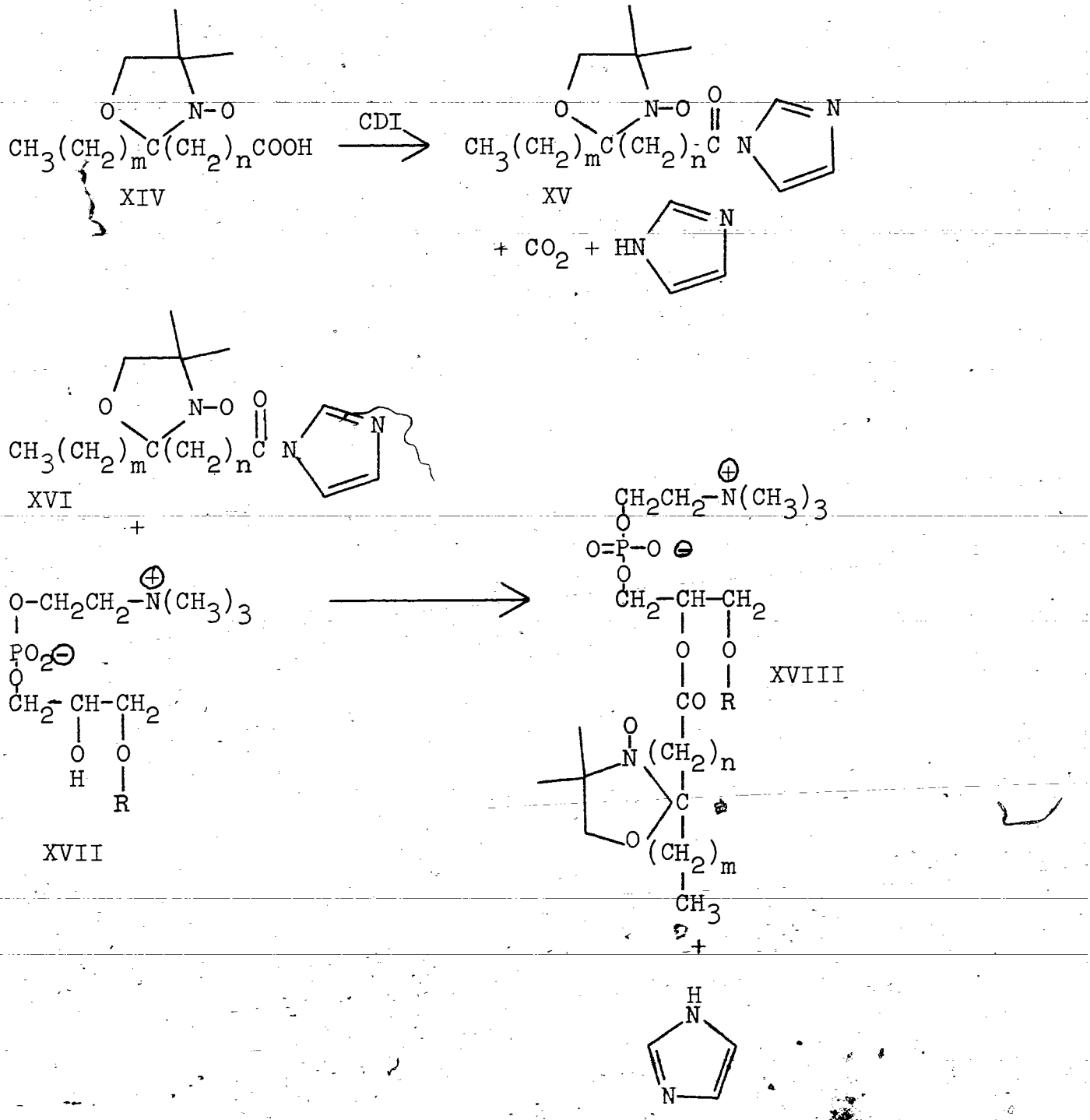


FIGURE 14 - Synthesis of PC(2)-5-PASL, PC(2)-8-PASL, PC(2)-12-SASL
(m,n)=(10,3), (7,6), (5,10)
R= Fatty Acid Residue



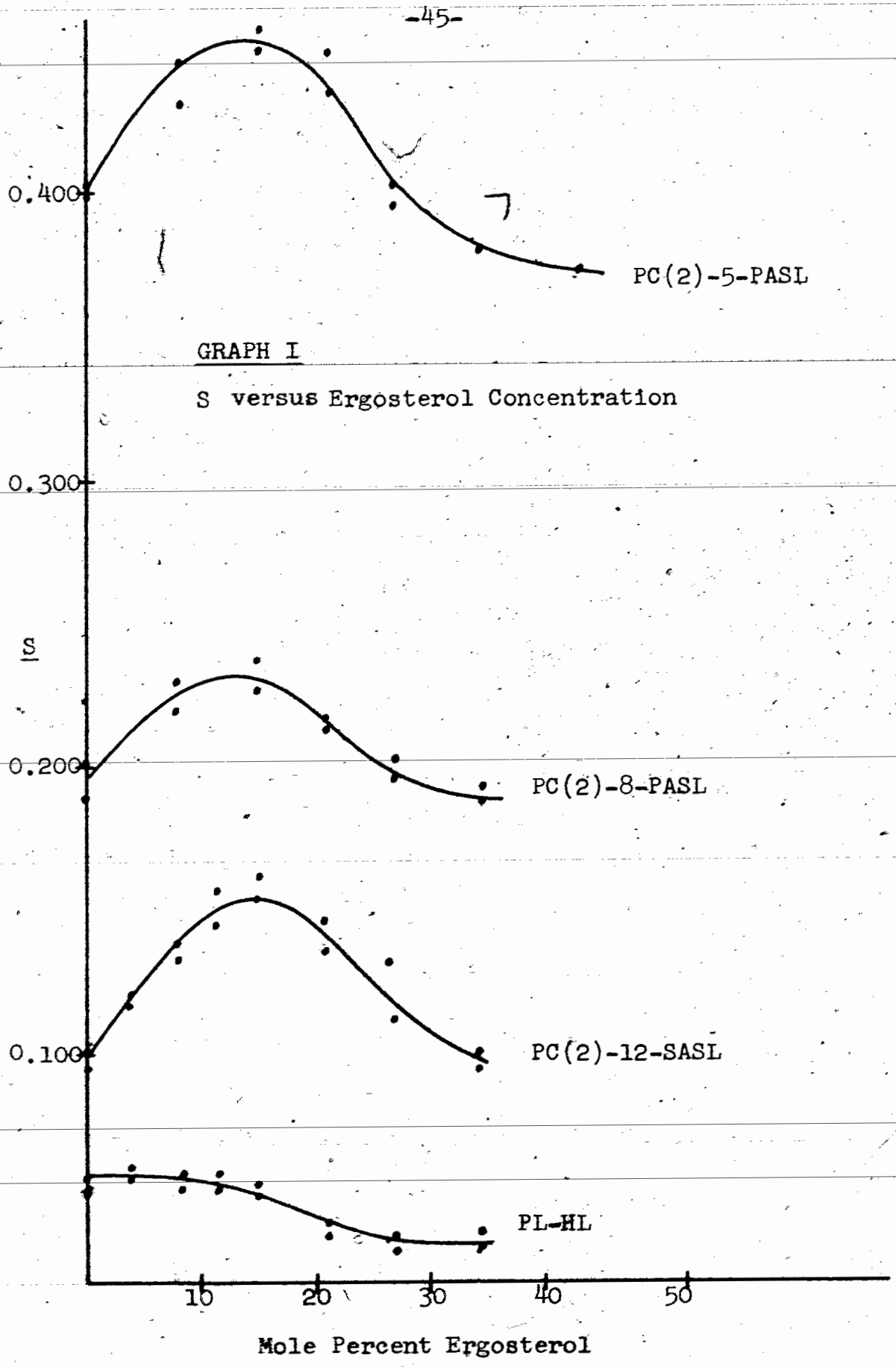
under dry N_2 with stirring to form the acyl-imidazole derivative. After the CO_2 effervescence had stopped (about 3/4 hr), 250 mg of lysolecithin was added and the solution stirred overnight under nitrogen. The benzene was removed by evaporation and the remaining viscous yellow liquid heated to 50° C in an oil bath under nitrogen for 3 days. During this time, imidazole released during the reaction sublimed to the cooler parts of the flask where it was mechanically removed as often as necessary. If most of the imidazole is not removed, it interferes with the purification procedure. The mixture was cooled to room temperature and 0.5 ml of water added to destroy any unreacted intermediate. The water was then azeotropically removed by repeated evaporations of 30% methanol/chloroform. The lecithin derivative was purified by preparative TLC on 0.75 mm silica gel plates run in a 65 chloroform/ 25 methanol/ 4 water solvent system. The yellow product co-chromatographs with EYL as well as having a very similar IR spectrum.

