

THE PHYSICAL STATE OF HYDROCARBONS AND CHLOROPHYLL a
INCORPORATED IN PHOSPHOLIPID BILAYERS AS DETERMINED
BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Thesis by
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In Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy

California Institute of Technology
Pasadena, California 91125
1980

(submitted February 13, 1980)

ACKNOWLEDGEMENTS

The support of the National Institutes of Health in the form of a Predoctoral Fellowship is gratefully acknowledged.

Professor Sunney Chan has provided both invaluable help and tremendous freedom during my tenure in his research group; I am grateful to him for both.

Ken Eigenberg, who initiated the chlorophyll work in our lab, has been an ideal co-worker and an example of creativity at work.

The ^2H NMR spectrometer could not have been built without the help of the gentlemen of the Caltech electronics shop, particularly Ken Bloch and Irv Moskovitz. It has been a privilege to learn together with them; no one at Caltech has encouraged me more.

The problem of the motional state of hydrocarbons in bilayers was suggested to us by Prof. Stephen White of U.C. Irvine, with whom we have had many useful discussions.

Diana Gamble typed the entire manuscript, fighting illegible handwriting and impending deadlines, without a single complaint. Thank you.

Most of all I want to thank my wife, Pat, for four and one-half years of constant support. She has done more for me than anyone could ever expect, and this thesis is as much hers as it is mine.

ABSTRACT

Several classes of non-protein lipophilic molecules interact with biological membranes in physiologically important ways. Nuclear magnetic resonance has been used to investigate the physical state of two model membrane systems composed of a phosphatidylcholine and a lipophile.

The motional state of straight-chain hydrocarbons in phospholipid bilayers has been studied by deuterium NMR. Wide-line ^2H spectra have been obtained for multilamellar dispersions of dipalmitoylphosphatidylcholine containing perdeuterated hydrocarbons and fatty acids ten and sixteen carbons in length. Hydrocarbon spectra show three or four resolved quadrupolar splittings as well as a central maximum whose intensity increases with hydrocarbon concentration. The maximum quadrupolar splitting is two to three times less than that observed for fatty acids. These data indicate two pools of hydrocarbon in the bilayer which are in slow exchange on the ^2H NMR timescale. The central maximum corresponds to hydrocarbons in an isotropic motional state and may be assigned to micro-droplets of hydrocarbon between the two monolayers of the bilayer. The various quadrupolar splittings indicate a flexibility gradient along the hydrocarbon chain and may be assigned to hydrocarbon

lying parallel to the fatty acid chains of the lipid. The magnitude of the splittings, and the fact that the two ends of the hydrocarbon show equivalent splittings may be accounted for by either a rapid end-for-end flip of the molecule within one monolayer or sliding of the molecule between monolayers of the bilayer. To conduct these experiments, a ^2H NMR spectrometer for observation of wide-line spectra utilizing a solid-echo pulse sequence and quadrature phase detection has been constructed.

Chlorophyll-containing phospholipid bilayers have been investigated as a model system for light-harvesting antenna chlorophyll in vivo. The red shift of the chlorophyll visible absorption band observed in vivo is best duplicated by the model system at temperatures below the gel to liquid crystalline phase transition of the bilayer. The phase diagram of the two component bilayer as determined by differential thermal analysis shows a double eutectic with compound formation around 40 mole % chlorophyll. ^{31}P , ^{13}C and ^1H magnetic resonance measurements confirm that phase separation occurs below the transition temperature and suggest that in the compound phase the chlorophyll Mg is coordinated by the phosphate of the lipid headgroup. The constant composition of the compound phase, the interaction of chlorophyll with lipid and the possibility of spatial ordering of chlorophylls in the compound make it an attractive antenna model.

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CHAPTER I. INTRODUCTION

It is increasingly apparent that much of the biological chemistry of a cell takes place on or in biological membranes. This is hardly surprising, since membranes endow the cell with the ability to compartmentalize, the ability to localize reagents and catalysts, and the opportunity to choose between aqueous and hydrophobic reaction media.

Biological membranes are composed primarily of two types of molecules: amphiphiles, called collectively lipids, and polypeptide chains composing various membrane-associated proteins.

Since the advent of the fluid-mosaic model for biological membranes (1), it has been popular to view the basic membrane structure as a lipid bilayer "sea" in which a large number and variety of intrinsic proteins float freely about. The structural properties of the membrane are ascribed to the lipid constituents, whose amphiphilic character dictates the bilayered structure. The chemical activity of the membranes is ascribed to the proteins, in a logical extension of the observation that nearly all cytoplasmic chemical activity is carried out by these macromolecules. In particular, protein polypeptides are thought to have catalytic functions, while cell surface recognition is accomplished by the sugar moieties of glycosylated membrane proteins.

. On the other hand, closer scrutiny suggests that a less simplistic view of membrane structure is required. The gel to liquid crystalline phase transition exhibited by some biological membranes (2) occurs over a wide temperature range which includes physiological temperatures, suggesting that while some regions of the membrane may be fluid, others may not be so fluid (3). The requirements of some proteins for specific lipids to maintain their activity (4) and the observation by ESR of boundary lipid to proteins (5) implies that lipids do more than just physically support the intrinsic proteins. They provide a specific chemical environment in which the protein can function. By the same token the observation of ordered protein arrays in membranes (6,7) and the prediction that protein clustering can cause lipid ordering (8) suggest a role for proteins in determining the membrane's physical properties. Finally, there are classes of small molecules which interact with biological membranes in physiologically important ways but probably do not interact with the membrane protein directly. Such molecules include antibiotics (9), anaesthetics (10), voltage-sensitive dyes (11), and perhaps some steroid hormones (12). Thus while it makes sense to ascribe specific catalytic activity to the membrane proteins, other important chemical properties such as permeability, transmembrane electrical potential, etc. depend primarily on the

lipids and on the interaction of the lipids with other small molecules.

This point of view makes the job of the membrane biophysicist both more difficult and more interesting. If the lipid component of the membrane is important only as a structural base for proteins, lipid bilayers may be studied from a macroscopic thermodynamic point of view, to determine dimensions, viscosity, elasticity, etc. In particular, for complex mixtures of different kinds of lipid molecules such as occur in natural systems only the average properties are important. On the other hand, if the interaction of specific lipid molecules with other small molecules is important then it becomes necessary to sort out the relationship between two or more individual components of the complex mixture on a molecular level. Model systems containing three components: water, lipid and the small molecule of interest, are used to isolate the interaction to be characterized. The continuing saga of cholesterol effects on model membranes (13-15) indicates how complicated these apparently simple three-component systems can be.

A particularly useful physical technique for the study of these systems is nuclear magnetic resonance (16). First, NMR provides selectivity, allowing the experimenter to observe particular molecules or particular functional groups in a complex system, either by a judicious choice of the nucleus to be observed or by labelling of specific

components. Second, magnetic resonance provides sensitivity to intermolecular effects via changes in chemical shift (first order effects) or relaxation times (second order effects). Third, motion in bilayer systems is anisotropic and tensor interactions between a nucleus and its neighbors are incompletely averaged, rendering NMR sensitive to molecular ordering. Fourth, bilayer membranes exhibit relatively slow molecular motions, and NMR is sensitive to motions in the frequency range near the Larmor frequency.

For instance, proton magnetic resonance has been used to study membrane permeability (17), examine the geometry of artificial bilayer vesicles (18), and to determine the correlation times of lipid hydrocarbon chain motions in artificial lipid bilayers (19). Phosphorous NMR has been applied to determine the motional state of phosphoryl choline headgroups (20,21) and to study the transmembrane distribution of phospholipids in mixed phospholipid vesicles (22). Fluorine serves as a specific label on fatty acid hydrocarbon chains and is sensitive to a number of membrane parameters due to its high sensitivity and wide chemical shift range (23). ^{13}C NMR has been used to estimate the number of gauche bonds in lipid hydrocarbon chains (24). Deuterium magnetic resonance (25,26) has also been applied to the determination of molecular ordering in model and natural membranes since deuterium possesses

an electric quadrupole moment and is a relatively nonperturbing label which can be substituted for hydrogen in virtually any molecule of interest.

This thesis describes the application of magnetic resonance techniques, particularly deuterium NMR, to two systems in which small molecules are dissolved in lipid bilayers. In the second chapter deuterium magnetic resonance is used to investigate the motional state of long-chain normal hydrocarbons dissolved in model membranes. In order to perform these experiments a broadline pulsed ^2H spectrometer is required; the construction of this spectrometer for the study of systems exhibiting incomplete motional averaging is described in chapter three. The fourth chapter describes the investigation of model and natural membranes containing chlorophyll a by magnetic resonance and other physical techniques, in an attempt to determine whether chlorophyll-lipid arrays serve as light-gathering antennae in photosynthetic membranes.

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CHAPTER II. ^2H NMR INVESTIGATION OF THE ORIENTATION AND
MOTIONAL STATE OF HYDROCARBONS DISSOLVED IN
PHOSPHOLIPID BILAYERS

A. Introduction

Even before the general acceptance of the fluid mosaic model for biological membranes it was believed that the membrane permeability barrier was formed by an oily layer separating two aqueous compartments (1,2). This implied that one could estimate the permeability of a molecule through the membrane by determining its partition coefficient between water and, for instance, a hydrocarbon. With the advent of the bilayer leaflet model for membranes (3), it was therefore natural to assume that the hydrophobic interior of the membrane acted like an isotropic hydrocarbon solvent for dissolved or permeant species. This notion was challenged by White (4) and by Simon (5), who reasoned that if the bilayer interior acts like a hydrocarbon solvent the free energy of transfer of a hydrocarbon from the bulk alkane to the membrane interior should be small. However when they measured this parametric directly they found a value about two orders of magnitude larger than expected. Thus it is evident that the behavior of the bilayer as a solvent for small molecules is not as simple as at first imagined, and a logical first step to understanding the solvent properties of the bilayer is to study the properties of bilayers containing dissolved hydrocarbons.

The interaction of hydrocarbons with bilayers is also important because hydrocarbons are known to affect membranes in physiologically important ways. For one thing, straight chain hydrocarbons of certain lengths act as anaesthetics (6), suppressing the transmission of action potentials along nerve fibers. Normal alkanes are also known to be promoters of carcinogenesis (7), significantly potentiating the effects of carcinogens in tumor induction when they are applied simultaneously. It has been tentatively suggested that this phenomenon may be related to the destabilizing effect of hydrocarbons on bilayer vesicle membranes near their phase transition temperature; vesicles containing hydrocarbon show an increased rate of lysis, or at least an increased permeability, near T_c (8).

Finally, it is important to understand the interaction of hydrocarbons with bilayers because hydrocarbons are present in the bilayer portion of black lipid films (BLMs). The black lipid film, which was one of the first model systems for the biological membrane, is formed by painting a solution of lipid in hydrocarbon across a small aperture in a thin septum between two compartments filled with aqueous buffer (9). The resulting droplet consists of a monolayer of lipid covering a droplet of hydrocarbon-lipid solution suspended between two aqueous phases. The drop thins spontaneously at the center until the two surface monolayers are directly apposed to form a

bilayer membrane between the two aqueous compartments. The bilayer portion in the center of the aperture is joined to the septum by a thicker annulus which consists of a monolayer-coated solution of lipid in hydrocarbon and is called the Plateau-Gibbs border.

Because the bilayer portion of the film is in thermodynamic equilibrium with the border (10), the bilayer portion is saturated with hydrocarbon. The amount of hydrocarbon in the bilayer may be estimated from measurements of membrane capacitance if a value is assumed for the dielectric constant of the hydrocarbon portion of the bilayer (11). Alternatively the proportion of lipid and hydrocarbon in the bilayer portion has been measured directly by dropping mercury droplets through a horizontal film made from radiolabelled components (12). Each droplet is coated with a single layer of the bilayer and the accumulated drops may be counted separately for the hydrocarbon and lipid labels to determine the ratio of lipid to hydrocarbon. For lecithin bilayers this ratio depends strongly on the chain length of the hydrocarbon used. BLMs made with decane have a hydrophobic portion containing up to 50% decane by volume, while bilayers made using hexadecane include only about 1% by volume of hexadecane in the hydrophobic portion.

BLMs have also been studied extensively by measurements of the membrane capacitance, which is smaller than the capacitance of natural membranes owing to the presence

of the hydrocarbon. Capacitance of BLMs also depends on the nature of the hydrocarbon used in forming the film. Capacitance of black lipid films increases with chain length of the hydrocarbon used from C_{10} through C_{16} (13). X-ray data confirm that the increased capacitance is due to a decrease in thickness of the hydrophobic portion of the bilayer (14).

One of the first and most fundamental questions addressed by workers investigating hydrocarbon-containing bilayers was how dissolved hydrocarbon is oriented in the bilayer. There are at least three ways in which normal alkanes could be inserted into the membrane. First, they could be aligned parallel to the hydrocarbon chains of the lipids in association with one of the two monolayers making up the membrane. Second, individual molecules could lie between the two monolayers in the center of the bilayer, with their long axis parallel to the plane of the membrane. Third, the alkane could form droplets, or microlenses of pure hydrocarbon sandwiched between the two monolayers and causing the bilayers to swell in thickness at the point where the droplet is located.

Of these three possibilities the third is the best established. Microlenses have been observed as bright spots in the otherwise black lipid films. The lenses gradually coalesce with one another and with the Plateau-Gibbs border. Microlenses do not account for all

of the hydrocarbon in black lipid films, however, since microlenses are observed for some membranes but not for others. Each of the other possibilities has been suggested in the literature on the basis of capacitance data and estimates of membrane thickness from x-ray diffraction measurements (14). These studies attempt to localize dissolved hydrocarbons indirectly by observing their effect on the membrane as a whole. Varying portions of chain-parallel and membrane parallel lipid have been postulated for different hydrocarbon chain lengths to account for the variation in membrane thickness with chain length.

A useful technique for observing the orientation and motional state of hydrocarbons directly is deuterium nuclear magnetic resonance, which has been applied with great success to the study of lipid hydrocarbon chains in model and natural membrane systems (15,16,17). The utility of ^2H NMR in these systems is based on the fact that deuterium, as a spin 1 nucleus, has a non-zero electric quadrupole moment which interacts with the electric field gradient at the nucleus arising from bonding with adjacent atoms. This interaction lifts the coincidence between the $m=+1$ to $m=0$ and $m=0$ to $m=-1$ transitions. Since the electric field gradient tensor (which is referred to a molecule-fixed coordinate system) is anisotropic, the strength of the interaction depends on the orientation of the molecule with respect to the

laboratory-fixed magnetic field $\vec{H}_0$. For molecules which are slowly reorientating two transitions are observed, whose separation provides information about the orientation of the molecule.

Let us review this problem in more detail. The total Hamiltonian of a quadrupolar nucleus in a magnetic field may be written (18):

$$H = -\gamma_n \hbar \vec{I} \cdot \vec{H}_0 + \frac{eQ}{4I(2I-1)} [V_0 (3I_z^2 - I^2) + V_{+1} (I_+ I_z + I_z I_- - I_- I_+) + V_{+2} I_+^2] \quad (\text{II-1})$$

where $\vec{I}$ is the nuclear spin operator, $\vec{H}_0$ the externally applied magnetic field, γ_n the gyromagnetic ratio of the nucleus, Q the electric quadrupole moment, e the elementary electronic charge, and V_0 , V_{+1} and V_{+2} are functions of the electric field gradient tensor:

$$\begin{aligned} V_0 &= V_{zz} \\ V_{+1} &= V_{xz} + iV_{yz} \\ V_{+2} &= \frac{1}{2}(V_{xx} - V_{yy} + 2iV_{xy}). \end{aligned} \quad (\text{II-2})$$

Note that the components of the field gradient tensor are referred to a molecule-fixed coordinate system. This tensor is traceless and symmetric so that in the principal axis system of the field gradient tensor we may let

$$V_{zz} = eq$$

$$\frac{V_{xx} - V_{yy}}{V_{zz}} = \eta \quad (\text{II-3})$$

and write

$$V_{ij} = 0, i \neq j$$

$$H = \gamma_n \hbar \vec{I} \cdot \vec{H}_0 + \frac{e^2 q Q}{4I(2I-1)} [3I_z^2 - I(I+1) + \frac{1}{2}\eta(I_+^2 + I_-^2)] \quad (\text{II-4})$$

For a C-D bond the field gradient tensor is axially symmetric about the bond axis. In this case the z axis of the principal axis system coincides with the C-D bond axis and $\eta \approx 0$.

Equation II-4 contains a Zeeman term, usually treated in a laboratory-fixed coordinate frame, and a quadrupolar term referred to a molecule-fixed frame. For deuterium the quadrupole interaction is at most about 2% the size of the Zeeman interaction so the quadrupole interaction may be treated as a perturbation. Using the laboratory-fixed frame of reference in which $\vec{H}_0$ lies along z' and transforming the quadrupolar Hamiltonian, which refers to a molecule-fixed coordinate system, into this reference frame we find (18)

$$H = \gamma_D \hbar H_0 I_{z'} + \frac{e^2 q Q}{4I(2I-1)} [3I_{z'}^2 \cos^2 \theta + 3I_{x'}^2 \sin^2 \theta + 3(I_{z'} I_{x'} + I_{x'} I_{z'}) \sin \theta \cos \theta - I^2] \quad (\text{II-5})$$

where θ is the angle between the H_0 field (z' axis) and the symmetry axis of the electric field gradient tensor (z axis).

To first order the energies corresponding to this Hamiltonian are

$$E_m = -\gamma_D \hbar H_0 m + \frac{e^2 q Q}{4I(2I-1)} \left(\frac{3\cos^2\theta - 1}{2} \right) [3m^2 - I(I+1)] \quad (\text{II-6})$$

For a ^2H nucleus there are two transitions, $m=+1$ to $m=0$ and $m=0$ to $m=-1$. In the absence of the quadrupolar interaction the two transitions are coincident. When the quadrupolar interaction is included the two transition frequencies are

$$\frac{1}{\hbar}(E_0 - E_{+1}) = \nu_{+1 \rightarrow 0} = \frac{\gamma_D}{2\pi} H_0 - \frac{3}{4} \frac{e^2 q Q}{\hbar} \left(\frac{1}{2} \right) (3\cos^2\theta - 1) \quad (\text{II-7})$$

$$\frac{1}{\hbar}(E_{-1} - E_0) = \nu_{0 \rightarrow -1} = \frac{\gamma_D}{2\pi} H_0 + \frac{3}{4} \frac{e^2 q Q}{\hbar} \left(\frac{1}{2} \right) (3\cos^2\theta - 1)$$

The quadrupolar interaction lifts the coincidence, and the two transitions are separated by

$$\Delta\nu_Q = \frac{3}{4} \frac{e^2 q Q}{\hbar} (3\cos^2\theta - 1) \quad (\text{II-8})$$

$\frac{e^2 q Q}{\hbar}$ is about 167 kHz for a C-D bond in a hydrocarbon chain (19). Recall that θ is the angle between the magnetic field $\vec{H}_0$ and the C-D bond vector. For a single crystal sample where θ is the same for all C-D bonds we

expect two resonance lines separated by $\Delta\nu_Q$. In the case of a liquid, where there is rapid, isotropic motion of the C-D bond vectors with respect to H_0 the quadrupolar interaction averages to zero and we observe a single line.

Now we consider the case of restricted or anisotropic motion of the C-D bond axis with respect to the externally applied magnetic field. By "restricted motion", we mean the following: Only motions which are rapid compared to $(\frac{3}{4} \frac{e^2 q Q}{h})^{-1}$ (the timescale of the 2H experiment) will be effective in averaging the quadrupolar interaction. If on this timescale, the C-D vector covers only a certain range of angles θ with respect to H_0 , then the motion is called restricted. By "anisotropic motion" we mean motion which allows the C-D vector to sample all angles θ in the timescale of the experiment, but with different probabilities for different angles θ (17). In the case where the motion has axial symmetry about some axis called a director, we may define an order parameter S_β :

$$S_\beta = \frac{1}{2} \langle 3\cos^2\beta - 1 \rangle \quad (\text{II-9})$$

where β is the angle between the C-D vector and the director and the brackets denote a time or ensemble average. If θ' is the angle between the director and H_0 then

$$\langle 3\cos^2\theta - 1 \rangle = \frac{1}{2} \langle 3\cos^2\beta - 1 \rangle (3\cos^2\theta' - 1) \quad (\text{II-10})$$

and

$$\Delta\nu_Q = \frac{3}{4} \frac{e^2 q Q}{h} S_\beta \left(\frac{1}{2}\right) (3\cos^2\theta' - 1) \quad (\text{II-11})$$

Thus as a result of the motion the quadrupole splitting is reduced by a factor S_β whose magnitude depends on the distribution of orientations of the C-D bond vector with respect to the director sampled during the timescale of the experiment. S_β varies from -0.5 to 1.0, with 1.0 indicating perfect ordering while 0.0 implies isotropic motion.

Recall that we have made two important assumptions in arriving at (II-11). Note that according to (II-11) we are able to determine for a sample in which all of the directors are parallel unambiguously a single order parameter simply by measuring $\Delta\nu_Q$. This results directly from the axial symmetry of the field gradient tensor. In the general case $\eta \neq 0$ and in the expression for $\Delta\nu_Q$ there are two order parameters which cannot be determined separately by a single measurement. Second, we have referred the order parameter S_β to the director. In the general case where the restricted motion does not have axial symmetry, the order parameters are referred to the laboratory axis $\vec{H}_0$.

So far we have considered the case of a sample in which all of the directors are parallel. For bilayers

this corresponds to a sample in which all of the membrane normals are parallel. Such oriented samples have been prepared. Much easier to prepare are multilamellar dispersions in which the directors are distributed randomly with respect to $\vec{H}_0$. The powder pattern lineshape $g(\nu)$ expected for these samples may be computed by substituting for $(3\cos^2\theta-1)$ in equation (II-7) according to (II-10), computing the probability of transition for a given θ' , integrating over θ' , and adding the contribution from the two transitions at a particular frequency (16). Taking into account the finite transverse relaxation time T_2 we find (20):

$$g(\nu) = \frac{1}{T_2} \int_0^\pi \sin\theta' \left\{ \left[\frac{1}{T_2} 2 + [2\pi\nu + A(\frac{1}{2})(3\cos^2\theta'-1)]^2 \right]^{-1} \right. \\ \left. + \left[\frac{1}{T_2} 2 + [2\pi\nu - A(\frac{1}{2})(3\cos^2\theta'-1)]^2 \right]^{-1} \right\} d\theta'$$

where

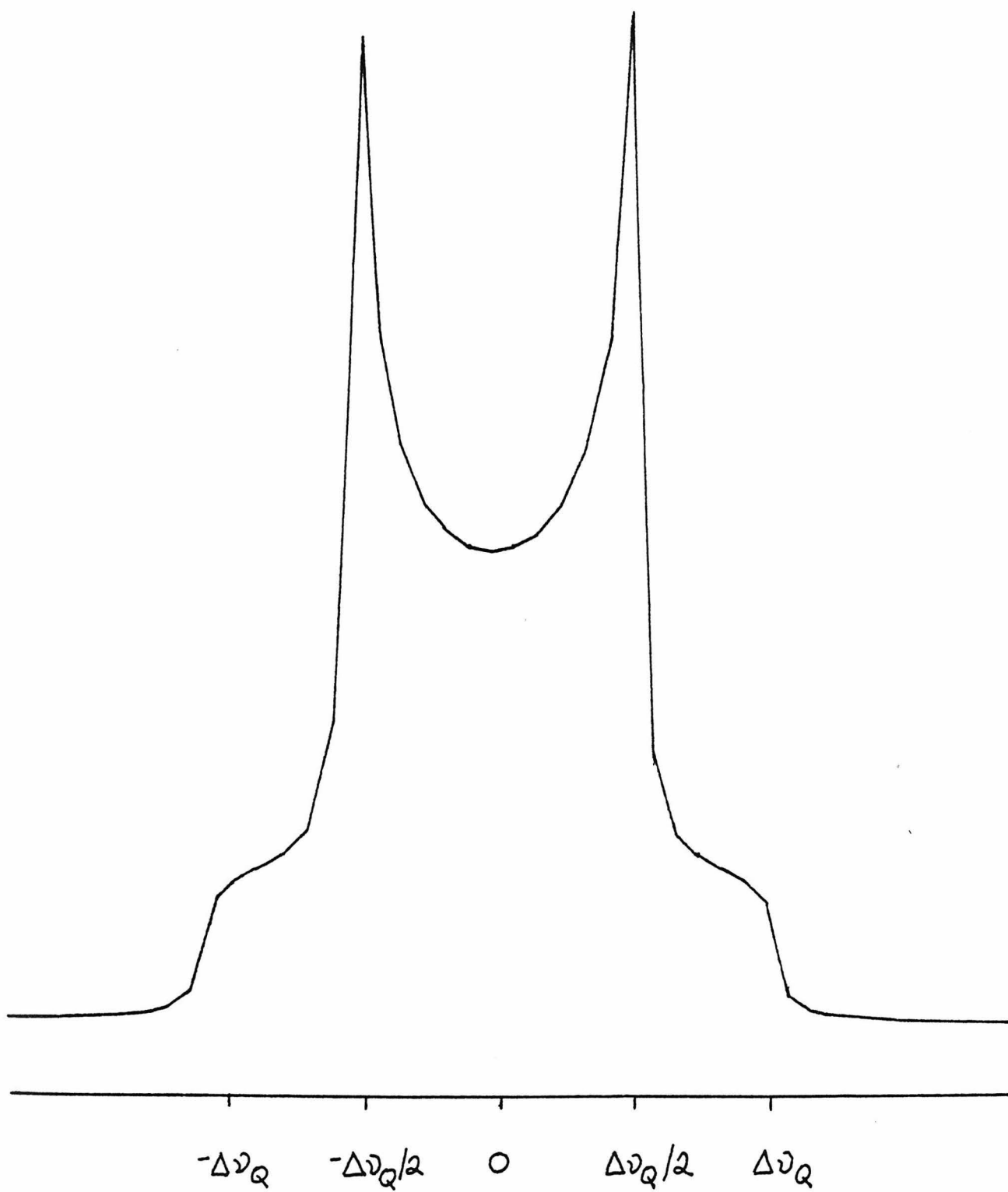
(II-12)

$$A = \frac{3}{8} \frac{e^2 q Q}{h} S_\beta = 2\pi\Delta\nu_Q$$

For T_2 infinite the pattern has two discontinuities separated by $\Delta\nu_Q$. For T_2 finite but long there are two sharp maxima, still separated by $\Delta\nu_Q$. When T_2 approaches the same order of magnitude as A , the two maxima are rounded off and separated by slightly less than $\Delta\nu_Q$. The powder pattern lineshape for long T_2 is illustrated in Figure II-1. Order parameters may be computed trivially after measuring

FIGURE II-1

Theoretical ^2H NMR powder pattern lineshape for an aliphatic deuteron undergoing anisotropic motion, computed according to equation II-12 for $2\pi/T_2 = 0.0025\Delta\nu_Q$. The discontinuities at $\nu = \pm \frac{1}{2}\Delta\nu_Q$ correspond to deuterons oriented with the C-D bond vector perpendicular to $\vec{H}_0$, while the discontinuities at $\pm \Delta\nu_Q$ correspond to deuterons oriented so that the C-D vector is parallel to $\vec{H}_0$.



$\Delta\nu_Q$ directly from the powder pattern. For short T_2 $\Delta\nu_Q$ is best determined by fitting the observed pattern to the theoretical lineshape (21).

Thus ^2H NMR provides information about the distribution of orientations of C-D bonds with respect to $\vec{H}_0$ in systems exhibiting restricted or anisotropic motion on a time scale of about 10^{-5} seconds. ^2H order parameters as a function of chain position have been measured for bilayers in the liquid crystalline phase containing phospholipids specifically deuterated at various positions along the hydrocarbon chain (22). Studies of oriented samples indicate that there is an axis of symmetry for the restricted motion, namely the bilayer normal, so that the above formation applies (23). S_β is constant at about -0.22 for the upper two-thirds of the chain ($\Delta\nu = \sim 28$ kHz), but decreases gradually for the last half dozen positions, reaching its minimum at the terminal methyl. Thus there is evidently a "flexibility gradient" along the chain. Similar results are obtained for long chain fatty acids intercalated in bilayers (24).

Order parameters for this system may also be derived from ^1H NMR measurements (25) and ESR studies on spin-labelled lipids (26). Each of these experiments exploits a different second-rank tensor interaction with a different set of principal axes and a different characteristic timescale. For the upper portion of the chain the three sets of data along with NMR relaxation measure-

ments, have been interpreted in terms of two characteristic motions (27): rapid hydrocarbon chain isomerization via β -coupled trans-gauche rotations (rate $\sim 10^{10} \text{sec}^{-1}$) and slower reorientation of the instantaneous chain axis relative to the director (rate 10^7sec^{-1}). Since the timescales of the two motions are different and the chain isomerization is axially symmetric with respect to the instantaneous chain position the order parameter S_β may be expressed as a product of two order parameters describing the two motions individually:

$$S_\beta = \frac{1}{2} \langle 3 \cos^2 \beta - 1 \rangle = \left[\frac{1}{2} \langle 3 \cos^2 \alpha - 1 \rangle \right] \left[\frac{1}{2} \langle 3 \cos^2 \gamma - 1 \rangle \right] = S_\alpha S_\gamma \quad (\text{II-15})$$

where α is the angle between the instantaneous chain axis and the director and γ is the angle between the molecular interaction vector and the instantaneous chain axis. The molecular interaction vector is the C-D bond vector for the ^2H experiment, the geminal interproton vector for the ^1H experiment, or the long axis of the p-orbital containing the unpaired electron for the ESR spin-label experiment. S_α is now an order parameter for chain reorientation referred to the director, while S_γ is an order parameter for chain isomerization referred to the instantaneous chain axis. By comparing ^2H and ^1H data, S_α may be estimated as $0.35 \leq S_\alpha \leq 0.53$ and S_γ^{CD} as $-0.5 \leq S_\gamma^{\text{CD}} \leq -0.40$ for the upper two-thirds of the chain.

