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Infrared spectrophotometric studies of thermotropic and lyotropic mesomorphism in some important membrane phospholipids

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INFRARED SPECTROPHOTOMETRIC STUDIES OF THERMOTROPIC AND LYOTROPIC MESOMORPHISM IN SOME IMPORTANT MEMBRANE PHOSPHOLIPIDS

by

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DISSERTATION

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ABSTRACT

Phospholipid-water systems in the form of thin films (1 m) of low water content (10%) were studied by infrared absorption spectroscopy in the region from 4000 to 900 cm^{-1}. The lipids included were: egg lecithin, dimyristoyl phosphatidyl choline, dipalmitoyl phosphatidyl choline and sphingomyelin. A cell and holder were designed which allowed simultaneous control and monitoring of both temperature and hydration. The hydration level was monitored by the absorbance of the O-H stretching band of water which in these films was at 3380 cm^{-1}. Since this band in free water is at 3490 cm^{-1}, all the water in these films was designated as "bound".

The intensity of the C-H deformation (scizzoring) band at 1470 cm^{-1} was found to reflect transitions from the gel to liquid crystalline phases. This is in agreement with infrared studies on other phospholipids. The intensity of the C=O stretching band at 1740 cm^{-1} was also found to be sensitive to these phase changes. This has not been previously reported.

The PO_2^- asymmetric stretching band at 1250 cm^{-1}, which is well known to undergo a frequency shift to lower wavenumbers on hydration, was resolved by computer into two overlapping bands. The shift was determined to be due in part to a change in intensity of these two bands, one increasing and one decreasing as hydration proceeds. It is suggested that these two bands represent a

hydrogen bonded and nonhydrogen bonded PO_2^- respectively. An equilibrium constant was calculated for the reaction between water and lipid to give the hydrated species.

The band at 1090 cm^{-1} is frequently assigned to the PO_2^- symmetric stretch. Evidence is presented to support Chapman's assignment of this band to the C-O-(P) vibration.

Dedicated to my wife, Ursula

with all my love

and to:

Tracy

Paul

Sean

& Noel, with more of the same.

ACKNOWLEDGEMENTS

A special dept of gratitude is owed my research director, Dr. I.D. Kuntz, who maintained the faith for both of us at times.

I will be forever grateful to my family for enduring both my absence and presence for the duration.

Thanks is due to my wife, Ursula, and my friend, Joan Kosinski, for the typing and to my son, Noel, for some of the graphics.

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INTRODUCTION

Life as we know it owes its existence to the unique structure of the biological membrane. Some of the important life processes which take place at or in the membrane include: protein synthesis, immunological reactions, photosynthesis, conversion of light into chemical and electrical energy, conduction of nerve impulses, active transport and selective permeability. The milieu of the biological membrane is the aqueous phase. It owes its structure to the unique properties of water and to the amphiphilic character of the lipids which comprise it. The differentiation of structure and function of the membrane from organism to organ to organelle is due largely to the protein associated with it, but common to all is the bimolecular leaflet with a hydrophobic core bounded by two hydrophilic layers first suggested by Gorter & Grindel (1) in 1925 (see Fig. 1A, B). The nature of the association of the protein is a matter of much conjecture and controversy but the bilayer structure of the lipid portion is generally accepted. Since membranes are largely composed of this bilayer structure, understanding many of their basic properties and the nature of their association with protein depends on understanding the chemical and physical properties of this system.

Two basic approaches are evident in biochemical studies: first, experiments on living organisms or structures derived directly from them and second, the development of analogous model systems.

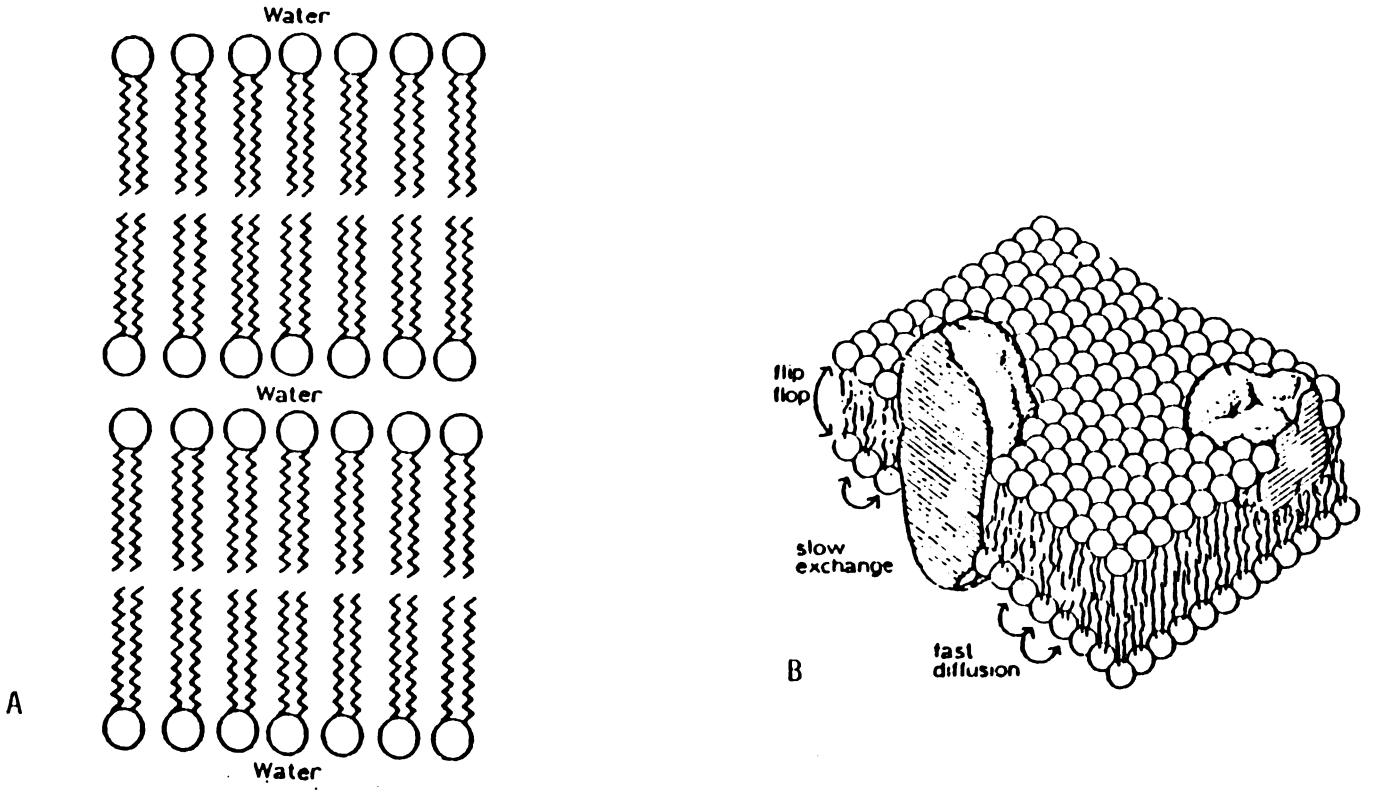


FIG. 1. A. Schematic view of phospholipids in lamellae. Circles represent head groups, lines represent hydrocarbon chains.

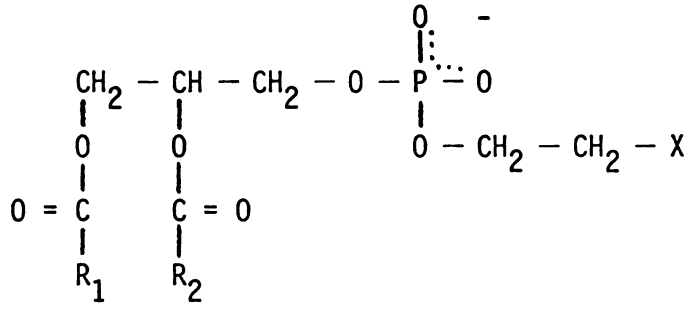
B. Perspective view of bimolecular leaflet showing associated protein. (From ref. 5.)

In the first approach the systems are so complex that interpretation of the results is often difficult. In the second, while the chemistry may be better understood, the relevance of the results to biological systems may be difficult to establish. Neither approach is better and both are necessary, the choice depends on the inclination and expertise of the investigator. Being a chemist, my approach is the model system.

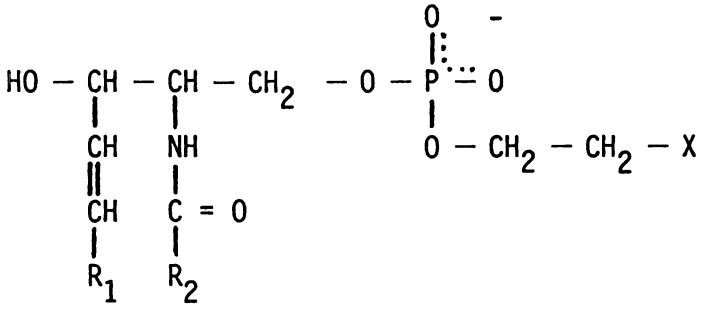
On reviewing the studies of membranes in the literature one cannot help but be reminded of the story of the nine blind men who set out to determine the nature of the elephant. One encountering a leg decided "The elephant is very like a tree." Another, holding the trunk, decided it was "...very like a snake," and so on. Bold statements are often made about the nature of the membrane based on a single type of experiment only to be contradicted by someone else using a different type. Since all types of experiments must be taken into account a brief survey of the properties of model membrane systems and the techniques used to study them will serve as a useful introduction to this study.

Two broad classifications of lipids are found in cell membranes, phospho- and sphingolipids, representatives of which are shown in Fig. 2, and cholesterol and its esters. This discussion will be concerned with the former. These amphiphiles vary widely in structural detail but all have in common the highly hydrophilic "head" group and the two long hydrophobic alkane or alkene chains. The head group may be neutral, ionic or zwitterionic. The hydro-

FIG. 2. Structural Formulas (for names and sources see Table 1)



I thru V



VI

		R_1	R_2	X
I	Lecithin	*	*	$-N(CH_3)_3^+$
II	DMPC	C_{13}	C_{13}	$-N(CH_3)_3^+$
III	DPPC	C_{15}	C_{15}	$-N(CH_3)_3^+$
IV	PEA	*	*	$-NH_3^+$
V	PE	*	*	-H
VI	Sphingomyelin	*	*	$-N(CH_3)_3^+$

*These compounds contain chains of varying lengths and some unsaturated chains.

carbon chains are from 14 to 24 carbons long and may be saturated or have from 1 to 4 double bonds. One of the most common, lecithin, from egg yolk (structure I, Fig. 2), on which a large number of studies has been done, is composed of molecules with one saturated and one unsaturated chain. Other sources are rich in one or another specific type. For example, lung tissue contains large amounts of dipalmitoyl lecithin (structure III, Fig. 2) while a bacterial cell wall is rich in phosphatidyl ethanolamine (structure IV Fig. 2). Other sources contain as many as a dozen different lipids, and most contain cholesterol in varying amounts.

All of these lipids are capable of forming lamellar structures spontaneously in the presence of water. Other structures are possible under certain conditions but these are probably not as important to biological systems. Electron micrographs of these dispersions reveal multilamellar structures with each lamella resembling the familiar "railroad track" seen in micrographs of cell walls. Some are extended and some are in the form of vesicles with concentric lamellae. Special techniques and treatment result in special systems. For example, dilute aqueous solutions will form monolayers (one half of a lamella) at the air water interface with the hydrophilic head dipping in the aqueous phase and the hydrocarbon chains protruding into the air. Treatment of aqueous dispersions of phospholipids with ultrasonic energy results in spherical cell-like vesicles a few hundred angstroms in diameter bounded by a single bilayer.

The phase behavior of these systems is different than most substances in that instead of the usual sequence of

$$\text{solid} \rightleftharpoons \text{liquid} \rightleftharpoons \text{vapor}$$

these compounds exhibit mesomorphism which can be represented as

$$\text{solid} \overset{T_t}{\rightleftharpoons} \text{mesophases} \overset{T_m}{\rightleftharpoons} \text{liquid} \rightleftharpoons \text{vapor}$$

These mesophases are referred to as liquid crystalline states in which there is considerable order but also considerable fluidity. Fig. 3 shows a phase diagram of a representative phospholipid-water system. The lateral line represents the temperature, T_t, at which the transition to liquid crystal takes place. As the diagram shows, increasing amounts of water lower T_t until a lower limit is reached at around 20% water.

T_t is also influenced by:

1) The type of head group; phosphatidyl cholines being lower than phosphatidyl ethanolamines.
2) Hydrocarbon chain length; shorter chains have lower T_t's.
3) Degree of unsaturation; unsaturated compounds being much lower than saturated.

There are many excellent and extensive reviews of the experimental work done to reveal the nature of these phases and the transition between them (2, 3, 4, 5). I will attempt only to give a brief overview of the evidence as it pertains to this study.

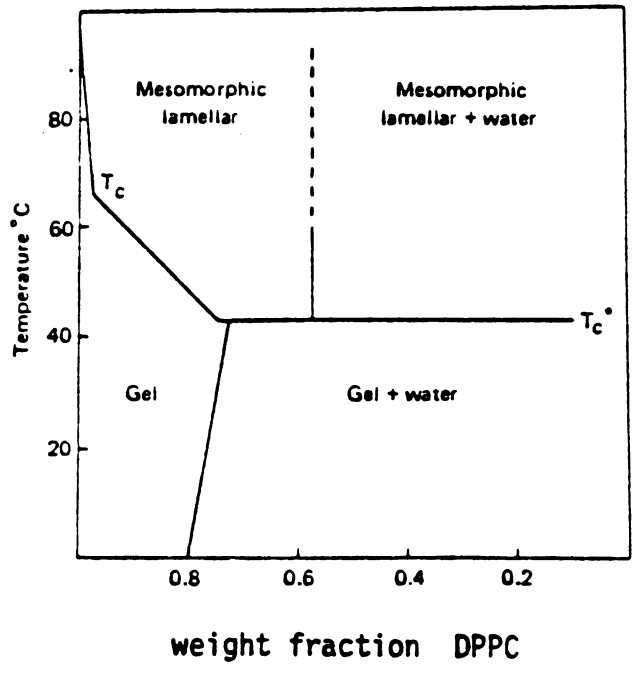


FIG. 3. Phase diagram of dipalmitoyl phosphatidyl choline (from ref. 24)

Our quantitative picture of the structure of these systems comes from X-ray diffraction. These studies reveal a lamellar structure in both the gel and liquid crystalline state with water intercalated between the bilayers. The width and spacing of the lamellae can be measured and an electron density profile can be constructed. In the gel phase the hydrocarbon chains are shown to be extended and packed in a paracrystalline array. In the liquid crystalline phase, increased fluidity is indicated and the width of the lamellae decrease. Introduction of water into the system swells the lamellae laterally (i.e. in the plane of the lamella) and increases their separation. The phase behavior revealed by these studies is much more complex than the simple diagram referred to earlier (Fig. 3).

Differential thermal analysis and differential scanning calorimetry (24) show an endothermic peak with increasing temperature at T_t, characteristic of a phase change. These studies also reveal no peak at $0^{\circ}C$ due to the melting of ice until the proportion of water reaches about 20% by weight. This indicates the absence of bulk water below this level of hydration so that any water present must be bound in some fashion.

Nuclear magnetic resonance (nmr) and electron spin resonance (esr) have also been brought to bear on this problem. Broadly, pmr studies by Chapman (6) showed liquid-like sharp line spectra above the transition temperature and solid-like broad line spectra below. Wide line analysis (6, 7) showed that the lines were narrowing under the influence of water near the transition temperature. More detailed

studies of the hydrocarbon chain by both nmr (8) and esr (9) reveal a gradient of motion along the hydrocarbon chain. The CH_2 groups near the head groups being much more restricted than those toward the terminal methyl group.

Many studies (10, 11, 12, 13) indicate that the bound water is present in several layers of hydration. For example, an nmr study (10) of the $N(CH_3)_3^+$ and water proton signals showed discontinuities in the relationship between linewidth and hydration in both signals. The stepwise decrease in linewidth indicates three distinct layers. Studies in D_2O of the nmr water line (12), however, indicate that there is rapid exchange between these layers and slow exchange with bulk water if it is present.

Raman and infrared spectroscopy are valuable tools in the study of these systems. Some advantages are: 1) the rapidity with which spectra can be taken, 2) the instantaneous reporting of the state of a given group, 3) the sensitivity of groups to the immediate nature of their environment, 4) the absence of exogenous probe molecules which can act as impurities, 5) the ability to work with small samples in thin films (with total internal reflectance only a few bilayers are needed), 6) the ability to look at diverse parts of the molecule at the same time under the same conditions.

Thus, Fringeli et al (14) have shown changes in the head group, the glycero-ester backbone and the hydrocarbon chains under rapidly fluctuating humidity. Lippert and Peticolas (15) with laser Raman spectroscopy have shown the influence of water and cholesterol on the hydrocarbon chains during the transition.

From all these and other studies a picture emerges of the bilayer structures as follows. In the dry state at low temperatures the headgroups form an ionic crystalline layer with the hydrocarbon chains lying perpendicular, extended and packed in a paracrystalline array. With increasing temperature a transition point is reached where the chains "melt" to a liquid-crystalline state. This liquid-like state is not the same over the length of the chain. The methylene groups close to the head group have a very restricted motion while those further along the chain are less and less restricted until at the terminal methyl groups a more liquid-like state exists. As water is added to the system the temperature of the transition drops. This is probably because the water disrupts the crystalline structure of the head groups. The configuration of the head group is less well characterized than the hydrocarbon chains, but indications are that the choline group lies parallel to the lamellae in the dry state and tends toward the perpendicular in the presence of water (5). The state of the various portions of the phospholipid molecule and its interaction with the closely bound water may be very important to the biological properties of membranes. It was with the hope of shedding more light on this state and the details of the interaction with water that these studies of infrared spectra during the early stages of hydration were undertaken.

EXPERIMENTAL

Conventional ir spectra were taken of several important membrane phospholipids in the gel and liquid crystalline phase. Specific lipids were selected because of their biological importance (lecithin, phosphatidyl ethanolamine {PEA}, dipalmitoyl phosphatidyl choline {DPPC} and sphingomyelin) or because they have structural features that might help differentiate certain aspects of the spectra. Sphingomyelin has the phosphoryl choline group, but does not contain the glycero-ester group, phosphatidyl ethanol (PE) is the same as lecithin but lacks the quaternary ammonium group, dimyristoyl phosphatidyl choline (DMPC) is similar to DPPC but has shorter hydrocarbon chains and a lower transition temperature.

There has been considerable improvement in the purity of commerically available phospholipids in recent years. The ones purchased were "chromatographically pure" (i.e. give a single spot on TLC) and hence were used without further purification.

A cell and holder were designed in which the temperature and amount of water present could be controlled and monitored in situ. This system is described in detail and a typical experiment is outlined. Certain parts of the spectra were anlyzed by a computer program. This procedure is also outlined in this section and the complete routine detailed in Appendix 1.