RESULTS

The results are summarized in Graphs I to XI. A division into two basic sections can be made. Graphs I to V show the responses of the spin labels to varying concentrations of purified ergosterol and amphotericin B in multilayers of EYL. The changes in order parameter with increasing concentrations of the two components individually, are shown in Graphs I and V. In the remaining graphs (II, III and IV), antibiotic was added to multilayers of EYL containing a constant amount of sterol. The interaction of the two components was studied up to the solubility limit of ergosterol in EYL. In the second phase (Graphs VI to XI), the solubility of ergosterol in EYL was raised by two methods; 1) using a dicetyl phosphate (DCP) - lecithin mixture as the supporting lipid and, 2) using ergosterol that had been slightly decomposed by standing in solution for one week. The very small amount of oxidation products present in the latter solution appeared to increase the sterol solubility dramatically.

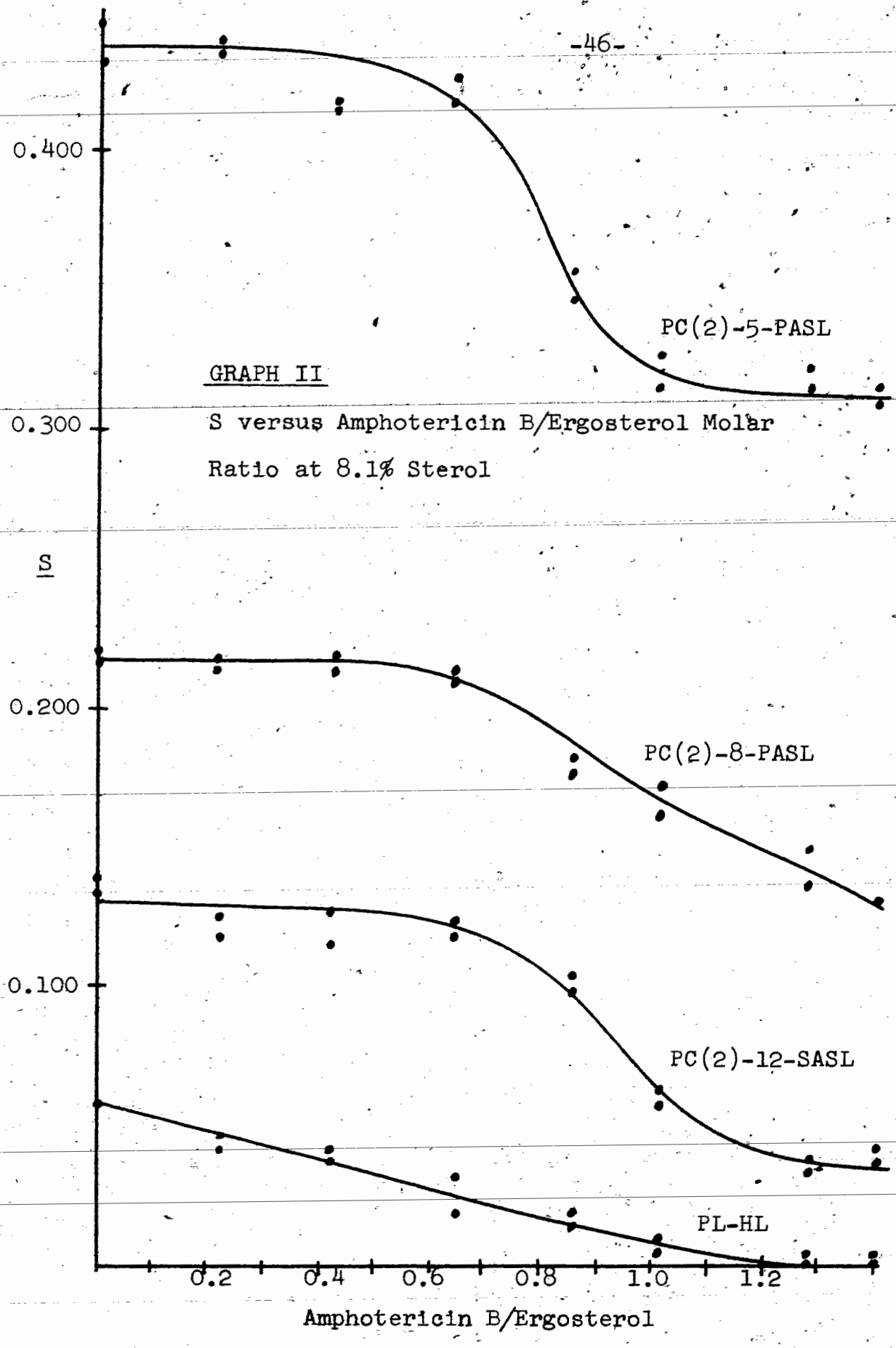
The responses of the probes to incorporation of ergosterol in the two altered types of bilayer are shown in Graphs VI and IX. The other graphs in this section show the changes in order parameter when antibiotic was added to multilayers containing a fixed concentration of ergosterol.

The responses of the four synthesized spin labels to ergosterol at different concentrations in pure EYL are shown in Graph I. Similar order parameter changes were