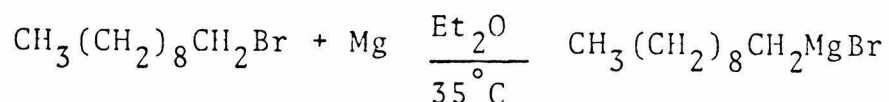
We describe in this chapter of the thesis the application of ^2H NMR to perdeuterated hydrocarbons dissolved in multilamellar phospholipid dispersions. Using quadrupolar splittings measured from powder pattern spectra as a function of temperature we may compare the motional state of the hydrocarbons to that of fatty acids or the lipid hydrocarbon chains themselves, and thus deduce information about the location and motional state of dissolved hydrocarbons in the membrane.

B. Experimental

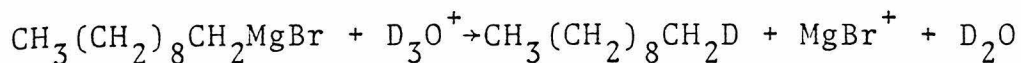
1. Materials

Phospholipids were obtained from Calbiochem and were used without further purification. Perdeuterated fatty acids and hydrocarbons were obtained from Merck, Sharp and Dohme of Canada, Limited and also used as received. As noted below, ^2H spectra of the C_{16} fatty acid intercalated in dipalmitoylphosphatidylcholine (DPPC) multilayers showed two anomalous splittings, indicating contamination with other deuterated species. Perdeuterated dimyristoylphosphatidylcholine (DMPC), from Serdary Research Laboratories, was pure by TLC and was used without purification.

1-deutero-decane was prepared from 1-bromo-decane by forming the Grignard reagent from the alkyl halide:



and then destroying it with dilute deuterated acid:



1-bromo-decane (Aldrich) was purified by passing the material over a short alumina column (aluminum oxide woelm neutral, grade 1, ICN) and stored over 4A molecular sieves (Linde). For the Grignard reaction a three-necked flask equipped with a heating mantle, magnetic stirring, a pressure equalized dropping funnel and a Friedrichs condenser was flushed with dry nitrogen admitted from the top of the condenser. All glassware was oven-dried (170°C, several hours) prior to use. While still warm the flask was charged with 2.7 g (0.11 mole) magnesium turnings. After the apparatus cooled 50 ml of dry diethyl ether (stored over 4A molecular sieves) was added to the flask and the dropping funnel was charged with 21 ml (0.1 mole) 1-bromo-decane and 30 ml diethyl ether. The apparatus was maintained under positive pressure of nitrogen throughout the reaction. To start the reaction diethyl ether in the reaction vessel was warmed to reflux, several milliliters of alkyl halide solution were added to the reaction vessel and a dry stirring rod was used to crush several of the magnesium turnings against the side of the vessel. Bubble formation on the magnesium surface indicated that the reaction had begun. The alkyl halide was added gradually over approximately 45 minutes while stirring and heating to maintain the ether at reflux. Stirring and heating were continued for

approximately 1 hour after alkyl halide addition was complete.

To destroy the Grignard reagent approximately one-quarter of the cooled reaction mixture was added to 50 ml of 0.5N DCl in D₂O (Bio-Rad Laboratories, Stohler Isotope Chemicals). The mixture was cooled in an ice bath to keep the reaction from becoming too vigorous. A small amount (~5 ml) of ether was added to the reaction mixture and the ether layer was separated from the aqueous phase. The crude product mixture was washed once with dilute HCl solution.

The product mixture was added to 50 ml TFTP, a high boiling ether (provided by T. Stevens), and the mixture distilled at reduced pressure (23 mm Hg) in a small-scale distillation apparatus. 1-deutero-decane (~2 ml) was collected as the fraction distilling at 68.5 to 71°C. The distilled product was further purified by passing it over a short alumina column.

The ¹H NMR spectrum of the product (Figure II-2) shows the expected methyl (0.89 ppm downfield from tetramethylsilane) and methylene (1.31 ppm) resonances with a CH₂:CH₃ intensity ratio of 3.15. The expected intensity ratio for CH₃(CH₂)₈CH₂D is 3.20. The intensity of the single impurity peak (2.13 ppm) indicates purity of >95%. The high resolution ²H spectrum of the product may be compared to that of d₂₂-decane (Figure II-3). The product shows a single ²H resonance in the methyl

FIGURE II-2

100 MHz Fourier transform ^1H NMR spectrum of synthetic $\text{CH}_2\text{D}(\text{CH}_2)_8\text{CH}_3$, with assignments and integration of results. Ratio of intensities of methylene protons to methyl protons is 3.15:1.

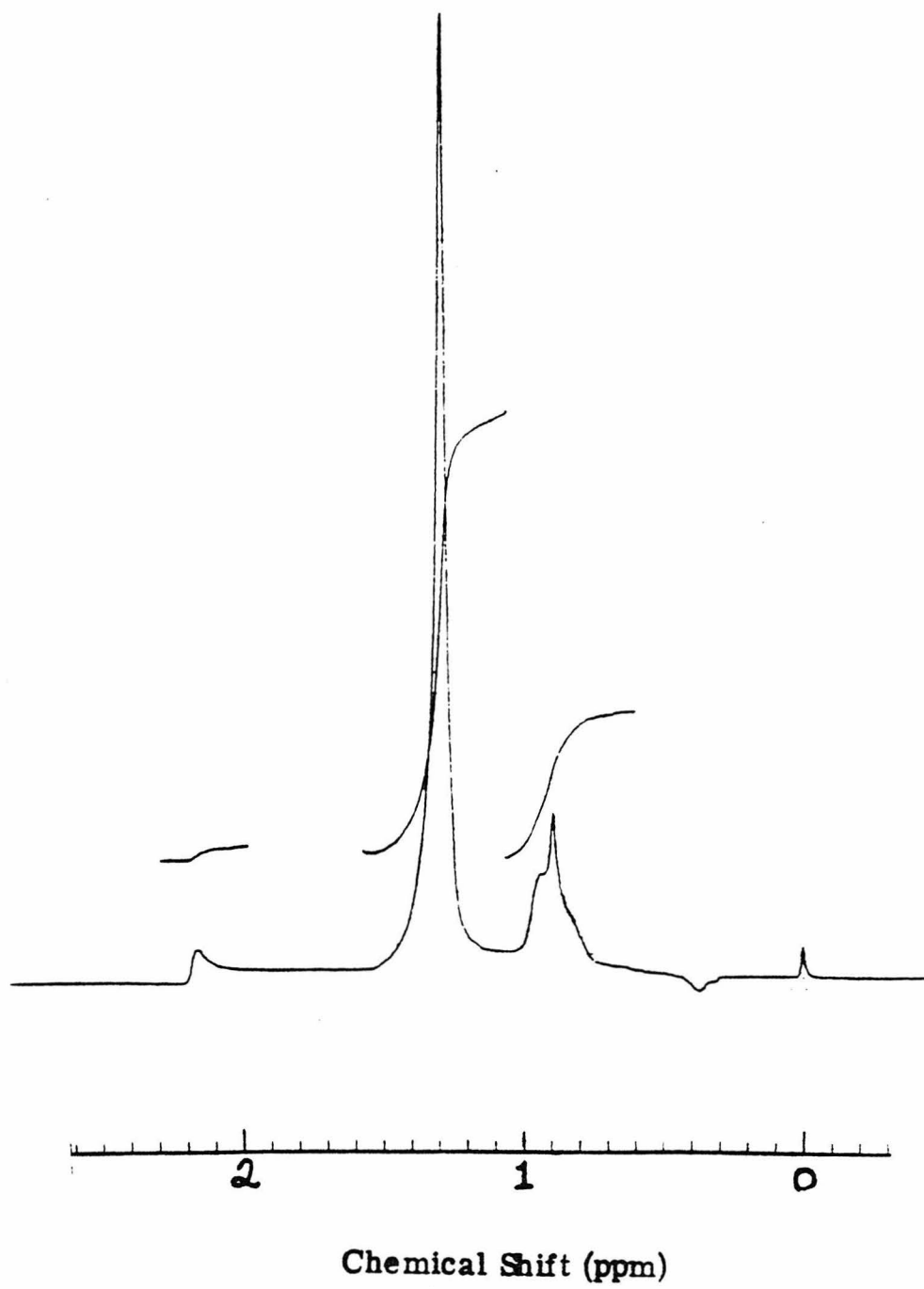
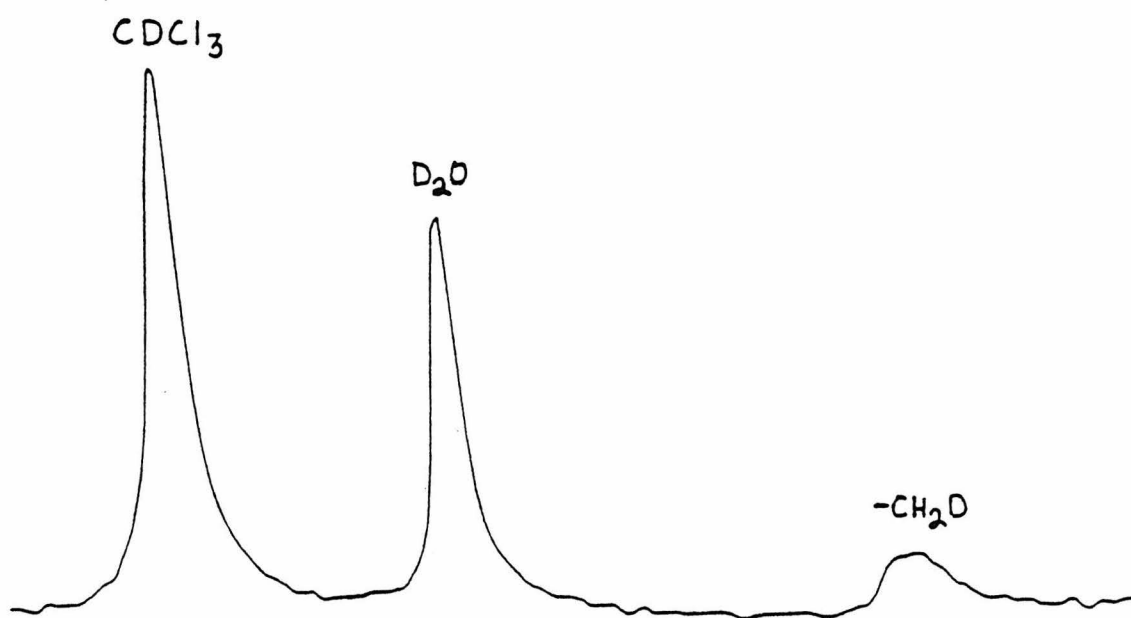


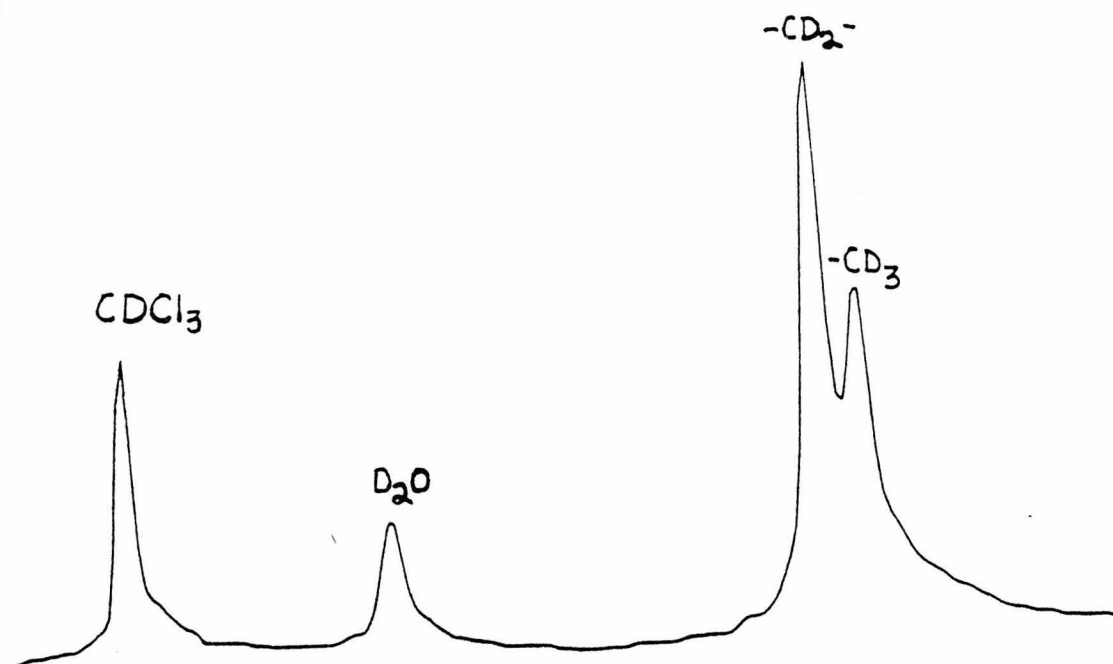
FIGURE II-3

9.21 MHz Fourier transform NMR spectra of (a) synthetic $\text{CH}_2\text{D}(\text{CH}_2)_8\text{CH}_3$ in CDCl_3 and (b) $\text{CD}_3(\text{CD}_2)_8\text{CD}_3$ (Merck, Sharp and Dohme) in CDCl_3 . Both samples were placed in a 5 mm NMR tube inserted as a coaxial capillary in a 15 mm NMR tube containing $\text{H}_2\text{O}/\text{D}_2\text{O}$.

(a)



(b)



1 ppm

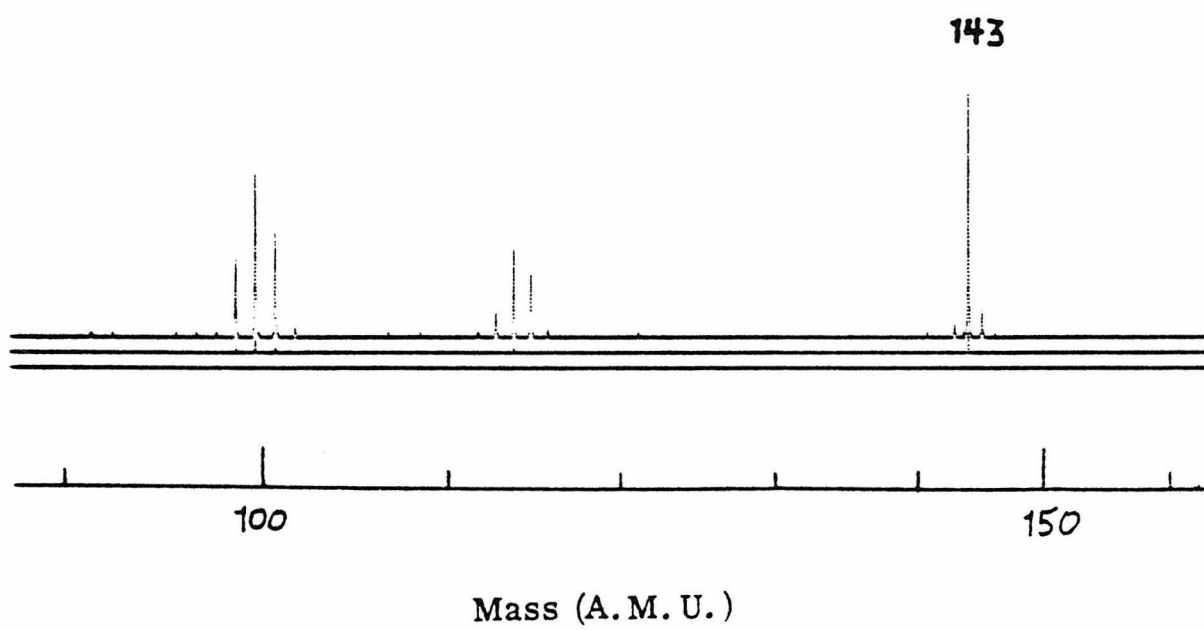
position, 6.40 ppm upfield from CDCl_3 . The mass spectrum of the product (Chemistry Division analytical laboratory) (Figure II-4) shows the predicted principal ion peak of mass 143 daltons. The peak at mass 142 may be assigned to $\text{CH}_3(\text{CH}_2)_8\text{CH}_3$. The relative intensities of these two peaks indicate that the product is approximately 95% deuterated.

2. Preparation of model membranes

Multilamellar dispersions of perdeuterated fatty acids in dipalmitoylphosphatidylcholine were prepared by dissolving the weighed, dry components (1 g DPPC + ~100 mg fatty acid) in CHCl_3 , evaporating the solvent under a stream of air, and evacuating the sample container to a pressure of 10 μm of mercury on a vacuum line. This procedure is designed to produce an intimate mixture of lipid and fatty acid. The dry mixture was placed in a 15 mm diameter flat-bottom NMR tube to which 1.5 ml H_2O was added. The contents of the tube were mixed lightly on a vortex mixer, a vortex plug was added to the sample tube, and the sample was incubated at 60-70°C, either in a water bath or directly in the NMR probe, for 1 hour before data accumulation was begun. Multilamellar dispersions of perdeuterated hydrocarbons in phospholipids were prepared by adding the hydrocarbon (20 - 100 μl) via a Hamilton microliter syringe to 1 g DPPC in an NMR tube. The contents of the tube were shaken and vortexed for several minutes

FIGURE II-4

Mass spectrum of synthetic $\text{CH}_2\text{D}(\text{CH}_2)_8\text{CH}_3$
in the region of the principal ion peak,
which appears at mass 143.



to insure thorough mixing and then the sample was hydrated exactly as described above. The sample of perdeuterated DMPC (~90 mg) was hydrated with ~1.5 ml H_2O in an ampoule sealed under N_2 , which was then placed in an NMR tube for recording of spectra.

3. Nuclear magnetic resonance spectra

Deuterium magnetic resonance spectra were recorded at 9.21 MHz on a wide line ^2H spectrometer operating in the Fourier transform mode. This instrument was assembled in our laboratory from a variety of commercial systems, commercial components, and home-built modules. Its construction is described in chapter 3 of this thesis. In the ^2H spectrometer, field frequency stabilization is provided by an external reference proton lock system. Wide-line spectra were recorded using quadrature phase detection and a solid-echo burst sequence ($90_x - \tau_{\text{echo}} - 90_y - \tau_{\text{echo}} - \text{data acquisition} - \text{recovery period} - \text{repeat}$). A phase-alternating burst pattern is employed to average phase and intensity differences between detector channels. The 90° burst was approximately 3.5 μsec in length and τ_{echo} was customarily 200 μsec . Transient data were filtered before digitization with a commercial filter having a Butterworth response, using a cutoff frequency 10 - 20% greater than one-half the data digitization rate. 10,000 to 200,000 free induction decays were accumulated per spectrum. After Fourier transformation zero and

first order phase corrections to the data were made so as to best approximate the ^2H quadrupolar powder pattern lineshape. For some of the spectra the spectrometer frequency was set exactly at the center of the powder pattern and only data from the 0° accumulated FID channel was used. The single-sided transform of this FID was reflected about zero frequency to produce the double-sided spectrum. This "spectrum reverse" method of handling the data, frequently employed in the literature (28), generates a plot with signal-to-noise improved by $\sqrt{2}$ and symmetry which is artificially perfect.

High resolution ^2H spectra were taken on the same instrument utilizing a simple free induction decay burst sequence (90°_x - acquire data - recover - repeat) and averaging on the order of 100 free induction decays per spectrum. Quadrature phase detection and the phase alternating pulse sequence were employed. High resolution samples were placed in a 5 mm NMR tube inserted as a coaxial capillary in a 15 mm tube. The larger tube contained approximately 1% D_2O in H_2O which provided a reference signal in the transformed spectrum.

High resolution ^1H spectra at 100 MHz were taken on a Varian XL-100 NMR spectrometer operating in the Fourier transform mode and employing a ^2H field/frequency lock using CDCl_3 as an internal reference standard.

4. Calorimetry

Calorimetry of lipid samples containing hydrocarbons

or fatty acids was performed on a Dupont model 900 Differential Thermal Analyzer (DTA). For the sample of hexadecanoic acid in DPPC, approximately 1 mg of the dry lipid and fatty acid mixture prepared for ^2H NMR was placed in a sample capillary, about 1 microliter of water was added, and the capillary was sealed and incubated for 1 hour at 60°C to hydrate the sample for DTA. For the hydrocarbon in DPL samples a portion of the NMR sample was removed from the NMR sample tube following the recording of spectra and placed in a DTA capillary for calorimetric investigation.

5. SIMULATIONS

The ^2H NMR powder pattern spectra were simulated using equation (II-12) on a Digital Equipment Corporation PDP-11 computer equipped with a dual floppy disk system and Fortran operating system from Advanced Electronic Design. Simulated spectra were plotted by a Hewlett-Packard x-y recorder interfaced to the PDP-11 by a digital interface from SLIP Systems, Inc.

C. Results

We have obtained ^2H spectra of multilamellar dispersions of dipalmitoylphosphatidylcholine (DPPC) containing perdeuterated hydrocarbons and fatty acids of ten and sixteen carbon chain length. For reference we have also examined multilamellar dispersions of dimyristoylphosphatidylcholine (DMPC) with perdeuterated

fatty acid chains. All systems were studied as a function of temperature at temperatures above the gel to liquid crystalline phase transition temperature. Hydrocarbon-containing multilayers were examined at several hydrocarbon concentrations. In addition, as an aid in assigning spectral components of different quadrupolar splitting we have studied the system 1-d₁-decane in DPPC. Finally we have examined d₂₂-decane in dioleoylphosphatylcholine (DOPC).

We consider the results beginning with the spectra of a multilamellar dispersion of chain-perdeuterated DMPC. Both specifically deuterated and perdeuterated lipids have been extensively studied in the literature (22,29,30) so this system allows us to assess the quality of our preparation and the performance of our spectrometer, as well as to point out the general features of ²H spectra of perdeuterated molecules in multilamellar dispersions. Next we look at perdeuterated palmitic acid in DPPC. This system, also studied in the literature (24) is similar to pure perdeuterated lipid in terms of information available from quadrupolar splittings, but shows important differences in quantitative details. To consider effects of shorter chain length we will compare this system to perdeuterated decanoic acid in a DPPC multilamellar dispersion. Then, with all of these systems as background, we examine perdeuterated hydrocarbons in DPPC.

1. d₅₄-Dimyristoylphosphatidylcholine

²H spectra of DMPC perdeuterated in the hydrocarbon chains are shown in Figure II-5 for a series of temperatures above T_c (18°C for d₅₄-DMPC). The superposition of quadrupolar powder patterns from various chain positions produces the central order parameter plateau characteristic of perdeuterated lipid dispersions at temperatures above T_c. Baselines are relatively flat and in the 35°C spectrum the 0° orientation shoulder is clearly visible. We resolve ten distinct quadrupolar pairs, as well as a central peak at $\nu=0$. Splittings $\Delta\nu_Q$ (kHz) for each pair are plotted as a function of temperature in Figure II-6. The gradual decrease in splitting with increasing temperature indicates a gradual decrease in order with temperature. The central peak has contributions from natural abundance deuterium in water and from any isotropically reorienting lipid, e.g., lipid in micelles. The relative intensity of this type of lipid is a small percentage of the total lipid in the spectrum. Individual splittings may be assigned to specific chain positions by comparison with data for specifically deuterated DPPC (22) in analogy with the procedure employed by Davis (29), in his study of perdeuterated DPPC. The tentative assignments are also shown in Figure II-6. Near the methyl end of the chain the two fatty acid chains show different splittings for the same position along the chain, with chain b slightly

FIGURE II-5

9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{54} -DMPC as a function of temperature ($^{\circ}\text{C}$), obtained with a solid-echo burst sequence. The double-sided spectrum is generated by pulsing on resonance and then folding back the single-sided transform from a single accumulated free induction decay (spectrum reverse presentation).

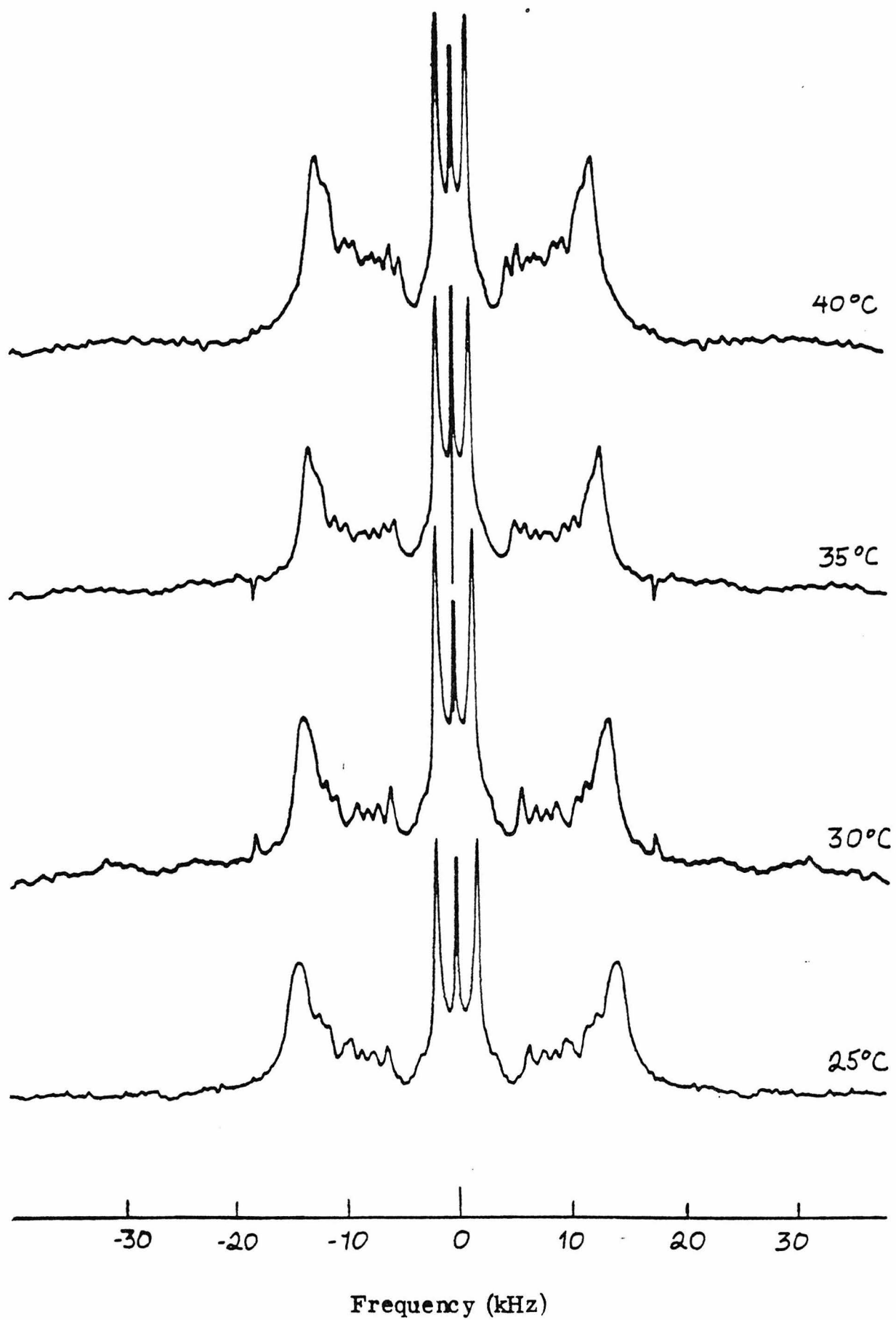
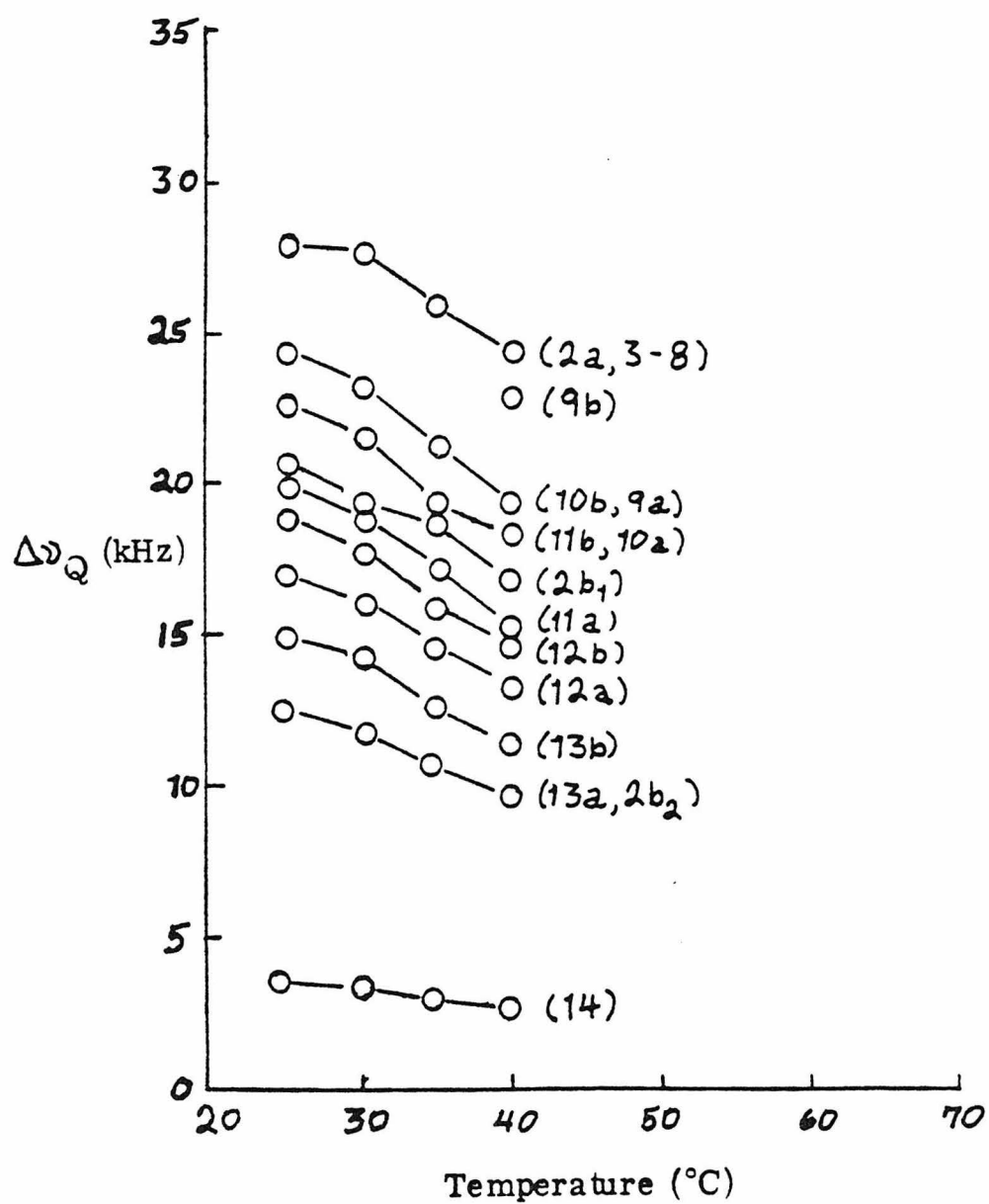


FIGURE II-6

Observed quadrupolar splittings $\Delta\nu_Q$ (kHz) as a function of temperature ($^{\circ}\text{C}$) for a multilamellar dispersion of d_{54} -DMPC. Tentative assignments are indicated in parentheses, where the numbers refer to the position of the deuteron along the hydrocarbon chain and the letters to the individual hydrocarbon chain, which give rise to different splittings for some positions along the chain.



more ordered than chain a. At position two not only do we resolve the two chains but also two inequivalent deuterons on chain b. The assignment of deuteron $2b_1$ is based primarily on the decreased intensity of this component in the spectrum relative to its neighbors. In Figure II-7 we plot the quadrupolar splitting versus chain position for chain-deuterated DML. At 25°C the upper portion of the chain (position 2-8) shows a nearly constant splitting of 27.7 kHz corresponding to an S_β of -0.22 for a temperature 7° above T_c . The order parameter falls off for position 9-14, reading 0.09 for the methylene deuterons at position 13. The three splittings assigned to position 2 (11.5 kHz, 20.8 kHz, 25.7 kHz) agree well with splittings measured from specifically deuterated DPPC at 50°C, 9° above T_c (12.0, 19.2, 27.8 kHz). The shape of the plot in Figure II-7 is comparable to the order parameter profile obtained by Davis for perdeuterated DPPC at 45°C (7° above T_c), Figure II-8. At positions equidistant from the methyl terminus of the chain, the DMPC has an order parameter 10 to 25% larger than for DPPC.