Table 1 is a list of the compounds along with their sources and the abbreviations used in the text. The structures are given in Fig. 2. Dispersions of these compounds were made using 10 to 20 mg of lipid (accurately weighed or in the case of solutions measured with a microlitre syringe) in distilled water. Dispersion was achieved by mechanically agitating the vial then placing it in an ultrasonic cleaning bath. These dispersions could usually be used for 1 or 2 weeks but they were discarded if they showed any discoloration or lack of homogeneity.

Films of the lipid were prepared by applying enough dispersion to the window of the cell to give approximately 2 mg of lipid to a 2 cm^2 area and removing excess water at reduced pressure. The exact amount of lipid in the film was calculated from the concentration and the volume dispensed.

The cell was assembled from two CaF_2* discs 1 × 41 mm and an "O" ring (see Fig. 4). A hypodermic needle was inserted through the "O" ring and connected through a series of valves to a water reservoir and a vacuum pump (see Fig. 5). The temperature was monitored by a thermistor in a hypodermic needle also inserted through the "O" ring. The thermistor was only a few mm from the

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*CaF_2 was chosen as the window material because it is water resistant, transparent to 900 cm^{-1} and has good thermal and mechanical stability. Quartz is opaque to many of the regions of interest, AgCl is too soft and KCl and NaCl, of course, are not water resistant.

Table 1. Compounds, Abbreviations and Sources -

for structures see fig. 3

I	Phosphatidyl Choline (lecithin)	Sigma, #P-5338 L Type III - E (from egg)
II	Dimyristoyl Phosphatidyl Choline (DMPC)	Sedary Research Laboratories (Canada) L-3 Cat. #B-68
III	Dipalmitoyl Phosphatidyl Choline (DPPC)	Grand Island Biological Co. (GIBCO) Cat. #100070
IV	Phosphatidyl Ethanolamine (PEA)	Grand Island Biological Co. (GIBCO) Cat. #100110 Cont. #C671080 (From E. Coli)
V	Phosphatidyl Ethanol (PE)	Grand Island Biological Co. (GIBCO) Cat. #800107 Cont. #C671080 (transphosphatidylated egg lecithin)
VI	Sphingomyelin	Grand Island Biological Co. (GIBCO) Cat. #100140 Cont. #A644664

LEGEND FOR FIG. 4

1. Rubber "O" ring
2. Thermistor
3. Hypodermic needle to vacuum pump
4. CaF_2 disc
5. Parafilm dam
6. Lipid film
7. Styrofoam holder

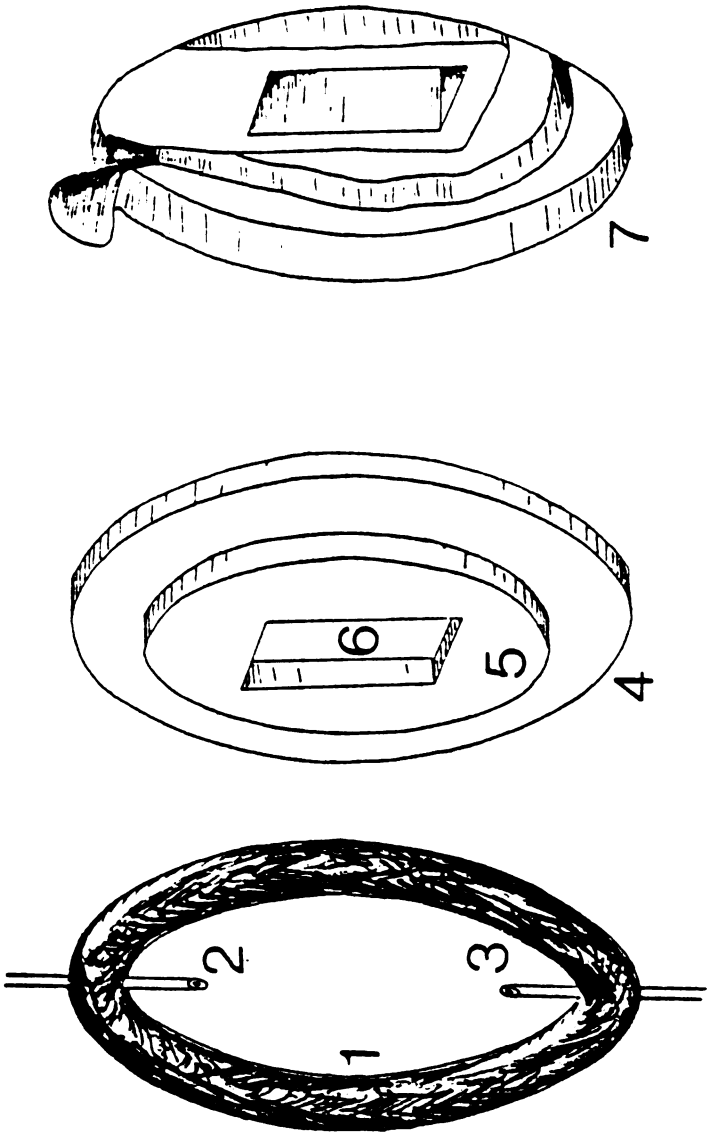

FIG. 4A. (For legend see page 14.) Exploded, perspective view of half of cell and holder. (B, C and D next page)

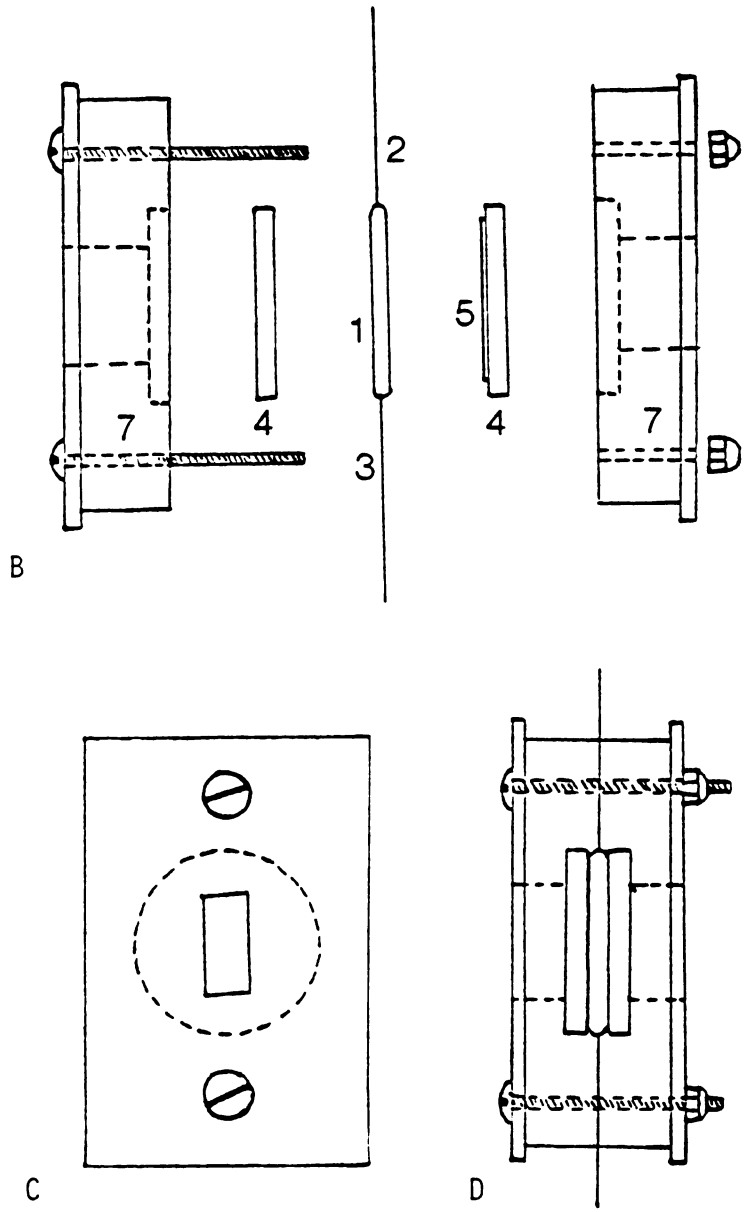

FIG. 4 (cont'd). B. Exploded view of cell and holder.
C. Front view of assembled cell and holder.
D. Side view of assembled cell and holder.

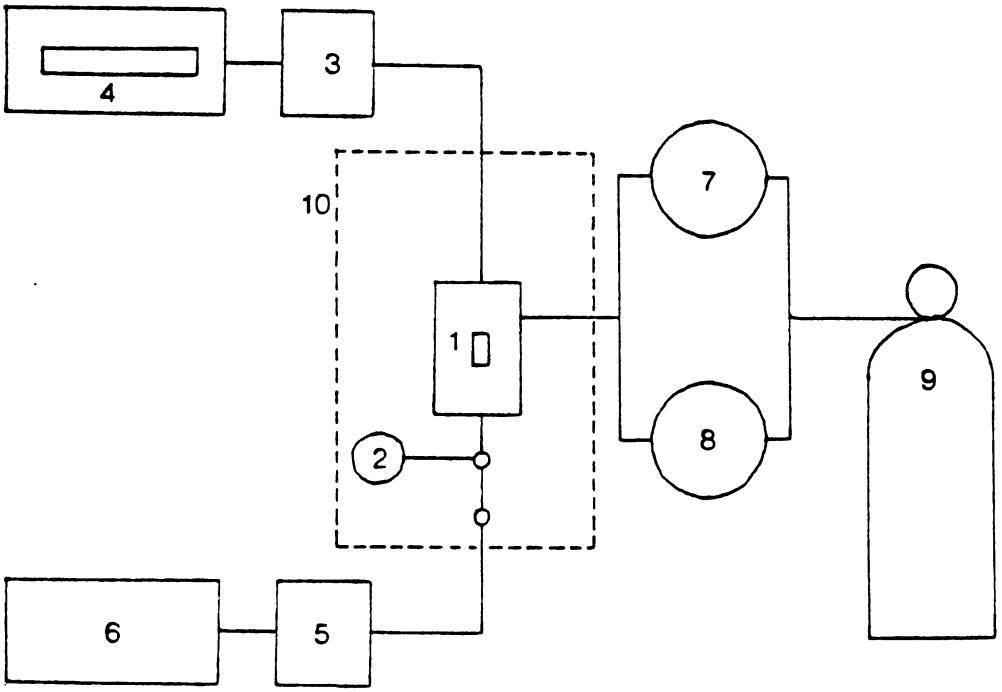

FIG. 5. Schematic view of experimental setup.

1. Cell
2. Water reservoir
3. Switch and resistor for thermistor
4. Electrometer
5. Cold trap
6. Vacuum pump
7. Heater
8. Cooler
9. N_2 gas
10. Spectrophotometer

film but was shielded from the spectrophotometer light beam. The cell was held in a specially designed styrofoam holder (see Fig. 4). Temperature was controlled by circulating heated or cooled nitrogen through passages in the holder. Temperatures from 0° to $90^{\circ}C$ were maintained with this system. The thermistor was calibrated against a quartz thermometer (Hewlett-Packard model 2850A). All spectra were taken on a Perkin Elmer model 180 double beam, grating spectrophotometer equipped with automatic gain and slit width control. The instrument provided abscissa expansion and continuously variable ordinate expansion. The actual amplification of the ordinate was calculated by scanning the strong carbonyl peak (1736 cm^{-1}) at the setting used and at × 1 and measuring the ratio of peak heights. The CaF_2 window restricts the spectrophotometer to frequencies above 900 cm^{-1}. The accessible region covers most of the readily assignable vibration frequencies of the compounds under consideration.

The data from the spectrophotometer was analyzed on the Prophet System. Prophet (Bolt Beraner and Newman Inc.) is the "Chemical/Biological Information-Handling Program of the NIH." It is a computer system located in Boston and connected by telephone to graphics terminals throughout the country. The terminal consists of a display screen for viewing pictures and text, a tablet and stylus for entering pictorial information, a keyboard for entering text and a device for obtaining permanent paper copies. The system has a wide variety of data handling and computational

procedures available and a relatively simple language system for accessing them. Use of these systems is described later in this chapter.

In a typical experiment 200 μl of a solution of lecithin (Structure I, Fig. 2, 0.1 gm/ml in hexane) was taken to dryness in a small glass vial with a stream of nitrogen. 1.0 ml of distilled water was added and the capped vial subjected to mechanical agitation on a vortex mixer for about 1 hour. The vial was then placed in an ultrasonic cleaning bath (Model 35, McKenna Laboratories) for another hour. A homogeneous, turbid, dispersion resulted. 100 μl of this dispersion was applied to the CaF_2 window with a microlitre syringe. This is a total of 2.0 mg or 2.56 micromoles of lipid. By manipulating the droplet with the tip of the syringe needle, the dispersion was made to cover the entire window (see Fig. 4) adhering to the walls of the dam. The CaF_2 disc was placed in a vacuum desiccator which was connected to a vacuum pump through a liquid nitrogen trap. Since the formation of bubbles due to boiling or degassing results in a poor surface on the film, the following precautionary procedure was used. After the trap was pumped down, the stopcock between the trap and the pump was closed and the stopcock to the desiccator opened. Since the volume ratio of the desiccator to trap is 10:1 or greater this resulted in only a small drop in pressure. The desiccator was then closed to the trap which was again pumped down and the process repeated thus lowering the pressure stepwise in the desiccator. If a bubble appeared in the droplet the

desiccator was repressurized and the process started again. The droplet was observed closely during the process for the first visible sign of reduction in size. At this point the desiccator can be left open to the trap (not to the pump) and the trap will remove the excess water leaving a thin film of gel. At this time the moisture content can be further reduced by pumping directly on the desiccator. In this fashion a film of low water content (<3%) can be prepared in 30 to 60 minutes. The cell was then assembled and placed in the holder (see Fig. 4). After applying a slight negative pressure in the cell, the screws were tightened and the assembly placed in the sample beam with 2 CaF_2 discs in the reference beam. During the preparation of the film the spectrophotometer had been warmed up and zeroed and phased. The temperature control system, the electrometer and the vacuum pump were then connected to appropriate leads (see Fig. 5). Warm (or cool) nitrogen was circulated through the cell until it stabilized at the desired temperature. During the heating period the cell was pumped on directly by the vacuum pump. The spectrophotometer was indexed to 3380 cm^{-1} (the maximum of the water peak in lecithin films) and the absorbance at this frequency was monitored vs. time to determine when all the water had been removed. (It is estimated that we could detect the presence of 3×10^{-7} moles water in the film.) The region from 3800 to 3000 cm^{-1} was scanned with an amplification of 2.63 and an abscissa expansion of x 2 (20 cm^{-1}/cm). The region from 3000 to 1800 cm^{-1} was scanned quickly at x 1 with no abscissa expansion. The region from 1800 to 900 cm^{-1} was scanned with an amplification

of 2.63 and an abscissa expansion of x 5 (10 cm^{-1}/cm). Voltage readings across the thermistor were noted periodically on the chart to detect temperature drift. In a typical run the temperature is stable to $\pm$ 1°C. The water region (3800 to 3000 cm^{-1}) was then scanned again to make sure the water content had remained the same. If there was any change the run was repeated. After the scan the cell was closed off to the pump and the water reservoir evacuated. The pump was then closed off and the cell and reservoir were connected while the spectrophotometer scanned the water peak at 3380 cm^{-1} vs. time. When the desired amount of water had been absorbed the cell was closed off and allowed to equilibrate as indicated by an unchanging absorbance at 3380 cm^{-1}. After equilibration the regions were scanned again as described above. In this fashion the water content was changed stepwise from dry to as much water as the film will absorb when exposed to 100% humidity. (For lecithin this is calculated to be 4.66 moles/mole of lipid at 15°C.) The temperature was then changed and the above process is repeated for example at 15°, 35°, 49°, and 67°C.

A series of curves obtained in this manner, for example, the 1300 to 1150 cm^{-1} region, was then analyzed on the Prophet System as follows. The chart paper is taped to the tablet at the computer terminal and the procedure AREA called on the computer. With prompting from the computer the origin and axes are indicated with the light pen touching each 2 cm^{-1} over the region. The data points are displayed on the screen and the computer compiles the coordinates of each point in a table. Successive curves are

affixed to the tablet and the data put into the same table in the same manner until all the curves over the range of hydration for a given compound at a given temperature have been tabulated. To have the computer resolve the complex curve, a function must be written representing the components.

Judging from the appearance of the bands from 1300 to 1150 cm^{-1}, I believe this region to be composed mainly of 4 bands, of which 2, (1170 cm^{-1} and 1280 cm^{-1}) do not seem to be affected by hydration and two (around 1230 cm^{-1} and 1255 cm^{-1}) that are affected. Each band is assumed to be a lorenzian which has the formula:

$$f(\nu) = \frac{A}{1 + (\nu_0 - \nu/\Delta\nu_{\frac{1}{2}})^2} \qquad (1)$$

where

ν = frequency

A = absorbance - at the maximum

ν_0 = the maximum -

$\Delta\nu_{\frac{1}{2}}$ = peak width - at ½ height

A first approximation is made for the parameters of the fixed curves and a function is written composed of 4 lorenzians. The computer recognizes the following representation of this function

$$F1 = B1/(1+B2\{B3-X\}^2) + B4/(1+B5\{B6-X\}^2) + 17/(1+.003\{1170-X\}^2) + 4/(1+.003\{1280-X\}^2) \qquad (2)$$

This function is declared to the computer, the procedure FITFUN is called, and the table holding the data is designated. Some guesses as to the unknown parameters are made to give the computer some place to start. The computer then tries to fit this function to the data using an iterative least squares approach. When the function has been fitted, the screen displays a graph of the data and the fitted curve and a list of parameters. A typical first try is shown in Fig. 6A. Refinements can then be made in the function to see if a single expression can be made to fit a series of curves well. A typical fitted curve is shown in Fig. 6B. A complete listing of the computer procedure is provided in Appendix 1.