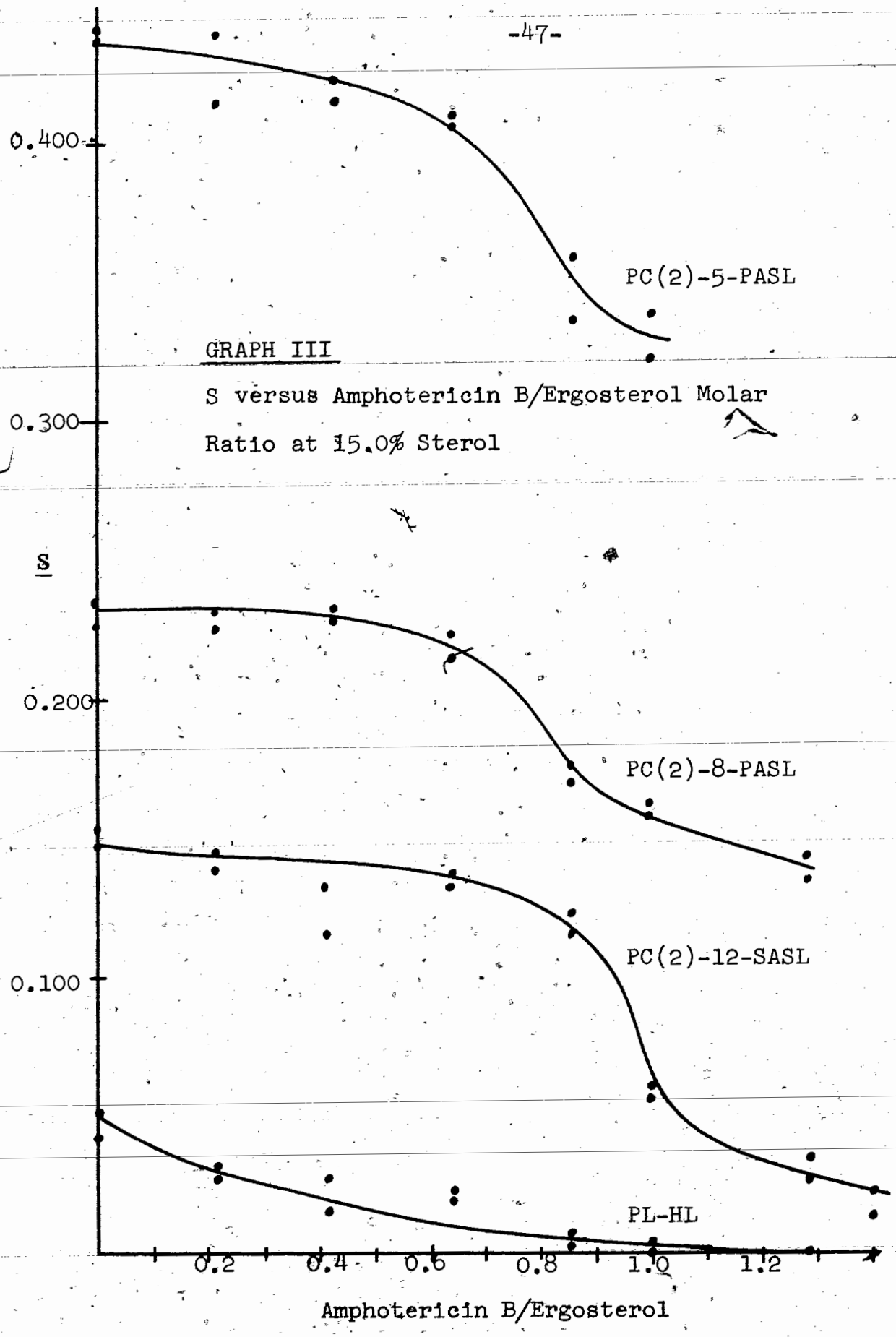
GRAPH I

S versus Ergosterol Concentration



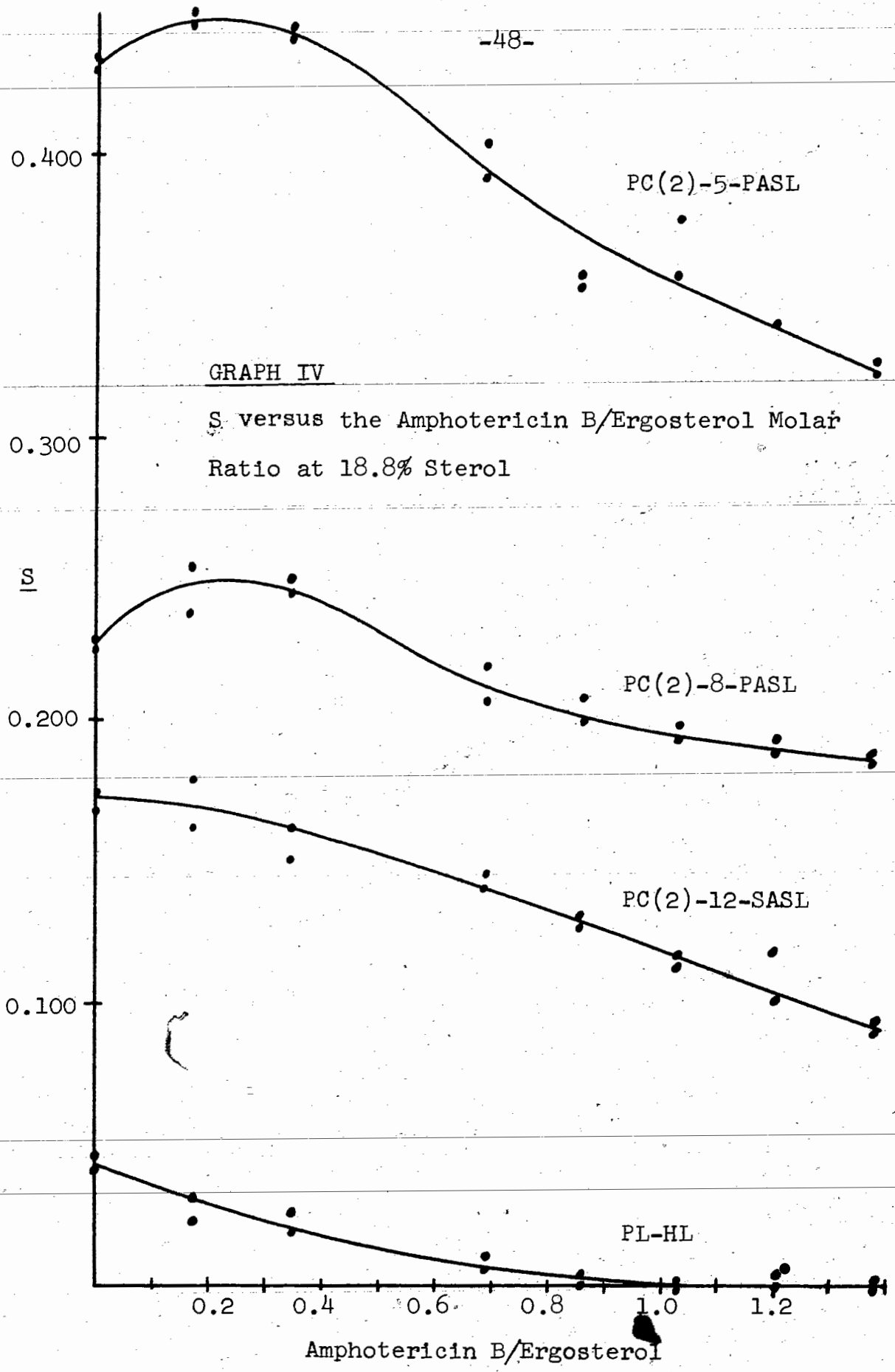
GRAPH II

S versus Amphotericin B/Ergosterol Molar Ratio at 8.1% Sterol



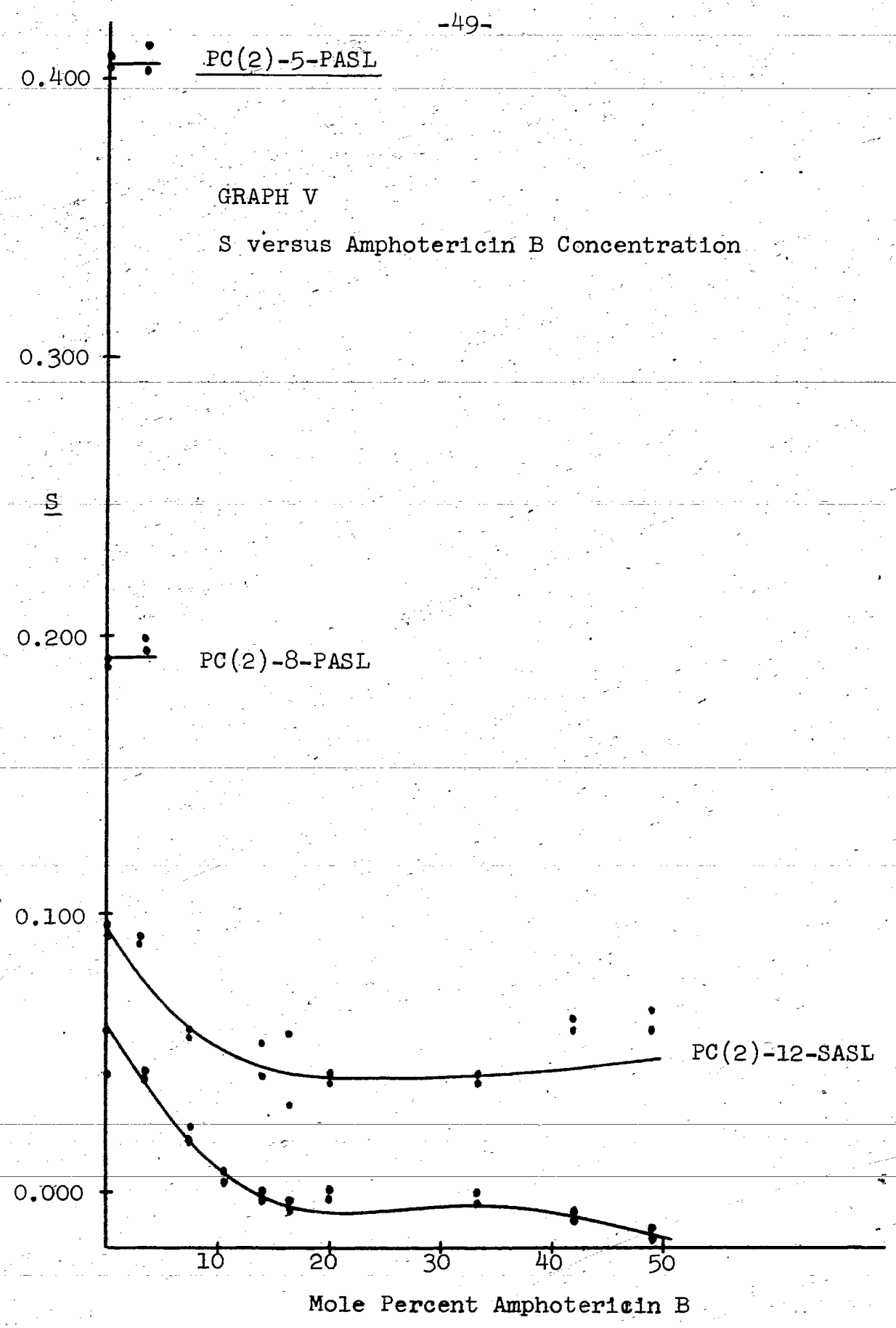
GRAPH III

S versus Amphotericin B/Ergosterol Molar Ratio at 15.0% Sterol



GRAPH IV

S versus the Amphotericin B/Ergosterol Molar Ratio at 18.8% Sterol



GRAPH V

S versus Amphotericin B Concentration

GRAPH VI

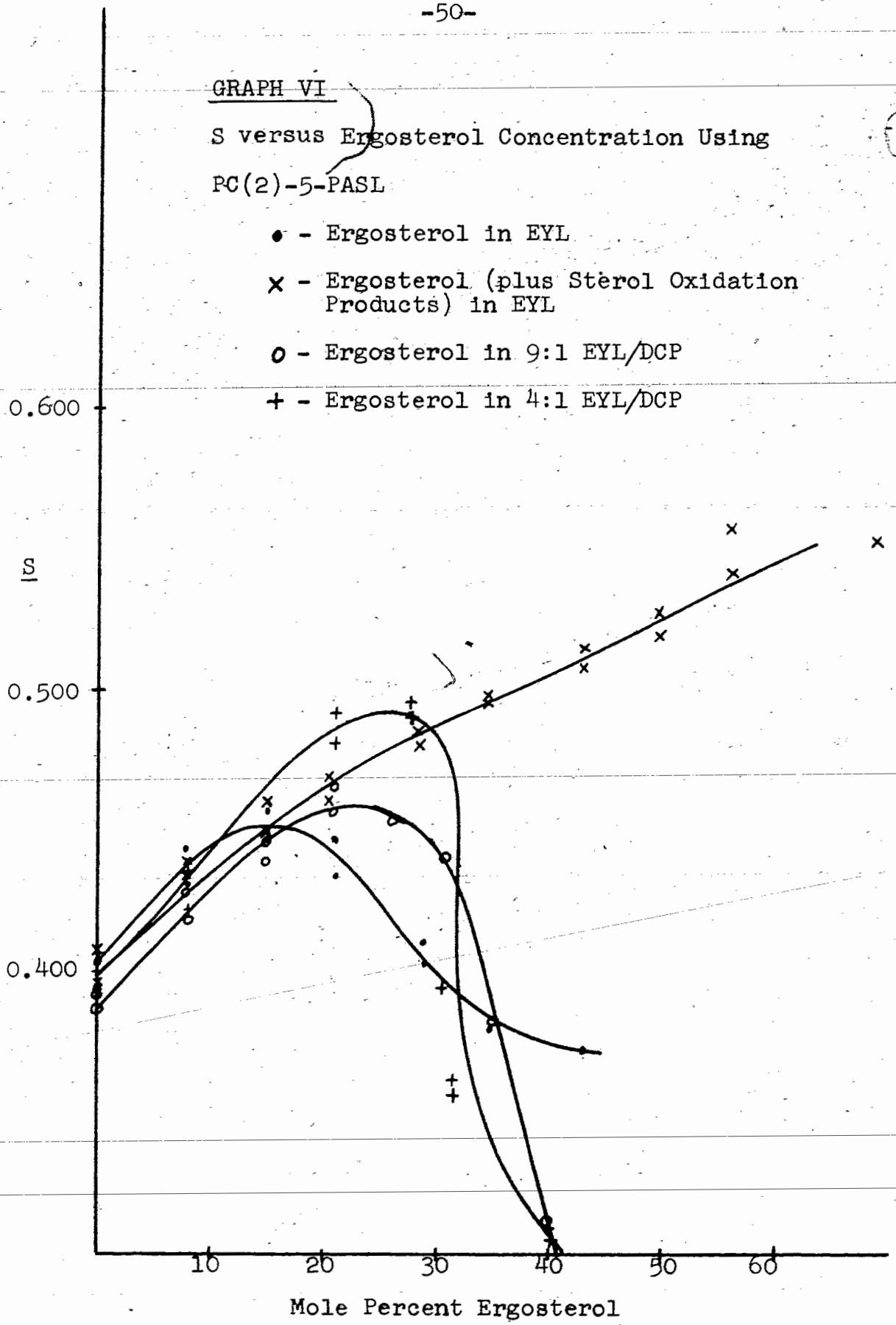
S versus Ergosterol Concentration Using PC(2)-5-PASL