2. d_{31} -Palmitic acid in DPPC

The ^2H NMR spectrum of a multilamellar dispersion of 16.7 mole % perdeuterated palmitic acid in DPPC at 60°C is shown in Figure II-9. Once again the order parameter plateau characteristic of a gradation in order parameter is observed and the 0° edges are clearly

FIGURE II-7

$\Delta\nu_Q$ (kHz) versus hydrocarbon chain position
for a multilamellar dispersion of d_{54} -DMPC
at 25°C.

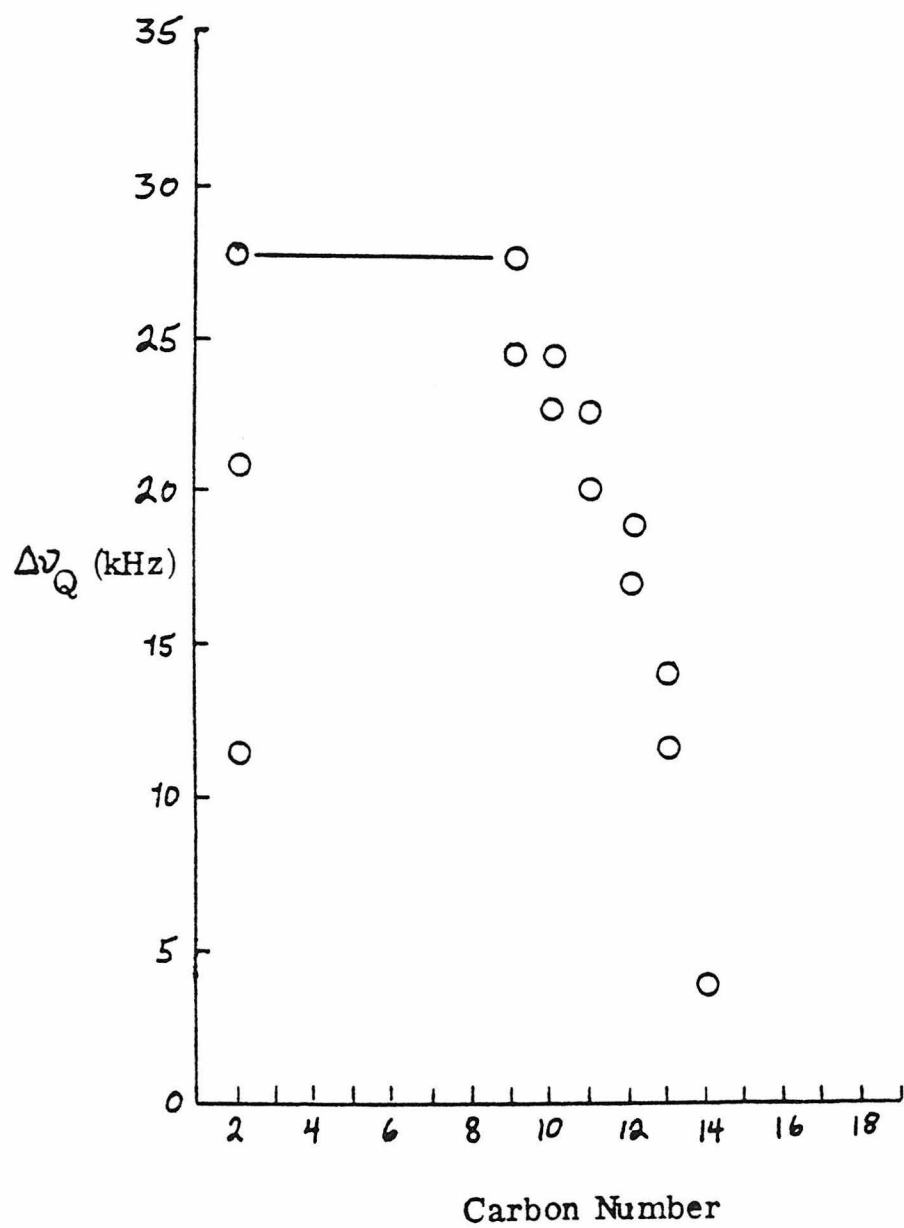


FIGURE II-8

$\Delta\nu_Q$ (kHz) versus hydrocarbon chain position
for a multilamellar dispersion of d_{62} -DPPC
at 45°C. Data are taken from the work of
Davis (29).

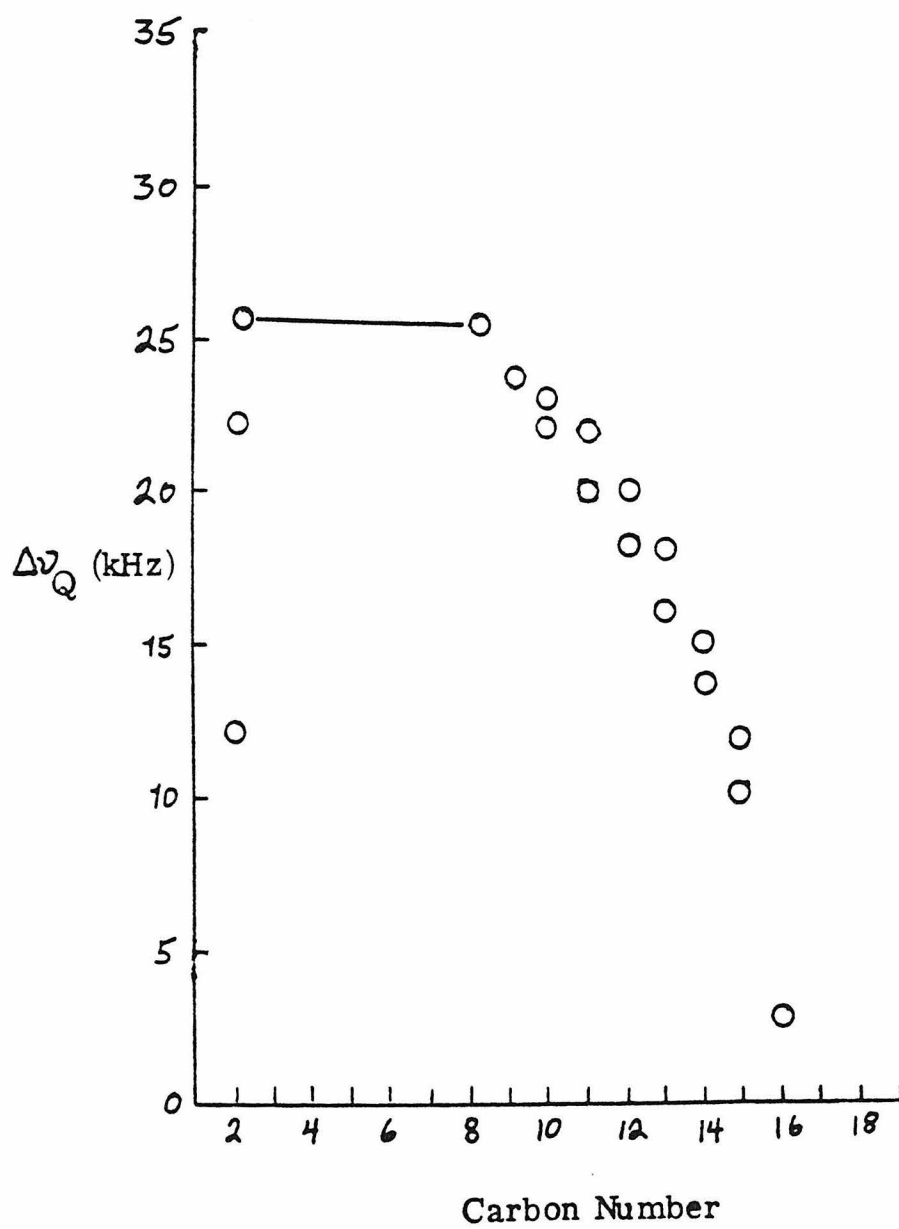
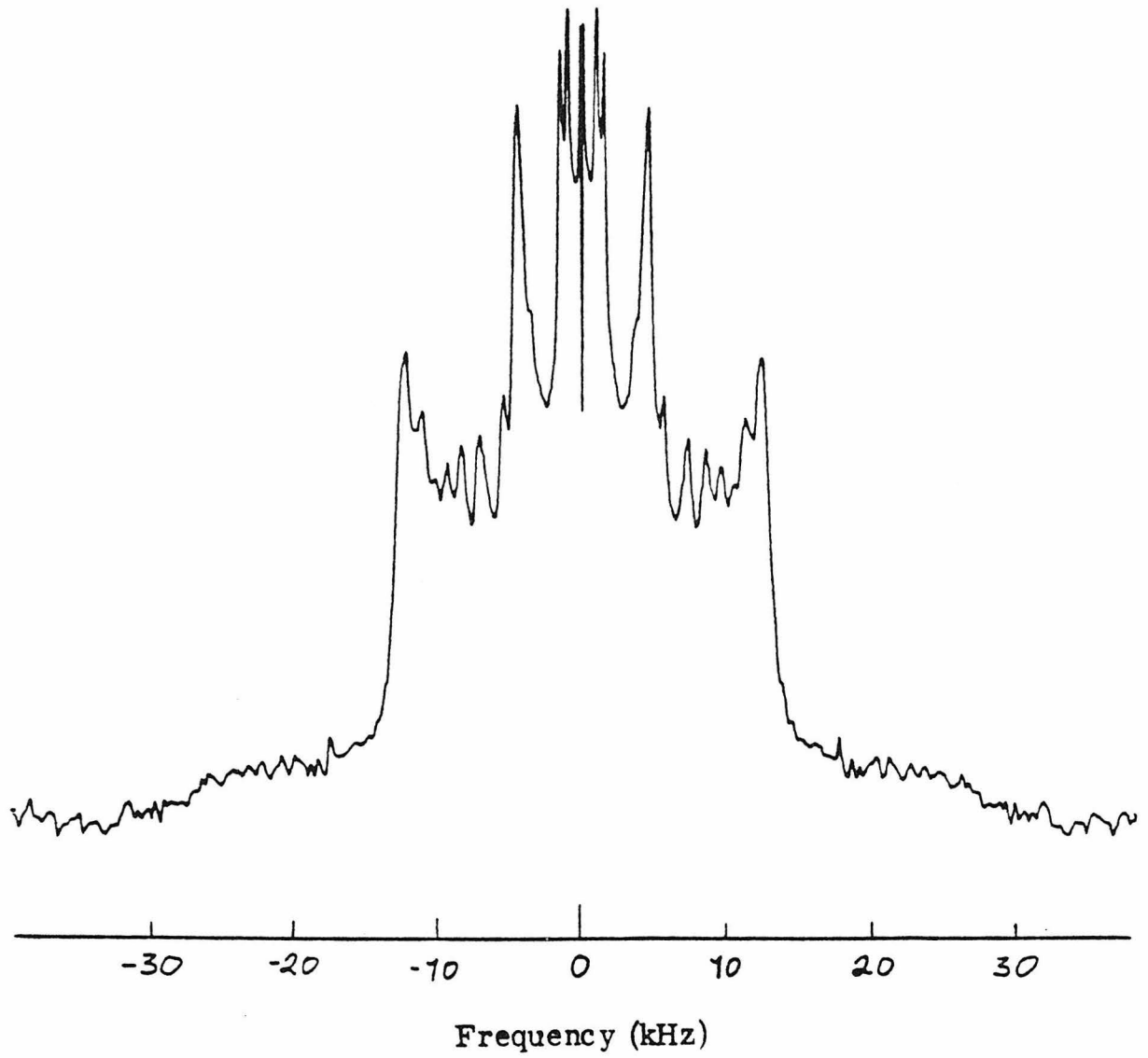


FIGURE II-9

9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{31} -palmitic acid (16.7 mole %) in DPPC at 60°C, obtained using a solid-echo burst sequence and spectrum reverse presentation. Additional splittings are present due to a deuterated contaminant in the fatty acid (see text).



visible. The greater height of the plateau for this spectrum in comparison to those for the deuterated lipid indicates better adjustment of the spectrometer transmitter and receiver phases in the present case. Eleven quadrupolar splittings may be distinguished; these are plotted at two temperatures in Figure II-10. As indicated above, the d_{31} -palmitic acid used in this experiment contains some contaminants; the appearance of a splitting in the narrowest quadrupolar pair, assignable to the methyl group, indicates that the contaminant is also deuterated. The uncharacteristically intense quadrupolar pair at a splitting of 9.5 kHz evidently corresponds to the bulk methylenes of the contaminant. The two splittings assignable to contaminant are distinguished in Figure II-10 by filled circles; the methyl peak assigned to the contaminant is the one of smaller intensity. The remaining 9 splittings may be assigned to individual chain positions of the fatty acid. The assignments are straightforward compared to the lipid case since there is only one kind of hydrocarbon chain here. The order parameter profile for palmitic acid in DPL is shown in Figure II-11. These data may be compared to those of Stockton et al. for specifically deuterated stearic acid in egg yolk lecithin, plotted in Figure II-12. The two profiles are in substantial agreement. Comparison of the fatty acid profile with that for pure lipid (Figures II-7 and II-8) indicates that the fatty acid provides qualitatively the

FIGURE II-10

Observed quadrupolar splittings $\Delta\nu_Q$ (kHz) as a function of temperature ($^{\circ}\text{C}$) for a multilamellar dispersion of d_{31} -palmitic acid (16.7 mole %) in DPPC. Splittings indicated by filled circles may be assigned to a deuterated impurity in the fatty acid (see text).

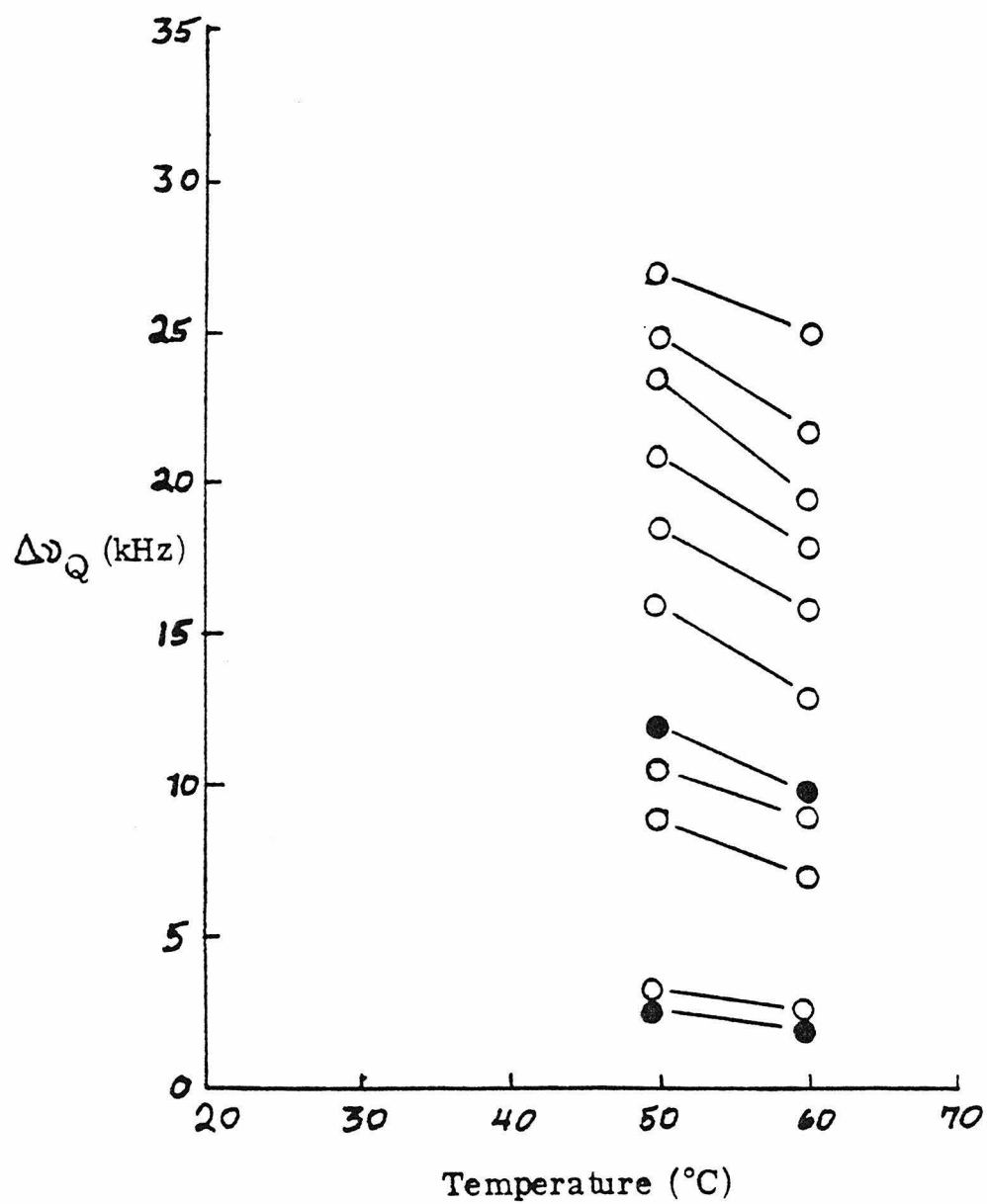


FIGURE II-11

$\Delta\nu_Q$ (kHz) versus hydrocarbon chain position
for a multilamellar dispersion of d_{31} -palmitic
acid (16.7 mole %) in DPPC at 60°C.

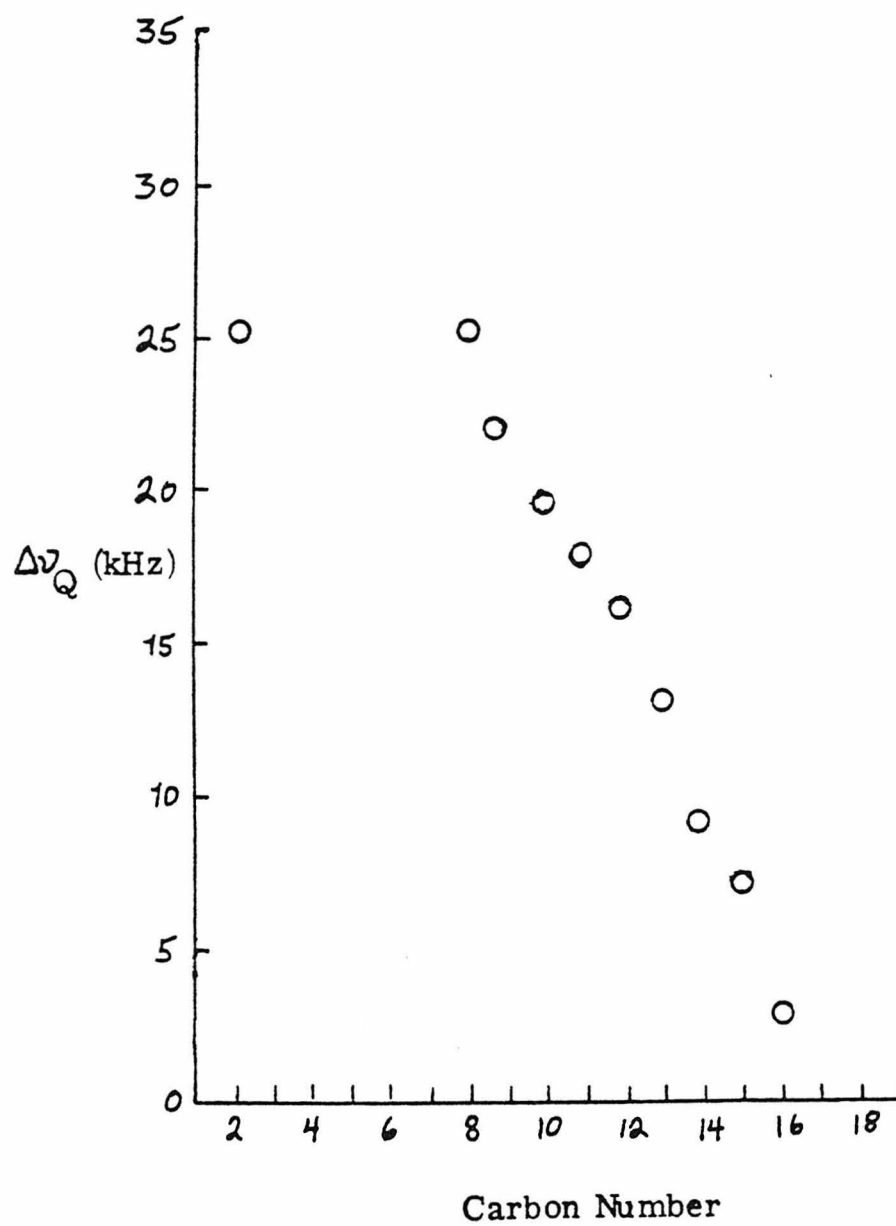
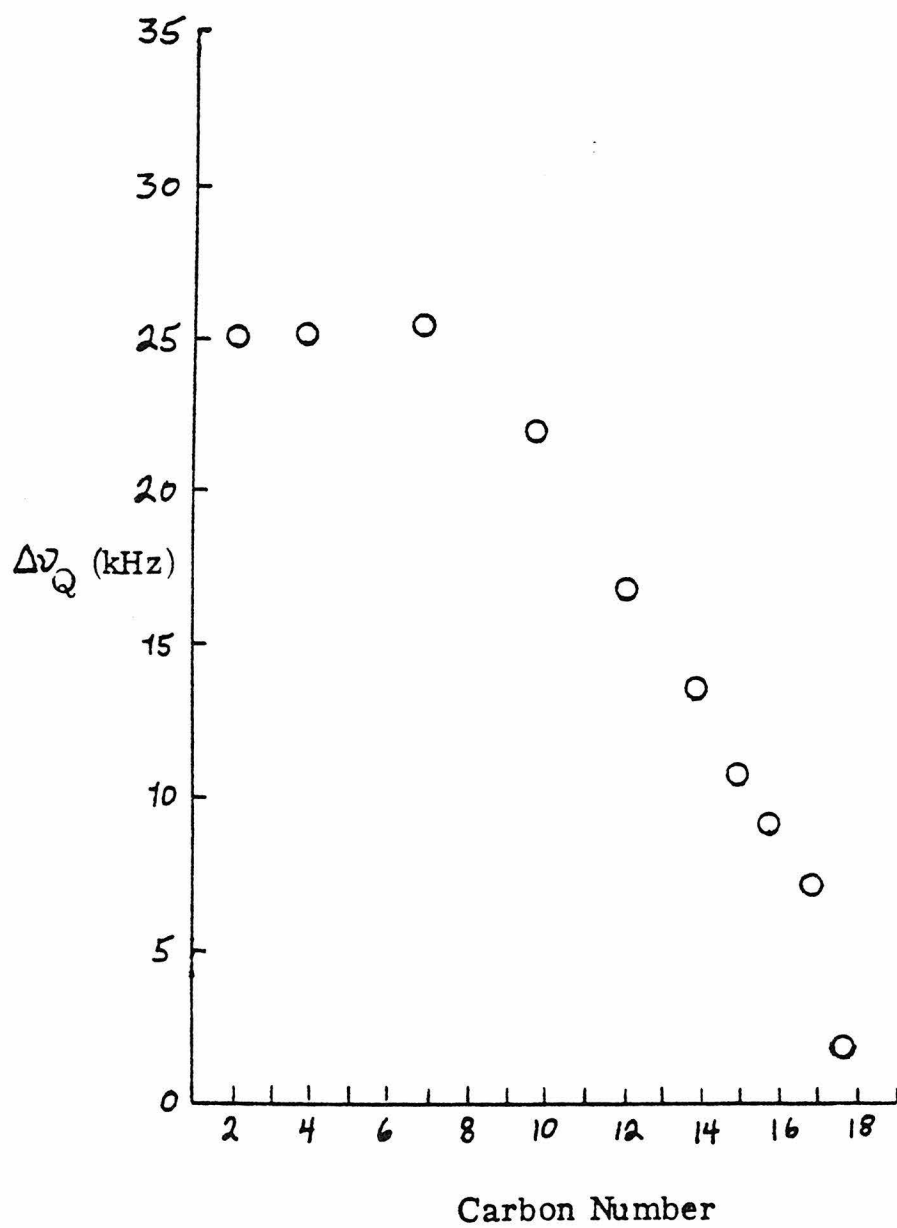


FIGURE II-12

$\Delta\nu_Q$ (kHz) versus hydrocarbon chain position
for multilamellar dispersions of specifically
deuterated stearic acid in egg yolk lecithin.
Data are taken from the work of Stockton,
et al. (24).



same information regarding order parameters as may be obtained from perdeuterated lipids, which are costlier and more difficult to prepare. However, there are quantitative differences between the two profiles, particularly near the methyl end of the chain, where the splitting for the fatty acid may be as much as 40% smaller than the splitting for the lipid at the equivalent position. Finally, there is once again a small central peak due to natural abundance deuterium in water and some fatty acid in an isotropic motional state.