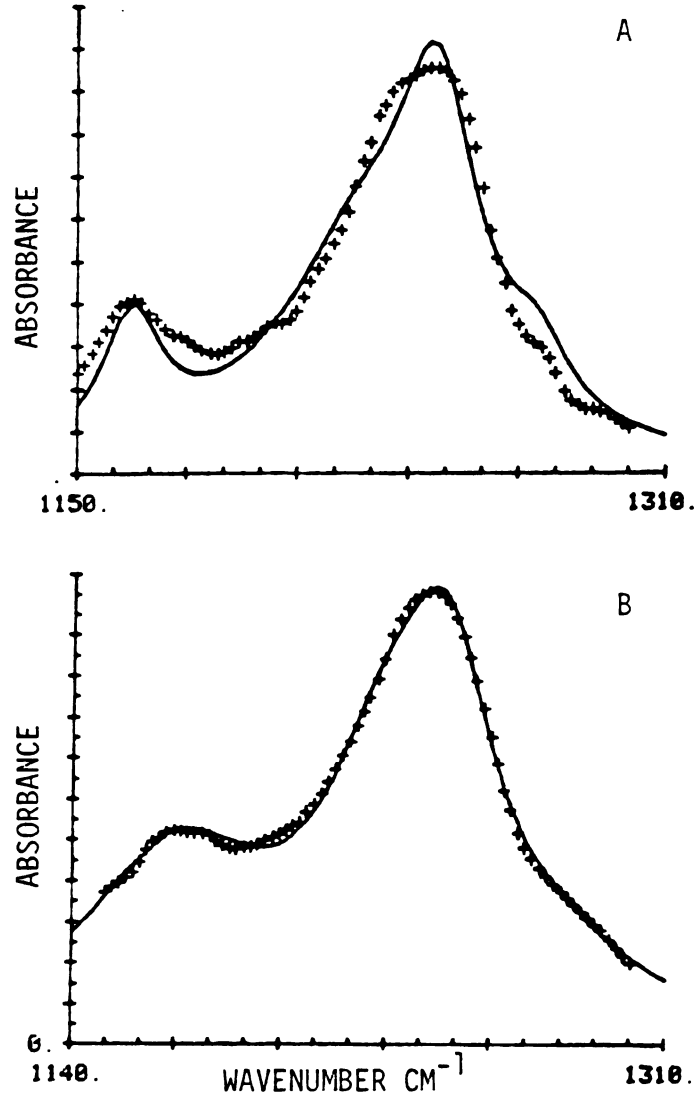

FIG. 6. A. A typical first try using constraints (fixed lorenzians)

B. Refined fit

+++ Data from spectrophotometer

—— Fitted curve from computer

RESULTS AND DISCUSSION

There are many infrared studies with extensive lists of assignments of specific vibrations of phospholipid molecules. Conflicts and ambiguities abound from list to list. We will depart from that format here and rather look at each region studied as a whole and try to determine the meaning of the changes seen and comment on specific assignments only if the available information warrants. Since the regions studied, taken in the order of decreasing frequency are also in the order of increasing difficulty of interpreation (in terms of the phospholipid molecules) this seems a logical order in which to discuss them.

I. 3800 - 3200 cm^{-1} Region

The frequency of the O-H stretching vibration is found in this region. The intensity maximum of pure water is at 3490 cm^{-1} (16). Although there is only one kind of bond in the water molecule, the absorbance band is complicated by coupling, Fermi resonance and the effect of hydrogen bonding (17). The complex band has been studied by Walrafren (18) who resolved it by computer into four gaussian curves. Attempts have been made to assign the frequencies to specific vibrations and explain the widths and intensities of the bands. The O-H stretching frequency in HOD is uncomplicated by coupling or Fermi resonance and Wall and Hornig (19) and Walrafren (18) have studied the effects of temperature and

hydrogen bonding. As expected there is a decrease in frequency due to hydrogen bonding and a slight increase with increasing temperature (36 cm^{-1} over a range of 40 - 80°C).

In our experiments the O-H absorbance is a broad (400 cm^{-1}) band with two distinct shoulders (see Fig. 7). The maximum lies between 3370 and 3390 cm^{-1} and could not be determined more precisely in these spectra. We were unable to detect any shift in frequency over a range of 15° to 80°C although with such a broad band a considerable shift could have gone undetected. Nor could we detect any shift due to varying amounts of water in these samples. However, as the peaks get smaller with decreasing amounts of water the maximum is even more difficult to determine and even larger shifts and changes in shape could have gone undetected. Davenport and Fisher (20) detected a shift from 3375 to 3385 cm^{-1} over the range of 0 to 5 moles of H_2O per mole of lecithin in inverse miceles in a water-lecithin-benzene system. This shift could not have been detected in our system.

Since we were working in a range of hydration well below that in which bulk water exists in these systems, (v.i.) the lower frequency of our water peak is consistent with the expected frequency of strongly hydrogen bonded water and indicates that all of the water in our system was "bound" in some fashion.

The absorbance of the water peak in sphingomyelin samples is complicated by the overlapping absorbances of the alcoholic O-H and the amide N-H stretching frequencies (see Fig. 8).

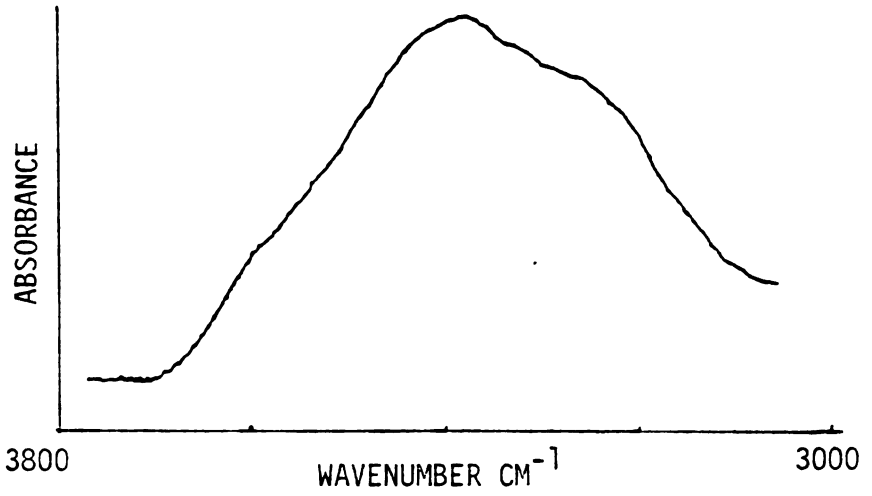

FIG. 7. Water peak in lecithin film at 32.5°C and 4.7 moles H_2O/mole lipid.

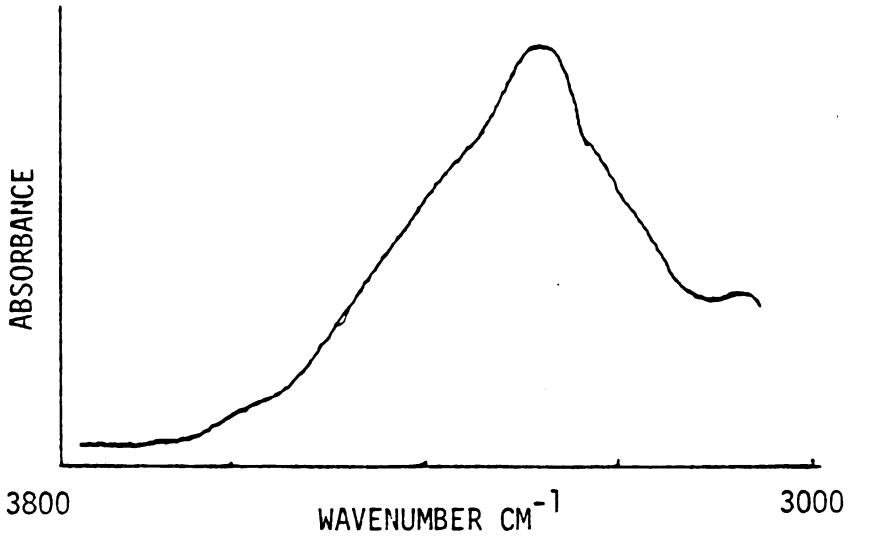

FIG. 8. Water peak in sphingomyelin film at 32°C and 3.0 moles H_2O/mole lipid.

The maximum appears to be at 3400 cm^{-1} when relatively large amounts of water are present but with lesser amounts it becomes a shoulder and the maximum cannot be determined. Again, however, the position of the peak does indicate strongly bound water.

Because of the complexities of the water peak we have elected to use this band solely to calculate the amount of water present in the films. This was done by measuring the absorbance at the intensity maximum and assuming as have others (26, 27) that the water in the films has the same extinction coefficient as that of pure water. This is not perfectly accurate because of the different water environment but the error should be less than 20% and will not affect our conclusions.

Then

$$A = \frac{\varepsilon c}{a} \qquad (3)$$

where A = absorbance (corrected for the amplication factor)

$\varepsilon = 64 \times 10^{-3} \ \frac{cm^2}{mole}$ (16)

a = area of the film

c = concentration in moles/cm^2

The amounts of water ranged from about 6×10^{-6} moles of water down to an undetectable amount, and we estimate we could detect as little

as 3×10^{-7} moles. Ratios of moles H_2O to moles of lipid are tabulated with the spectroscopic data.

II. 1800 - 1400 cm^{-1} Region

The peaks of interest in this region are the C=O stretch at 1740 cm^{-1} and the C-H deformation (scissoring) at 1470 cm^{-1}. In the phosphatidyl cholines the C=O is the ester carbonyl and in sphingomyelin it is the amide I band.

A. The C-H deformation band.

The C-H peak in phospholipids has been used as a monitor of hydrocarbon chain mobility by Bulkin *et al* (21) who found sharp decreases in intensity in this peak that correlate well with phase changes determined by other methods in PEA:H_2O systems. Figs. 9A and 9B show plots of the height of this peak vs. hydration for lecithin and DMPC at various temperatures. In lecithin at low temperature there is a distinct drop with increasing hydration. At 32° the overall level has dropped considerably but the effects of hydration are minimal. At 50° there is a further overall drop but again little effect of hydration while at 67° no further drop and no evident effect of hydration. In DMPC the low temperature sample shows little effect of hydration while at 49° there is a sharp drop. At 81° there is a further drop and the dry sample is considerably higher than the hydrated samples. A look at the phase diagrams (Figs. 10 & 11) offers a possible explanation for this behavior.

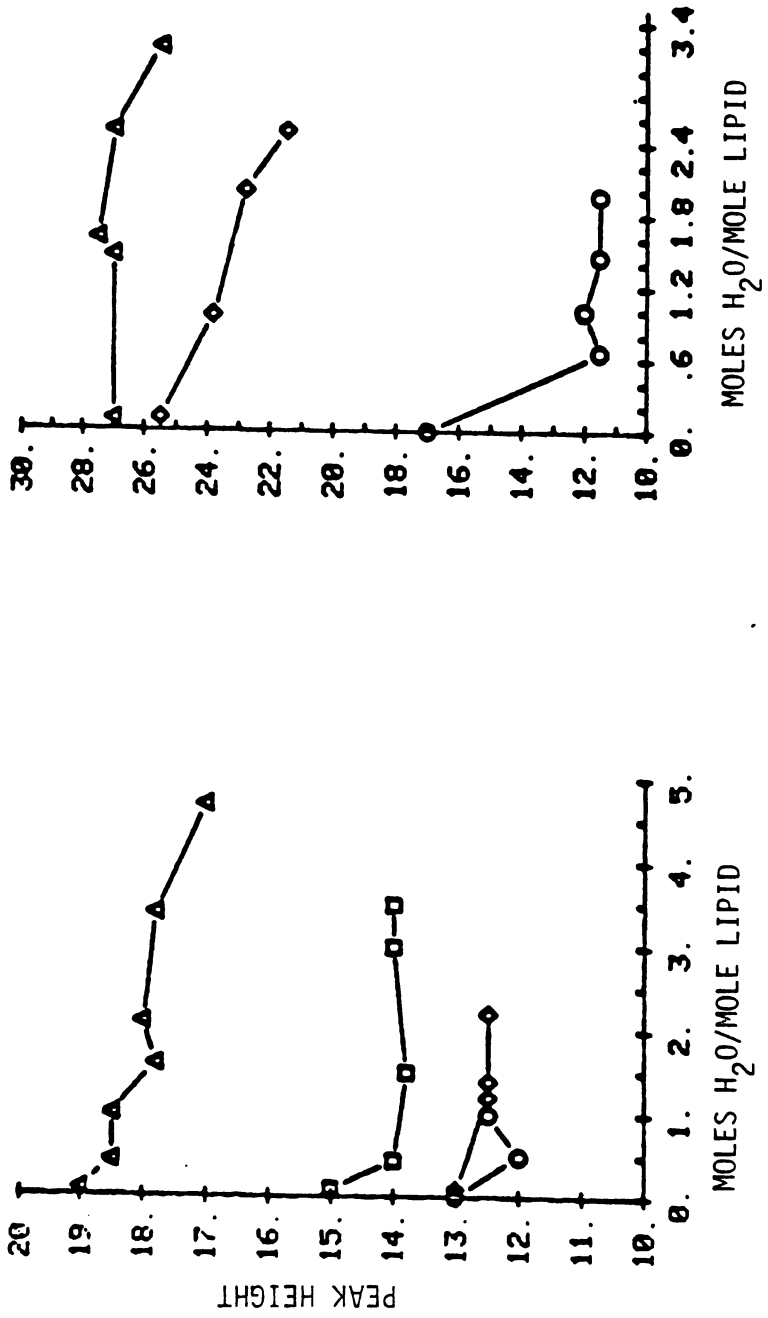

FIG. 9. A. Lecithin C-H peak height vs. hydration
(Δ) 13.5°C, (□) 32.5°C, (◇) 50°C, (○) 67°C

B. DMPC C-H peak height vs. hydration
(Δ) 15.6°C, (◇) 49.1°C, (○) 81°C

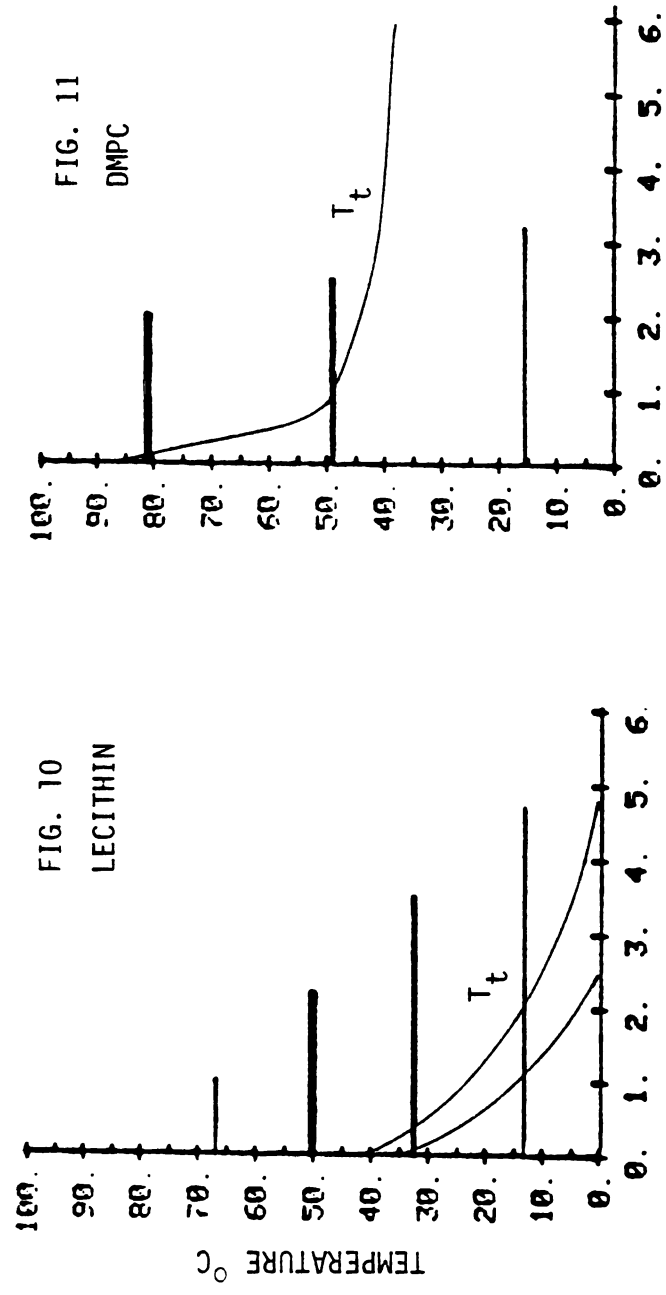

FIG. 10 & 11. Phase diagrams for lecithin and DMPC showing isotherms for experiments and transition temperature lines.

In DMPC it is seen that both the higher temperature isotherms cross the T_t line while the lower temperature experiment is completely within the gel phase. In lecithin the 15° and 32° experiments are near a phase change but the 50° and 67° experiments are completely in the liquid crystalline phase. Fig. 12 shows a plot of the mean peak height at various temperatures for lecithin, DMPC and DPPC along with the T_t for the monohydrate of the compounds. The peak height is seen to diminish with increased motion of the hydrocarbon chains. The increased motion occurs going from gel to liquid crystal. This can be accomplished by a change in temperature or hydration if the sample is near the transition temperature line.

B. The carbonyl stretching band.

In all the spectra of lecithin, DMPC and DPPC at all temperatures and at all levels of hydration the C=O peak lies between 1736 and 1737 cm^{-1}. The frequency of the C=O peak is known to be sensitive to hydrogen bonding (22). The lack of any shift indicates that water is not directly H-bonded to this moiety.

The peak height of this band does, however, show effects of both temperature and hydration. Figs. 13A and 13B show plots of peak height vs. hydration for lecithin and DMPC at different temperatures. At 15° and 49°, DMPC shows little change with increasing hydration, at 81° the dry sample is at a fairly high level and then drops sharply after hydration. In lecithin the low temperature sample shows a steady drop with increasing hydration.

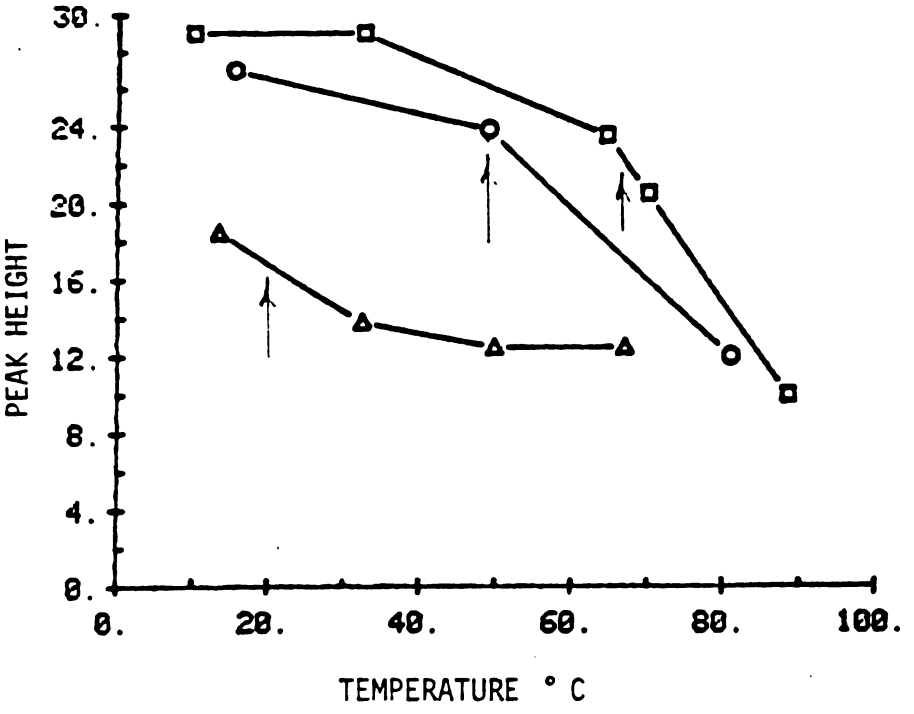

FIG. 12. C-H peak height vs. temperature at a hydration level of ca. 1 mole H_2O/mole lipid for (△) lecithin, (○) DMPC and (□)DPPC.
Arrows indicate transition temperatures for the monohydrates.