• - Ergosterol in EYL

× - Ergosterol (plus Sterol Oxidation Products) in EYL

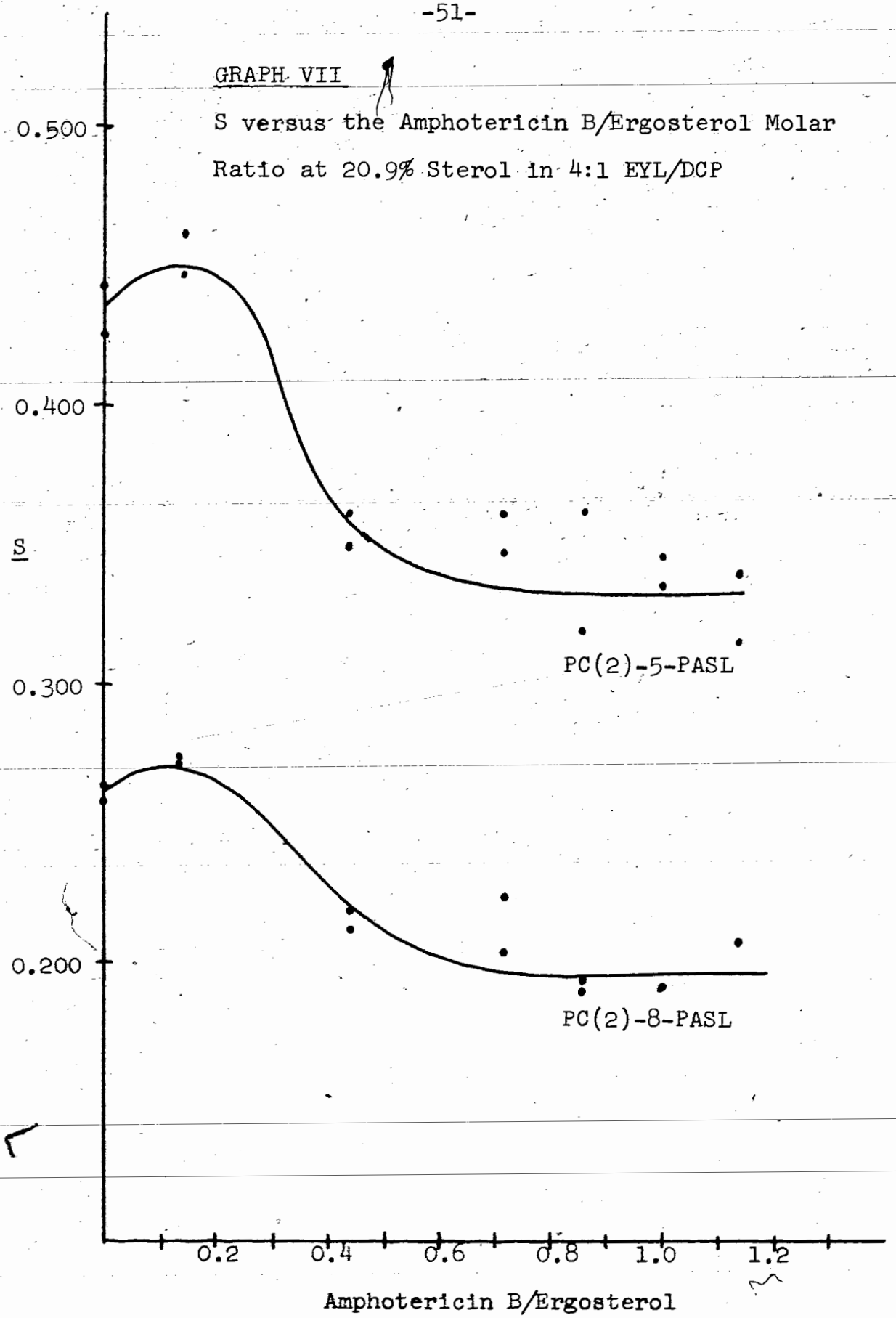
o - Ergosterol in 9:1 EYL/DCP

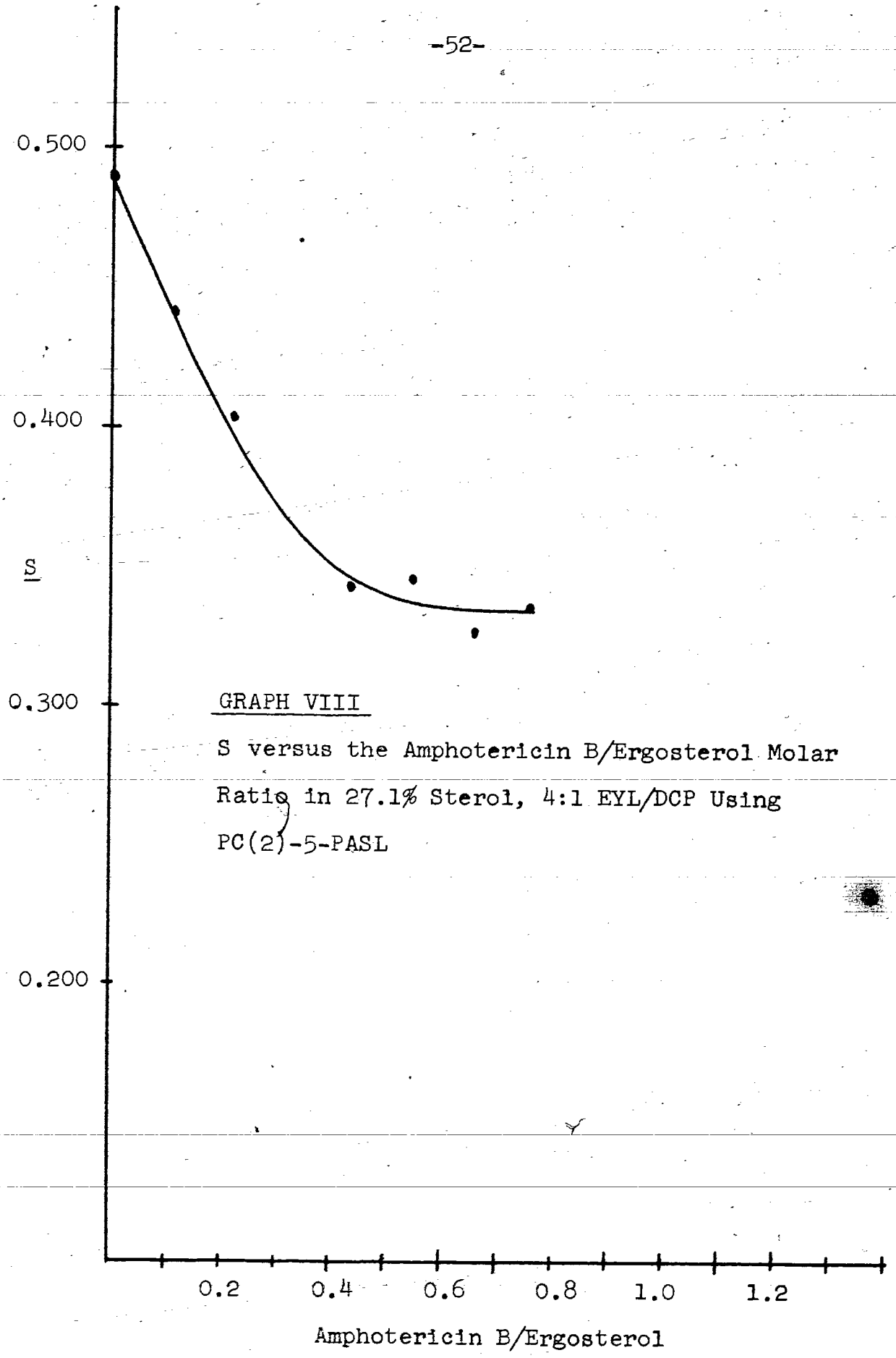
+ - Ergosterol in 4:1 EYL/DCP



GRAPH VII

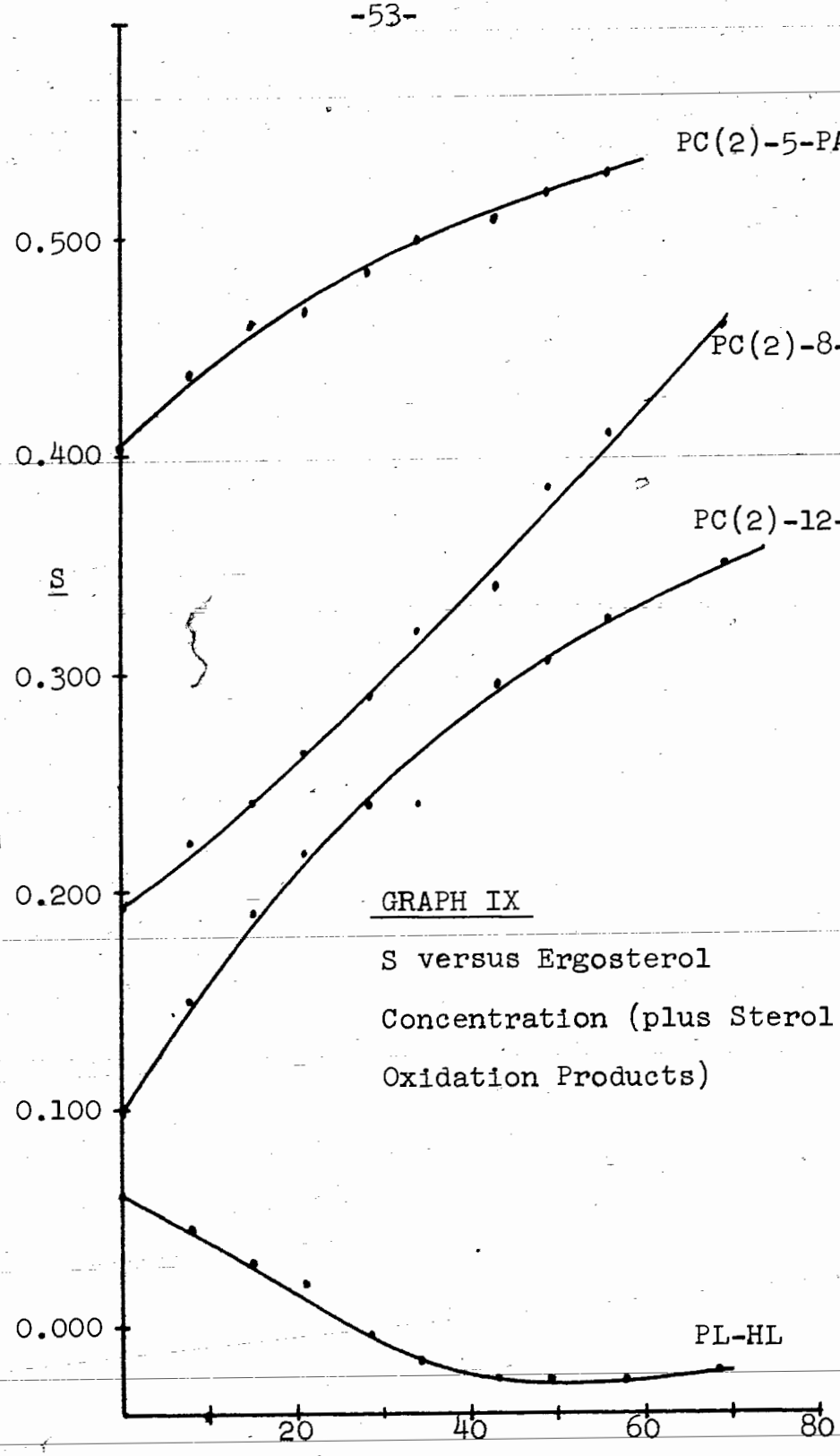