3. d_{19} -Decanoic acid in DPPC

We turn now to systems not yet investigated in the literature. As a bridge between the previous system and data for decane-containing DPPC multilayers we consider DPPC multilayers containing perdeuterated decanoic acid. Figure II-13 illustrates the ^2H spectrum of d_{19} -decanoic acid (12.3 mole %) in DPPC at 50°C. Once again the order-parameter plateau is observed but now we resolve only five individual quadrupolar pairs. Note that this spectrum is presented in the standard quadrature phase detection manner, rather than by the "spectrum reverse" technique used in the spectra already presented. Thus it shows some asymmetry and reduced signal-to-noise. Nevertheless the various components of the spectrum are readily identified. The identifiable splittings are plotted as a function of temperature in Figure II-14. Note that in comparison to the d_{31} -palmitic

FIGURE II-13

9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{19} -decanoic acid (12.3 mole %) in DPPC at 50°C, obtained using a solid-echo burst sequence, and displaying the full quadrature spectrum.

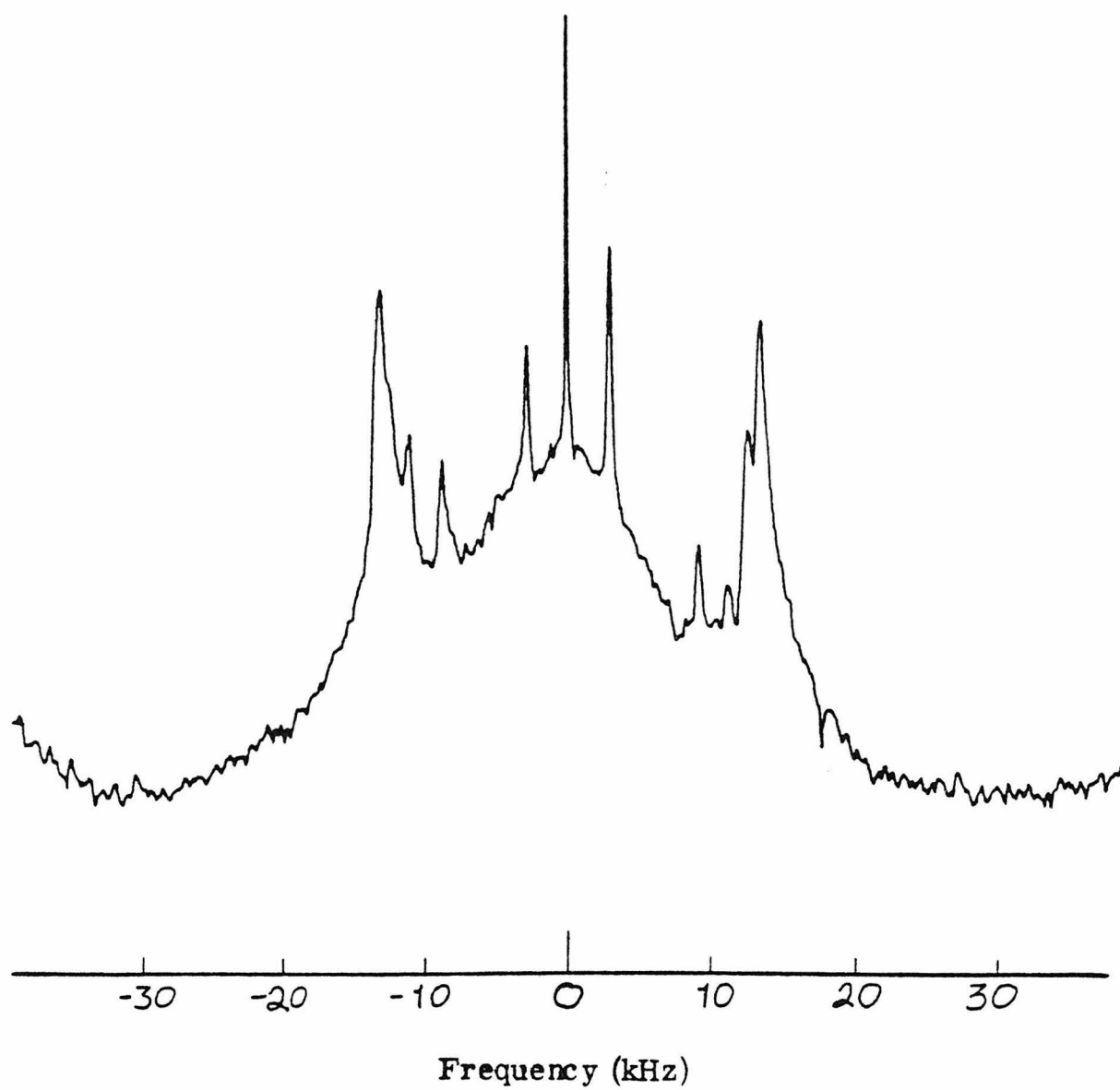
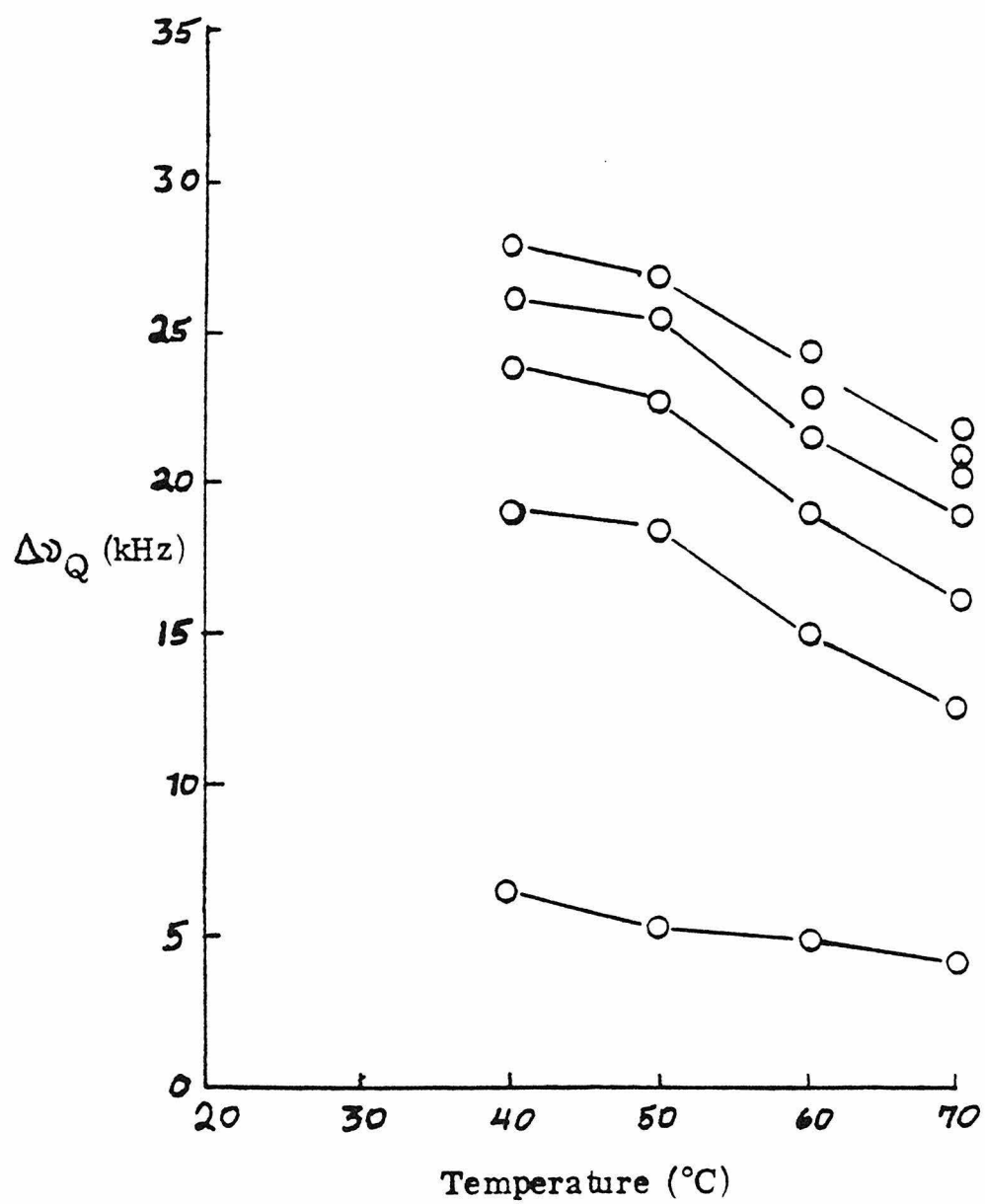


FIGURE II-14

Observed quadrupolar splittings $\Delta\nu_Q$ (kHz)
as a function of temperature ($^{\circ}\text{C}$) for a
multilamellar dispersion of decanoic
acid (12.3 mole %) in DPPC.



acid (Figure II-10), there are fewer splittings at values below 15 kHz. The temperature profile for individual splittings is substantially the same as for other systems. The central maximum is proportionally larger in this set of spectra, as illustrated in II-13. This may indicate an increased tendency toward micellization in this system. The relative size of the central peak and the wings did not appear to change substantially when the sample temperature was cycled from 60°C to 35°C or less several times over a 12-day period.

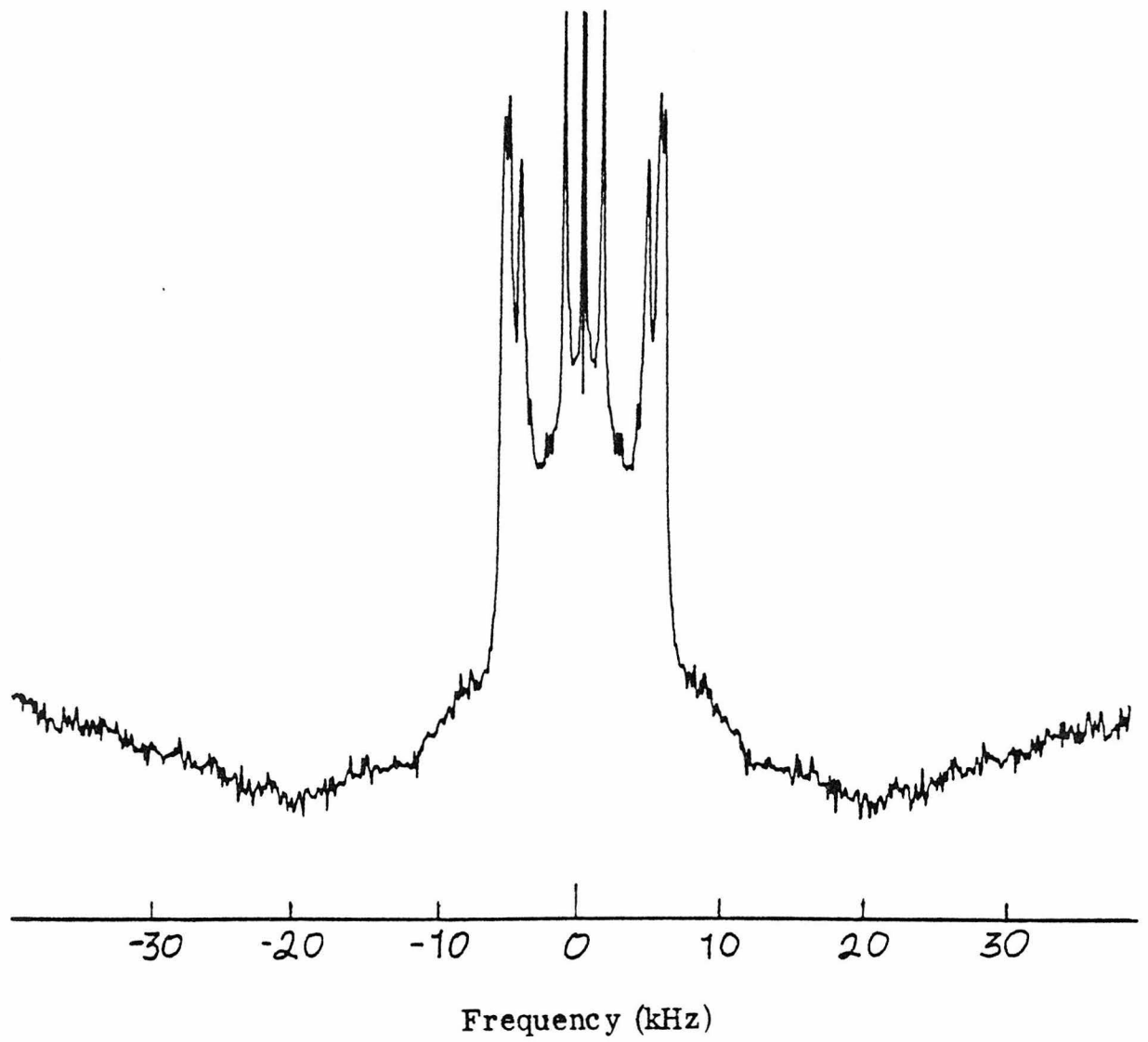
4. d₂₂-Decane in DPPC

Finally we examine DPPC multilayers containing straight-chain hydrocarbons. We begin by considering decane containing dispersions. Decane was one of the most popular solvents for the preparation of black lipid films (BLMs) when these systems were first prepared as bilayer models. Decane is known to be more soluble in the bilayer portion of BLMs than longer hydrocarbons (12).

Figure II-15 shows the ²H magnetic resonance spectrum of DPPC multilayers containing 10.3 mole % perdeuterated decane at 47°C. As in the case of deuterated fatty acids and lipids we see an order parameter plateau indicative of a flexibility gradient along the hydrocarbon chain. The lineshape is in excellent agreement with the theoretical powder pattern for restricted or anisotropic motion, and the 0° edges for both the narrowest quadrupolar pair and the widest pair are clearly

FIGURE II-15

9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{22} -decane (10.3 mole %) in DPPC at 47°C, obtained using a solid-echo burst sequence and spectrum reverse presentation.



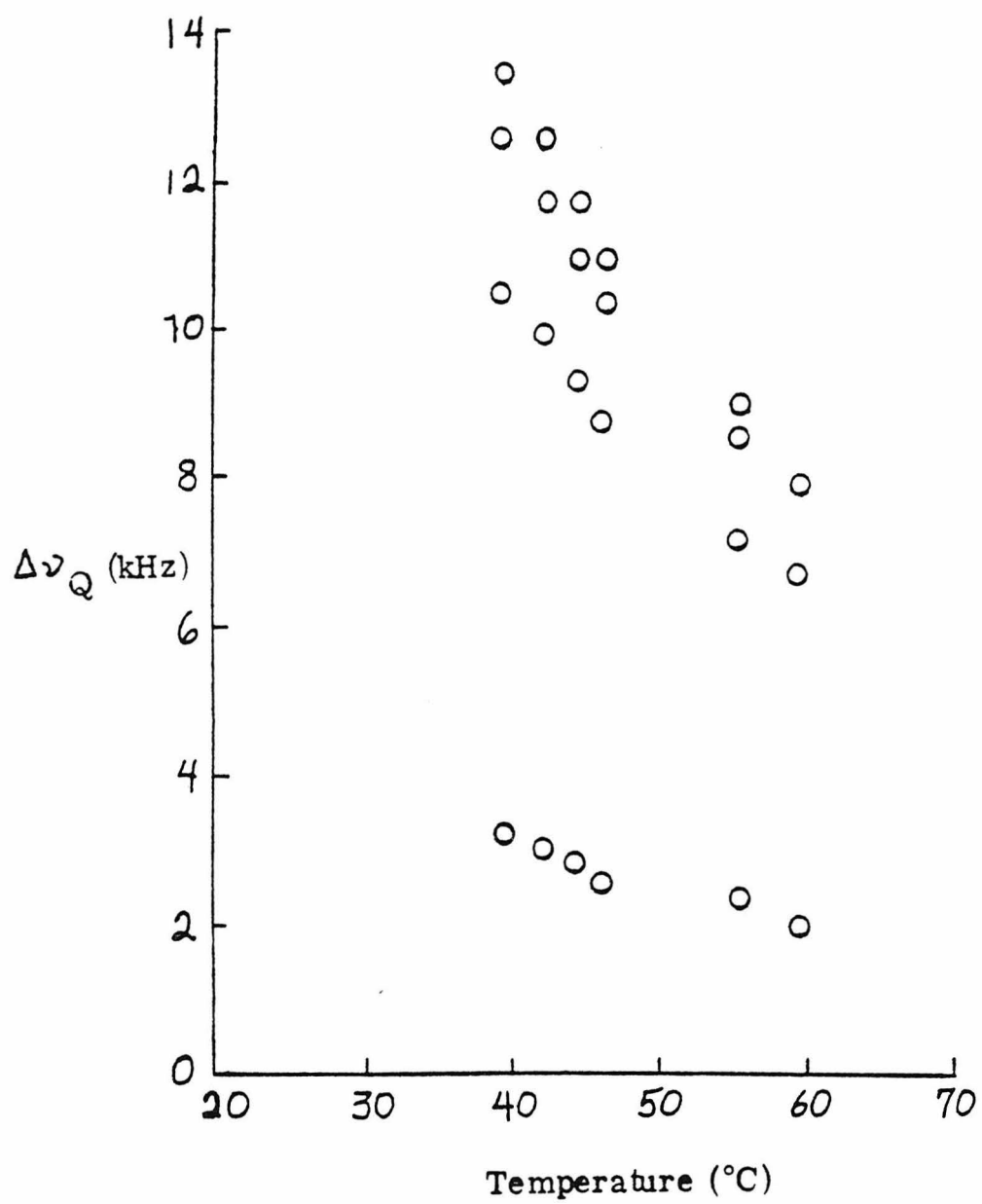
observed. We resolve a total of four quadrupolar pairs. There is a slight baseline roll indicative of a small error in either spectrometer phasing or burst sequence timing. Finally, a small central maximum with integrated intensity only a few percent of the total spectrum intensity is observed.

We note also several obvious differences between this spectrum and those observed previously. First, the largest splitting in this spectrum is only 10.9 kHz, compared to about 27 kHz for C_{10} fatty acid at 47°C; thus the maximum splitting is reduced by a factor of between two and three over spectra observed previously. Second, there is a large difference between the magnitude of the smallest splitting and the size of the other three splittings, which are similar in magnitude but not equal. In this regard the hydrocarbon spectrum is most like that for decanoic acid of the spectra we have already studied. In the present case the division into two sets of splittings is even more pronounced. Third, although there are potentially ten inequivalent kinds of deuterons corresponding to ten carbon atom positions only four pairs are resolved. (At higher temperatures we resolve only three pairs.) In the case of perdeuterated palmitic acid in DPPC and perdeuterated DMPC half the individual deuteron positions are resolved.

The temperature dependence of the observed quadrupolar splittings is illustrated in Figure II-16. The

FIGURE II-16

Observed quadrupolar splittings $\Delta\nu_Q$ (kHz)
as a function of temperature ($^{\circ}\text{C}$) for a
multilamellar dispersion of d_{22} -decane
(10.3 mole %) in DPPC.



same increase in splitting with decreasing temperature is seen here as is observed for the systems discussed earlier, but the magnitude of the effect is larger. For decane the largest splitting changes by 34% per 10°C drop in temperature; for decanoic acid the rate is 8% per 10°C. The ratios of the quadrupolar splittings of the various pairs do not change substantially with temperature.

We have also studied the DPPC-decane system as a function of concentration of decane. Figure II-17 illustrates the concentration dependence of the three splittings observed at 60°C. There is evidently a decrease in order with increasing concentration of the hydrocarbon. In the absence of data near 20 mole % decane it is impossible to say whether this decrease occurs smoothly as a function of concentration or begins more abruptly at a threshold concentration. The single point available at 18.2 mole % suggests the latter but is inconclusive. Figure II-18, the ^2H NMR spectrum of 26.7 mole % decane in DPPC, illustrates another important effect on the spectrum which results from increasing the hydrocarbon concentration. (Note that the quality of this spectrum is reduced due to the use of a straight free induction decay (90° - collect data - recover) sequence as opposed to the solid-echo sequence used in the other spectra displayed thus far). Notice that while the same spectral features are present here as are seen

FIGURE II-17

Observed quadrupolar splittings $\Delta\nu_Q$ (kHz)
as a function of concentration (mole %
hydrocarbon in DPPC) for multilamellar
dispersion of d_{22} -decane in DPPC.

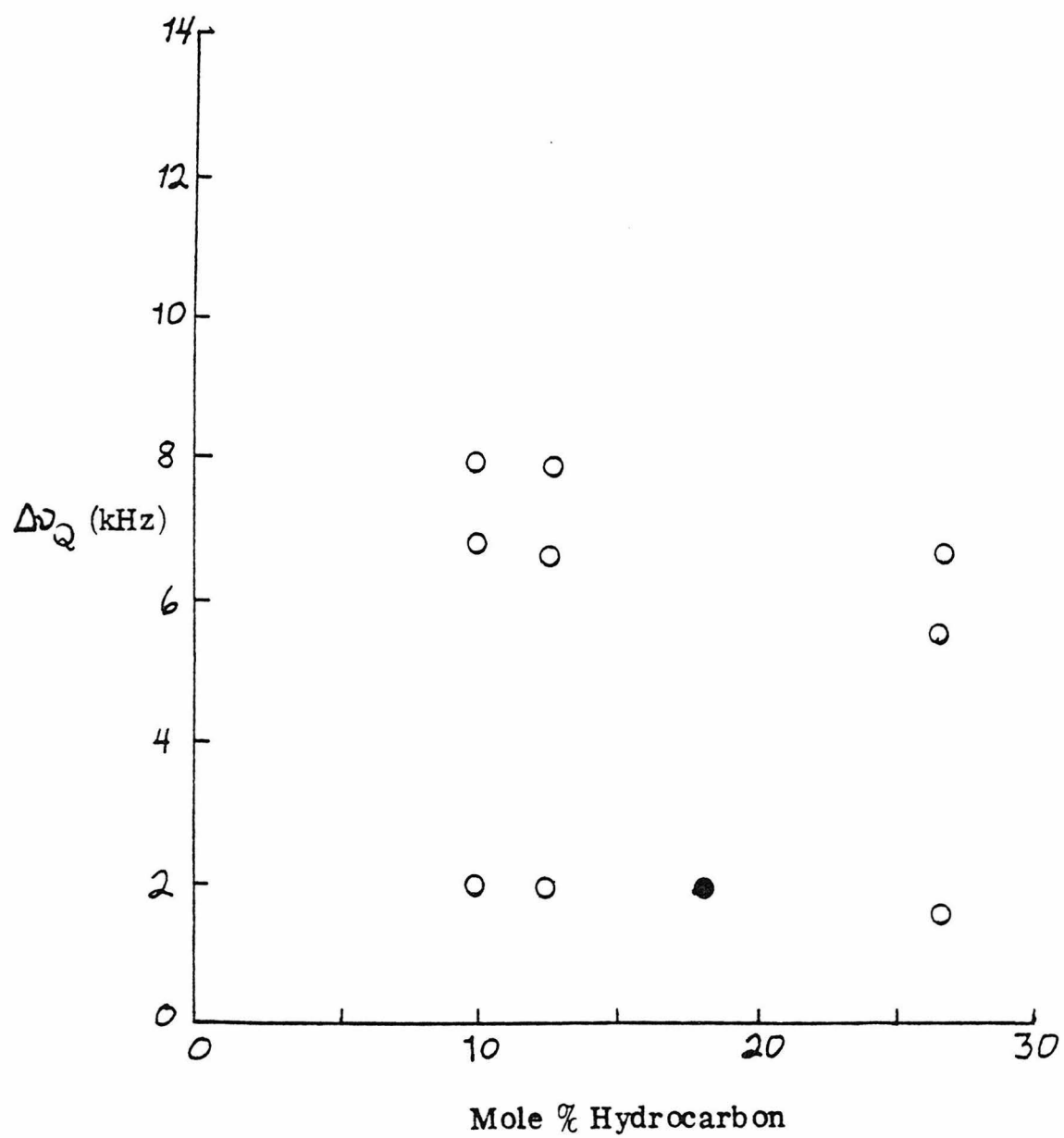
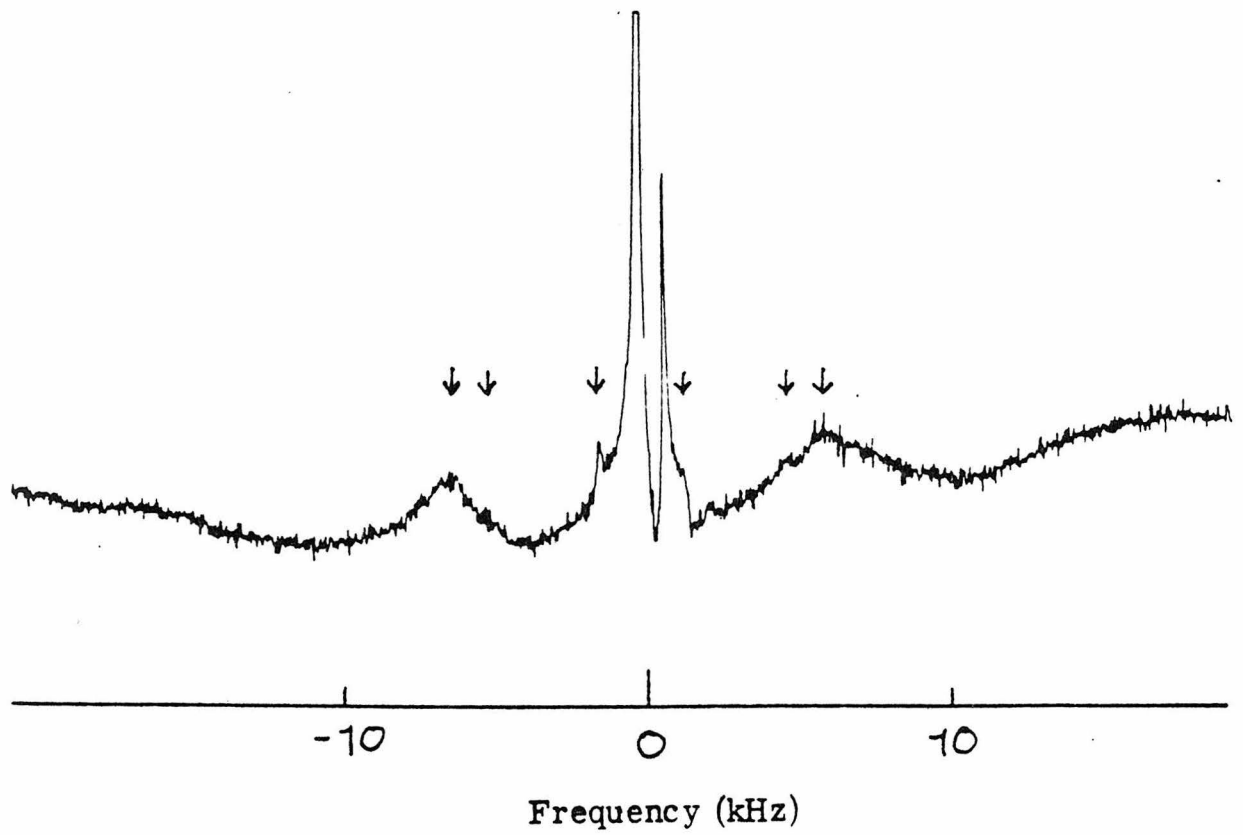


FIGURE II-18

9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{22} -decane (26.7 mole %) in DPPC at 35°C, using a simple free induction decay burst sequence and presenting the full quadrature spectrum.

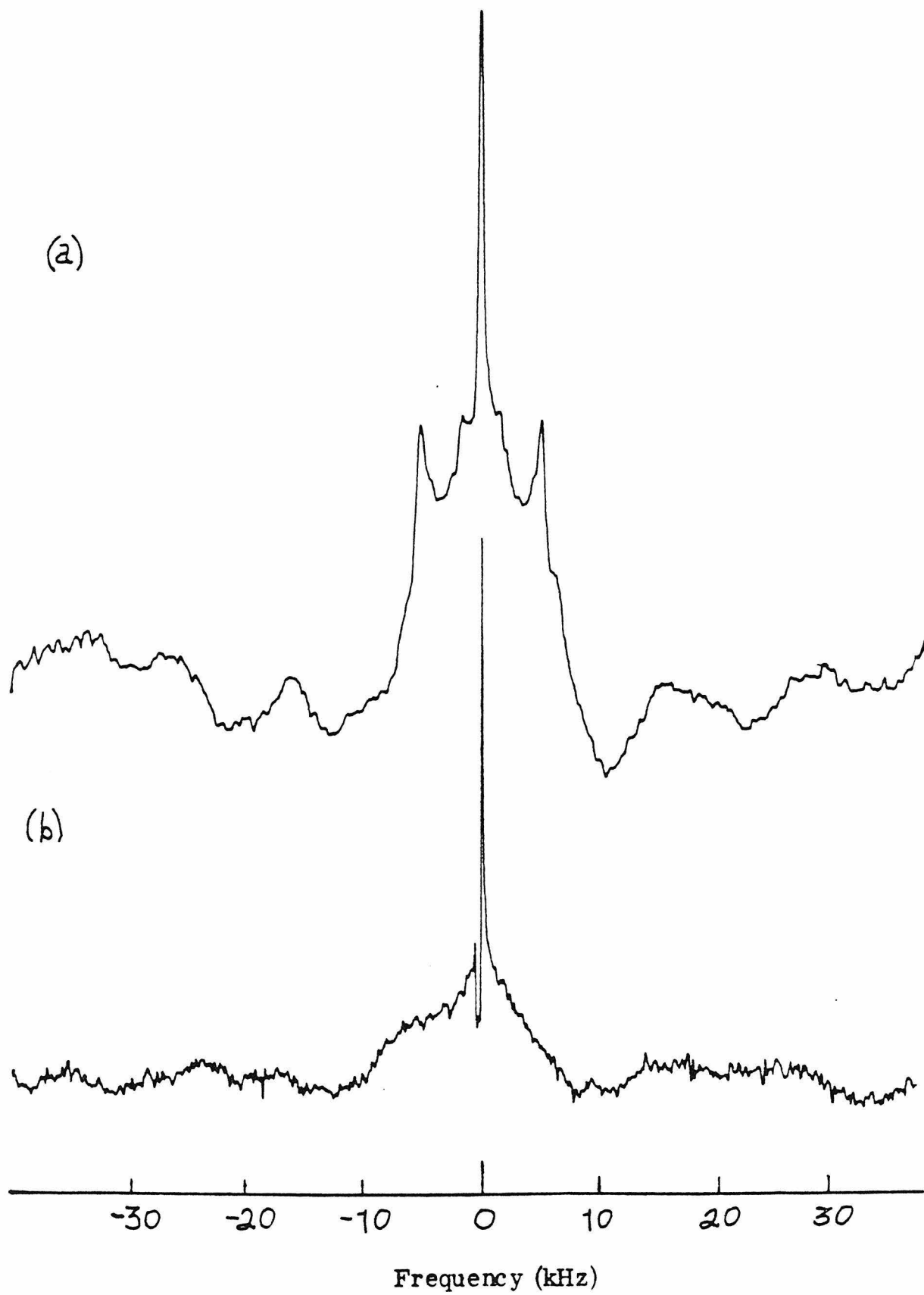


at lower decane concentration, namely three resolved quadrupolar splittings and a central line, the intensity of the central line is relatively much larger at higher decane concentration. The linewidth of this central line is approximately 100 Hz.