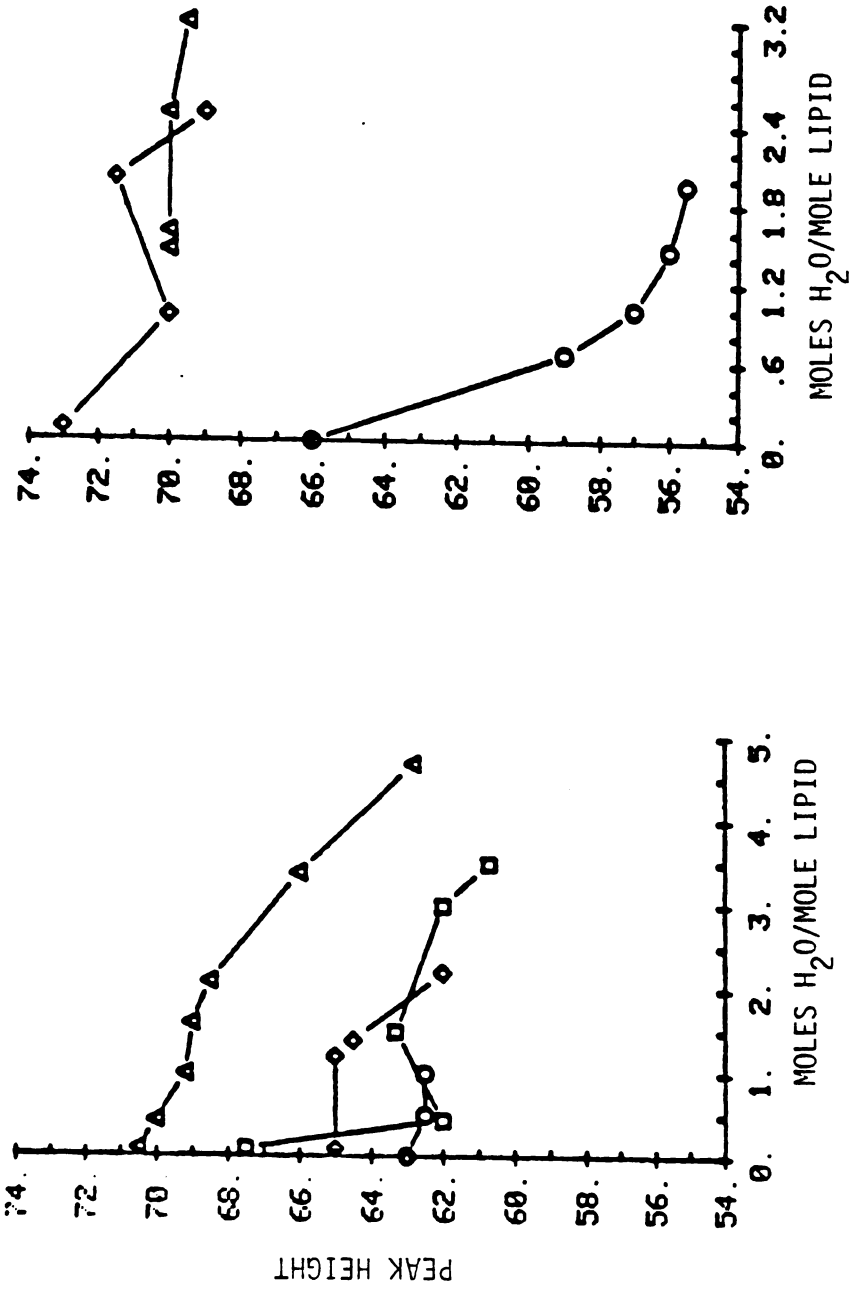

FIG. 13. A. Lecithin C=O peak height vs. hydration
(Δ) 13.5°C, (□) 32.5°C, (◇) 50°C, (○) 67°C.

B. DMPC C=O peak height vs. hydration
(Δ) 15.6 C, (◇) 49.1 C, (○) 81°C.

At higher temperatures the height is generally lower but the effects of hydration are much less. Along with the diminution in height there is a slight broadening of the peak indicating that the integrated intensity is probably not changing. This, along with the fact that there is no shift, indicates that this is only a change in the motional state and not any change in the H-bonding environment.

Thus, the behavior of the C=O peak is somewhat similar to that of the C-H peak and seems to reflect the increased motional state of the hydrocarbon chains. This is reasonable since the hydrocarbon chains are anchored to the glycero-ester backbone but somewhat surprising since there is apparently no mention of such changes in the literature.

In sphingomyelin there is a distinct difference in the C=O peak. In a freshly prepared film the carbonyl peak is skewed and after standing overnight splits into a doublet with maxima at 1639 cm^{-1} and 1655 cm^{-1}. Since sphingomyelin has an O-H and N-H group there is the possibility for both intra- and intermolecular hydrogen bonding. Schmidt, et al (23) have presented convincing evidence for the existence of both. There are two indications from this study which tend to confirm this. First, the alcoholic O-H stretching band lies at 3300 cm^{-1} (see Fig. 8) instead of at 3600 cm^{-1} where the free O-H usually is. The magnitude of the shift suggests that it is H-bonded to a powerful proton acceptor such as the PO_2^- group. Second the PO_2^- asymmetric band is at 1240 cm^{-1}

instead of 1260 cm^{-1} as it is in the lecithins. The doublet in the 1650 cm^{-1} region could represent the hydrogen bonded and nonhydrogen bonded C=O groups.

There seem to be no effects of hydration on either the C=O or C-H peaks in a freshly prepared film of sphingomyelin but the same trend is seen in the C-H peak due to temperature.

III. 1300 to 1150 cm^{-1} Region

The strong band between 1260 and 1230 cm^{-1} is generally agreed to be due to the PO_2^- antisymmetric stretch vibration. Some authors refer to it as P=O but this species should not be present in any of the compounds of this study. This region also contains the C-O-C aliphatic ester stretch (except in sphingomyelin) which lies at about 1170 cm^{-1}. The region is complicated slightly by a series of CH_2 bands characterized by Chapman (24) which are most evident at lower temperatures when the fine-structure of these spectra is increased.

In the presence of water the PO_2^- band is shifted to lower wavenumbers and broadens considerably (see figs. 14 to 20). Chapman (24) states that it shifts 14 wavenumbers with one mole of water and then shifts no further. In our hands the shift continues up to almost 5 moles of water. Davenport and Fisher (13) found the shift to continue up to 10 moles of water. Chapman's spectra were taken in KBr pellets which could account for the different results.

The possibility that this band is actually two overlapping bands led to an attempt to resolve the band using a computer program

LEGEND FOR FIGS. 14 THROUGH 20

Fitted curves for PO_2^- assymmetric stretch region (1300-1150 cm^{-1}) of lecithin and DMPC

+++ data from spectrophotometer

—— fitted curve from computer

.... resolved lorenzians (only peaks which change with hydration are shown)

Temperature and hydration are given with each fig.