S versus the Amphotericin B/Ergosterol Molar Ratio at 20.9% Sterol in 4:1 EYL/DCP





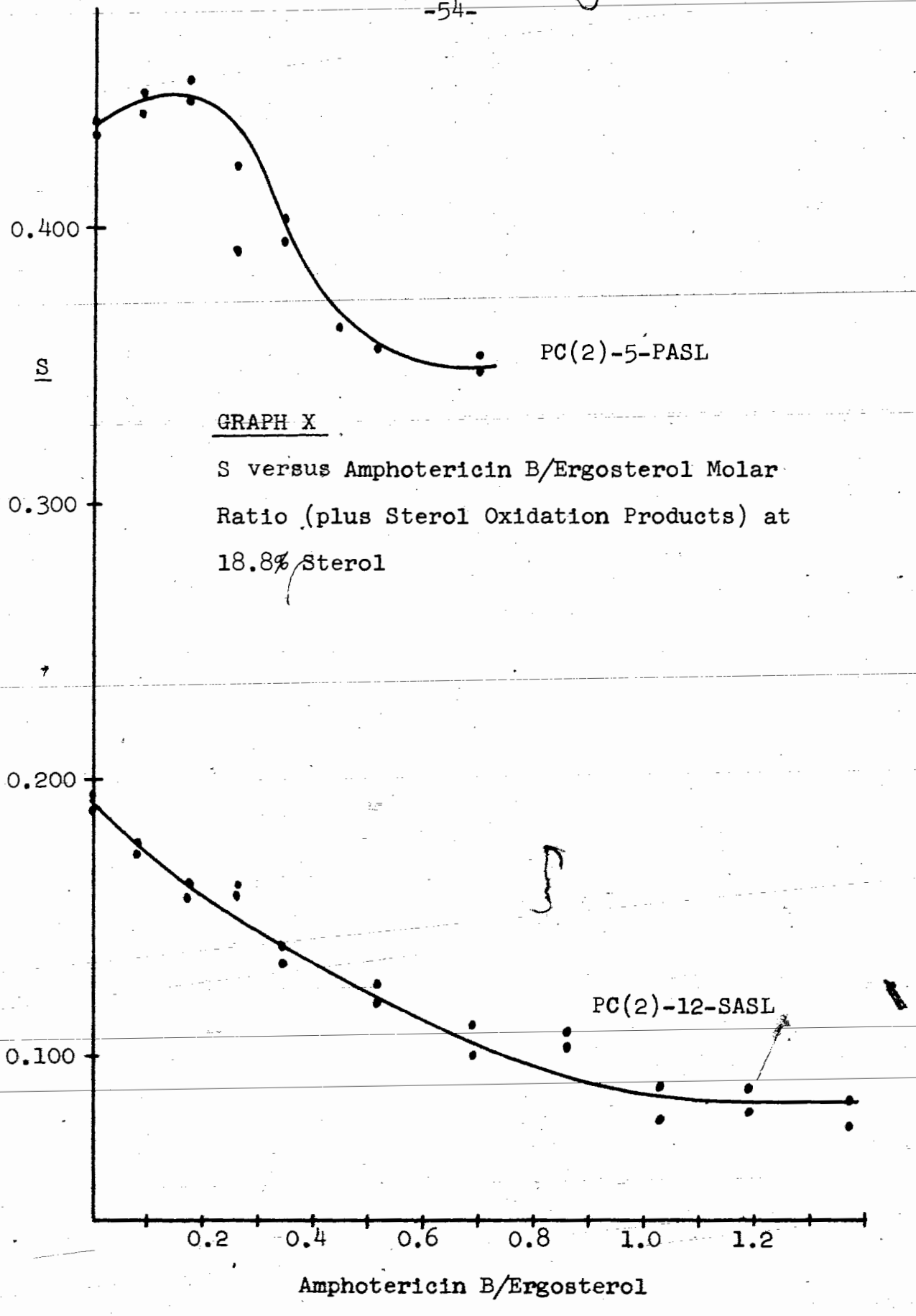
GRAPH VIII

S versus the Amphotericin B/Ergosterol Molar Ratio in 27.1% Sterol, 4:1 EYL/DCP Using PC(2)-5-PASL



GRAPH IX

S versus Ergosterol Concentration (plus Sterol Oxidation Products)