The relative magnitude of the central line depends not only on the concentration of the hydrocarbon in the multilamellar dispersion but also on the temperature history of the sample. For all of the samples hydration was carried out by incubating at 60°C for an hour or more. Spectra were then taken as a function of temperature beginning at 60°C and working down in temperature to a temperature at or below T_c . In most cases samples were then reheated for a repeat spectrum at 60°C. Since each spectrum takes from 8 to 24 hours to run, several days elapsed between the initial and final 60° spectrum. In the case of the 10.3% decane sample the minimum temperature was 36°C; at this temperature the order parameter plateau losses its square shape and the individual components are less distinct, though still visible (Figure II-19a). When reheated the sample showed a spectrum essentially identical to the initial one. The 12.8% decane sample was also cooled to 40°C and reheated with no evident effect on the spectrum. It was then cooled to 22°C, where the spectrum (Figure II-19b) shows a central line superposed on a broad one. Upon reheating the spectrum consists of a single line

FIGURE II-19

9.21 MHz Fourier transform spectra of (a) a multilamellar dispersion of d_{22} -decane (10.3 mole %) in DPPC at 36°C (solid-echo, full quadrature presentation) and (b) a multilamellar dispersion of d_{22} -decane (12.8 mole %) in DPPC at 22°C (solid-echo, full quadrature presentation).



at the center frequency position with a linewidth on the order of 100 Hz. Thus cooling the sample well into the gel phase temperature region causes the decane signal to coalesce irreversibly; this effect has been observed for other decane samples but is not observed for fatty acid-containing samples.

5. d_{22} -Decane in DPPC

Because black lipid films are commonly formed from glycerol monooleate or dioleoylphosphatidylcholine (DOPC) we investigated the system consisting of DOPC multilayers containing perdeuterated decane, to see whether the presence of a double bond roughly halfway up the lipid hydrocarbon chains had any effect on the ^2H spectrum. As illustrated in Figure II-20, at 50°C and 18.5 mole% decane we resolve only two splittings and a central maximum. The magnitude of the splittings is small but increases with decreasing temperature as illustrated in the temperature profile in Figure II-21. At room temperature four splittings are resolved, in ratios reminiscent of decane in DPPC. Even at 22°C the system is still about 44°C above T_c (-22°C for pure DOPC), so it is hardly surprising that the splittings are still smaller in magnitude than for decane (26.7 mole %, Figure II-17) at 60°C, 18° above T_c for pure DPPC. Extrapolation of the decane-DOPC data to lower temperatures suggests that for temperatures an equal number of degrees above T_c the decane-DOPC and decane-DPPC systems give

FIGURE II-20

9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{22} -decane (18.5 mole %) in DOPC at 50°C, obtained using a solid-echo burst sequence and presenting the full quadrature spectrum.

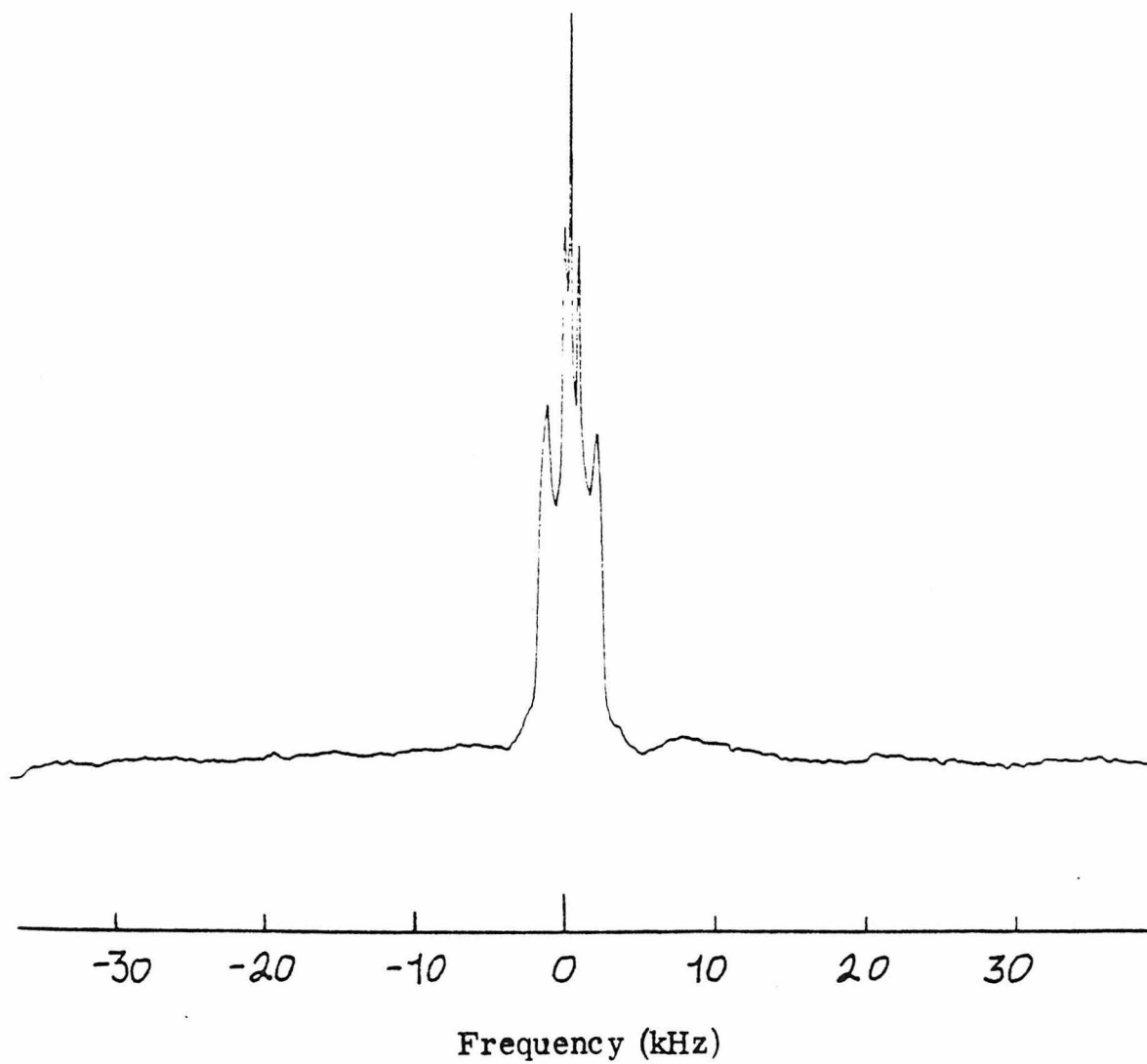
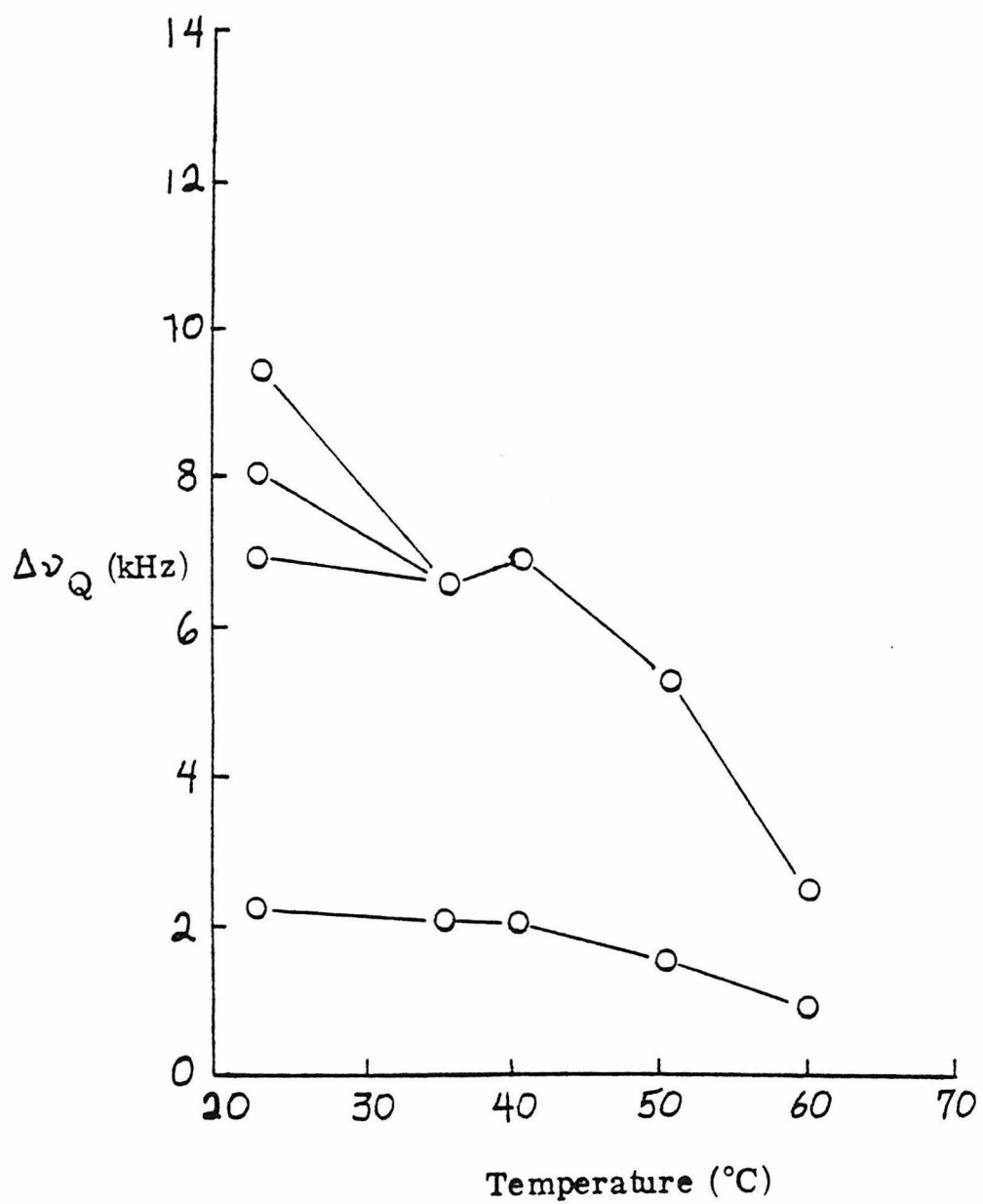


FIGURE II-21

Observed quadrupolar splittings $\Delta\nu_Q$ (kHz)
as a function of temperature ($^{\circ}\text{C}$) for a
multilamellar dispersion of d_{22} -decane
(18.5 mole %) in DOPC.



essentially equivalent results. Also, the relative magnitude of the central peak for decane in DOPC is intermediate between the 10.3 mole % (Figure II-15) and 26.7 mole % (Figure II-18) decane in DPPC cases.

6. d₁-Decane in DPPC

As an aid in assigning the various components of the d₂₂-decane in DPPC spectrum we have prepared multilamellar dispersions of DPPC containing decane labelled with a single deuteron in one of the two methyl groups. The ²H spectrum of 1-d₁-decane (18.2 mole %) in DPPC is shown in Figure II-22. It consists of a single quadrupolar powder pattern with well-resolved 0° edges surrounding a central peak of moderate intensity. The quadrupolar splitting is equal to the smallest splitting observed for perdeuterated decane in DPPC. The temperature profile, Figure II-23, is very similar to that for the smallest splitting of d₂₂-decane-DPPC, Figure II-16. The splitting at 60°C fits neatly into the plot of the concentration dependence of d₂₂-decane-DPPC splittings in Figure II-17, where the 1-d₁-decane-DPPC datum is indicated by the filled circle. Finally, the relative intensity of the central maximum for this 18.2 mole % sample is intermediate between the 10.3 mole % and 26.7 mole % samples illustrated earlier.

7. d₃₄-Hexadecane in DPPC

When it became evident that the large proportion of decane in the bilayer portion of black lipid films

FIGURE II-22

9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of 1-d₁-decane (18.2 mole %) in DPPC at 45°C, obtained using a solid-echo burst sequence and spectrum reverse presentation.

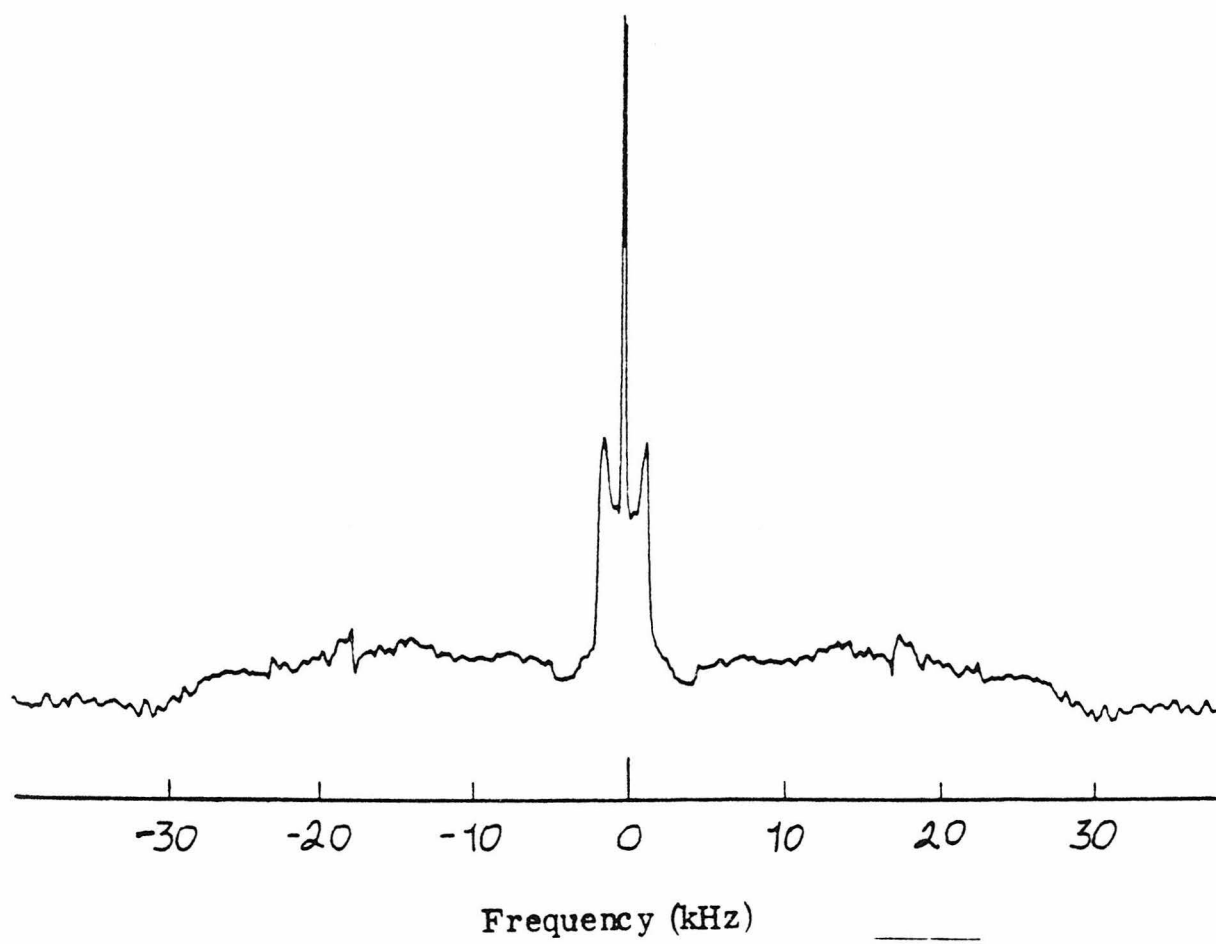
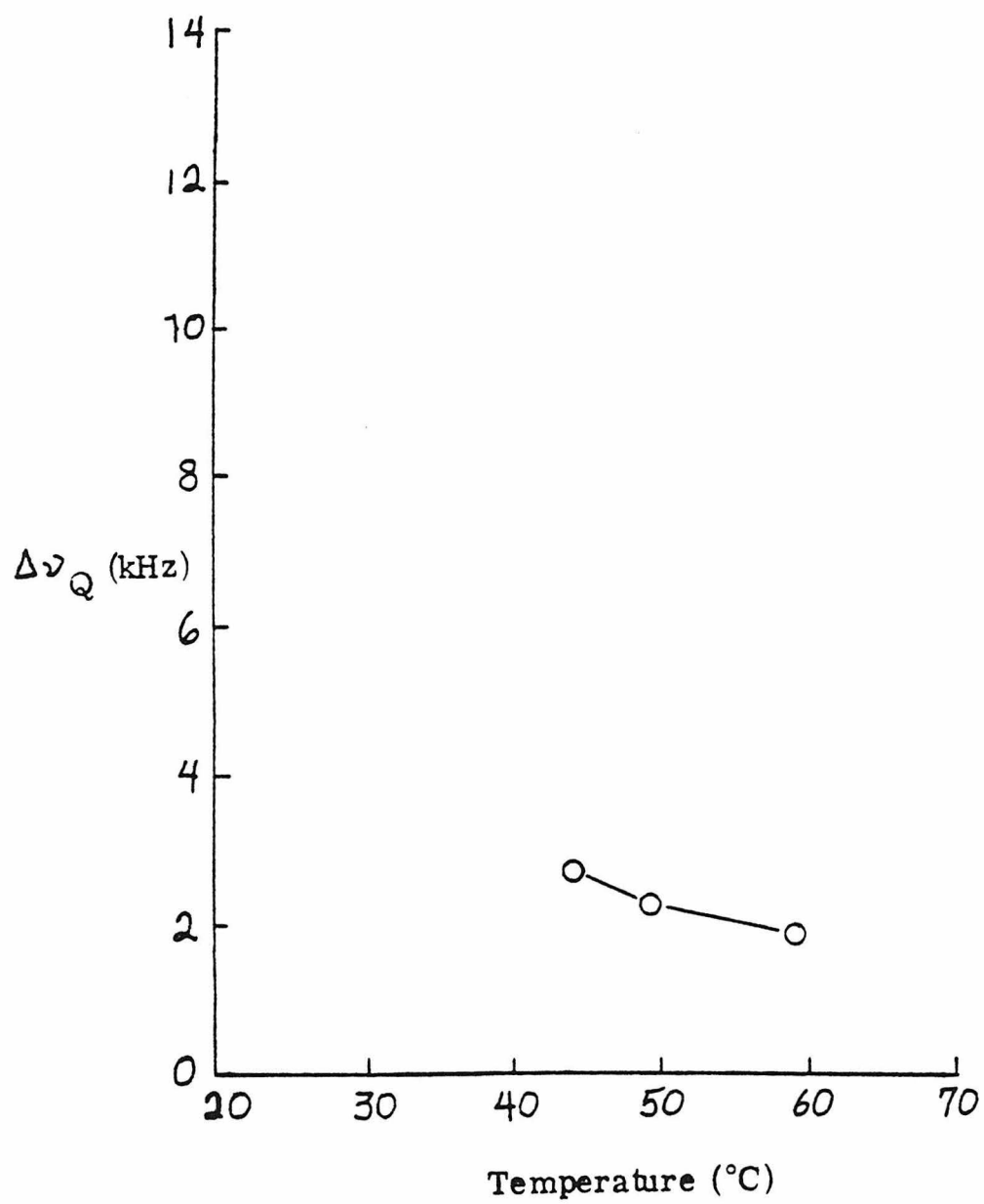


FIGURE II-23

Observed quadrupolar splitting $\Delta\nu_Q$ (kHz)
as a function of temperature ($^{\circ}\text{C}$) for a
multilamellar dispersion of 1- d_1 -decane
(18.2 mole %) in DPPC.



made from decane-lipid solution was perturbing the properties of the membrane, it was suggested that hexadecane or other longer chain hydrocarbons be used as solvents, since their concentration in the bilayer region of the resulting BLMS is relatively small (31). Accordingly, we have investigated multilamellar dispersions of d_{34} -hexadecane in DPPC to see what is the effect of lengthening the hydrocarbon chain on the 2H spectra of alkane-containing multilayers. We will describe the results by pointing out how they are similar to or different from the decane results.

Figure II-24 shows the 2H NMR spectrum of d_{34} -hexadecane (4.4 mole %) in DPPC at 60°C. As in the decane case we resolve three quadrupolar pairs and a central maximum. The small number of resolved splittings is unexpected since the hexadecane has a larger number of potentially inequivalent carbons. The intensity of the central maximum is relatively large for a small concentration of hydrocarbons; it appears most similar to the 18.2% d_1 -decane case. The temperature profile for 4.4 mole % d_{34} -hexadecane in DPPC is shown in Figure II-25. Additional quadrupolar pairs are resolved at lower temperatures. Figure II-25 is very similar to Figure II-16, the temperature profile for 10.3 mole % d_{22} -decane in DPPC. However for hexadecane the data do not extend below 43°C; at 40°C the order-parameter plateau has lost its square shape and none of the

FIGURE II-24

9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{34} -hexadecane (4.4 mole %) in DPPC at 60°C, obtained using a solid-echo burst sequence and full quadrature data presentation.

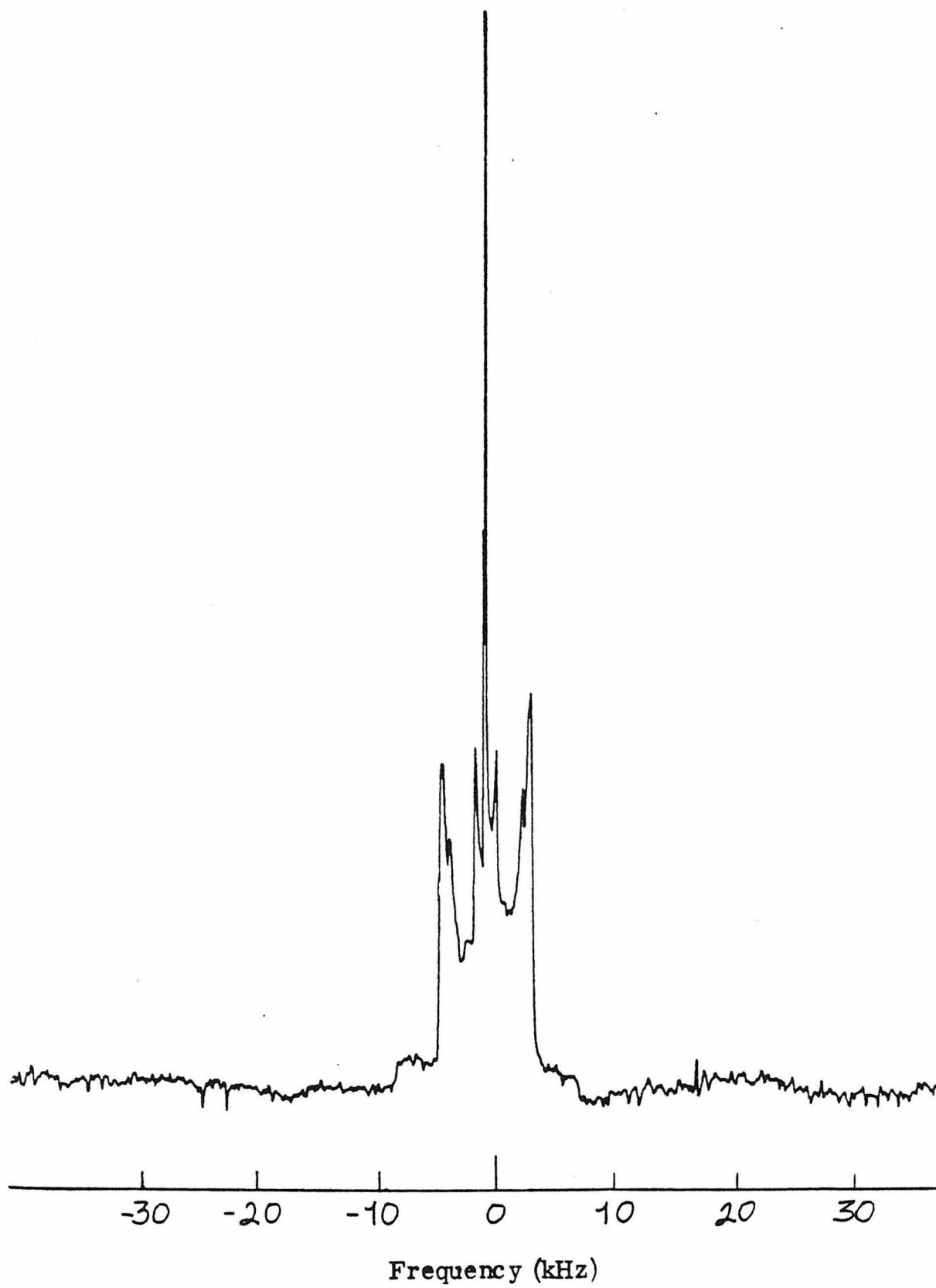
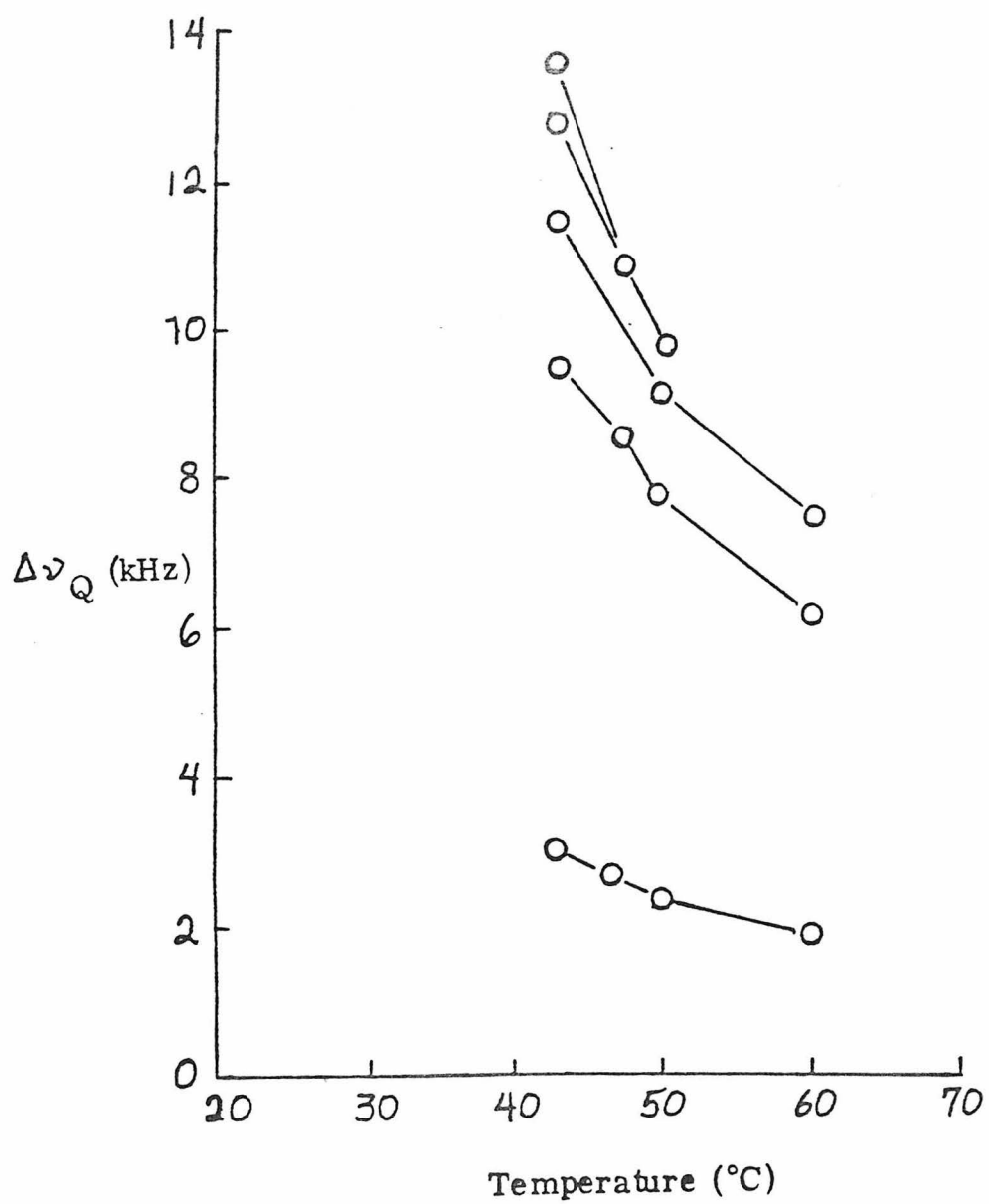


FIGURE II-25

Observed quadrupolar splittings $\Delta\nu_Q$ (kHz)
as a function of temperature ($^{\circ}\text{C}$) for a
multilamellar dispersion of d_{34} -hexadecane
(4.4 mole %) in DPPC.



quadrupolar splittings can be identified. We have also examined the hexadecane-DPPC system at 16.4 mole % hydrocarbon. Figure II-26 illustrates the concentration dependence of the quadrupolar splittings. Hexadecane at 60°C and 16.4 mole % gives splittings approximately equal to those for decane at 60°C and 26.7 mole %. Figure II-27 shows the ^2H spectrum of d_{34} -hexadecane-DPPC at 50°C and 16.4 mole % hydrocarbon. The central maximum completely dominates the spectrum and it is only after a large increase in the vertical scale that we identify the three quadrupolar splittings. Thus the central maximum is much larger for hexadecane than for decane at equal hydrocarbon concentrations. Finally, where the two hexadecane-containing DPPC samples were cooled to 40°C and reheated to 60°C, no change in the relative intensity of the central maximum was observed.