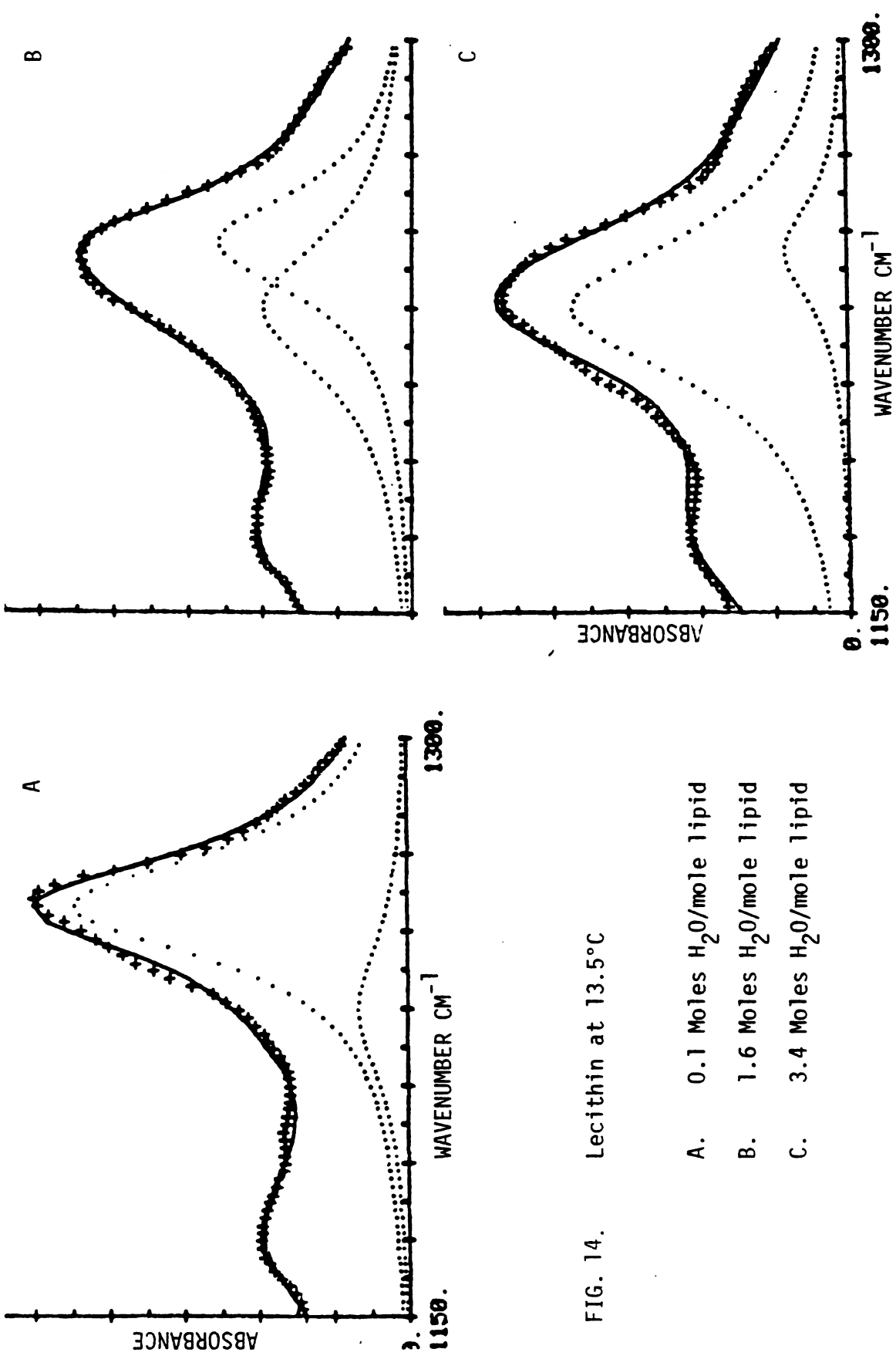

FIG. 14. Lecithin at 13.5°C

A. 0.1 Moles H_2O/mole lipid

B. 1.6 Moles H_2O/mole lipid

C. 3.4 Moles H_2O/mole lipid

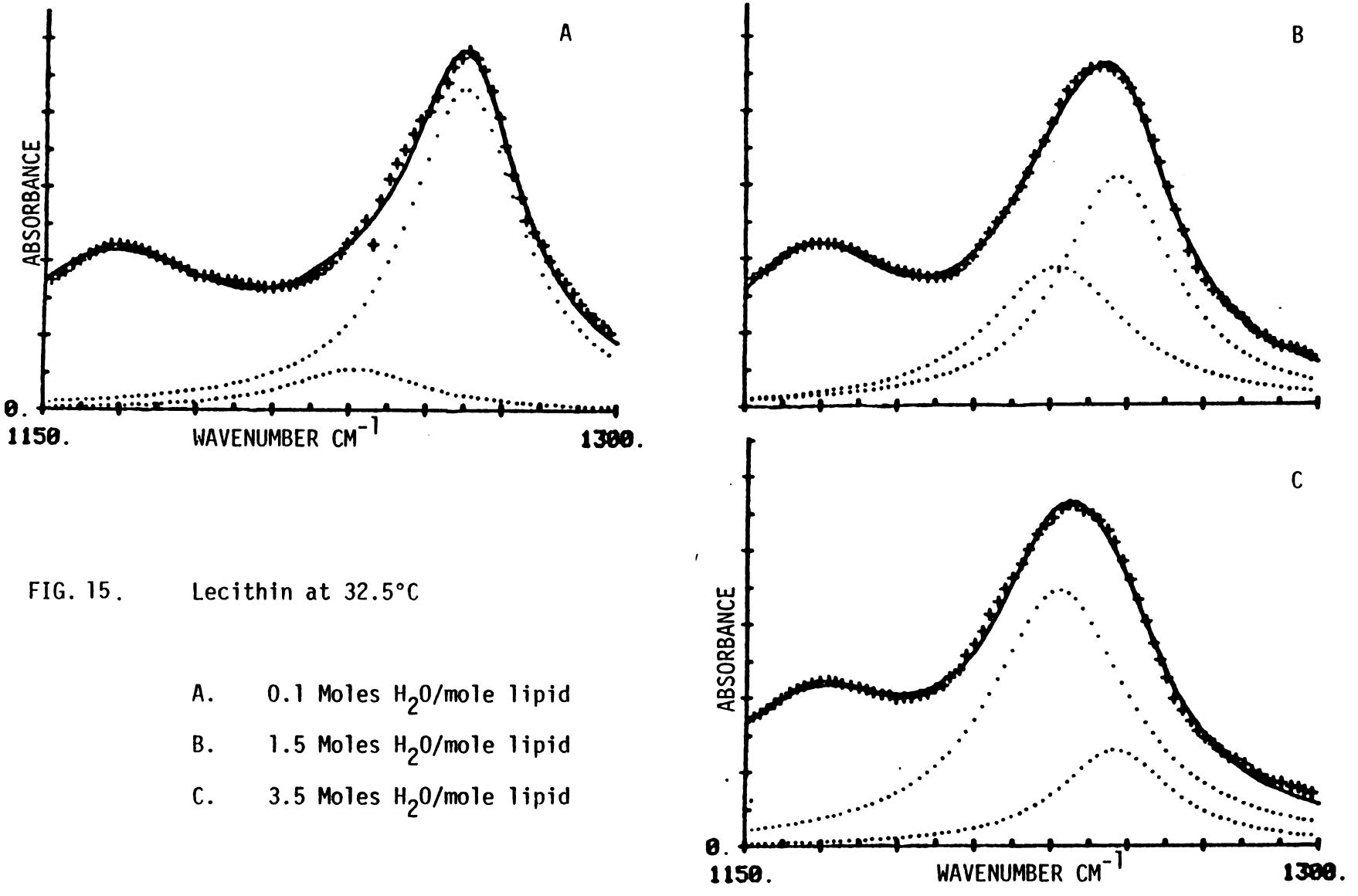

FIG. 15. Lecithin at 32.5°C

A. 0.1 Moles H_2O/mole lipid

B. 1.5 Moles H_2O/mole lipid

C. 3.5 Moles H_2O/mole lipid

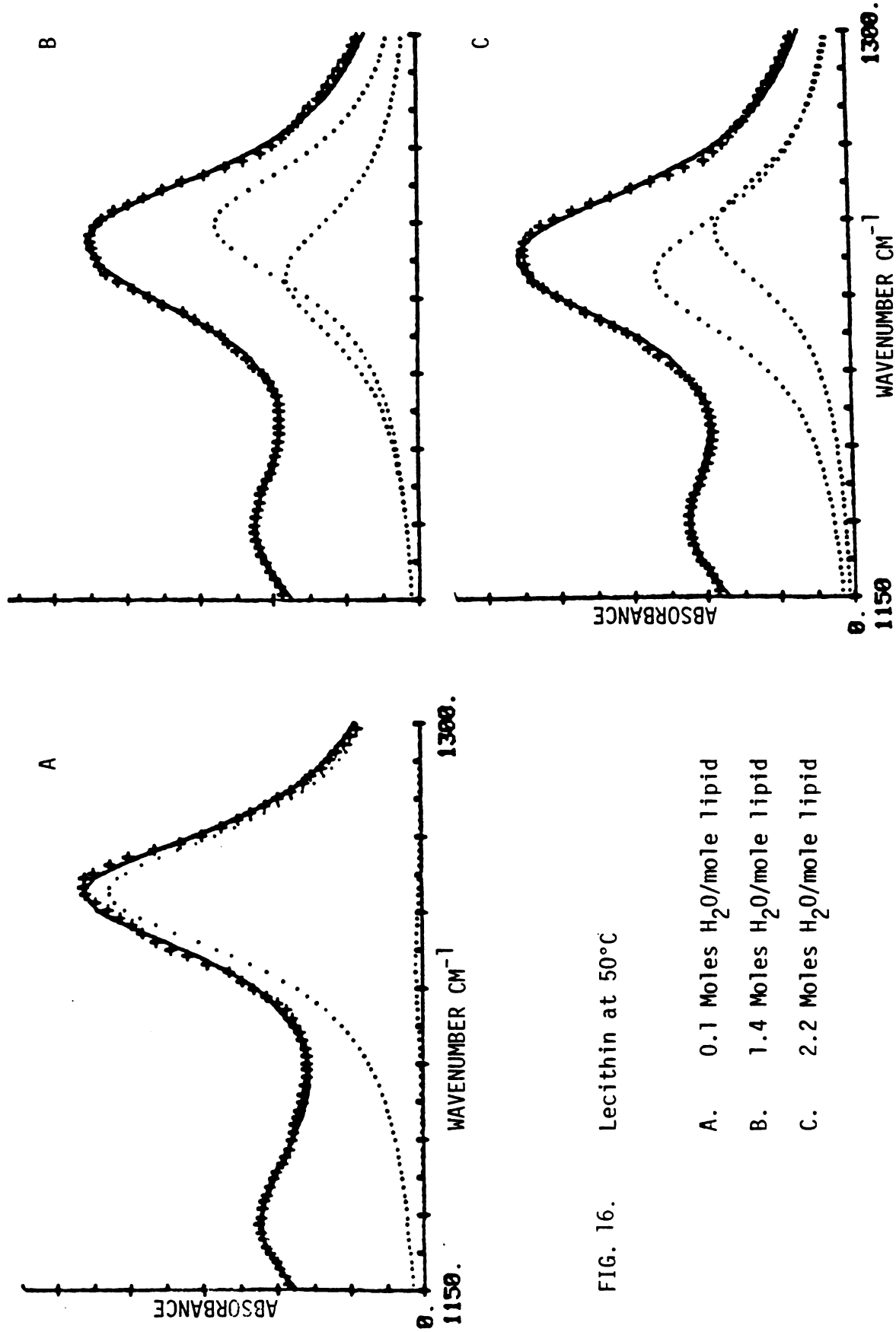

FIG. 16. Lecithin at 50°C

A. 0.1 Moles H_2O/mole lipid

B. 1.4 Moles H_2O/mole lipid

C. 2.2 Moles H_2O/mole lipid

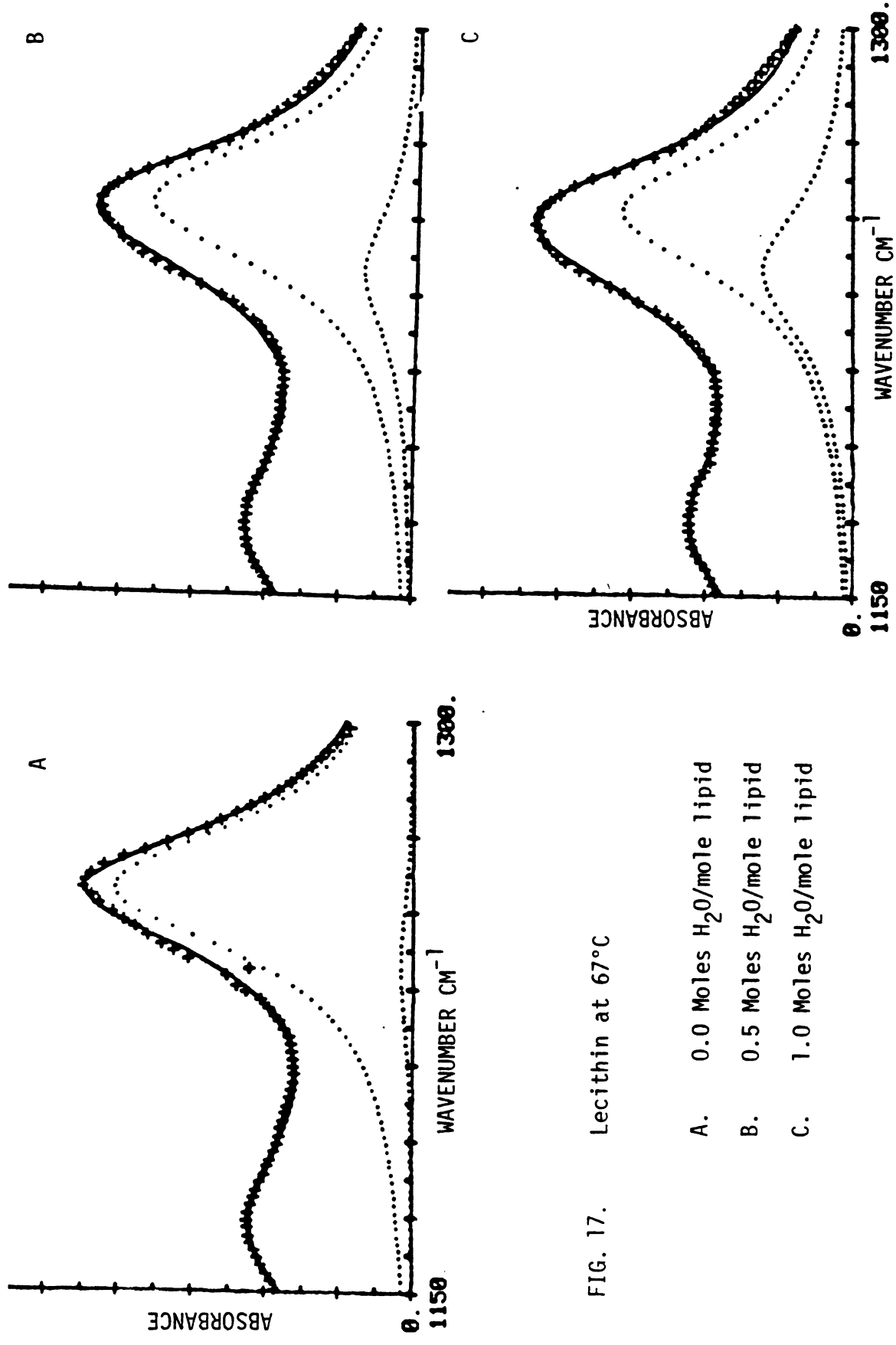

FIG. 17. Lecithin at 67°C

A. 0.0 Moles H_2O/mole lipid

B. 0.5 Moles H_2O/mole lipid

C. 1.0 Moles H_2O/mole lipid

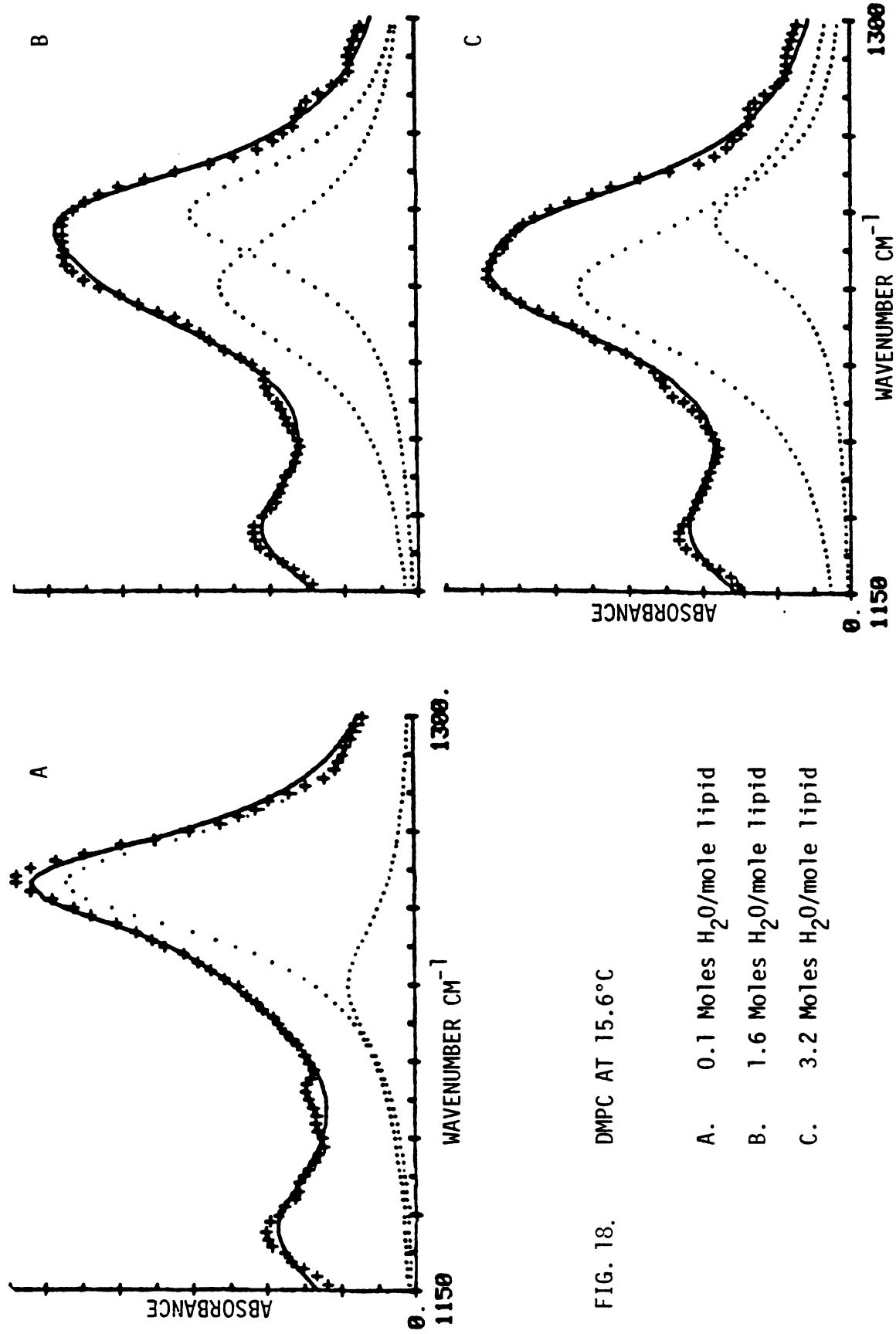

FIG. 18. DMPC AT 15.6°C

A. 0.1 Moles H_2O/mole lipid

B. 1.6 Moles H_2O/mole lipid

C. 3.2 Moles H_2O/mole lipid

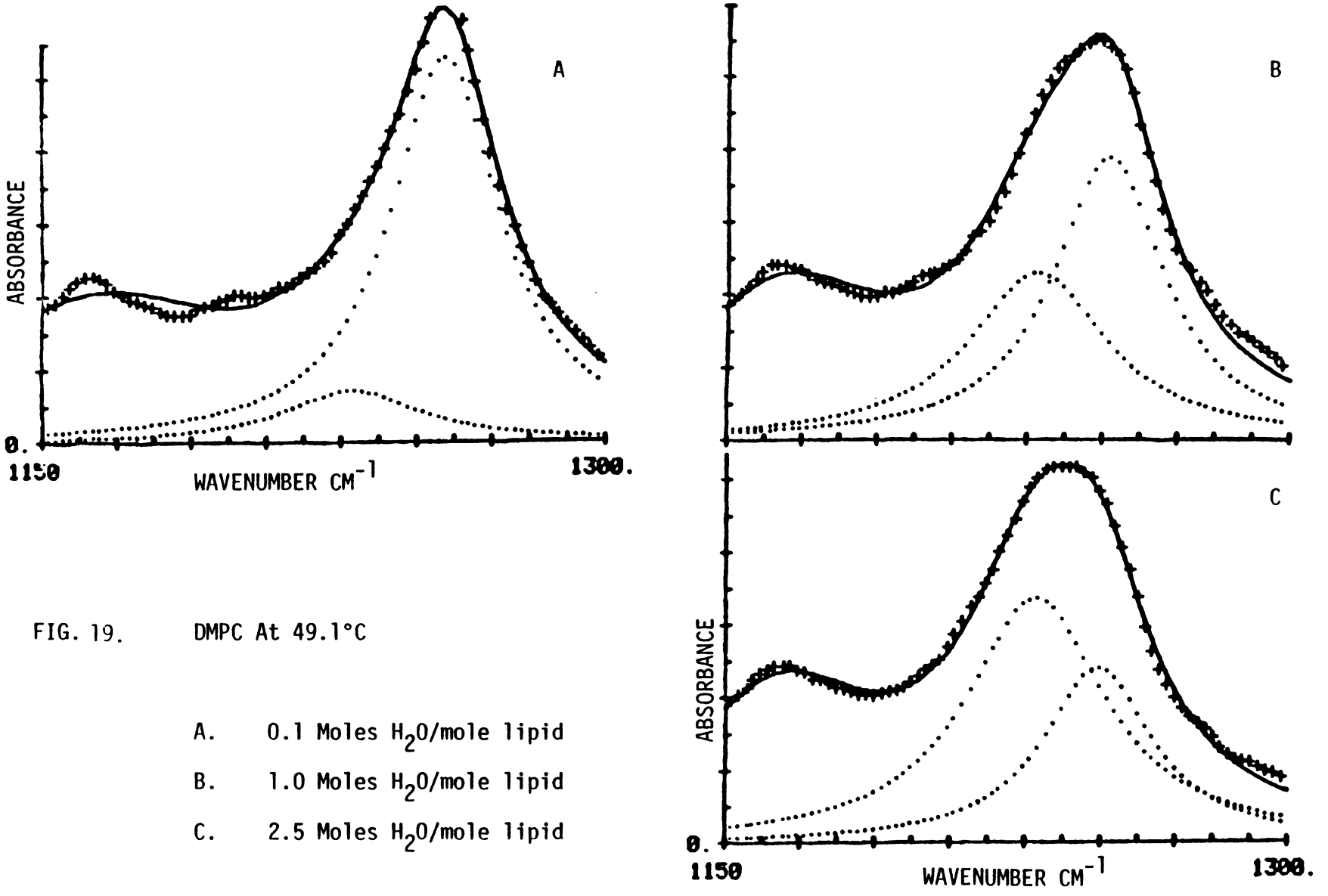

FIG. 19. DMPC At 49.1°C

A. 0.1 Moles H_2O/mole lipid

B. 1.0 Moles H_2O/mole lipid

C. 2.5 Moles H_2O/mole lipid

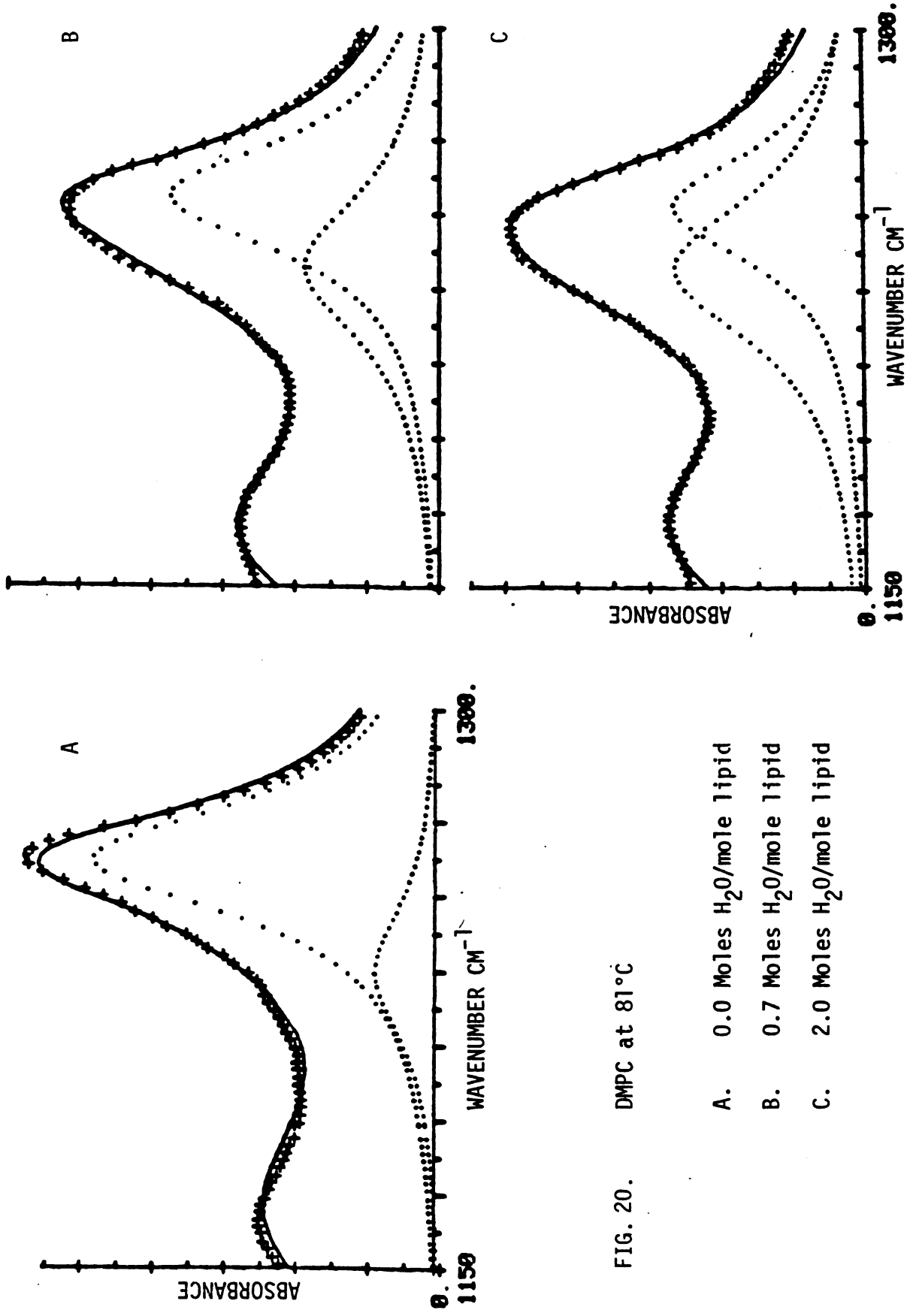

FIG. 20. DMPC at 81°C

A. 0.0 Moles H_2O/mole lipid

B. 0.7 Moles H_2O/mole lipid

C. 2.0 Moles H_2O/mole lipid

available on the Prophet system (described in the experimental section and Appendix 1). The results of these analyses can be seen in figs. 14 to 20 which show the curves of the functions fitted to the ir spectra. When one fixed Lorenzian is used to represent the ester band at 1170 cm^{-1} and another small fixed band about 1280 cm^{-1} the main peak could be resolved into two changing Lorenzians at about 1250 cm^{-1} and 1230 cm^{-1}. The linewidth of both peaks was held constant and the frequency of the 1230 cm^{-1} band was held constant. In egg lecithin, dimyristoyl lecithin and dipalmitoyl lecithin, the same pattern is seen. The 1250 cm^{-1} peak diminishes steadily with increasing water while shifting slightly to lower frequencies and the 1230 cm^{-1} band concomittantly increases in intensity. The behavior of these curves as a function of both temperature and hydration is quite different from that of the C-H and C=O peaks discussed earlier. Changes do not appear to be associated with phase transition phenomena. Figs. 21 and 22 show plots of the areas of the two peaks vs. hydration in lecithin and DMPC. The similarity of the curves at all temperatures is striking. The main difference being the decrease in level of hydration as the temperature is increased.