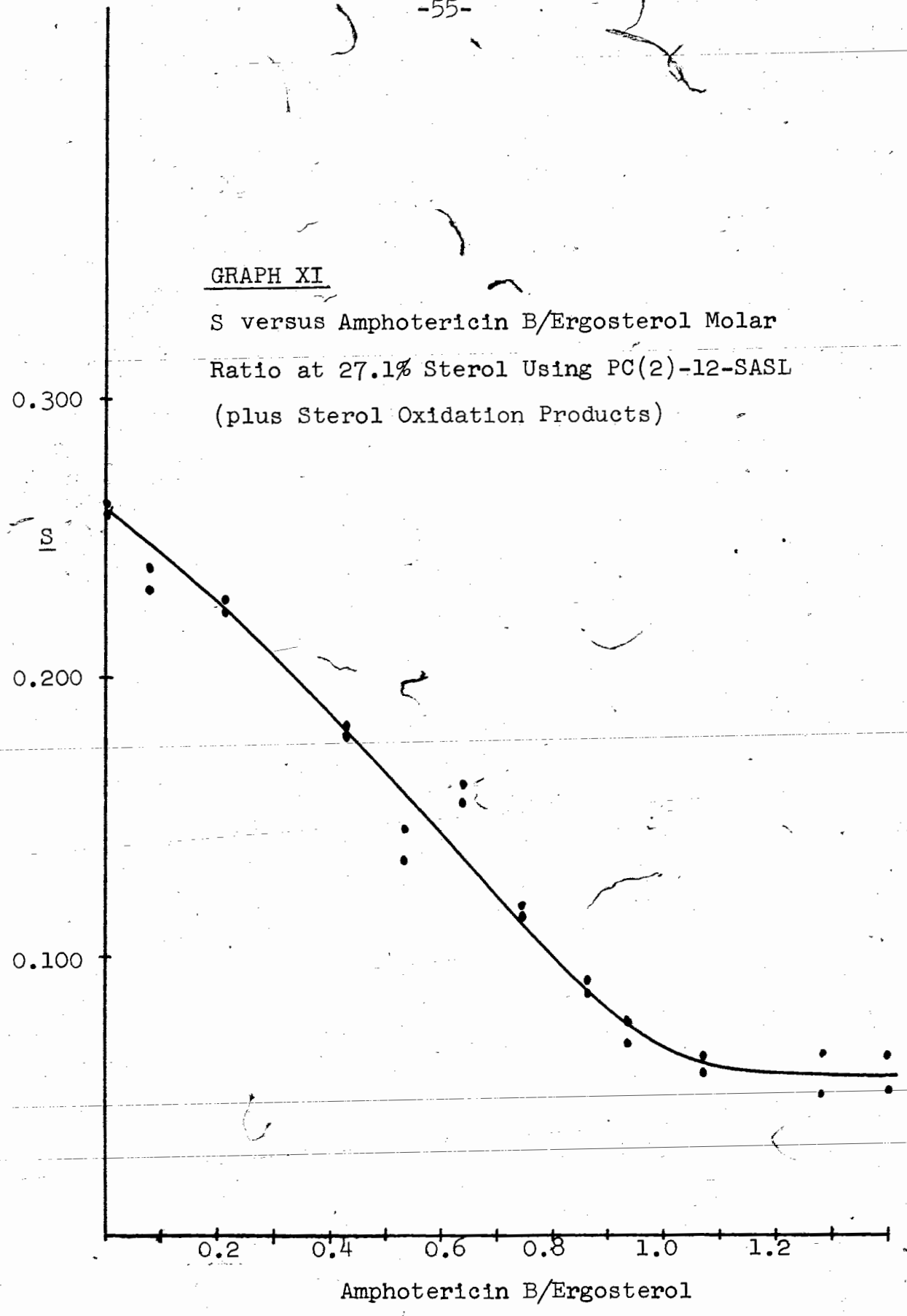


GRAPH X

S versus Amphotericin B/Ergosterol Molar Ratio (plus Sterol Oxidation Products) at 18.8% Sterol

GRAPH XI

S versus Amphotericin B/Ergosterol Molar Ratio at 27.1% Sterol Using PC(2)-12-SASL (plus Sterol Oxidation Products)



observed for the three fatty-acid type probes (PC(2)-5-PASL, PC(2)-8-PASL and PC(2)-12-SASL). For these spin labels, S (the order parameter) reached a maximum around 15 mole percent ergosterol. The increase in order parameter from zero to 15% sterol was approximately 0.05 units for the three chain probes. The order parameter of the head-label (PL-HL) was constant up to 15% ergosterol after which it decreased slightly.

In pure EYL multilayers containing a fixed concentration of sterol, addition of amphotericin B caused changes in the order parameters of the spin labels. At a concentration of 8.1% ergosterol in the multilayers (Graph II), the three fatty-acid derived probes showed a similar order parameter response to changing antibiotic concentrations. For these labels S was approximately constant up to a antibiotic:sterol ratio of 0.6 after which it decreased. PL-HL exhibited a linear decrease in S up to an antibiotic:sterol ratio of 1.3. When the ergosterol concentration was 15%, (Graph III) similar results were obtained except the decrease in S after a antibiotic:sterol ratio of 0.6 was more extreme. With multilayers containing 18.8% ergosterol , quite different changes in S were observed. There was an initial increase in order parameter for the PC(2)-5-PASL and PC(2)-8-PASL probes with the maximum occuring at an antibiotic:sterol ratio of 0.25. The initial increase in S was larger for the

first label (0.035 S-units), than for the latter (0.020 units). The label PC(2)-12-SASL, showed only a decrease in S over the antibiotic:sterol ratio investigated (0 to 1.5). The order parameter of the head-label also decreased up to a antibiotic: sterol ratio of 0.9, after which it was constant with S equal to zero.

The results from control experiments in which amphotericin B was added to multilayer preparations containing no ergosterol are shown in Graph V. No trends in order parameter variation were observed with the PC(2)-5-PASL and PC(2)-8-PASL probes at antibiotic concentrations less than 4%. The spectra were distorted at higher concentrations of antibiotic and no order parameters could be calculated. The other two labels showed a steady decrease in S up to 15 mole percent antibiotic, after which the order parameter was approximately constant.

As described above, the solubility of ergosterol in EYL was increased by two methods. The order parameter responses of PC(2)-5-PASL to increasing sterol concentration under the different conditions, are shown in Graph VI. Dicetyl phosphate in 9:1 and 4:1 EYL/DCP molar ratios raised the maximum ordering ergosterol concentrations to 25% and 30% respectively. The order parameters derived from multilayers containing slightly oxidized ergosterol continuously increased until a sterol concentration of 70 mole percent was attained. This was the highest ergosterol content examined.

At 20.9% sterol in a 4:1 EYL/DCP multilayer (Graph VII), the PC(2)-5-PASL order parameter curve with respect to increasing antibiotic concentration was very similar to that observed for 15.0% ergosterol in pure EYL. The main differences were that the maximum S occurs at a smaller amphotericin B:ergosterol ratio (0.20 as compared to 0.25 in the latter case), and the subsequent decrease in S was more extreme. At a higher concentration of ergosterol (27.1%) in the 4:1 EYL/DCP multilayer (Graph VIII), the maxima in the order parameter curves of PC(2)-5-PASL seen in Graphs IV and VII were not observed. The addition of antibiotic to 27.1% sterol in 4:1 EYL/DCP multilayers resulted in a PC(2)-5-PASL order parameter curve that initially decreased sharply, then flattened out to become approximately constant after an antibiotic:sterol ratio of 0.5 was reached.

The solubility of ergosterol in EYL was very large when the sterol oxidation products were present (Graph IX). PC(2)-8-PASL and PC(2)-12-SASL exhibited the same type of response to increasing ergosterol concentration under these conditions as described above for PC(2)-5-PASL. The order parameter increased up to a sterol concentration of at least 70%. The order parameter curve of PL-HL under these conditions decreased up to 45% ergosterol, after which there was little change.

Amphotericin B added to a multilayer system containing 18.8% ergosterol (plus oxidation products) (Graph X) resulted in PC(2)-5-PASL and PC(2)-12-SASL order parameter curves similar to those observed for antibiotic addition to 18.8% sterol in pure EYL (Graph IV) and 20.9% sterol in 4:1 EYL/DCP multilayers (Graph VII). In the sterol oxidation products/EYL system, an S maximum was observed similar to that seen in the other systems, followed by a sharp decrease in S, then a levelling off when the antibiotic:sterol ratio reached 0.5. The PC(2)-12-SASL order parameter curve of Graph X shows a decrease in S down to a antibiotic:sterol ratio of 1.0, after which the order parameter is constant. At a higher concentration of ergosterol with this system (27.1%, Graph XI), the initial change in S with added amphotericin B was larger than at the lower concentration but with the same change of slope to zero at an antibiotic:sterol ratio of 1.0.

DISCUSSION

In the presence of ergosterol and amphotericin B, the spin label probes incorporated in the multilayers give three basic types of response depending on the concentrations of the sterol and antibiotic. At low initial concentrations of ergosterol (8.1% and 15.0%), the three fatty-acid derived probes show similar order parameter curves (Graphs II and III). At 18.8% and 20.9% ergosterol (IV, VII and X), only PC(2)-5-PASL and PC(2)-8-PASL exhibit similar order parameter dependance, with the PC(2)-12-SASL probe showing quite a different response to changes in ergosterol concentration. At higher concentrations of ergosterol (27.1%, Graphs VIII and XI), there is another change in the PC(2)-5-PASL behaviour while the stearic acid derived label order parameter curve is essentially the same as at 18.8%.