8. Differential thermal analysis

As an aid in interpreting the ^2H NMR data we have examined the gel to liquid crystalline phase transition in several of the systems discussed above by differential thermal analysis. For reference, in Figure II-28 we show the DTA heating and cooling traces for pure DPPC. We see as usual the main endothermic transition at 41.5°C with a width of approximately 1.3°C (full width at half height). In addition there is a small pretransition at 36.5°C which is observed upon heating but not seen on cooling. This trace is repeatable

FIGURE II-26

Observed quadrupolar splittings $\Delta\nu_Q$ (kHz)
as a function of concentration (mole %
hydrocarbon in DPPC) for multilamellar
dispersions of d_{34} -hexadecane in DPPC.

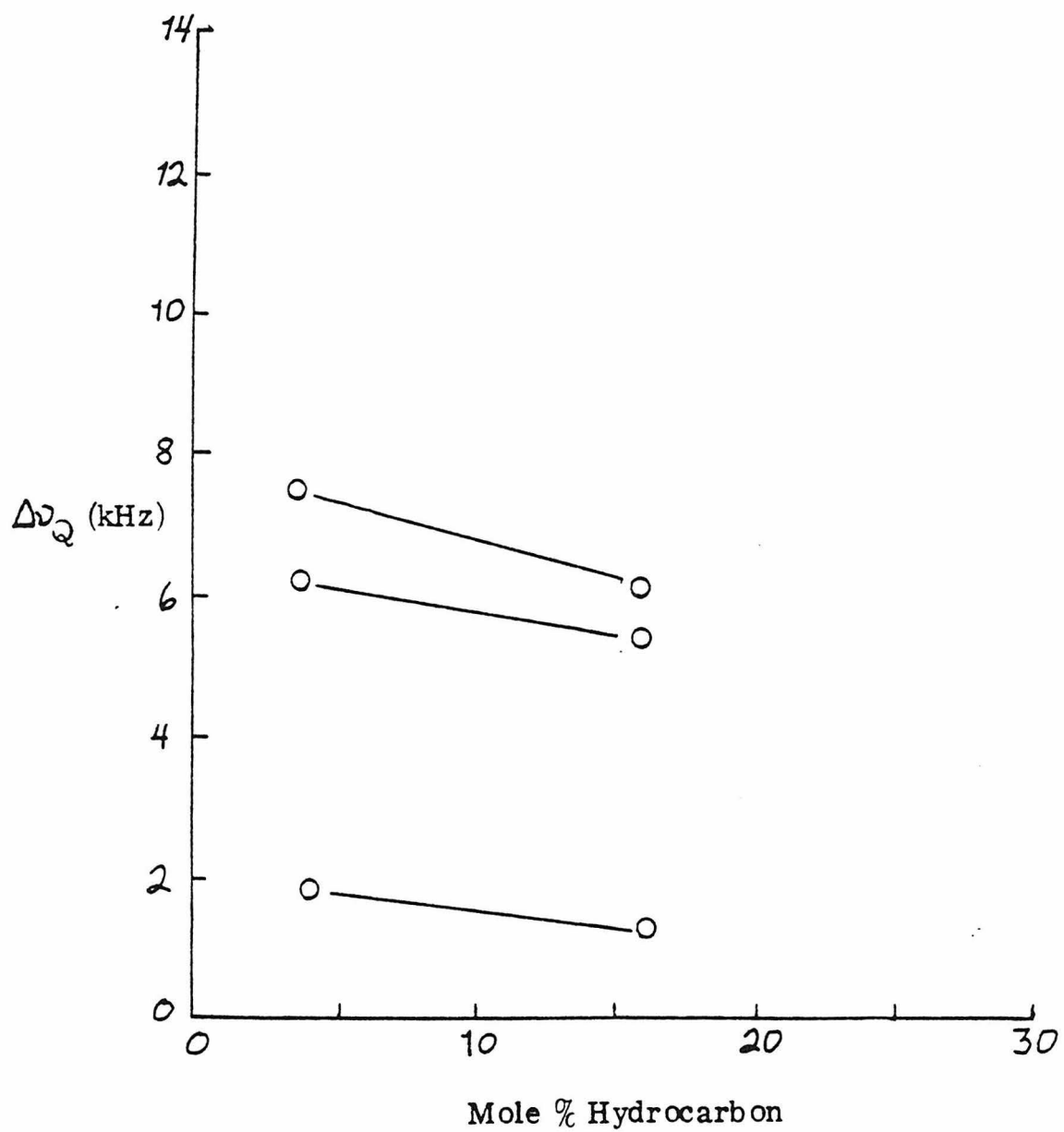


FIGURE II-27

- (a) 9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{34} -hexadecane (16.4 mole %) in DPPC at 50°C, obtained using a solid-echo burst sequence and spectrum reverse data presentation.
- (b) Same as (a) with vertical scale increased by a factor of 40.

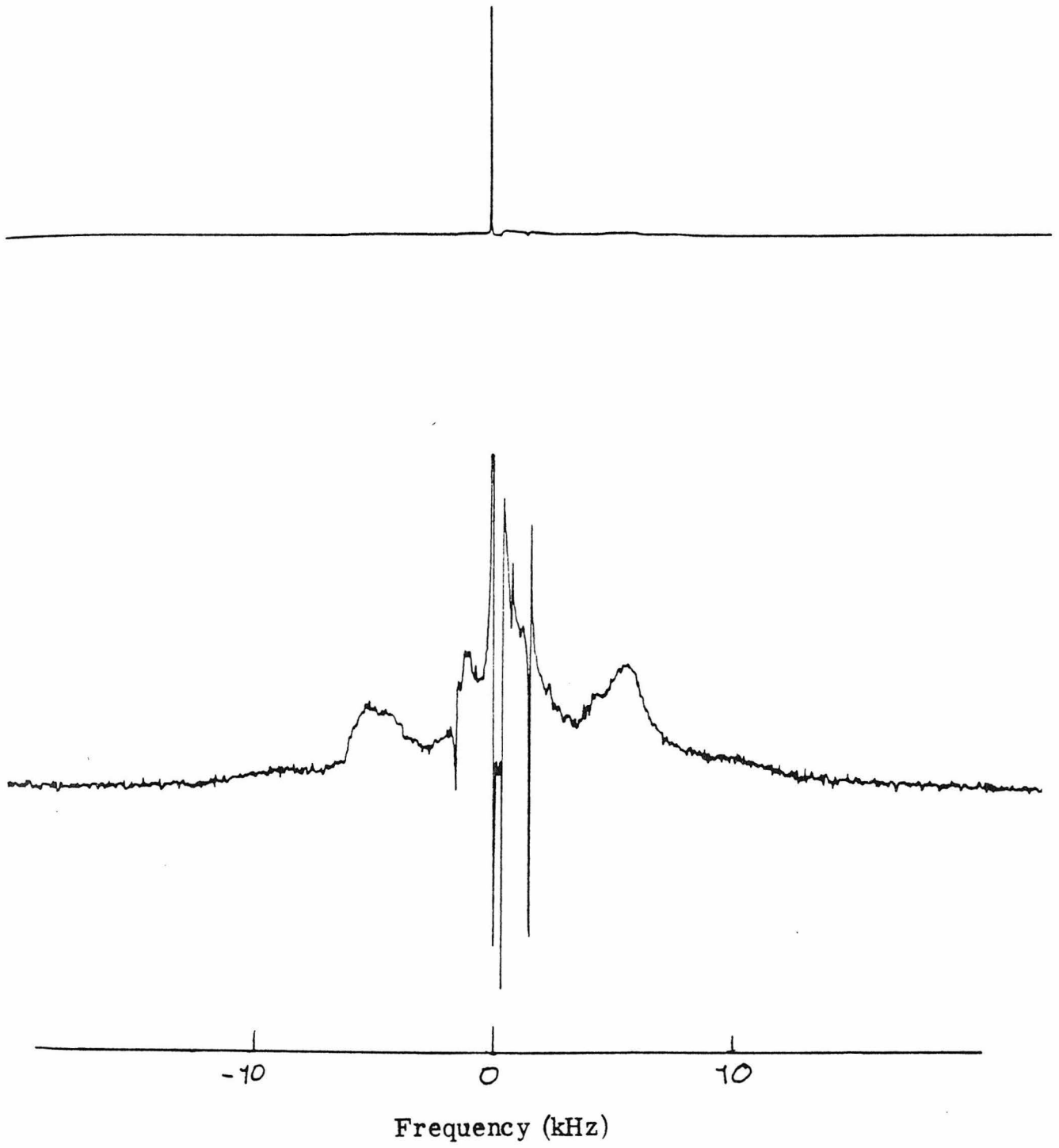
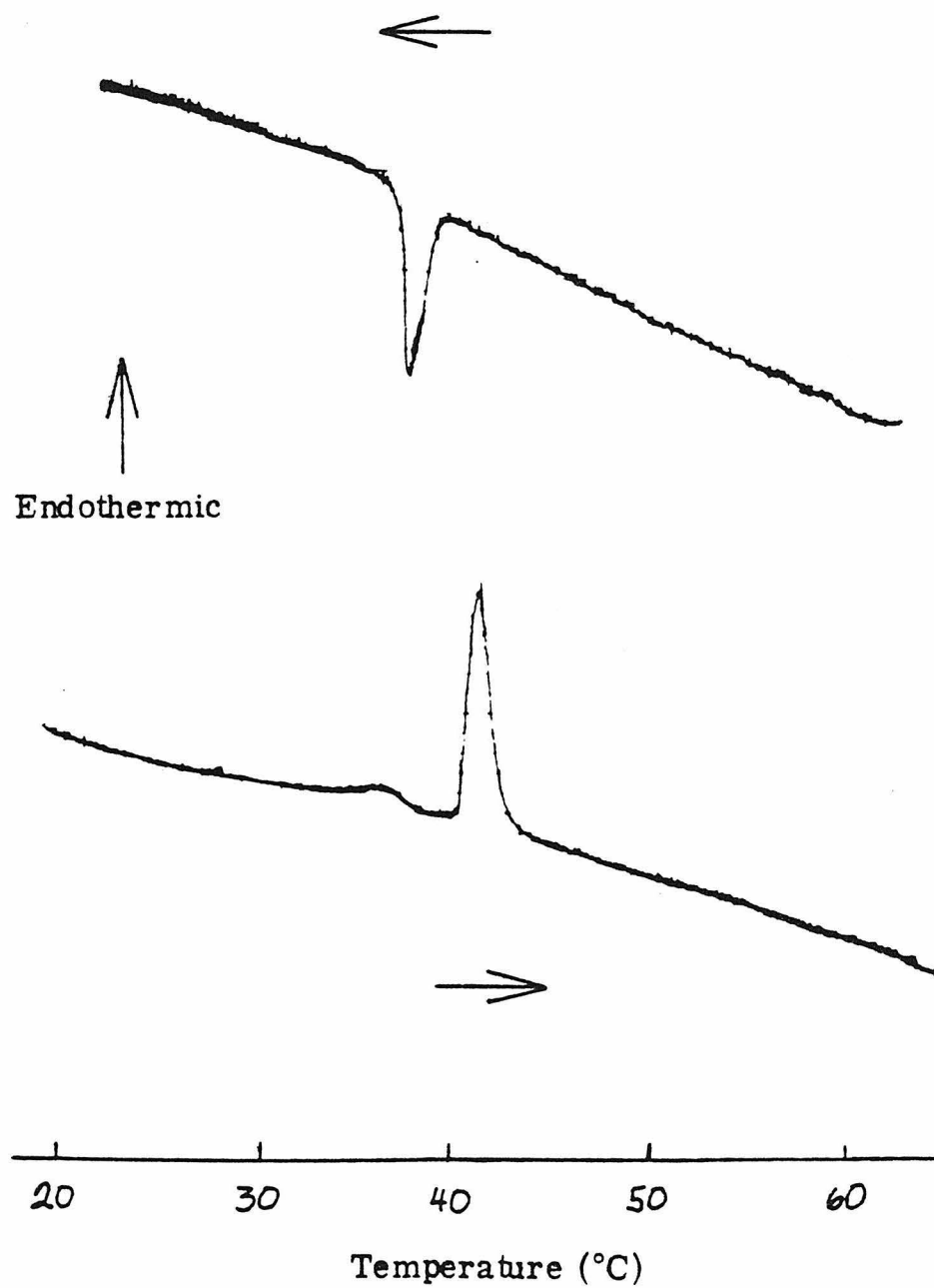


FIGURE II-28

Differential thermal analysis (DTA) thermogram for a multilamellar dispersion of DPPC. Arrows indicate heating and cooling traces; heating rate is 7.5°C per minute and cooling rate is variable.



but the magnitude of the pretransition decreases upon continued temperature cycling.

Figure II-29 shows the corresponding DTA traces for a DPPC dispersion containing 16.7 mole % perdeuterated palmitic acid. On heating we see a single endothermic transition about 4.5°C in width and centered at 50°C (average of these traces); on cooling we see two endothermic transitions, one at 48.0°C and the other at 41.6°C. No pretransition is evident. These traces are also repeatable. The increase in T_c upon addition of fatty acid, corresponding to an ordering effect of the fatty acid on the lipid chains, was first observed by Prestegaard (32).

The "Prestegaard effect" is also observed for DPPC dispersions containing hexadecane. Figure II-30 shows DTA heating and cooling curves for d_{34} -hexadecane-DPPC multilayers at 16.4 mole % hydrocarbon. As in the fatty acid case the transition temperature upon heating is raised, in this case to 47.5°C (average of five traces). This transition is about 7°C wide, with a pretransition at 40.5°C. The cooling curve shows a superposition of at least two endothermic transitions, with the principal components centered at 43.5°C and 36°C.

Finally in Figure II-31 we show the DTA behavior of DPPC multilayers containing 26.7 mole % decane. The endothermic transition observed on heating is broadened

FIGURE II-29

DTA thermogram for a multilamellar dispersion of d_{31} -palmitic acid (16.7 mole %) in DPPC. Arrows indicate heating and cooling traces; heating rate is 7.5°C per minute and cooling rate is variable.

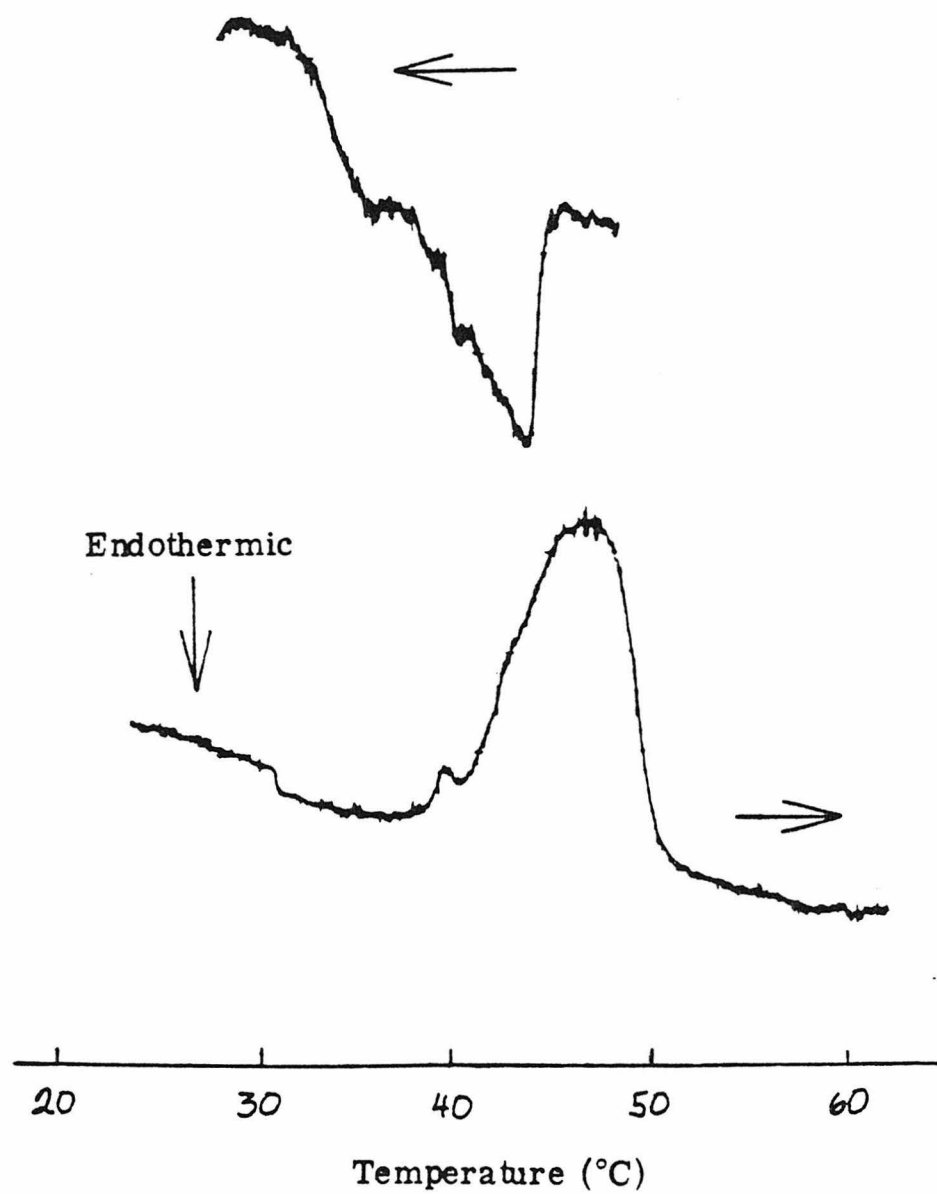


FIGURE II-30

DTA thermogram for a multilamellar dispersion of d_{34} -hexadecane (16.4 mole %) in DPPC. Arrows indicate heating and cooling traces; heating rate is 7.5°C per minute and cooling rate is variable.

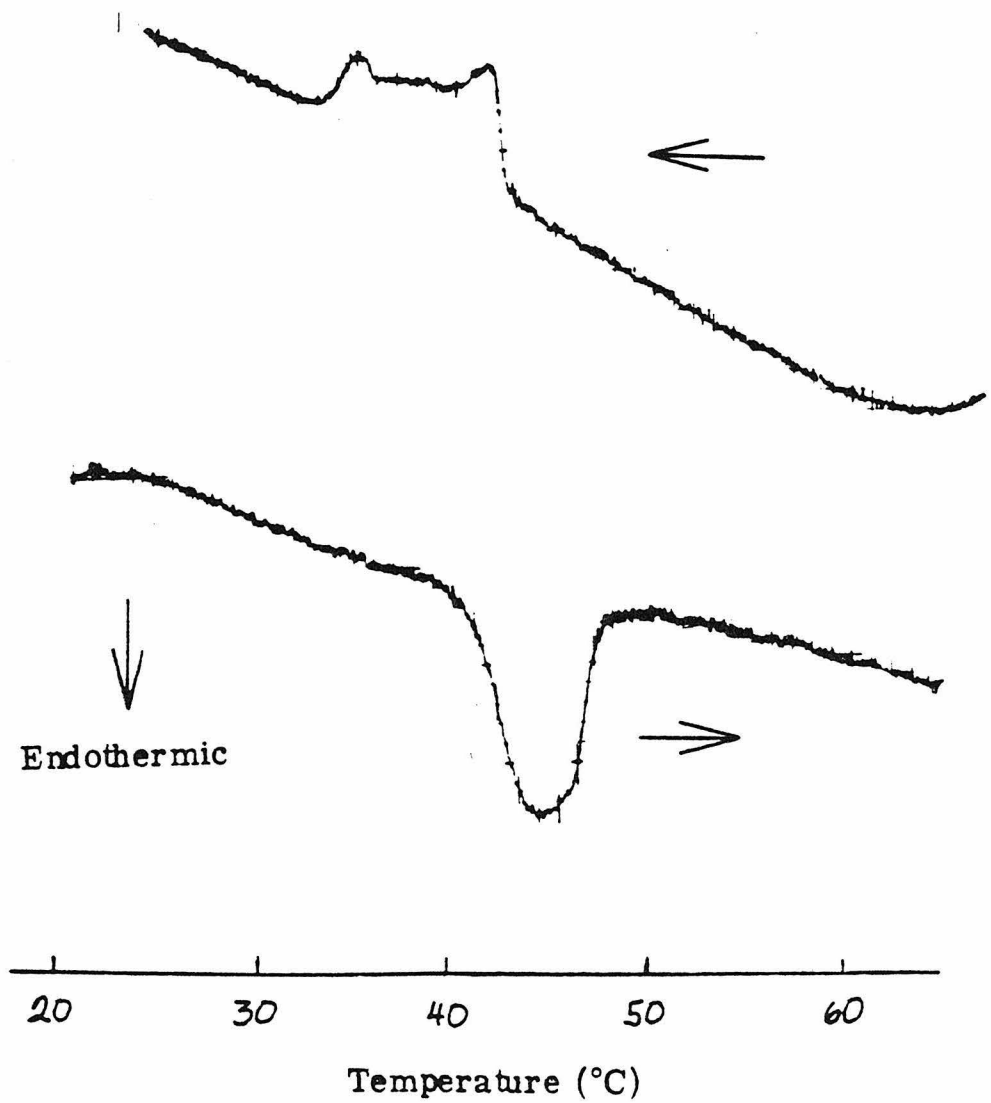
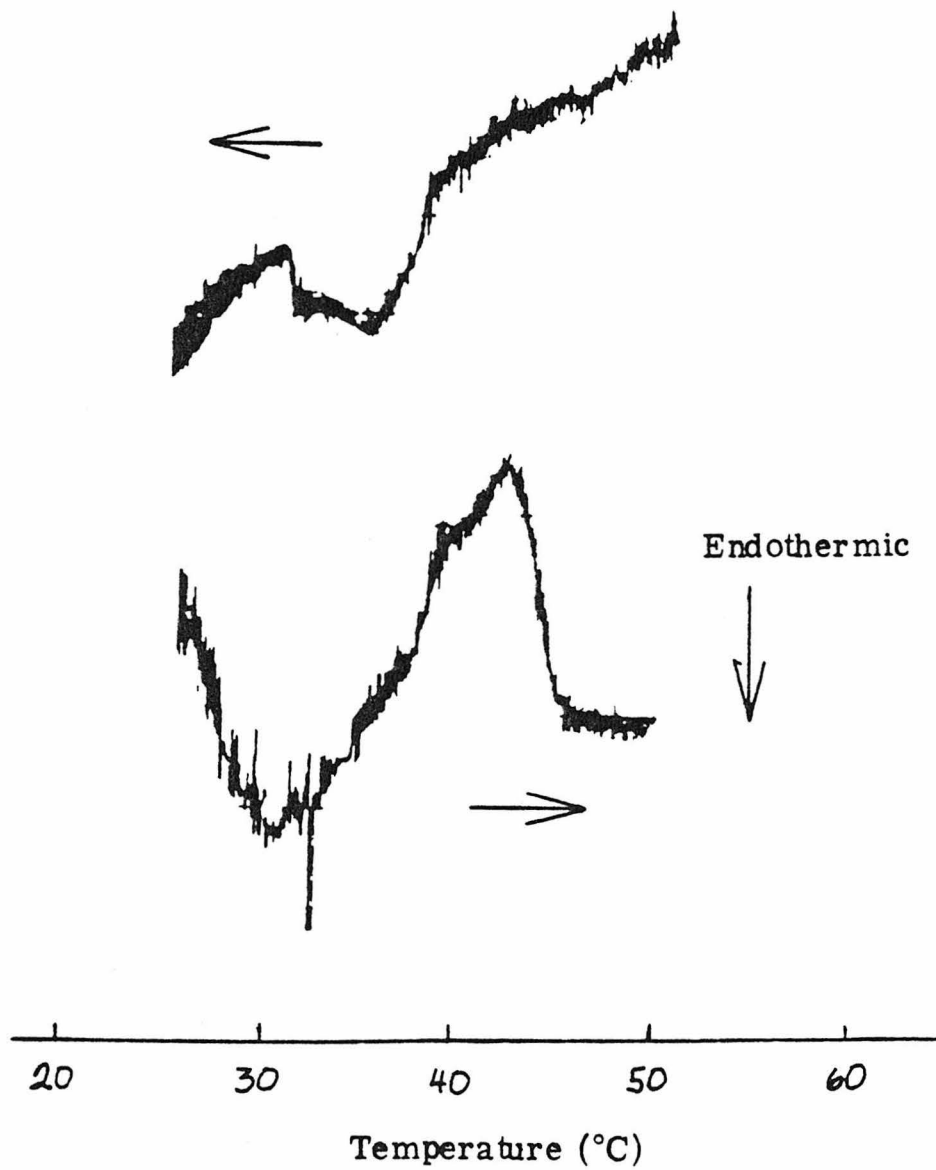


FIGURE II-31

DTA thermogram for a multilamellar dispersion of d_{22} -decane (26.7 mole %) in DPPC. Arrows indicate heating and cooling traces; heating rate is 7.5°C per minute and cooling rate is variable.



compared to the trace for pure DPPC, beginning at a lower temperature but still centered at 41.5°C. The Prestegard ordering effect is not observed. On cooling there are once again two transitions, one at 40°C and the other at 36.5°C.

D. Discussion

In interpreting ^2H order parameters for fatty acids and hydrocarbons in bilayers we will make frequent use of the Petersen-Chan model for the motional state of lipid hydrocarbon chains. Therefore we begin by reviewing this model in more detail.

To interpret order parameters for molecules such as bilayer lipids, which undergo severely restricted motions, we begin with a description appropriate for solids and then introduce those motions believed responsible for reducing the order parameter from its maximum possible value. Thus to develop a motional model for lipids we first consider the hydrocarbon chains to be in a rigid, all trans configuration parallel to the bilayer normal and then introduce a simple set of motions deemed most favorable energetically. In other words, in terms of a static picture we allow the molecule to sample only a certain well-defined subset of conformations and orientations out of all the possible conformations and orientations of the molecule.

For bilayer lipids at least two motions with

different characteristic timescales are required to explain the temperature and field dependence of the ^1H relaxation data. The Petersen-Chan model proposes that the slower motion (timescale $\sim 10^7/\text{sec}$) is a reorientation of the hydrocarbon chain as a whole with respect to the bilayer normal. This motion is thought to be locally cooperative, with approximately 100 molecules moving together. The faster motion (timescale $\sim 10^{10}/\text{sec}$) is a chain isomerization describing the equilibrium between trans and gauche conformations of the carbon-carbon bonds along the chain. Petersen and Chan describe chain isomerization in terms of "kink diffusion". They hypothesize that isolated gauche bonds occur only rarely, since a gauche bond high in the chain produces a tilt in the remaining portion of the chain, which is sterically unfavorable owing to the presence of the neighboring chains. Since β -coupled gauche $\pm$ rotations produce no change in the overall direction of the chain they are sterically less hindered, and Petersen and Chan suppose that gauche bonds always occur in β -coupled pairs, called kinks, which can diffuse up and down the chains. Kink formation is supposed to occur near the methyl end of the chain, where steric restrictions from neighboring chains are the smallest.