It is not possible to make unequivocal assignments based on this type of analysis alone but a reasonable explanation is that the 1250 cm^{-1} band represents a nonhydrogen bonded PO_2^- and the 1230 cm^{-1} band a hydrogen bonded PO_2^-. Since the time scale of absorption of ir radiation is small compared to the time scale of

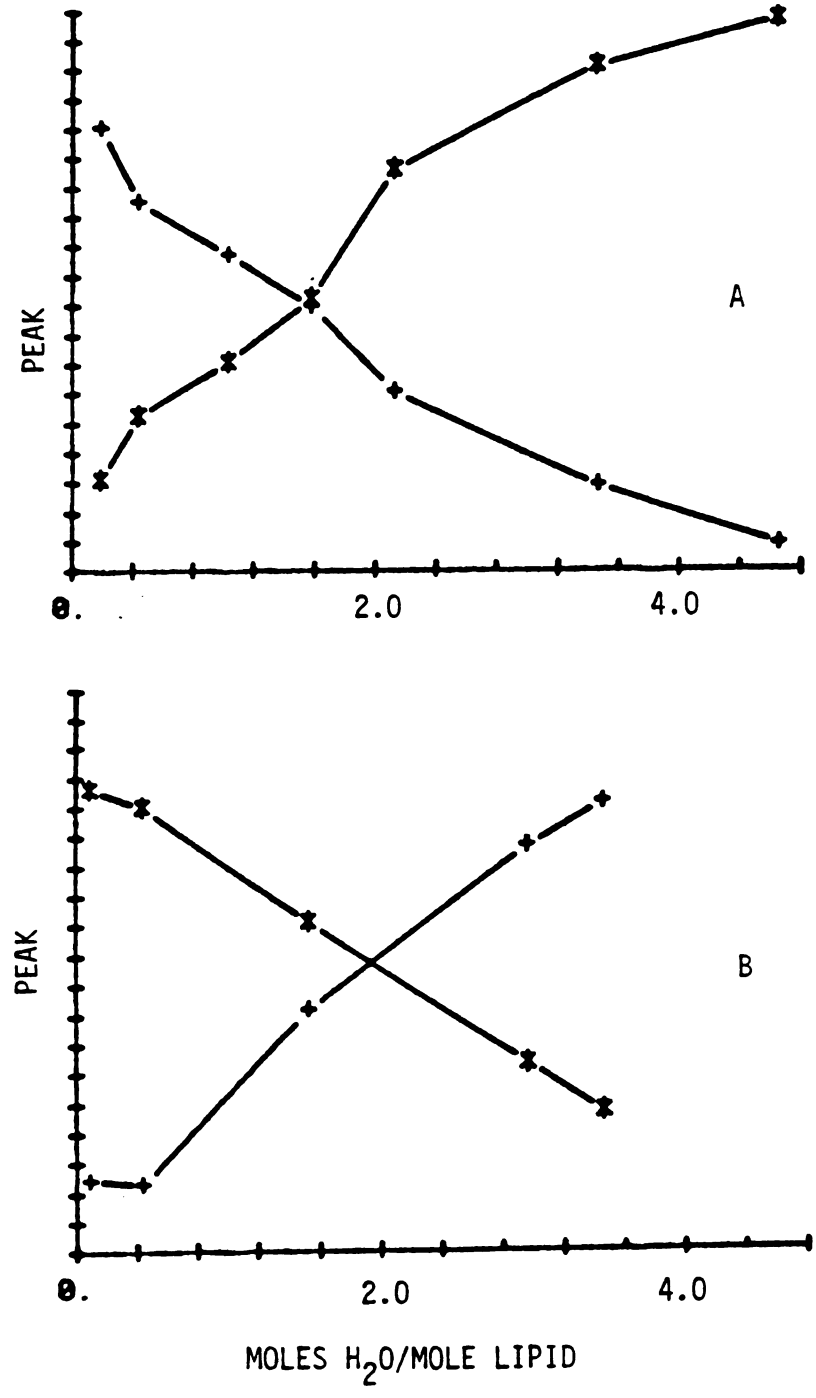

FIG. 21. Area of PO_2^- peaks in lecithin vs. hydration
(*) nonhydrogen bonded
(+) hydrogen bonded

A. 13.5°C. B. 32.5°C. (C and D next page)

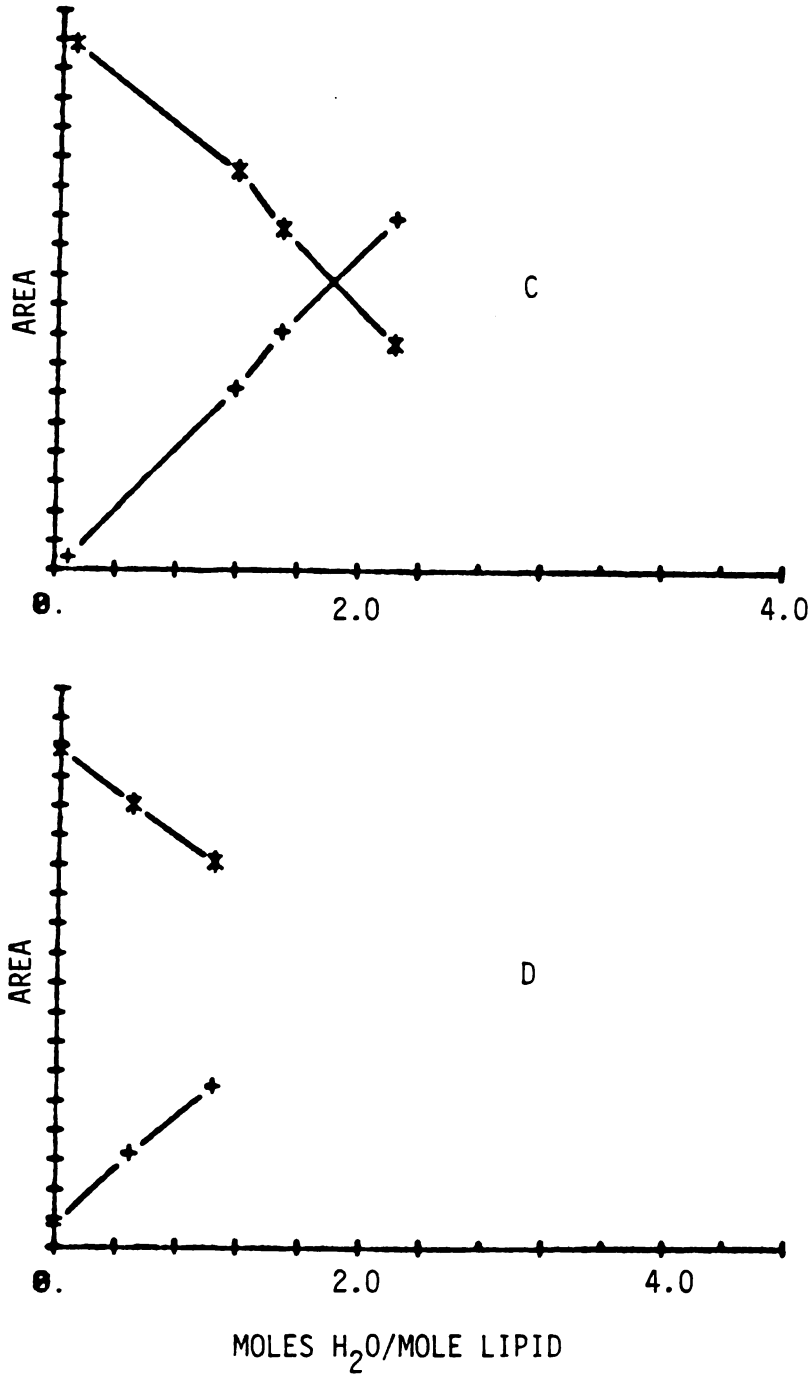

FIG. 21. (continued) C. 50°C. D. 67°C

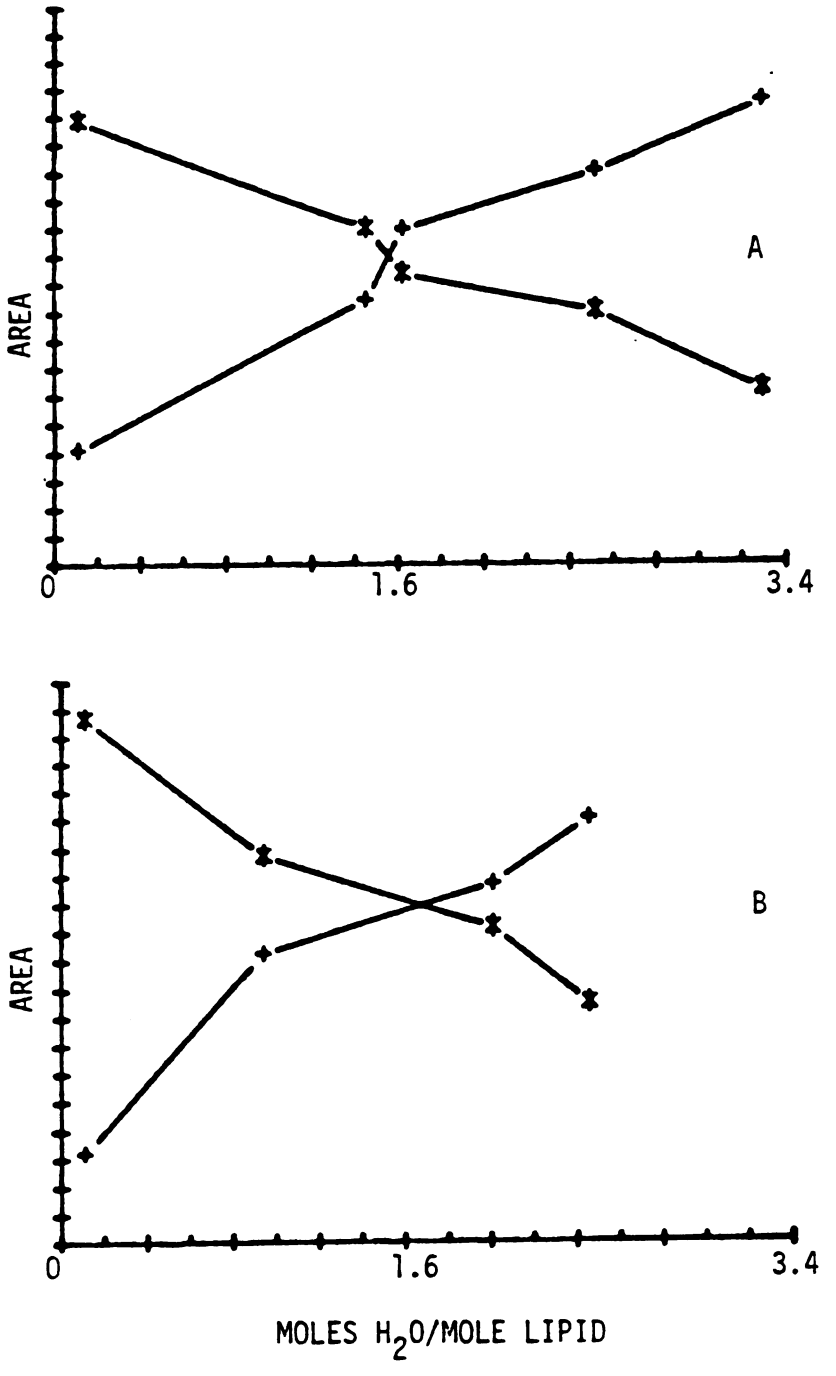

FIG. 22. Area of PO_2^- peaks in DMPC vs. hydration.
(*) nonhydrogen bonded
(+) hydrogen bonded

A. 15.6°C. B. 49.1°C (C next page)

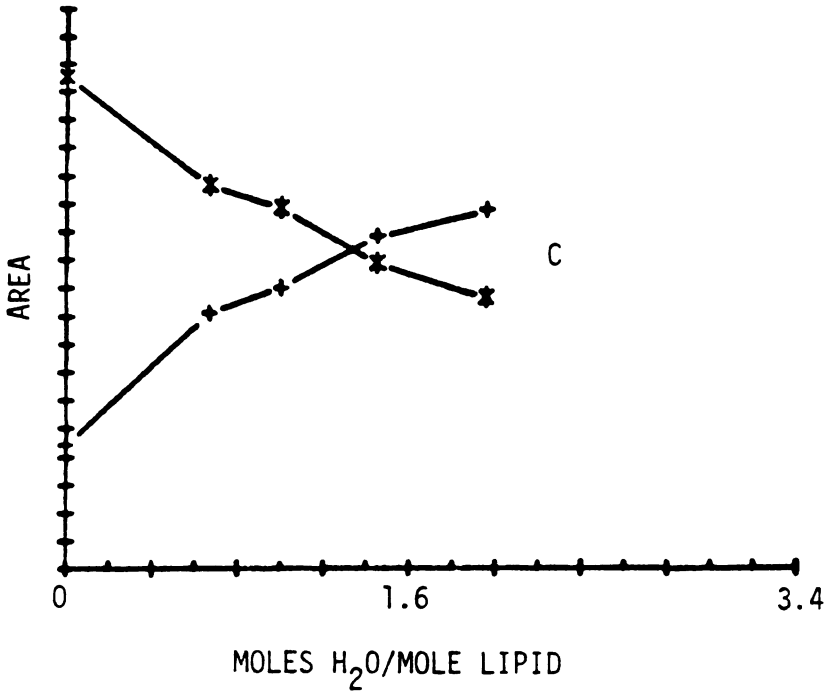

FIG. 22. (continued) C. 81°C.

molecular displacements, it is reasonable to expect two peaks representing the two states. Since the nonbonded species is still present even after more than one mole of water has been absorbed, water not bonded to the PO_2^- must be present and we can imagine an equilibrium of the sort

$$(H_2O) + (lip) \rightleftharpoons (lip \cdot H_2O) \qquad (4)$$

where (H_2O) is water not H bonded to PO_2^-, (lip) represents dry lipid and $(lip \cdot H_2O)$ is lipid with water bonded to the PO_2^-. The concentration of each species can be calculated from the area of the peaks, the total lipid present and the total water present, and the equilibrium constant, K, calculated.

$$K = \frac{(lip \cdot H_2O)}{(H_2O)\ (lip)} \qquad (5)$$

These calculations involve a number of assumptions but a plot of ln K vs. 1/T (see fig. 23) yields a fairly straight line, and the ΔH and ΔS values from this line are not unreasonable for the addition of a single water molecule to a partial charge (i.e. a zwitterionic head group) in a medium of moderate dielectric constant. Table 2 shows the values plotted and the resulting ΔH and ΔS.

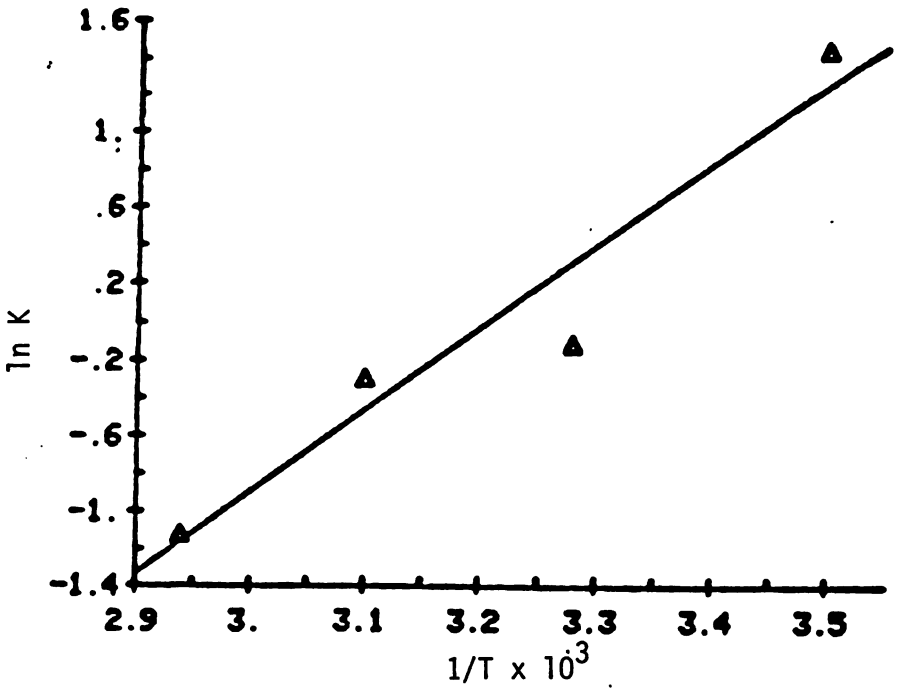

FIG. 23. Ln K vs. 1/T plot for equilibrium constant (eqn 5) for lecithin water reaction (eqn 4). See Table 2 for data.

Table 2. Values of the Equilibrium Constant at Different Temperatures for Lecithin-Water System and ΔH and ΔS Values from Plot (see fig. 23).

T	$1/T \times 10^3$	K	ln K
286	3.50	4.17	1.428
306	3.28	0.882	-0.128
323	3.10	0.746	-0.302
340	2.94	0.323	-1.131

ΔH = -8.5 Kcal/mole, ΔS = -28 e.u.

IV. 1150 - 900 cm^{-1} Region

The main features of this region are a strong, complex band between 1100 and 1050 cm^{-1} and a strong band at about 970 cm^{-1}. There is considerable confusion in the literature about the assignments of these bands. It is believed that the PO_2^- symmetric stretch is contained in this band and the sharp peak at around 1095 cm^{-1} is usually assigned to this vibration. Contributions have been suggested to come from C-O-P, P-O-H, $C-C-N^+$ and others. The characteristics of the band change markedly on hydration (see Figs. 24 to 30). In the presence of water the band seems to be two strong overlapping peaks but when dry, it is evident that at least three bands are present. Spectra taken by Chapman (24) of anhydrous distearoyl lecithin at low temperatures also show three distinct peaks. Using the curve fitting procedure described above also reveals that the peak must be composed of at least 3 bands as no 2 lorenzians could be made to fit any of the spectra in this region. Three lorenzians, however, do achieve good fits (see Figs. 24 to 30). In lecithin and dimyristoyl lecithin, analysis of this type yields a peak at ca. 1090 cm^{-1} which gradually increases in intensity and shifts slightly with increasing water, a peak at 1050 cm^{-1} which does not change at all and a smaller peak at 1070 cm^{-1} which decreases and shifts markedly with increasing water (see Fig. 24 to 30).

Many authors (14, 29, 30) assign the 1090 cm^{-1} band to PO_2^- symmetric stretch. Chapman (24) assigns the band to C-O-(P) but

LEGEND FOR FIGS. 24 THROUGH 30

Fitted curves for PO_2^- symmetric stretch region (1150 to 1000 cm^{-1}) of lecithin and DMPC.

+++ data from spectrophotometer

—— fitted curve from computer

.... resolved lorenzians (only peaks which change with hydration are shown)

Temperature and hydration are given with each fig.