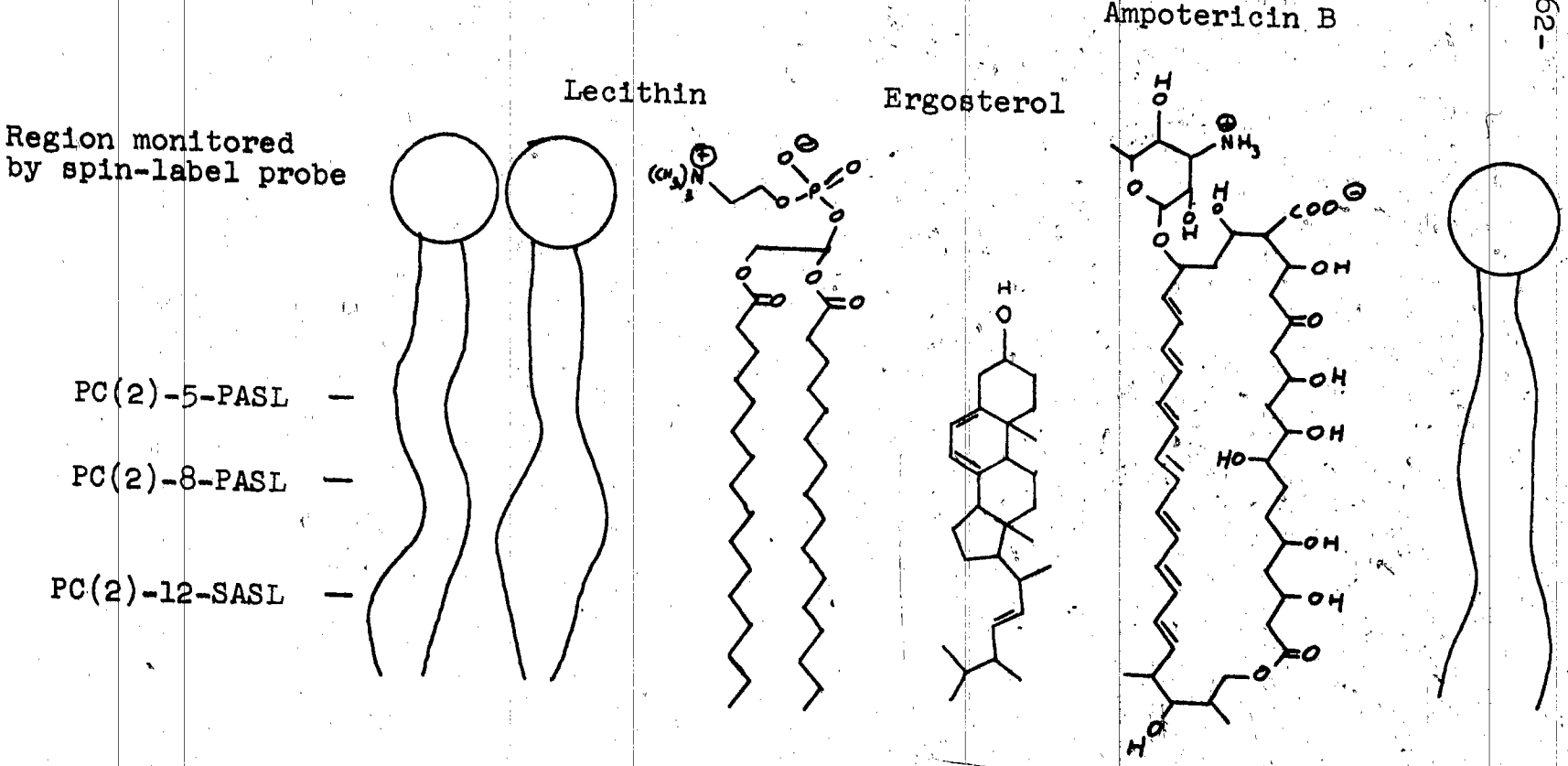
In order to determine if there were any effects on the amphotericin B - ergosterol interaction by DCP or the sterol oxidation products other than raising the solubility of the sterol in EYL, similar experiments were done with the different multilayer systems(ergosterol/EYL, ergosterol/4 EYL:1DCP and ergosterol with oxidation products/EYL). Antibiotic was added to the multilayer types containing sterol at concentrations around 20%(Graphs IV, VII and X). Similar order parameter curves were observed. The less extreme slope changes seen in the pure EYL system are probably due to the fact that at 18.8%, ergosterol is already past its solubility

maximum (~15%) in pure egg yolk lecithin. The DCP and ergosterol oxidation products allow the sterol:antibiotic interaction to be studied at higher concentrations of ergosterol.

All three chain - labelled probes show similar responses to increasing concentration of ergosterol in EYL with approximately equal order parameter increases at the same sterol concentration (Graph I). This observation infers the ergosterol is situated in the bilayer such that the portion of the fatty acid residue containing the three labels is affected equally by the sterol. Figure 15 shows that at least part of the sterol must extend past C_{12} of a lecithin fatty acid residue, as this is the position of the PC(2)-12-SASL nitroxide.

Stockton and Smith (60) have calculated segmental order parameters (S_{mol}) for perdeuterated stearic acid in cholesterol/EYL vesicles. Upon addition of cholesterol, S_{mol} increases linearly with the same slope for each carbon down to about C_{13}. Starting at C_{14}, the rate of increase in S_{mol} is not as large and gets progressively smaller as one approaches the terminal methyl group. At a given concentration of sterol, S_{mol} is constant up to about C_{11} and then decreases. The slope of the latter region is greater for larger concentrations of cholesterol. The authors show that in space-filling models of cholesterol and lecithin, the steroid phenanthrene nucleus extends to about C_{10} of a lecithin

FIGURE 15


Region monitored
by spin-label probe
PC(2)-5-PASL
PC(2)-8-PASL
PC(2)-12-SASL
Lecithin
Ergosterol
Ampotericin B

fatty acid residue. The cholesterol (or ergosterol) C_{21} methyl group makes the initial portion of the sterol chain quite bulky. The latter characteristic could account for the fact that the rigid portion of the bilayer extends past C_{10} to approximately C_{12}. There would be relatively little ordering effect from the rest of the sterol side chain as it is probably quite flexible (58). The fluidity of the lecithin fatty acid residues would increase as one moves down the chain from the last rigidly held carbon, C_{12}.

There is evidence that the carboxyl end of the perdeuterated acid described above is situated in the same region as the ester linkage of the EYL (59, 60). If this is the case and the above interpretation is correct, then PC(2)-5-PASL, PC(2)-8-PASL and PC(2)-12-SASL will measure the order within the region affected by the ergosterol ring system with C_{12} label very near the boundary of this region. This assumes cholesterol and ergosterol have similar locations within EYL bilayers.

It is possible that an amphiphilic compound such as amphotericin B could be incorporated into a multilayer and produce the effects observed without the requirement of a sterol. However, in the present case, amphotericin B must be interacting with ergosterol, since a different dependency of PC(2)-12-SASL order parameter on added antibiotic is observed when antibiotic is added to a sterol-free multilayer (Graph V) as compared to sterol-containing multilayers.

In the sterol-free system, S drops to a minimum at around 12% antibiotic in lecithin. An equivalent amount of antibiotic in ergosterol-containing multilayers results in a much higher order parameter which is about the same as in a multilayer of EYL only. In Graph III the multilayer contains 12% antibiotic at a amphotericin B:ergosterol ratio of 0.8.

Studies using vibrational Raman spectroscopy as a probe of amphotericin B in vesicles of EYL and dimyristoyl lecithin - cholesterol (4:1) have shown a difference that can be interpreted as being due to the formation of ordered complexes by the antibiotic in the presence of cholesterol. In the absence of cholesterol, no antibiotic was incorporated into the bilayer (61).

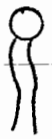
When antibiotic is added to multilayers containing low concentrations of ergosterol (8.1% Graph II and 15.0% Graph III), there is an initial concentration range of antibiotic where there is little change in the order parameter of the three chain probes. This type of behaviour would be expected if a complex composed of a single antibiotic-sterol pair is formed having the same ordering characteristics as a single ergosterol molecule. Although a 1:1 complex would be expected to be the predominant species under these conditions, it would probably be in equilibrium with larger assemblies, possibly the eight and eight unit hypothesized by De Kruijff (21) as the biologically - active form of the complex. As larger amounts of amphotericin B are incorporated

into the membrane, S decreases. The equilibrium described above could account for this. As the concentration of the 1/1 units increase, aggregates of these could begin to form, reducing the number of "ordering units" present, resulting in an increase in fluidity.

When the initial ergosterol concentration is higher, a differential effect on PC(2)-12-SASL and the other two tail labelled probes is observed. Graphs IV, VII and X show that at 18.8% and 20.9% ergosterol, the latter labels show an ordering effect upon addition of antibiotic up to a antibiotic:sterol ratio of 0.15, while the former shows a constant decrease over the same range. The abrupt change of slope to zero at an amphotericin B:ergosterol ratio of 0.5 for the PC(2)-5-PASL and PC(2)-8-PASL order parameter curves in graphs VII and X suggests that at these initial ergosterol concentrations a 1/2 complex is the predominant species. Observations similar to this were described in the introduction for amphotericin B and cholesterol in aqueous ethanol solution (13). In that case, the polyene UV chromaphore stopped changing at a ratio of 1:2 antibiotic/cholesterol and led to the conclusion there are two sterol binding sites per antibiotic molecule.