To predict an order parameter S_β from the Petersen-Chan model, we first note that because the two motions are independent, because they have different timescales,

and because chain isomerization (along with rotation of the chain about its long axis) is axially symmetric with respect to the instantaneous chain axis, we may compute an order parameter for each of the two motions separately. The product of these two order parameters should correspond to the experimentally observed S_β . To compute the order parameter for chain reorientation S_α , we specify a distribution function $g(\alpha)$ for the angle α between the instantaneous chain axis and the director (the bilayer normal). Then S_α is simply

$$S_\alpha = \frac{\frac{1}{2} \int_0^\pi (3\cos^2\alpha - 1) g(\alpha) \sin\alpha \, d\alpha}{\int_0^\pi g(\alpha) \sin\alpha \, d\alpha} \quad (\text{II-14})$$

Note that S_α is constant along the chain for a given α . To predict the order parameter S_γ for chain isomerization we use a model for chain isomerization to predict the probability of trans, gauche(+) and gauche(-) conformations at each chain position. Then S_{γ_j} for hydrocarbon chain position j is simply

$$S_{\gamma_j} = \frac{1}{2} \sum_i p_j(\gamma_i) (3\cos^2\gamma_i - 1) \quad (\text{II-15})$$

where γ_i is the angle between the C-D bond axis and the instantaneous chain axis for conformation i and $p_j(\gamma_i)$ is the probability of observing this conformation at

chain position j . Finally S_{β_j} is the product of the two order parameters:

$$S_{\beta_j} = S_{\alpha} S_{\gamma_j} \quad (\text{II-16})$$

By comparing ^1H and ^2H NMR data, Petersen and Chan were able to determine S_{α} and S_{γ} independently for positions at the upper end of the chain. Thus they could compute the probability of a trans conformation p_t and suggest distribution functions $g(\alpha)$ consistent with the observed S_{α} .

Petersen and Chan did not attempt to apply their model to the methyl end of the hydrocarbon chain, since in this region the restriction that gauche bonds appear in β -coupled $\pm$ pairs is not necessarily valid; single gauche bonds or "bends" should be allowed near the end of the chain. (Actually the subset of conformations which are allowed near the end of the chain may be larger still, but we will find this three-motion model sufficiently complicated to preclude going farther. Near the end of the chain the large reduction in order parameter indicates that the deviation from a solid-like system is great, and describing such a system is difficult.) How may we incorporate this new set of conformations, namely single gauche bonds, in our model? Since kink formation and bend formation are not independent motions we cannot consider them separately or derive separate order parameters for them. On the other hand, in order to include bend

formation along with kink formation in the order parameter for chain isomerization we must assume that both bend formation and kink formation are fast with respect to chain reorientation. Since we hypothesize that bend formation occurs only near the methyl end of the chain, which resides in relatively disordered surroundings, we will make this assumption and proceed to consider kinks and bends together. We will, however, indicate the chain isomerization order parameter as S_Y' as a reminder that for the terminal region of the chain we are considering two types of motion, lumped together under the term "chain isomerization".

How is S_Y predicted in this model? Seeling and Seelig (22) and Petersen and Chan (27) have pointed out that a single gauche bond at a particular chain position causes all the chain positions below to appear as gauche orientations with respect to the chain axis above. Thus in computing the probabilities $p_j(\gamma_i)$ for the various conformations at position j we need to consider not only the probability of a kink at location j but also the probability of a bend at position j or above. We will not attempt to make a detailed calculation of the contribution to the $p_j(\gamma_i)$ from bend formation and annihilation. We note, however, that since S_α is constant along the chain for a given angle α , S_α merely scales the entire order parameter profile, while the shape of the profile is due to bend and kink forma-

tion represented in the new chain isomerization order parameter S_Y' .

Keeping this background in mind, we will discuss the results beginning with the perdeuterated lipid system and continuing with the data on fatty acid-containing bilayers. Then, using these systems as a point of reference, we consider in detail the ^2H NMR data for hydrocarbons in bilayers.

1. d_{54} -Dimyristoylphosphatidylcholine

Spectra of this system are useful in the context of ^2H studies of hydrocarbon-containing multilayers for several reasons. First, this system illustrates the appearance of spectra of perdeuterated fatty acid chains in bilayer systems. The overlapping of a number of quadrupolar powder patterns of varying order parameters produces a relatively square plateau in the spectrum centered about the zero frequency. The nearly vertical edges of the plateau arise from the outer edges of the 90° orientation discontinuity of the largest quadrupolar splitting. Since the outermost component accounts for a large number of deuterons this edge remains sharp, and at twice the distance of the outermost 90° edge from the center frequency the 0° edge of the outermost component is also observed. The top of the plateau is scalloped by a number of peaks corresponding to the 90° discontinuities for the various quadrupolar components making up the plateau. The relatively flat top of the

plateau is a result of the relatively even spacing of the various quadrupolar splittings corresponding to a flexibility gradient along the hydrocarbon chain.

Second, the DMPC data indicate the quality of the spectra that may be obtained with our homemade ^2H spectrometer, whose construction is described in chapter III of this thesis. The agreement of the overall shape of the spectra with the theoretical powder pattern, the resolution of the 0° shoulder, and the relatively flat baseline are possible only with the use of a solid-echo RF burst sequence and careful adjustment of the transmitter phase, receiver phase and burst sequence timing. These features are not resolvable on high resolution NMR spectrometers due to finite receiver dead time. In spite of the small amount of lipid in the sample (90 mg) and the low H_0 field (14 kGauss) the signal-to-noise after 40,000 free induction decays is sufficiently good to allow us to identify with confidence all of the various quadrupolar splittings expected on the basis of spectra in the literature. The spectral width (~ 80 kHz) necessary to encompass without distortion the entire powder pattern including the 0° edges required the short 90° burst and rapid data digitization provided by the homemade instrument.

Third, since both perdeuterated and specifically deuterated lipids have been extensively studied in the literature the DMPC data allow us to calibrate our

spectra against results from other laboratories. As indicated in Figures II-6 and II-7, there is excellent qualitative agreement between the DMPC order parameter profile and Davis' data (29) on perdeuterated DPPC. Quantitatively, the order parameter for DMPC is about 10 to 25% larger than for DPPC at a chain position an equal number of carbons from the methyl group. The maximum S_{β} for DMPC 7°C above T_c is -0.22 compared to -0.20 for DPPC.

Finally, the lipid bilayer ^2H data serve as a reference point with which we may compare the data from other systems. We first investigate fatty acid-containing systems to see how single-chain species behave in the phospholipid bilayer environment.

2. d_{34} -Palmitic acid

For purposes of discussion in the context of bilayer systems containing hydrocarbons palmitic acid may be considered as a hydrocarbon sixteen carbons long with an anchoring group at one end which must be located at the hydrophilic/hydrophobic interface region of the membrane. Thus palmitic acid must necessarily have its hydrocarbon chain parallel to the fatty acid chains of the lipid. Since it has the same chain length as the lipid it differs from the lipid, for our purposes, mostly in having only one chain, rather than two chains coupled at one end. This basic similarity in length, environment and orientation is reflected in

the similarity of the order parameter profiles for d_{34} -palmitic acid in DPPC (Figure II-11) and perdeuterated lipids (Figures II-7 and II-8). Moreover the temperature dependence of the ^2H order parameter S_β in the two types of systems shows approximately the same slope, suggesting that the same process may control the temperature dependence in the two cases. The order parameter profiles differ only in that near the methyl end of the chain S_β is as much as 40% smaller for the free fatty acid chain than for the lipid chains. This may be due to the fact that the lipid chain is constrained by the second fatty acid chain on the same molecule. This restriction via an "intramolecular" steric interaction should be felt most at the upper (carbonyl) end of the chain. However, chain isomerization has little effect on S_β near the top of the chain, so that the phospholipid and fatty acid agree well near the top of the chain. An alternative explanation for the discrepancy at the bottom of the chain is that the fatty acid chain has some translational mobility parallel to the membrane normal, so that the methyl terminal region of the chain is occasionally permitted to slide into the relatively disordered interlayer space.

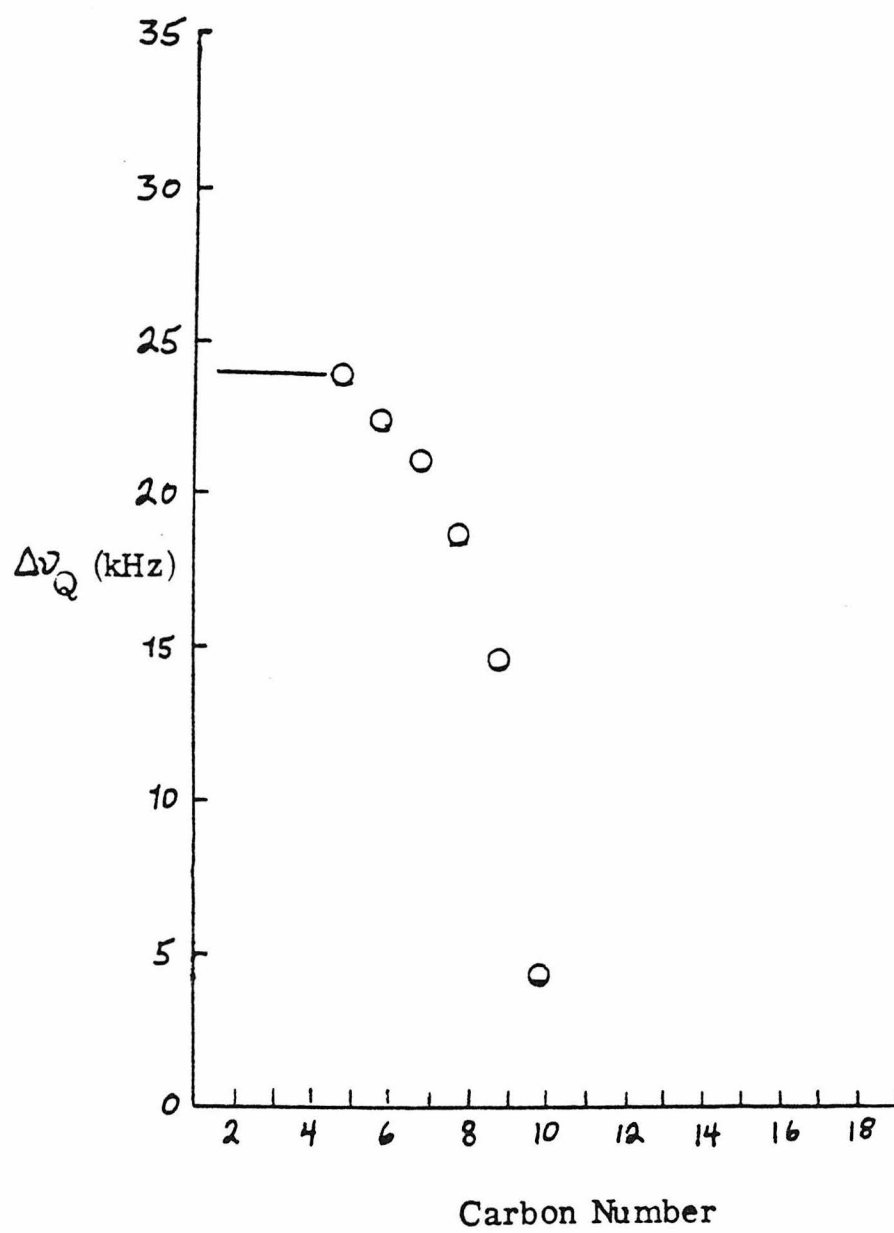
3. d_{19} -Decanoic acid

Next we examine the case of an interface anchored molecule with a hydrocarbon chain 10 carbons in length. The order parameter profile for perdeuterated decanoic

acid in DPPC at 60°C is illustrated in Figure II-32, where by analogy with the palmitic acid case we assign the smallest quadrupole splitting to the methyl group, the next smallest splitting to the penultimate methylene group, and so on up the chain. This plot should be compared with Figure II-11, the order parameter profile for perdeuterated palmitic acid in DPPC. At the uppermost end of the fatty acid hydrocarbon chain the two systems give an identical S_β of -0.20. Proceeding down the chain S_β for decanoic acid begins to fall off at carbon 6, halfway down the chain, whereas for palmitic acid the drop in S_β begins at carbon 9, also halfway through the chain. The terminal methyl for decanoic acid has $S_\beta = -0.035$, whereas for palmitic acid the ^2H order parameter for the methyl group is -0.020. For the penultimate methylene the discrepancy is even larger; S_β for the decanoic acid C_9 is -0.12 while S_β for the palmitic acid C_{15} is only -0.053. As a matter of fact, S_β for the terminal portion of the decanoic acid chain is larger than for equivalent positions in the terminal end of the fatty acid chains of perdeuterated lipid. Since the carbonyl of the fatty acid is constrained to lie in the interface region, when the decanoic acid chain lies parallel to the lipid hydrocarbon chains the terminal methyl must be in the neighborhood of carbons 9 to 11 of the lipid chain. In this region the lipid chains are nearly as ordered as

FIGURE II-32

$\Delta\nu_Q$ (kHz) versus hydrocarbon chain position for a multilamellar dispersion of d_{19} -decane acid (12.3 mole %) in DPPC at 60°C.



they are at the carbonyl end. The order profile of the decanoic acid reflects this ordering in the relatively high S_β of its methyl terminal region and the relatively rapid rise of S_β from the methyl towards the carbonyl end.

This system illustrates an important, if obvious, point about the use of fatty acids as probes for bilayer order. On the one hand decanoic acid in this system does not reflect quantitatively the order of its environment. Deuterons at decanoic acid C_9 have $S_\beta = -0.14$ at 50°C while C_9 for DPPC at 45°C has a $^2\text{H } S_\beta$ of -0.18 . On the other hand, C_9 and C_{10} are certainly more ordered than expected for the methyl terminus of a fatty acid in the region of the lipid methyl terminus. Thus both the nature of the labelled molecule and the relative order of the environment play a role in determining the order parameter profile. This is just another way of saying that both intermolecular and intramolecular effects play a part in determining S_β .

It is interesting to consider this observation in the light of our extended Petersen-Chan model. To understand the present data in light of this model we note that shortening the fatty acid chain changes the shape of the order parameter profile without changing the absolute value of S_β at the upper end of the chain. Thus chain reorientation (S_α) is unaffected while chain

isomerization (S_{γ}') is altered. This is just as expected: chain reorientation is supposed to be a cooperative phenomenon and thus (at least for relatively small fatty acid concentrations) the chain reorientation of the fatty acid reflects that of the bilayer as a whole. On the other hand, when the methyl terminal of the fatty acid resides in a relatively ordered lipid chain environment, bend formation and kink formation will be severely restricted, and chain isomerization will be less effective in reducing S_{β} near the methyl end of the chain. No alteration of S_{β} at the upper end of the chain results from reduced kink and bend formation since chain isomerization has a relatively small contribution to the reduction of the order parameter in this part of the bilayer.

4. Deuterated decane and hexadecane in DPPC

We turn finally to the discussion of the ^2H NMR spectra of DPPC bilayers containing deuterated hydrocarbons. We consider first the assignment of the various components in the spectra. Then we evaluate in detail various proposals for the motional state of the hydrocarbon which gives rise to the observed splittings. Next we examine the equilibrium between various pools of hydrocarbon, and finally we consider briefly the effect of added hydrocarbon on the gel to liquid crystalline phase transition of the bilayer.

a. Assignment of spectral components

To understand the ^2H data we first need to decide how many different motional states of decane are represented in the spectra. The most obvious supposition is that there are two motional states of decane which are in slow exchange with one another, one component having isotropic motion and represented by the central maximum and the other component having restricted motion and represented by the various powder pattern components, whose different splittings indicate a flexibility gradient along the hydrocarbon chain. Alternatively, it is possible that more than one motional state is contributing to the various powder pattern components of the spectrum. The specifically labelled decane experiment allows us to identify the number of motionally restricted components very easily. With a single label in the molecule we observe just two of the components which are present in the d_{22} -decane in DPPC spectrum, namely the central maximum and the narrowest powder pattern component. Thus there are two motional states for the methyl group and thus two pools of decane in the membrane. One pool has isotropic motion on the timescale of the ^2H experiment while the other undergoes a restricted motion. In the spectra of perdeuterated decane in DPPC, the narrowest powder pattern component is assigned to the methyl deuterons. Again, by analogy with the preceeding data we may assign the next to

narrowest powder pattern component to the penultimate methylene group of that portion of the decane whose motion is restricted. The third and broadest powder pattern component contains contributions from the bulk of the methylene deuterons of the ordered decane. Occasionally at low temperatures we resolve the methylene deuterons on the second carbon in from the methyl from the bulk methylene deuterons. Thus we conclude that the order parameter plateau observed for deuterated hydrocarbons intercalated in DPPC bilayers does indeed correspond to an order parameter gradient along the hydrocarbon chain for a portion of the decane in the system.

To what orientation of hydrocarbon in the bilayer do these two decane components correspond? We assume that the isotropically reorienting component does not represent hydrocarbon parallel to the lipid chain since isotropic reorientation seems highly unlikely in such a system. We postulate, rather, that the central line in the spectra represents hydrocarbon sequestered between the lipid layers in the center of the bilayer. The data are insufficient to permit detailed evaluation of the motional state of this component of the decane, except to note that the linewidth of the central line is about one order of magnitude larger than for neat d_{22} -decane liquid. While we have not made relaxation measurements and thus cannot speculate on the mechanism

of the line broadening or even say whether it originates from first order or second order effects, we can say that the motional state of this hydrocarbon component is different from hydrocarbon in the liquid.

There are two alternate possibilities for the origin of the central line: First, this component of the hydrocarbon could be associated with micelles of lipid. We consider this unlikely since decane, which increases the ratio of hydrocarbon volume to hydrophilic surface area of lipid structure, should retard rather than promote micellization. We can check whether hydrocarbon-containing dispersions exhibit increased micellization by examining the relative intensity of the central maximum in dispersions of protonated alkane in deuterated DPPC. Second, this hydrocarbon component could be completely dissociated from lipid. Due to the low solubility of hydrocarbons in water pure alkane could exist only in macroscopic droplets, which we have never observed in our samples.

b. Motional state of the ordered component

Next we examine the motional state of that fraction of hydrocarbon giving rise to the order parameter plateau. There are several observations for which any model for this component must account.

(i) Since in the experiment with methyl-labelled decane only a single methyl quadrupole splitting is observed, we conclude that for the ordered hydrocarbon component

either the two ends of the molecule are in equivalent environments or, if the two methyl environments are different, there is rapid end for end exchange of the molecule. (The exact timescale for the exchange depends on the difference in quadrupolar splitting between the two environments.) This observation is significant since, if the hydrocarbon lies parallel to the fatty acid chains of the lipid, the two methyl environments should indeed be different. This observation suggests that the smallest quadrupolar splitting in the ^2H spectrum of d_{22} -decane-DPPC dispersions corresponds to 6 methyl deuterons, while the next smallest component results from the 4 C_2 and C_9 deuterons and the third and largest splitting results from a composite of quadrupolar patterns from the 12 remaining deuterons at positions C_3 to C_8 . Figure II-33 shows the ^2H spectrum of a d_{22} -decane (10.3 mole %) in DPPC dispersion at 47°C , along with a spectrum simulated according to equation (II-12). The broadest splitting has been simulated as a superposition of three different quadrupolar powder patterns. T_2 is chosen as 1.77 msec for the methyl deuterons and 0.82 msec for the methylenes. There is good agreement between the simulated spectrum and the data, supporting our assignment of the various spectral components and indicating that the T_2 values for hydrocarbons are very similar to those for the lipid chains (29).

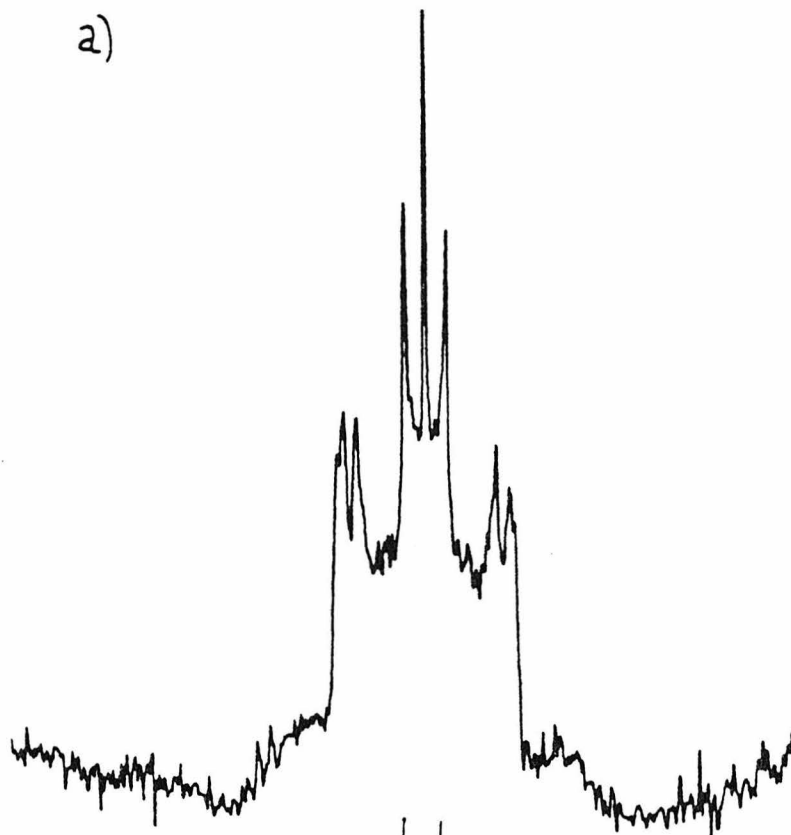
FIGURE II-33

(a) 9.21 MHz Fourier transform NMR spectrum of a multilamellar dispersion of d_{22} -decane (10.3 mole %) in DPPC at 47°C, obtained using a solid-echo burst sequence and presenting the full quadrature spectrum.

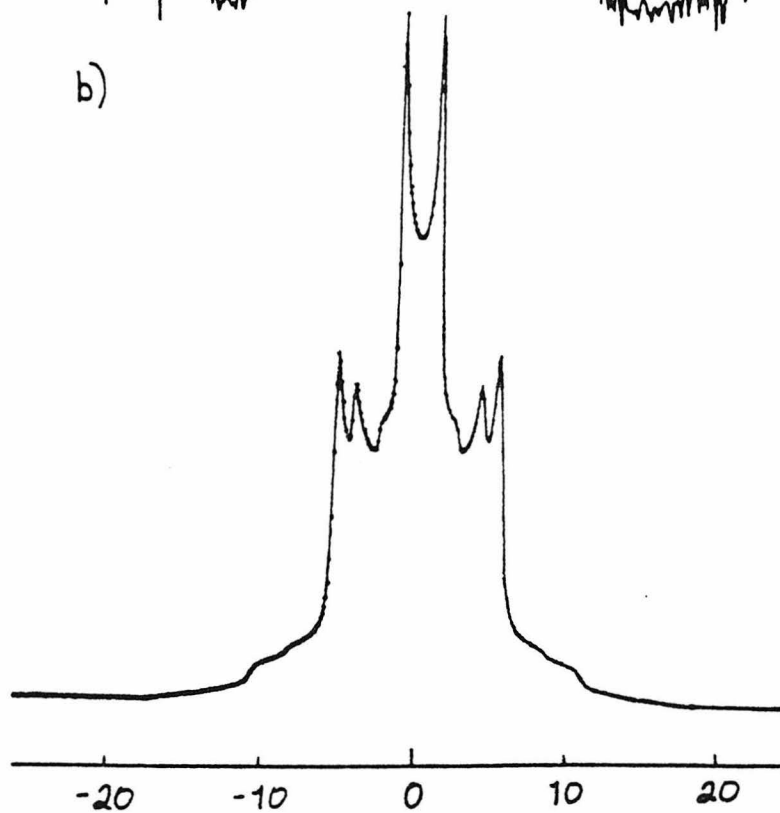
(b) Simulation of spectrum in (a) according to equation (II-12) using five quadrupolar components:

	$\Delta\nu_Q$ (kHz)	T_2 (msec)	number of deuterons
1)	10.9	0.82	4
2)	10.7	0.82	4
3)	10.6	0.82	4
4)	8.4	0.82	4
5)	2.6	1.77	6

a)



b)



Frequency (kHz)

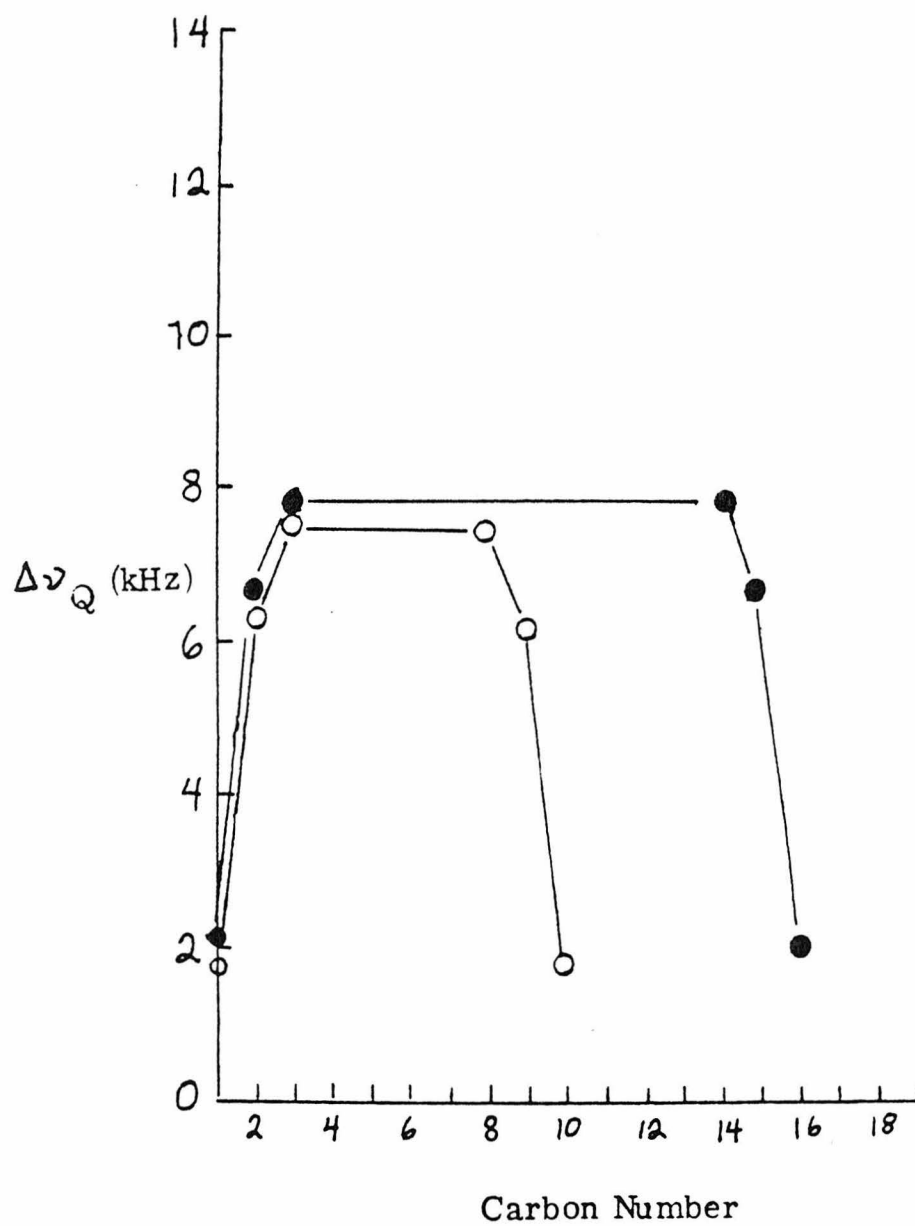
- (ii) The magnitude of S_β is reduced by a factor of two to three compared to the corresponding value for lipids or fatty acids, with the exact proportionality depending on temperature.
- (iii) The order parameter gradient is fairly steep at the ends of the hydrocarbon chain, but in the central portion of the molecule the order parameter is relatively constant. This is especially true for the hexadecane in DPPC system; the observation that hexadecane in DPPC gives only three resolved quadrupolar splittings at high temperatures is rather remarkable. The order parameter profile for the two hydrocarbons in DPPC is shown in Figure II-34.
- (iv) The temperature dependence of the order parameter for the various positions along the hydrocarbon chain is about three times steeper for hydrocarbons than for lipids or fatty acids. This implies that the process responsible for the temperature dependence in the hydrocarbon case is not the same as the processes governing the lipid and fatty acid cases. In other words, some motion other than chain reorientation, kink formation and bend formation is occurring.

We will consider three motional models for decane in DPPC multilayers:

- (i) First consider the possibility that the decane lies not parallel to the lipid chains but rather parallel to the membrane plane in the center of the bilayer.

FIGURE II-34

$\Delta\nu_Q$ (kHz) versus hydrocarbon chain position for a multilamellar dispersion of d_{22} -decane (10.3 mole %) in DPPC at 60°C (open circles) and a multilamellar dispersion of d_{34} -hexadecane (4.4 mole %) in DPPC at 60°C (filled circles).