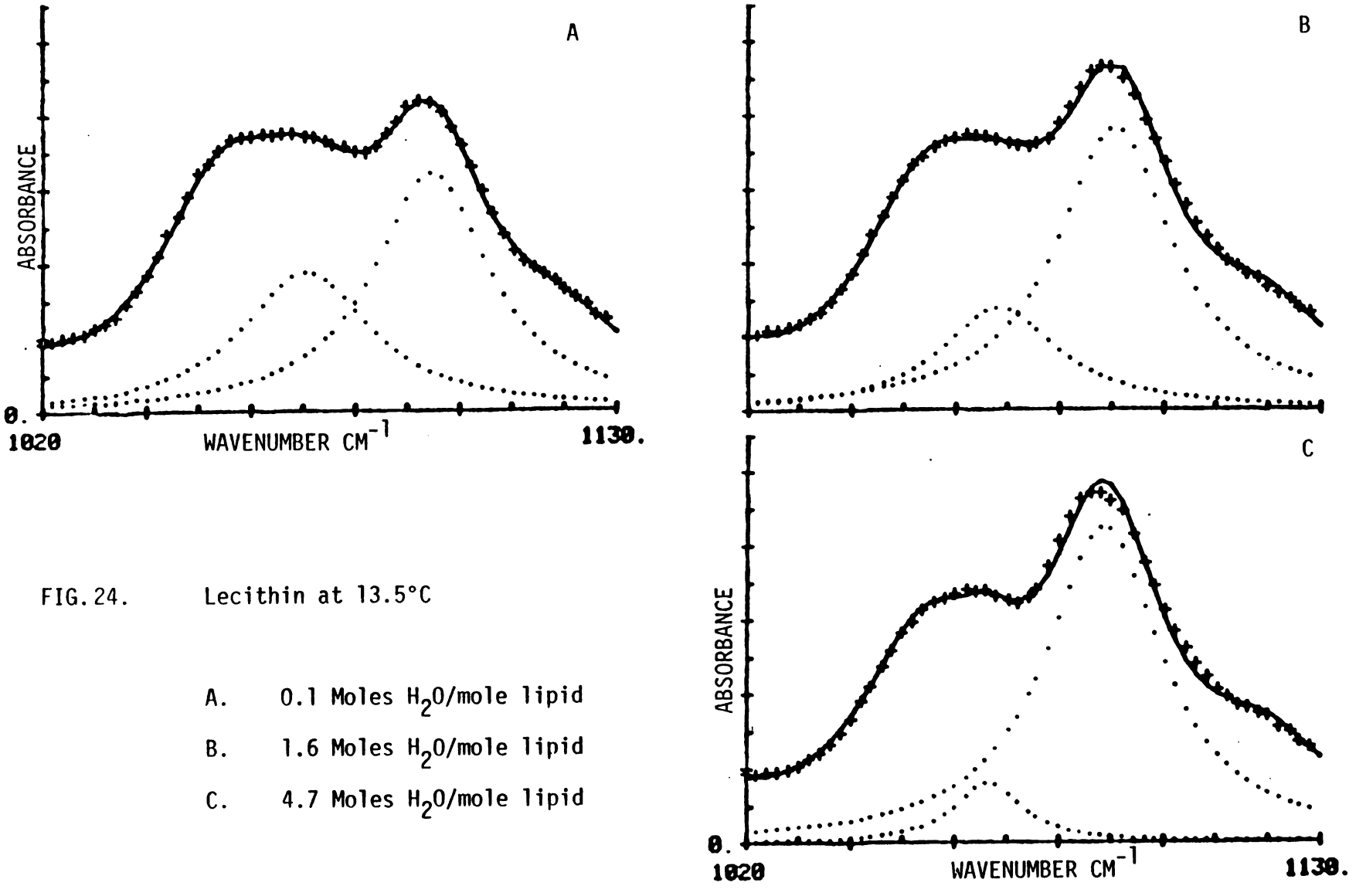

FIG. 24. Lecithin at 13.5°C

A. 0.1 Moles H_2O/mole lipid

B. 1.6 Moles H_2O/mole lipid

C. 4.7 Moles H_2O/mole lipid

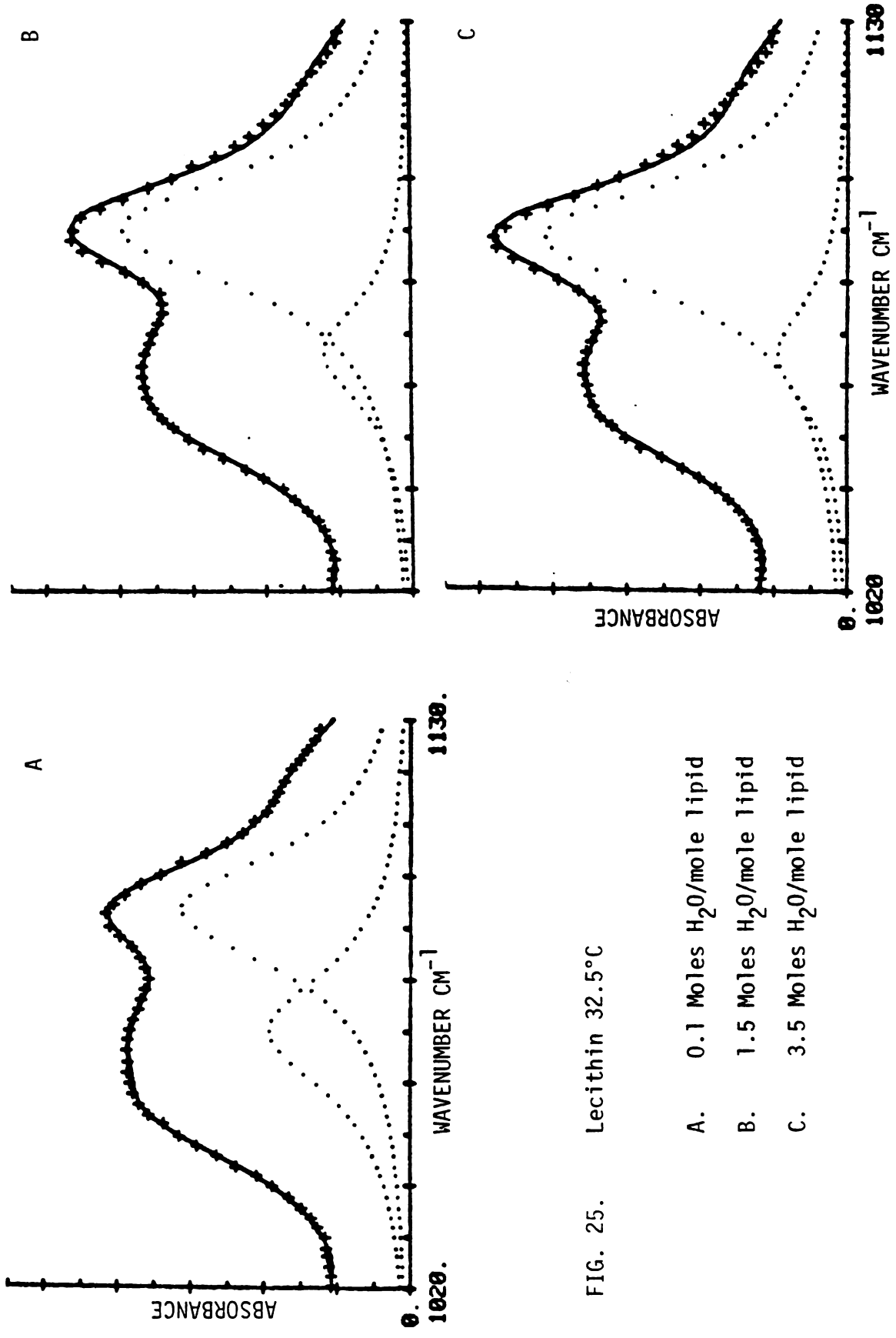

FIG. 25. Lecithin 32.5°C

A. 0.1 Moles H_2O/mole lipid

B. 1.5 Moles H_2O/mole lipid

C. 3.5 Moles H_2O/mole lipid

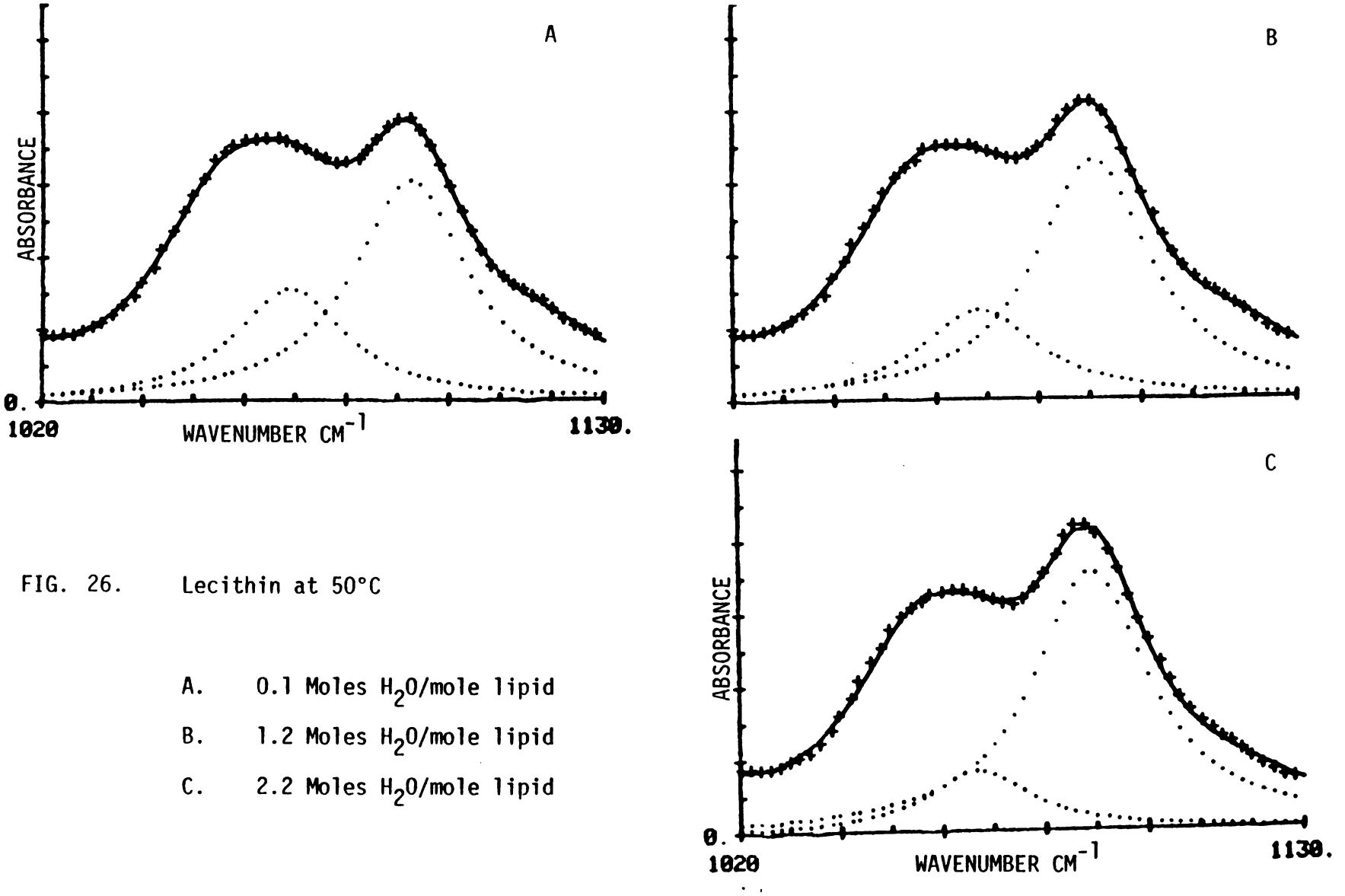

FIG. 26. Lecithin at 50°C

A. 0.1 Moles H_2O/mole lipid

B. 1.2 Moles H_2O/mole lipid

C. 2.2 Moles H_2O/mole lipid

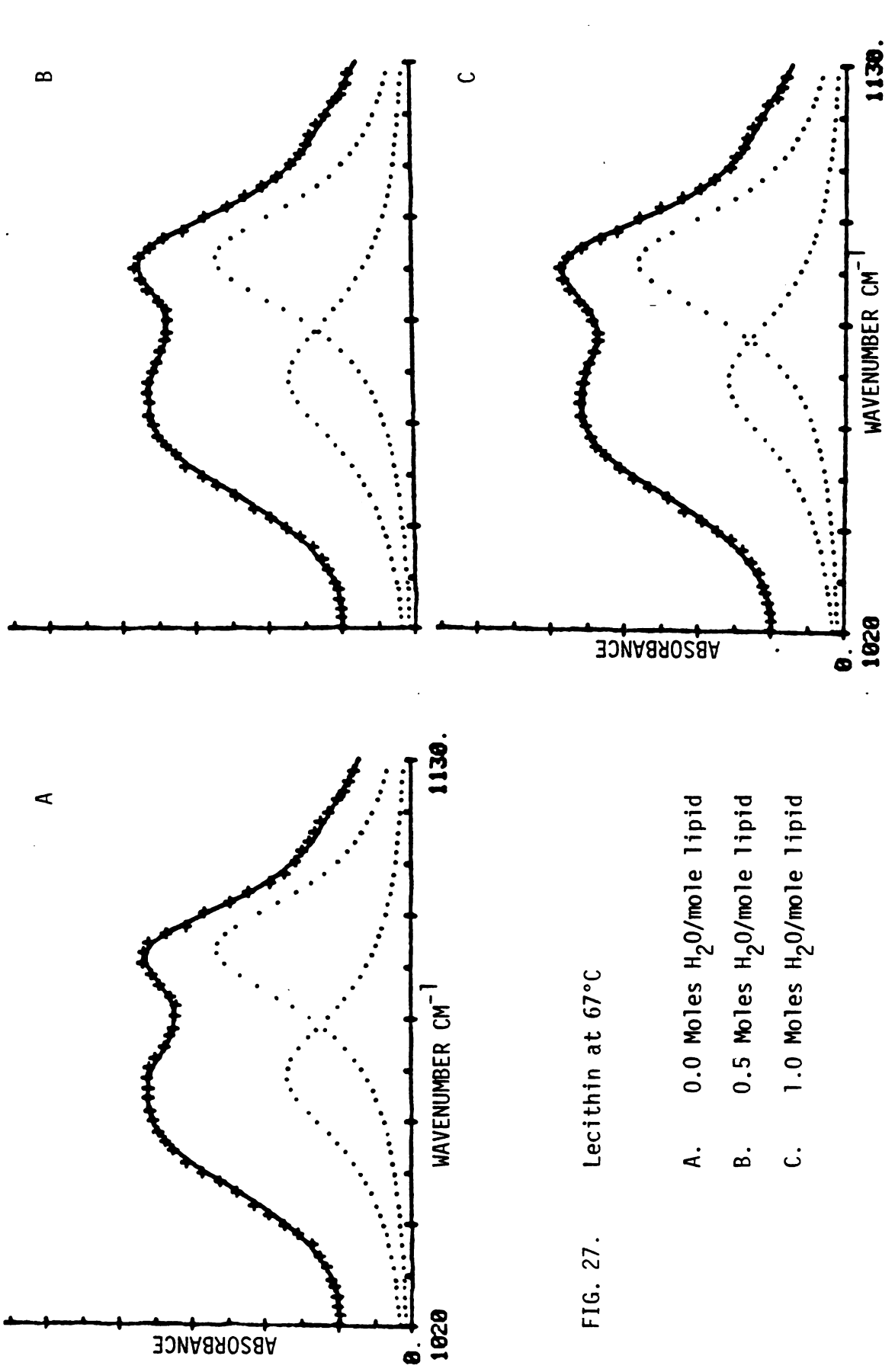

FIG. 27. Lecithin at 67°C

A. 0.0 Moles H_2O/mole lipid

B. 0.5 Moles H_2O/mole lipid

C. 1.0 Moles H_2O/mole lipid

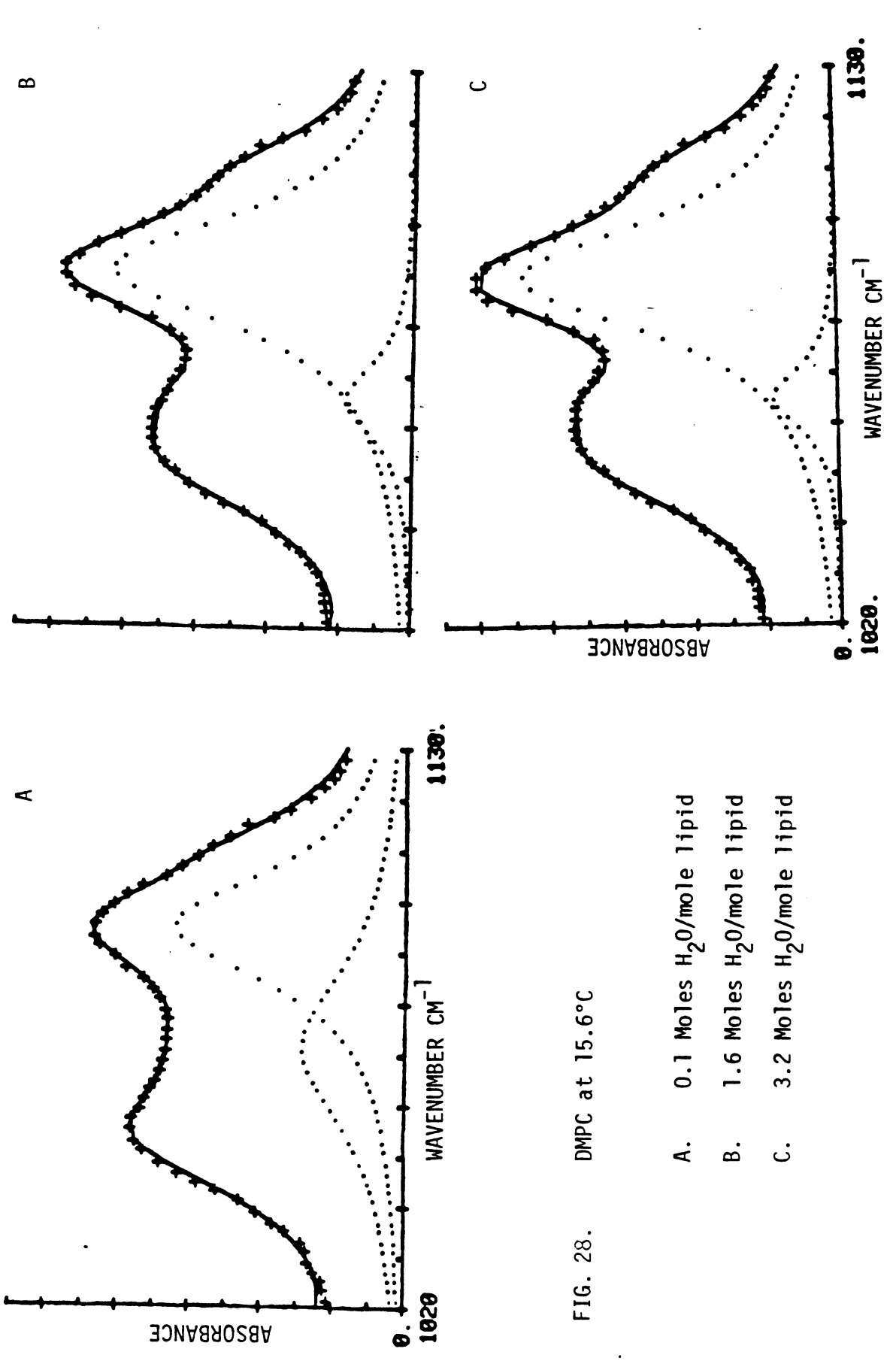

FIG. 28. DMPC at 15.6°C

A. 0.1 Moles H_2O/mole lipid

B. 1.6 Moles H_2O/mole lipid

C. 3.2 Moles H_2O/mole lipid

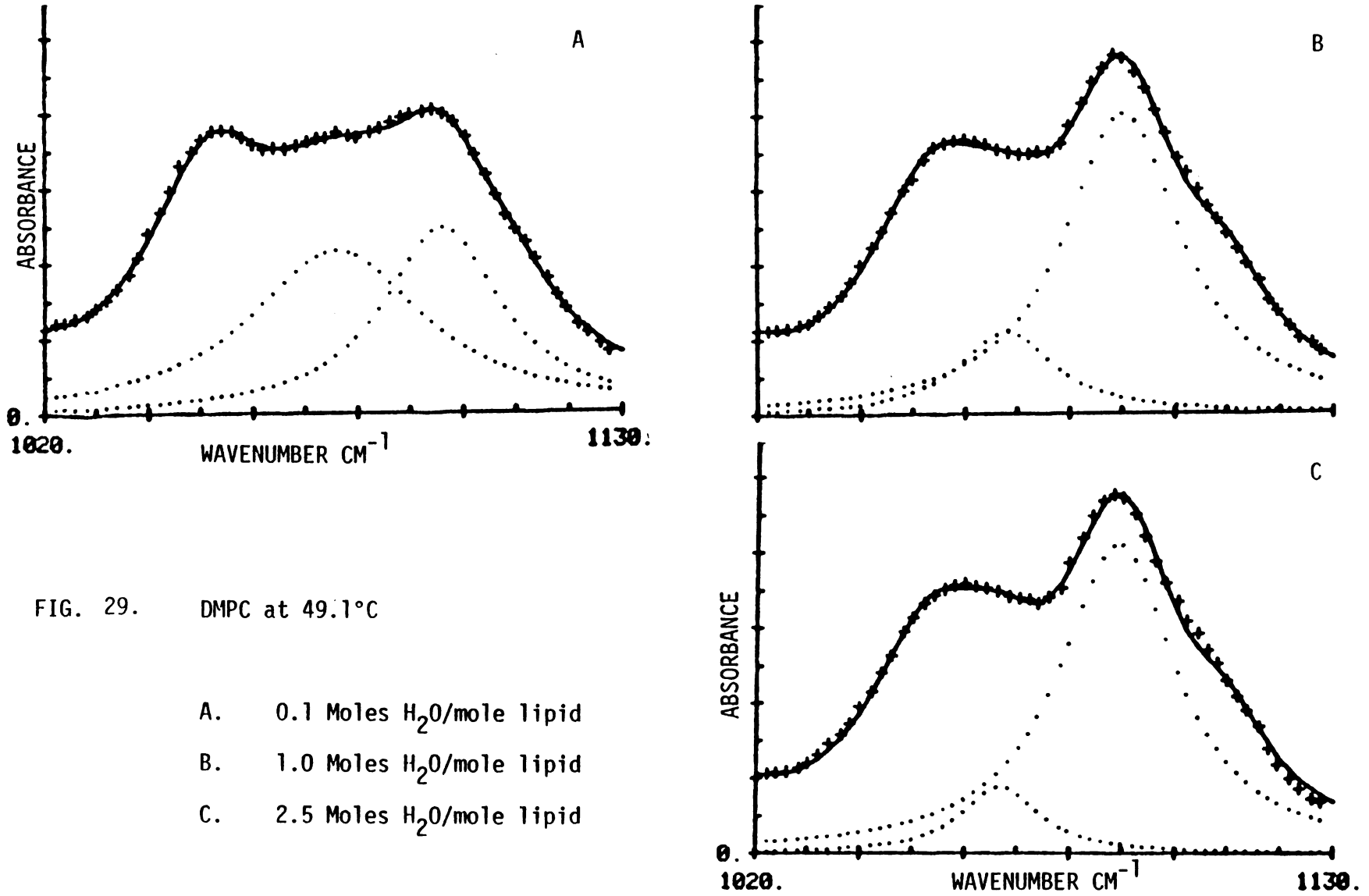

FIG. 29. DMPC at 49.1°C

A. 0.1 Moles H_2O/mole lipid

B. 1.0 Moles H_2O/mole lipid

C. 2.5 Moles H_2O/mole lipid

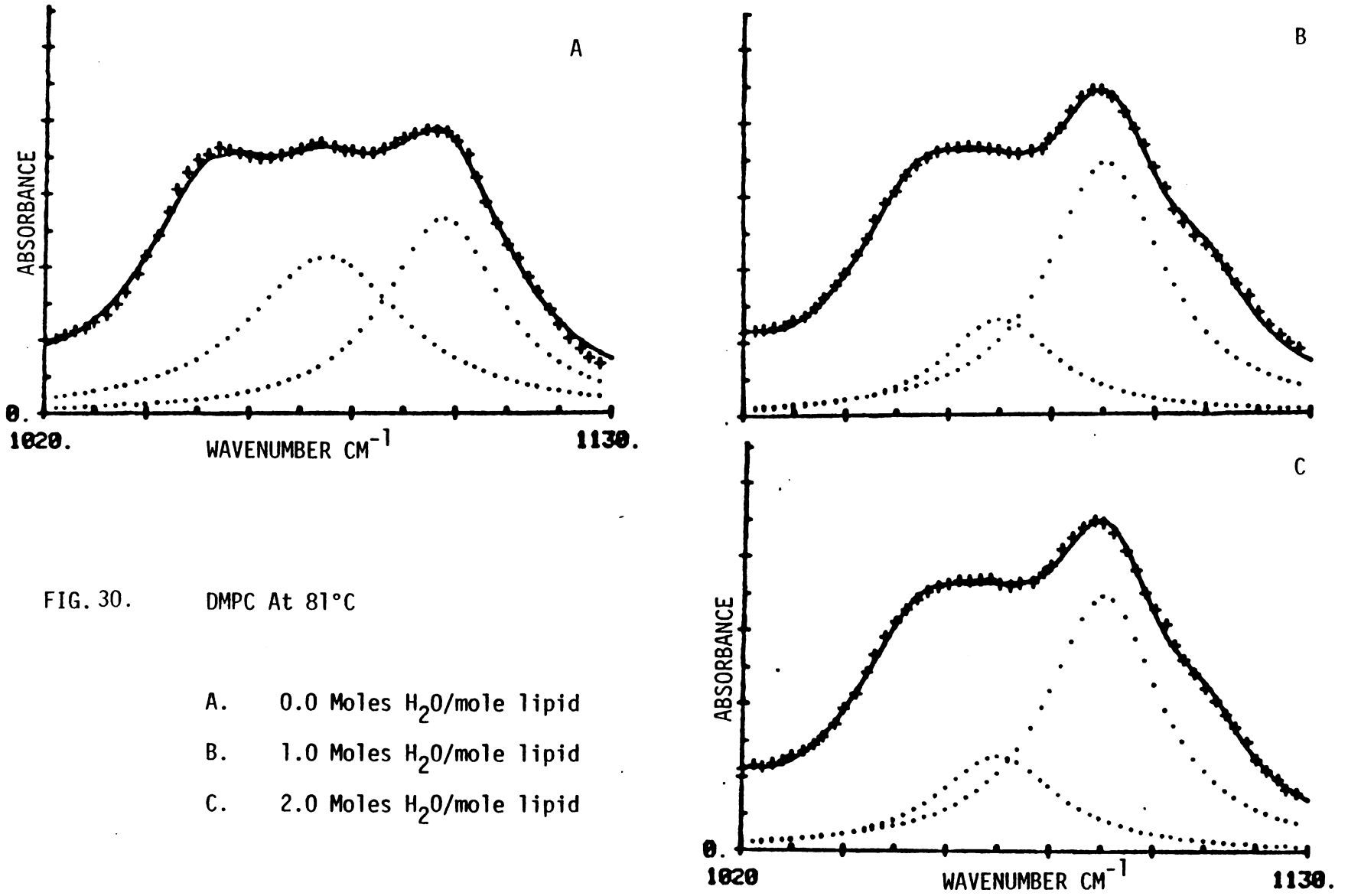

FIG. 30. DMPC At 81°C

A. 0.0 Moles H_2O/mole lipid

B. 1.0 Moles H_2O/mole lipid

C. 2.0 Moles H_2O/mole lipid

does not cite his reasons. We would like to concur with Chapman for the following reasons.

Baer et al (25) synthesized the phosphono analogue of DMPC. In comparing the spectra (see Fig. 31) of this coumpound with the phosphatidyl compound it is noted that the bands at 1090 cm^{-1} and 970 cm^{-1} are both reduced by about one half in the phosphono compound. Since the phosphono compound has just half as many C-O-(P) bonds, this is strong evidence that the 1090 cm^{-1} and 970 cm^{-1} bands are due to vibrations of this moiety. The phosphate group is undoubtedly the site of the first layer of hydration (10, 13, 20, 24) and thus, it would become increasingly hydrogen bonded as this layer is filled. We would not expect to see, as we do, an <u>increase</u> in intensity of the 1090 cm^{-1} band if it were due to the PO_2^- symmetric stretch. The band at 1070 cm^{-1} which decreases and shifts with increasing water is more likely to be due to this vibration.

Analysis of this region in spectra of sphingomyelin reveal some similarities and some differences. The 1090 cm^{-1} peak again increases markedly with increasing hydration (see Fig. 32), and again there is a peak at 1050 cm^{-1} which does not change. There is, however, no peak at 1070 cm^{-1} as there is in the lecithins. If the 1070 cm^{-1} band in lecithins is due to the symmetric PO_2^- stretch as suggested earlier then intra- or intermolecular hydrogen bonding could explain its absence.

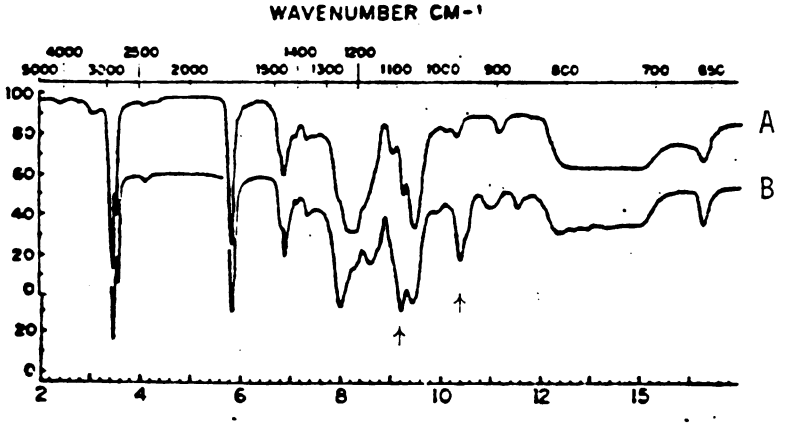

FIG. 31. Infrared spectra (from ref. 25) comparing DMPC (B) to its phosphono analogue (A). Arrows indicate changes in bands at 1090 cm^{-1} and 970 cm^{-1}.

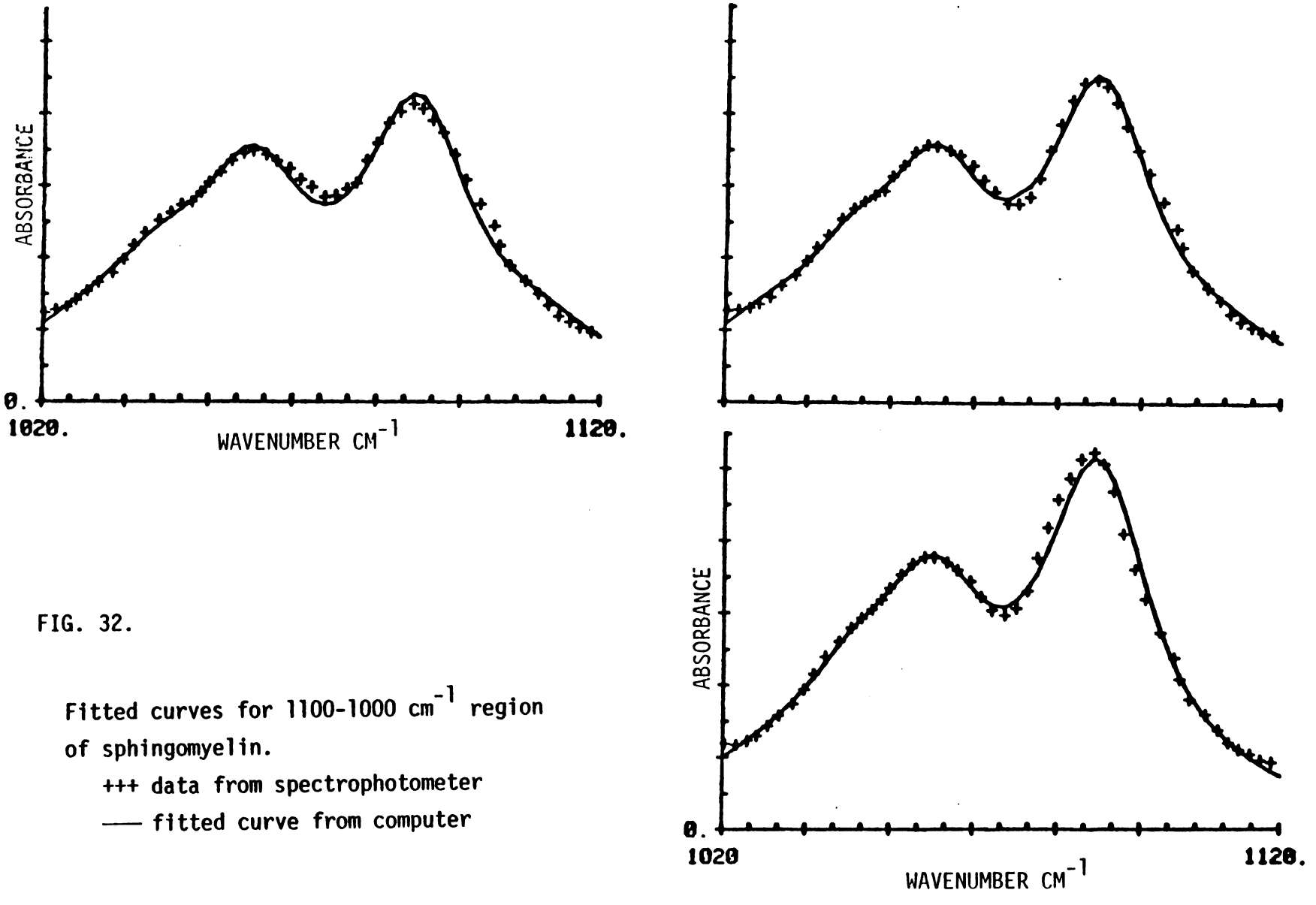

FIG. 32.

Fitted curves for 1100-1000 cm^{-1} region of sphingomyelin.

+++ data from spectrophotometer

—— fitted curve from computer

SUMMARY AND CONCLUSIONS

The ir spectra of several important phospholipids in the gel and liquid crystalline phases were studied. A unique aspect of this work is the simultaneously variable temperature and hydration. The amount of water associated with the lipid was calculated from the absorbance of the water peak at 3380 cm^{-1}. The position of this peak is consistent with strongly hydrogen bonded and hence bound water.

The intensity of the C-H deformation band at 1470 cm^{-1} was found to signal the phase transitions in lecithin, DMPC and DPCC by increased motion of the hydrocarbon chains. This is in agreement with studies on other phospholipids (21).

The intensity of the carbonyl peak was also found to be sensitive to both thermotropic and lyotropic phase changes. This has not been reported previously. The fact that there was no accompanying frequency shift shows that there is no penetration of water to this region at these levels of hydration.

Computer analysis of the PO_2^- asymmetric region showed it to be composed of two bands which rise and fall at the expense of one another as a function of water content but not of temperature. These two bands were tentatively assigned to a nonhydrogen bonded PO_2^- and a hydrogen bonded PO_2^-. Equilibrium constants were calculated at several temperatures for the reaction

$$(H_2O) + (lip) \rightleftharpoons (lip \cdot H_2O)$$

Enthalpies and entropies calculated from this data were seen to be reasonable for this reaction.