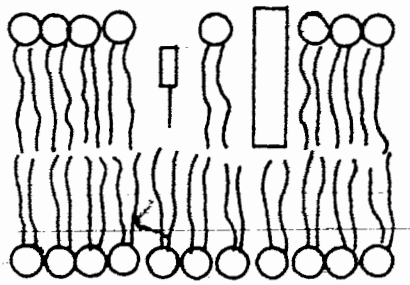
The difference in the responses of the different chain probes could be the result of a small change in sterol location in the bilayer (Figure 16). If the sterol moves toward the

FIGURE 16

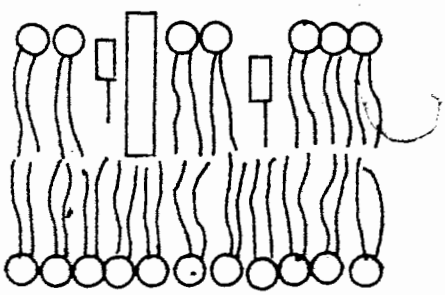

- Lecithin


- Ergosterol

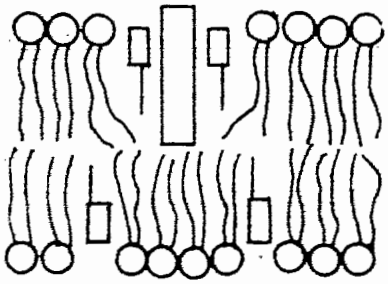

- Amphotericin B



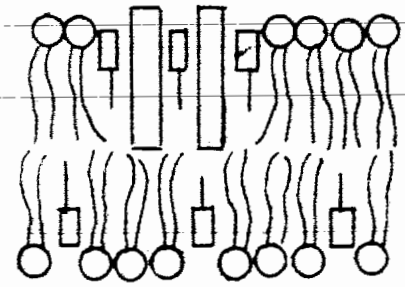
Free, uncomplexed ergosterol and amphotericin B in EYL bilayers



Low concentrations of antibiotic in bilayers of 8.1% or 15.0% sterol in EYL. A 1/1 complex is formed, where the complexed sterol is closer to the surface than uncomplexed ergosterol.



In bilayers of 18.8% and 20.9% ergosterol a 1/2 complex (Antibiotic/Sterol) is formed. There is greater steric crowding in outer portions of the bilayer.



At 27.1% ergosterol larger complexes are formed, possibly up to the 8/8 species hypothesized by De Kruijff.

bilayer surface it could then be out of the region monitored by the PC(2)-12-SASL probe. As mentioned previously, this label is probably on the boundary of the area affected by the steroid nucleus. If amphotericin B-associated ergosterol is in a position nearer the bilayer surface than the free sterol, the differential responses of the chain labels observed could be explained. As seen in Figure 16, the 1/2 complex would have more bulk in the surface region of the bilayer, possibly constraining the chain movements in this area and causing the observed small increase in S for the PC(2)-5-PASL and PC(2)-8-PASL probes as the antibiotic: sterol ratio increases in Graphs IV, VII and X to 0.20. The subsequent decrease in S upon incorporation of more antibiotic would be caused by the reduction in total free ergosterol concentration due to complexation. At an antibiotic:sterol ratio of 0.5, most of the free ergosterol would be used up forming the 1/2 complex hypothesized above. Further addition of antibiotic could result in condensation of 1/2 complexes into larger units with little change in the bilayer region monitored by the 5 and 8 PC(2)-PASL probes.

The order parameter curve of PC(2)-12-SASL in Graphs IV and X show a reasonably constant decrease in S over the 0 to 1.0 amphotericin B:ergosterol molar ratio range. Up to a ratio of 0.5, the decrease would be due to a reduction in free ergosterol concentration as the 1/2 complex is

formed. As described above, the complexed sterol seems to occupy a position closer to the bilayer surface as compared to the free ergosterol, so the C_{12} label would not be affected by the increased steric crowding in the C_1 to C_{10} region. At ratios higher than 0.5, the number of complexes in a given area decreases so the order in the C_{10} - CH_3 region decreases as well. At an amphotericin B:ergosterol ratio of 1.0, no more antibiotic is incorporated and the PC(2)-12-SASL order parameter does not change significantly after this point in Graph X. This levelling effect is not seen with the PC(2)-5-PASL probe. It is known from the control experiment that this label is more sensitive to antibiotic alone than the inner probe. The continual decrease is probably another aspect of this behaviour.

At the highest concentration of ergosterol investigated (27.1%, Graphs VIII and XI), similar behaviour to that seen at 18.8% and 20.9% is observed. No initial increase in order occurs in the third of the bilayer near the surface, probably because the order parameter is larger to begin with due to the greater sterol concentration. The levelling out of the order parameter in the PC(2)-12-SASL curve at a ratio of 1:1 antibiotic/sterol is more obvious due to the larger initial slope.

The hypothesized change in the molecular architecture of the ergosterol/amphotericin B/EYL system are summarized in Figure 16. The results from the use of the head-labelled probe are not used in the analysis as they show only a small dependence on the antibiotic - sterol interactions investigated in these experiments. The low order parameters ($S \leq 0.05$) and narrow line widths observed in the spectra indicate the PL-HL oxazolidine ring has a large amount of motional freedom. On the average, the ring makes an angle smaller than 45° with respect to the multilayer surface. This is consistent with the current view of the head group conformation in EYL, where its long axis is oriented approximately parallel to the bilayer surface (44, 45).

Dicetylphosphate and the ergosterol oxidation products probably increase the ergosterol solubility by changing the polarity of the bilayer. The latter are probably ketones, aldehydes and acids attached to a hydrophobic hydrocarbon residue. Compounds such as these, as well as DCP, would give the bilayer surface a net negative charge. Lecithin is a zwitterion and so has no net charge. The specific reason why surface charge should affect the ergosterol solubility in EYL is not known.

CONCLUSION

The work described here leads to several conclusions concerning the composition and molecular architecture of the amphotericin B - ergosterol interaction in EYL multilayers. A complex of the two components in a ratio of one to one does appear to predominate when the monomers are present in sufficient concentrations. At higher ergosterol concentrations, a complex composed of one amphotericin B and two ergosterol molecules seems to occur, but only if there is insufficient antibiotic present to form the 1:1 complex. The absolute number of molecules in the latter complex type could not be determined in this experiment except at low concentrations of the two constituents. Under these conditions, a complex of one molecule each of the components was indicated. At higher concentrations, a numerically larger complex appears to be formed. An 8:8 complex as described by De Kruijff (21) as the active species would not be inconsistant with the results.

The interactions described above can be summarized as follows:

$$A + E \overset{①}{\rightleftharpoons} AE \overset{②}{\rightleftharpoons} EAE \overset{③}{\rightleftharpoons} (AE)_X$$

A and E symbolize amphotericin B and ergosterol. X is an integer greater than one. The equilibria occur in the EYL multilayers under the following conditions:

① ergosterol concentration < 18.8% with an antibiotic:sterol ratio less than 0.6

② ergosterol concentration ≥ 18.8%, where there are two or more ergosterol molecules present for every antibiotic molecule.

③ when the antibiotic:sterol ratio is > 0.6 at 18.8% sterol or less and at higher ergosterol concentrations when the ratio is > 0.5.

The overall picture obtained is of a very fluid association of amphotericin B and ergosterol molecules, causing the predominant complex species to vary with conditions. In order to determine which is the biologically active form, the experiments would have to be reproduced using black lipid membranes with the same compositions and testing for conductance across the bilayer. At higher concentrations, the complexed sterol appears to influence a slightly different region in the bilayer as compared to the free ergosterol. The differences in the labels' responses can be explained by the ergosterol moving towards the bilayer surface upon interaction with the amphotericin B.

The research presented in this thesis could be extended in a variety of ways. As mentioned in the introduction, some sterol-containing yeast strains are resistant to the polyene antibiotics. This could be due to the observed differences in the sterol content of the yeasts. By using fecosterol or other sterols in simular experiments, more information about the interaction of amphotericin B

with sterols *in vivo* could be obtained. Of course, the work could be extended to other polyene antibiotics as well.

Deuterium-NMR would appear to be the ideal method to study systems of this type since there is no perturbation of the system by the probe. However, as can be seen in this thesis, a large amount of preliminary work needs to be done which may not be practical using deuterated probes since much higher concentrations of the latter are necessary in the system due to the lower sensitivity of the method. In addition, the use of liposomes, which would be required in an NMR study, could put artificial restraints on the formation of these relatively large complexes. This argument can be used against the applicability of other spectroscopic techniques such as Raman and UV which also require the use of liposomes, to the amphotericin B-ergosterol system. Spin-label probes other than those used here could be employed in this study. However, the problem with labelled sterol or antibiotic probes is that the relatively large nitroxide group could affect the binding constant or result in a totally different type of complex than the naturally occuring one. It has been reported that the binding constant for filipin and cholestane spin label is smaller than for the antibiotic and cholesterol(62).

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