Computer analysis of the complex band between 1100 and 1050 cm^{-1} under the influence of changing hydration gave supporting evidence for Chapman's assignment of the 1090 cm^{-1} band to C-O-(P) rather than the PO_2^- symmetric. Suggestions were made for possible assignment of other bands in this region.

The role of water in the structure and phase behavior of membranes has been widely discussed and the implications are important to biological properties. Water is believed to influence the state of the membrane in two ways: 1) by weakening the ionic interactions between the head groups (i.e. acting as a lubricant or plasticizer), and 2) by increasing the distance between molecules by intercalation, thereby weakening the Van der Waals interaction of the hydrocarbon chains. Conversely, the state of the hydrocarbon chains can have an effect on the association of water. For example, the condensing effect of cholesterol has been shown to extrude water from the head group region (28).

The question is: What is the nature of the interdependence of thermotropic and lyotropic mesomorphism? The hydration of the phosphate group proceeds smoothly above, below and through the transition temperature as shown by the plots of the H bonded PO_2^- peak vs. water in lecithin and DMPC. The only difference is the total amount of water in the system. In all cases the proportion of the H bonded species is dependent on the amount of water present.

As shown by this study the carbonyl absorbance is sensitive to the state of the phospholipid molecule. Further studies are indicated to determine why this is so. Whether this effect precedes, coincides with or follows the chain melting may help clarify the role water plays in lowering the T_t. It is significant that Lippert and Peticolas (15) showed with Raman spectroscopy that the phase transition of anhydrous DPPC is broad but the monohydrate undergoes a sharp cooperative transition. This means that the water or some hydrated species is not merely acting as an impurity and lowering the "melting" point but that some new system with different properties is being formed.

Infrared spectroscopy is seen to be able to report suble changes in the state or environment of various sites throughout the lipid molecule. Much of the data from nmr and esr are in conflict with one another or are subject to more than one interpretation. Although ir is not without ambiguities and conflicts it is clear that it has not been adequately exploited in the problem of membrane structure. More studies on these systems, both in greater depth and breadth, could do much to help resolve the questions touched on by this work. As always, only careful coordination of data from different techniques will eventually make the answers clear.

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APPENDIX 1.

The following is a listing of the interactive procedure with the computer to have a function fitted to spectrophotometer data. FITFUN is the name of the procedure. DSALD is a table of data from the spectrophotometer. GUESS and ANS are arrays to hold the guesses for the parameters and the computed parameters respectively. F1 is the function.

```
      ]DEF PUBLIC FITFUN← →

      DCL(GUESS,ANS)FLOAT ARRAY← →DCL(F1,F2)TEXT← →

      F1='B1÷(1+B2×(B3-X)**2)+B4÷(1+B5×(B6-X)**2)'← →

      FILL ALL GUESS                1:    1=10←
                                    1:    2=.005←
                                    1:    3=1280←
                                    1:    4=40←
                                    1:    5=.005←
                                    1:    6=1245:E

      CALL FITFUN(DSALD,1,2,F1,GUESS,ANS,F2)
```

On the following page is a table with the computed parameters and a graph of the fitted function.

NUMBER OF DATA POINTS = 76

FIT FOR FUNCTION B1/(1+B2*(B3-X)**2)+B4/(1+B5*(B6-X)**2)
MULTIPLE R-SQUARED = .9952617, STD. DEV. OF REGRESSION = .9763125

: PARAMETER TABLE 6R X 5C

	1 COEFFICIENT	2 VALUE	3 STANDARD DEVIATION	4 T-VALUE	5 SIG. LEV.
1	B1	3.603162	.6055472	5.950258	.001
2	B2	.02031368	.01178025	1.724384	.0891
3	B3	1290.525	1.163286	1109.379	.001
4	B4	46.89789	.3340632	140.3863	.001
5	B5	.001945991	$4.866169X10^{-5}$	39.99021	.001
6	B6	1244.334	.1758828	7074.789	.001

: ANALYSIS OF VARIANCE DATA 2R X 6C

	1 SOURCE	2 SUM OF SQUARES	3 D.F.	4 MEAN SQUARE	5 F VALUE	6 SIG. LEV.
1	REGRESSION	14014.97	5.	2802.995	2940.658	.0001
2	RESIDUAL	66.72303	70.	.9531861		

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