In this model we differentiate the ordered component from the isotropically reorienting component in that the isotropic component is located in a microdroplet where its nearest neighbors are hydrocarbon molecules, whereas the oriented component represents an isolated hydrocarbon in the environment of the methyl ends of the lipid chains. It is not difficult to reconcile such a model with the observations cited above. First, in such a model the two methyl groups necessarily lie in equivalent environments. Second, since the motional state of the hydrocarbon chain may be very different from that of the lipid fatty acid chains, there is no reason whatsoever to assume that the magnitude of S_β should be similar to that for lipids. Third, since the environment for each carbon atom of the hydrocarbon chain is the same, it is reasonable to expect that the order parameter be relatively constant for the central portion of the chain. Finally, since an entirely different set of motions may obtain for hydrocarbons in this model as opposed to lipids, a different temperature dependence is not surprising.

This model is unsatisfactory for the following two reasons: First, it fails to explain why any flexibility gradient at all is observed in the hydrocarbon. In the decane case at lower temperatures we resolve four splittings. Because of the end-for-end symmetry of the molecule we expect to resolve at most five

splittings; there is thus only one pair of unresolved splittings. In the case of the present model we may again divide the possible motions into two groups: chain isomerizations, which are referred to the instantaneous chain axis, and chain reorientations, which in this case consist of rotations of the chain axis about the bilayer normal. We again note that the shape of the order parameter profile depends on differences in the probability of observing a gauche bond at various carbon atom positions. Since all carbon atoms are in essentially the same environment, there is no basis on which to predict such a difference. Second, it is not at all clear on the basis of this model why the two pools of hydrocarbons, both lodged in the interlayer space, should be in slow exchange. Surely they would be in physical proximity to one another, especially in a bilayer which is more than 25 mole % decane. The exchange would have to be slow indeed, slower than 2 kHz, to account for the data.

It is instructive to consider whether the present hypothetical situation may be distinguished from the case where hydrocarbons align parallel to the lipid chains by means of a ^2H experiment utilizing oriented multilayers. An oriented sample experiment serves to identify the director for the motion. Thus we can distinguish membrane-parallel hydrocarbon from chain parallel hydrocarbon only if the two cases result in

different directors for the motion. For chain parallel hydrocarbon the bilayer normal serves as director. For membrane-parallel hydrocarbon, in the case where no chain reorientation takes place, the chain axis serves as a director. In an oriented sample in which the bilayer normals are all parallel we have a director distribution which is cylindrically symmetric about the bilayer normal. In this case an oriented sample experiment will distinguish membrane-parallel hydrocarbon from hydrocarbon oriented parallel to the lipid chains. In the case of rapid chain reorientation on the NMR timescale the membrane normal serves as a director for the overall motion and the two hydrocarbon cases cannot be distinguished by looking at an oriented sample. Finally, consider the case where reorientation occurs, but slowly on the timescale of the experiment. In this case there is no director for the motion, since there is no reason to expect the chain reorientation to be symmetric about the average chain position. Once again in this case an oriented sample experiment can distinguish membrane-parallel and chain-parallel hydrocarbon. Thus the outcome of an experiment with oriented multilayers depends on the rate of chain reorientation in the membrane parallel case. Observation of a director parallel to the bilayer normal for an oriented sample of DPPC containing deuterated hydrocarbon would not conclusively eliminate the possibility of membrane

parallel hydrocarbon.

At any rate, principally because of the observation of a flexibility gradient along the hydrocarbon chain, we conclude that it is likely that the decane showing restricted motion is oriented parallel to the lipid fatty acid chains rather than parallel to the membrane plane between layers. In this case it is necessary to invoke rapid exchange between the two methyl environments to explain the end-to-end symmetry apparent in the spectra. There are two mechanisms by which this exchange may take place. We consider these two cases as our two remaining motional models:

(ii) Suppose that the hydrocarbon lies parallel to the lipid fatty acid chains and undergoes essentially the extended Petersen-Chan motions, with two additions. First we permit rotation of the molecule about an axis parallel to the membrane plane. This rotation brings about end for end exchange of the hydrocarbon molecule. We consider that the exchange is a jump-type process between two states differing in having the orientation of the hydrocarbon parallel or antiparallel to the lipid chains. That is, we assume that there is some maximum angle α limiting chain reorientation so that chain reorientation retains axial symmetry about the bilayer normal, but that occasionally the molecule "flips" between parallel and antiparallel orientations. To accomplish the flip the molecule must be located

for some time at angles $\alpha > \alpha_0$ but we neglect the contribution of these angles to reduction of S_α . In other words, we assume that the distribution function $g(\alpha)$ has $g(\alpha) = g(\pi - \alpha)$ and $g(\pi - \alpha_0 > \alpha > \alpha_0) \ll g(\alpha < \alpha_0, \alpha > \pi - \alpha_0)$. Second, suppose that the hydrocarbon is free to slide along the bilayer normal, so that the methyl group for decane samples environments from C_2 to C_7 and C_{11} to C_{12} of the lipid, while the penultimate methylene group samples environments C_3 to C_8 and C_{10} to C_{15} and so on for the other three inequivalent deuteron positions. For our simple model we assume that both sliding and jumping occur such that the various environments are sampled with equal probability. Finally we assume that, except for these two additional motions, the hydrocarbon behaves essentially as a fatty acid. Then to predict the hydrocarbon S_β values from the fatty acid values we merely average the fatty acid values for the environments sampled by the particular hydrocarbon deuterons. Since the flip motion, together with rotation about the hydrocarbon chain axis, generates inversion symmetry, we need also to divide our averages by two to account for the added symmetry. Doing the averaging and dividing by two we predict the following values for the quadrupolar splittings for d_{22} -decane in DPPC at 60°C:

$$\begin{aligned}
\Delta\nu_Q(C_1 \text{ and } C_{10}) &= 9.0 \text{ kHz} \\
\Delta\nu_Q(C_2 \text{ and } C_9) &= 9.7 \text{ kHz} \\
\Delta\nu_Q(C_3 \text{ and } C_8) &= 10.1 \text{ kHz} \\
\Delta\nu_Q(C_4 \text{ and } C_7) &= 10.6 \text{ kHz} \\
\Delta\nu_Q(C_5 \text{ and } C_6) &= 10.8 \text{ kHz}
\end{aligned}
\tag{II-17}$$

Hexadecane has the same hydrocarbon chain length as DPPC. Thus for hexadecane no sliding parallel to the bilayer normal is allowed and we need average only two environments per position. For a temperature of 60°C we predict:

$$\begin{aligned}
\Delta\nu_Q(C_1 \text{ and } C_{16}) &= 7.0 \text{ kHz} \\
\Delta\nu_Q(C_2 \text{ and } C_{15}) &= 8.2 \text{ kHz} \\
\Delta\nu_Q(C_3 \text{ and } C_{14}) &= 8.5 \text{ kHz} \\
\Delta\nu_Q(C_4 \text{ and } C_{13}) &= 9.5 \text{ kHz} \\
\Delta\nu_Q(C_5 \text{ and } C_{12}) &= 10.2 \text{ kHz} \\
\Delta\nu_Q(C_6 \text{ and } C_{11}) &= 10.7 \text{ kHz} \\
\Delta\nu_Q(C_7 \text{ and } C_{10}) &= 11.0 \text{ kHz} \\
\Delta\nu_Q(C_8 \text{ and } C_9) &= 11.7 \text{ kHz}
\end{aligned}
\tag{II-18}$$

This sort of averaging is an oversimplification since it neglects the importance of additional gauche bond formation beyond what is present in a lipid or a fatty acid chain of the same length as the lipid. In the Petersen-Chan model in the upper half of the chain S_β is determined essentially by S_α . This is because

kinks formed at the methyl end do not reach the top of the chain and single gauche bonds are sterically forbidden in this region. Thus $p_j \cong 1$ and $S_\gamma \cong -0.5$. However in the present model gauche bonds formed at the lower end of the hydrocarbon chain can effectively jump to the top of the chain by means of an end for end flip. Thus, averaging fatty acid values overestimates the Δv_Q contribution from the top of the chain. This means that Δv_Q will be reduced for positions near the end of the hydrocarbon relative to positions near the middle. Also note that we have neglected the effect of the methyl rotor in reducing S_β for the terminal position. These two corrections produce a steeper order parameter gradient than listed in equations II-17 and II-18.

We may evaluate this model as follows: First, the model overestimates S_β near the middle of the chain by about 40%. This presents no serious difficulty. The reduction of S_α by the large angles α sampled during a flip may account for the discrepancy. Second, the model evidently predicts a fairly shallow, extensive order parameter gradient, especially for the hexadecane (compare C_8 and C_9 to C_3 and C_{14}), which is in contrast to the plateau-like order parameter profile observed experimentally at 60°C. Third, we must consider the temperature dependence of S_β . In the extended Petersen-Chan model the temperature dependence of S_β

evidently arises via both the temperature dependence of S_α (since there is a change in S_β with temperature in the upper portion of the chain) and the temperature dependence of S_α (since the rate of change for the upper portion of the chain is less than for the lower portion of the chain). By contrast, for both d_{22} -decane and d_{34} -hexadecane the temperature dependence is steeper at the top of the chain (Table II-1). Some additional process must therefore be contributing to the temperature dependence. The present model can account for this if we attribute the steeper dependence to the effect of temperature on chain flip, since the flip controls the transport of gauche bonds to the top of the chain.

In summary this model can account for much of the data, although it evidently predicts an order parameter profile too shallow and elongated to fit the data.

(iii) There is another way in which hydrocarbon parallel to the lipid fatty acid chains can undergo end for end exchange. If the hydrocarbon is permitted to move from one monolayer to the other by sliding parallel to the bilayer normal, then the two ends of the molecule effectively exchange environments. This model is more attractive than the previous one in that the energy barrier to this interlayer shuttling should be less than the barrier for rotation within one layer.

TABLE II-1 Change in S_{β} with temperature as a function of hydrocarbon chain position for (a) d_{54} -DMPC multilamellar dispersion (b) a multilamellar dispersion of d_{22} -decane (10.3 mole %) in DPPC and (c) a multilamellar dispersion of d_{34} -hexadecane (4.4 mole %) in DPPC.

(a) DML - d_{54}

Carbon #	$S_{\beta}(25^{\circ}\text{C})/S_{\beta}(40^{\circ}\text{C})$
14	1.30
13a	1.28
13b	1.30
12a	1.27
12b	1.29
11a	1.29
2b	1.13
11b, 10a	1.23
10b, 9a	1.25
2a, 3-8, 9b	1.13

(b) Decane - d_{22} (10.3 mole %) in DPPC

Carbon #	$S_{\beta}(40^{\circ}\text{C})/S_{\beta}(60^{\circ}\text{C})$
1, 10	1.60
2, 9	1.57
3, 8	1.58
4-7	1.70

(c) Hexadecane - d_{34} (4.4 mole %) in DPPC

Carbon #	$S_{\beta}(43^{\circ}\text{C})/S_{\beta}(60^{\circ}\text{C})$
1, 16	1.52
2, 15	1.52
3, 14	1.52
4, 13	1.69
5-12	1.80

This shuttling motion does not produce inversion symmetry and thus does not reduce Δv_Q by a factor of two. Rather we propose that the reduction in order parameter comes about because, in addition to the averaging of the two environments, as the hydrocarbon passes from one layer to the other, the entire length of the molecule passes through the interlayer space. In contrast to the preceding model we do not consider this transfer as a jump process. Rather, the transfer time is long enough (of the same timescale as chain isomerization) to allow gauche bonds to form at various positions along the chain. The gauche bonds are then transferred to a relatively more ordered environment by the shuttling process. While we are unable to make quantitative predictions based on this model, we can evaluate it in general terms. Model (iii) can explain the temperature dependence in the same way as model (ii). The reduction in order parameter depends on a new motion, which, in turn, affects the gauche bond distribution. The new motion depends on temperature in a new way, affecting the order parameter in central portions of the chain more than at the ends. The model accounts for the relatively flat central portion of the order profile since all portions of the molecule have an opportunity to form gauche bonds. The model predicts some order gradient at the end of the molecule since the ends still spend more time near the interlayer

region than the rest of the molecule. Finally it is interesting to consider in terms of this model why exchange between the two pools of hydrocarbon should be slow. We may speculate that, while the hydrocarbon traverses the interlayer space in shuttling between layers, one end or the other of the hydrocarbon always remains between lipid chains, so that the lipid chains hinder rotation of the hydrocarbons to a membrane-parallel orientation.

Summarizing the discussion on the motional state of the ordered portion of the hydrocarbon, the observation of an order parameter gradient along the hydrocarbon chain suggests that the hydrocarbon is aligned principally parallel to the fatty acid chains of the lipid rather than parallel to the plane of the bilayer between layers. To account for the end for end symmetry of the molecule in the ordered state and the reduction in $\Delta\nu_Q$ compared to lipids or intercalated fatty acids, it is necessary to invoke a new type of motion, either a rotation of the entire molecule about an axis parallel to the membrane plane or a shuttling of the molecule between layers or both. While both mechanisms apparently account for the data, quantitative difficulties with the rotation model and the steric restrictions to rotation of the molecule in one layer make the interlayer shuttling model the most attractive.

c. Equilibrium between hydrocarbon pools

We can draw several conclusions from the data concerning the nature of the equilibrium between the two motional states of the hydrocarbon.

- (i) The observation of two types of signals from a bilayer system containing decane labelled in a single position indicates that the two pools are in slow exchange on a timescale corresponding to the narrowest splitting observed for the hydrocarbon, about 2 kHz.
- (ii) The observation of two pools of hydrocarbon apparently results from a limited solubility of hydrocarbon parallel to the lipid chains, since at low hydrocarbon concentrations the central line contains only a few percent of the total intensity of the spectrum, whereas at higher alkane concentration an appreciable fraction of intensity is accounted for by the isotropic component. Apparently as the hydrocarbon concentration is increased the parallel component is filled in first with excess hydrocarbon going to the interlayer space. The "parallel solubility" of decane in DPPC is greater than that of hexadecane. While we have insufficient data to quantify this statement, the similarity of the 26.7% decane and 4.4% hexadecane spectra with respect to the relative intensity of the central maxima suggests a difference of about an order of magnitude in solubility. In black lipid films the volume fraction of hydrocarbon in the hydrophobic part of the bilayer portion of the

film is 0.5 for decane and 0.01 for hexadecane. The solubility difference may be due to greater packing difficulties at the carbonyl regions of the two monolayers (14) caused by hexadecane, which has a length comparable to the hydrocarbon chain length. The shorter decane can dissolve parallel to the fatty acid chains in one layer without occupying the interface region. The concentration dependence of the splittings $\Delta\nu_Q$ for the ordered component of alkane suggests that the presence of a pool of hydrocarbon in the interlayer space causes a reduction in the order parameter of the ordered component. If the droplets between layers cause a bulge in the bilayer, they would produce a local change in S_α , causing an overall reduction in order parameter.

(iii) For decane-containing DPPC multilayers, incubation at temperatures below T_C causes an irreversible shift of hydrocarbon from the chain parallel component to the interlayer component. The effect apparently depends on reaching temperatures in the gel phase region of the phase diagram, not merely on reaching temperatures in the phase transition region. We have not observed this effect for hexadecane although in the limited number of samples tested we may not have incubated at a sufficiently low temperature. White has postulated that when black lipid films of glycerol monoleate are incubated at temperatures below T_C , solvent (hexadecane

in this case) "freezes out" of the bilayer portion into microdroplets, leaving regions of hydrocarbon-free bilayer (33). This observation is consistent with our results.

More recently Simon (14) has studied DPPC bilayers containing various alkanes by x-ray diffraction. He interprets changes in the low angle pattern observed for all alkanes as indicating a removal of "chain tilt", changing the average angle of the chains with respect to the bilayer normal from 30° to 0° . Bilayer spacings observed from hexadecane-containing bilayers (0.3 to 0.9 mole fraction hexadecane) are consistent with two fully extended hydrocarbon chains, while for decane and the shorter alkanes spacings are longer, indicating a fraction of alkane between the layers. Simon concluded that decane orients both parallel to the chain and between the layers, while hexadecane is found only parallel to the lipid chains. Our results are in conflict with this interpretation, in that we clearly observe two different pools of hexadecane (and decane). No distinction can be made between the two alkanes from our data, except perhaps that hexadecane is less susceptible to "freeze out". Since the presence of kinks and bends in the lipid chains means that they are not fully extended for temperatures above T_c , we do not believe that observation of a bilayer spacing equivalent to the length of two fully extended chains implies that there

is no room for hexadecane to be located in the inter-layer space.

d. Effect of hydrocarbon on the gel to liquid crystalline phase transition

We have observed changes in the phase transition behavior as observed by differential thermal analysis (DTA) for DPPC bilayers containing both decane and hexadecane. For hexadecane the transition temperature increases relative to that for pure DPPC, and two endothermic peaks are observed on cooling. Simon (14) has recently studied these systems as well, using differential scanning calorimetry (DSC), and reports similar results, except that he observes a transition temperature 2.5°C higher than observed by DTA. For decane-containing multilayers we observe no change in T_c as observed by heating although the endothermic transition is broadened. On cooling two transitions are observed, one of which is at a lower temperature than the transition observed for DPPC. Again Simon sees similar results by DSC, except that he finds a lowering of T_c as observed in the single transition of the heating curve.

The general trend of a lower transition temperature for decane-containing multilayers and a higher temperature for those containing hexadecane can be seen in the NMR spectra, where the phase transition appears (upon cooling) first as a loss of shape in the order

parameter plateau and then disappearance of the plateau altogether. For decane in DPPC at 35°C all of the quadrupolar splittings are still identifiable, whereas for hexadecane in DPPC at 43°C splittings are observable but relative intensities have begun to change and by 40° most of the splittings are no longer distinguishable, with the plateau shape very distorted. These changes in T_c due to added hydrocarbon indicate a disordering effect of decane on the bilayer, while hexadecane appears to have an ordering effect. Sturtevant (34) has interpreted this Presteggaard effect of fatty acids as indicating that added fatty acid makes up for the discrepancy in cross-sectional areas between head-group and chains for phosphatidylcholines, noting that the transition temperatures for phosphatidylcholine bilayers containing fatty acids approach T_c for the corresponding phosphatidylethanolamines. Simon gives a similar explanation for the effect of hexadecane, and notes that since decane cannot replace a lipid chain in packing of the hydrocarbon region of the bilayer decane must have a disordering effect.

The observation of two transitions in the DTA (and DSC) on cooling indicates that phase separation is occurring. It is not possible to interpret with any confidence this information about the phase behavior of the system in the absence of systematic calorimetry data as a function of composition and other supporting data.

Simon has some DSC data not only as a function of composition but also as a function of chain length, but offers only cursory explanations of the phase separation, except to speculate that one phase is hydrocarbon rich and the other essentially hydrocarbon free. It is not clear how the calorimetry observations may be interpreted in the light of NMR evidence indicating two pools of hydrocarbon. In chapter IV of this thesis we consider in great detail another two-component bilayer system where phase separation occurs, and make use of both NMR data and systematic calorimetry to interpret the phase behavior of the system and suggest its physiological importance.

E. Summary

We have been able on the basis of ^2H NMR evidence to draw several important conclusions regarding the orientation and motional state of hydrocarbons dissolved in lipid bilayers. We see by NMR two pools of hydrocarbon, one of which shows rapid reorientation on the ^2H NMR timescale, while the other yields an order parameter plateau characteristic of hydrocarbon chains showing a flexibility gradient. The two pools are in slow exchange. The rapidly reorienting pool probably corresponds to microdroplets of hydrocarbon lying between the two monolayers of the bilayer. Using ^2H NMR spectra of perdeuterated lipids and intercalated fatty

acids as reference points, we have also considered several models for the motional state of the oriented component. The observation of an order parameter gradient along the chain implies that this component of the alkane dissolves parallel to the fatty acid chains of the lipid. Reductions of the magnitude of the order parameter and changes in the profile shape with respect to fatty acid probes, along with the equivalence of the two ends of the molecule in the NMR spectra, imply that the hydrocarbon undergoes a new motion in addition to those included under chain reorientation and chain isomerization. Either end for end flip of the molecule within a single monolayer or shuttling of the molecule between layers may account for the data. Spectra as a function of temperature and composition indicate that there is a limited solubility of hydrocarbon parallel to the fatty acid chains of the lipid, so that excess hydrocarbon enters the interlayer space. Incubation of the bilayer dispersion at temperatures below T_c causes irreversible transfer of hydrocarbon oriented parallel to the chains to the interlayer hydrocarbon pool.

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CHAPTER III CONSTRUCTION OF A ^2H NMR SPECTROMETER FOR OBSERVATION OF WIDE-LINE SPECTRA IN THE FOURIER TRANSFORM MODE.

A. Introduction

In Chapter II we have illustrated the great utility of deuterium nuclear magnetic resonance in investigating the motional state of various components of model membrane systems. The first applications of this technique to membranes came about shortly after Fourier transform NMR spectrometers became commercially available and the inherently low sensitivity of the ^2H nucleus for NMR detection could be compensated for by signal averaging in the time domain (1). The theory of first order spectral effects arising from incomplete motional averaging of the electric field gradient tensor at the quadrupolar ^2H nucleus is relatively straightforward (2). However, early experimenters were limited by the restrictions imposed by applying Fourier transform spectrometers designed for high resolution work to systems requiring large spectral widths (3,4). Thus in succeeding years expansion of the range of applicability of ^2H NMR to membrane systems has come about principally in response to improvements in wide-line Fourier transform ^2H NMR techniques and spectrometers, as experimentalists have attacked the problems associated with performing wide-line experiments in the time domain (5).

In this chapter we describe the construction of a ^2H NMR spectrometer for wide-line Fourier transform studies, utilizing an existing gated RF transmitter, receiver and pulse programmer, and adding other components already on hand, newly purchased components, and home-built components. The resulting spectrometer is relatively simple in design, was relatively inexpensive to assemble, and incorporates most of the recent techniques for generating high-quality wide-line ^2H spectra from a spectrometer operating in the Fourier transform mode. It produces spectra of quality greater than or equal to that of many of the ^2H spectra in the literature.

We begin the chapter by outlining the requirements for a ^2H NMR spectrometer to be applied to membrane systems and indicating the various techniques which have been developed by others for meeting these criteria. In the following two sections we indicate how these techniques have been implemented in the spectrometer hardware and computer software of our machine. In the last section we describe the performance of the resulting instrument, including sample spectra.

B. General requirements for a ^2H spectrometer for investigation of membrane systems

The principal requirements for a Fourier transform deuterium nuclear magnetic resonance spectrometer for the observation of lipid bilayer systems exhibiting incomplete motional averaging may be summarized as follows:

(i) Spectral width. Since $\frac{e^2qQ}{h}$ for aliphatic ^2H is approximately 170 kHz (6), the total width of the powder pattern for a perfectly ordered system is $2 \cdot \frac{3e^2qQ}{4h} = 255 \text{ kHz}$, and thus very large spectral widths may be required for systems exhibiting a high degree of order.

(ii) Sensitivity. The NMR sensitivity for detection of the ^2H nucleus is about 1/100 that for an equivalent number of protons. Moreover, the quadrupolar powder patterns cover a large spectral width, and thus high fields, large samples and/or long time-averaging are required.

(iii) Resolution. For membrane systems spectra consist of overlapping quadrupolar powder patterns of various splittings $\Delta\nu_Q$. The individual splittings are measured by identifying the two peaks in the spectrum corresponding to the 90° orientation discontinuities of a particular powder pattern component and measuring the frequency differences between the discontinuities. Resolving two closely spaced $\Delta\nu_Q$ s requires resolving the corresponding discontinuities; the sharpness of the discontinuities

depends on the isotropic linewidth (6), which in turn depends on the effective transverse relaxation time T_2^* . For some bilayer systems in which there is a relatively small degree of ordering, T_2 approaches its value in solution, so that spectrometer resolution on the order of 10 Hz or less may be useful. If, as in our case, the same spectrometer is to be used occasionally for high resolution work, the maximum possible resolution is required for these experiments since the low gyromagnetic ratio (about one-sixth that for protons) and small chemical shift range (10 parts per million) of deuterium make the entire chemical shift range only 92 Hz in a 14 kilogauss field.

1. Spectral width

By far the most difficult of instrumental criteria to meet is the need for large spectral widths. For one thing, in order to insure that a transmitter RF burst rotates all components of the magnetization through the same angle in the rotating frame, a large H_1 field, and thus a high transmitter RF power, are required. The origin of this requirement may be seen as follows: Consider a system with a single resonance at frequency ω_0 . The Hamiltonian of interest during the RF burst has two components, one due to the large field H_0 and one due to the smaller RF-generated field H_1 . Transforming from a laboratory reference frame to a rotating reference frame which rotates at frequency $\omega_0 = \gamma H_0$ the Hamiltonian contains only the H_1 field, and

thus the magnetization precesses about $\vec{H}_1$ in this frame. (The frequency of rotation of the rotating frame is the detector frequency.) If, however, the rotating frame rotates at frequency $\omega_r \neq \omega_0$ (i.e. we pulse off-resonance), then the Hamiltonian in the rotating frame still has two components, $\vec{H}_1$, due to the RF burst and $(\omega_r - \omega_0) \frac{1}{\gamma}$ due to the static magnetic field, whose effects are not completely removed by transforming to this rotating frame. In order to avoid distortion in the spectra, we wish the magnetization still to precess strictly about $\vec{H}_1$. This requires that $|\vec{H}_1| \gg \frac{1}{\gamma} (\omega_r - \omega_0)$. Clearly, the farther off-resonance we wish to observe the larger $|\vec{H}_1|$ must be to prevent distortion. The larger we make $|\vec{H}_1|$, the shorter the time required to rotate the magnetization through 90° . Since $\gamma |\vec{H}_1| t_{90} = \frac{\pi}{2}$, the criterion for distortion-free spectra may be expressed (7):

$$t_{90} \ll \frac{\gamma \pi}{2(\omega_r - \omega_0)} .$$

The need for large H_1 fields generated by high transmitter power also implies careful probe design. Since the probe contains a resonant circuit, input RF voltages of hundreds of volts produce potentials of thousands of volts in the probe tuning network. High voltage components must be used in probe construction and care must be taken to prevent electrical arcing.

A second problem for experiments in the time domain is that the large spectral width required

necessitates a rapid analog-to-digital conversion rate. With the Varian 620i minicomputer and Varian data acquisition hardware available, careful programming was required to achieve a maximum data conversion rate (see the program description in section D of this chapter). A more fundamental difficulty is that for a 100 kHz conversion rate, for instance, the digitized data points fall 10 μ sec apart, and to avoid phasing difficulties and baseline distortions the first point should be taken within 10 μ sec after the end of the RF burst. Another way of looking at this is to note that wide spectral lineshapes correspond to short free induction decays, so that if the initiation of data acquisition is delayed the broad components of the spectrum will be attenuated relative to the narrower components or lost altogether. In order that transmitter RF power be used as efficiently as possible, to maximize the strength of the H_1 field, a single coil probe is customarily used in wide-line time domain experiments. With this system, in order to initiate data acquisition, it is necessary that the transmitter signal should first decay off of the sample coil since the transmitted RF burst is about 10^7 times more intense than the free induction decay. The decay time constant τ_{coil} depends on the RF frequency ω_R and the quality factor Q of the coil (8):

$$\tau_{\text{coil}} = \frac{Q}{2\omega_R} \cdot$$

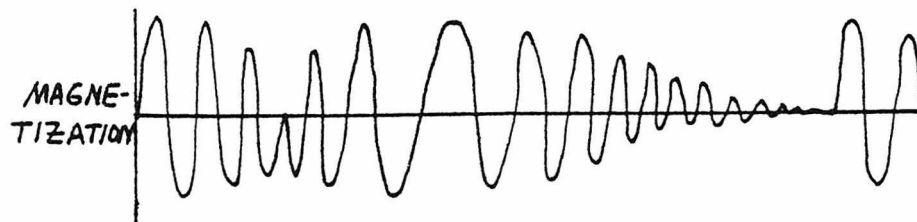
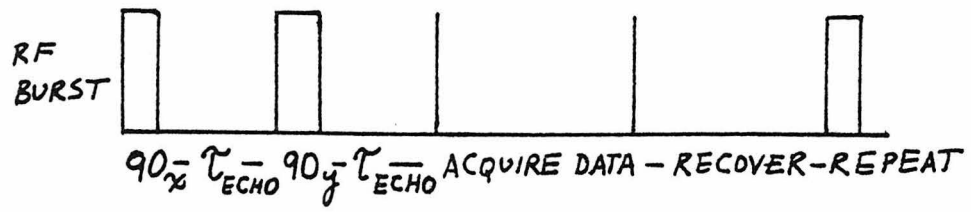
Shorter decay constants are produced by coils of lower Q operating at higher frequency. For our purposes the availability of a 14 kG magnet fixes ω_R at 9.210 MHz. Even for a fairly low Q , say $Q=50$, γ_c is about 2.7 microseconds. Since a delay of about ten to fourteen times γ_c is required before data acquisition can begin, about 30 to 40 microseconds recovery time is minimal. Even with more elaborate Q -switched probes, recovery times can be cut in half at best. Another method is to use a high Q system with a negative feedback amplifier to damp the ringing of the system; this is effective but reasonably complex (9). The usual approach is simply to gate the receiver off during the recovery period. With this approach, for a recovery time of 40 microseconds, any digitization rate faster than 25 kHz produces a digitized FID with one or more data points missing at the beginning. Thus spectra of width 25 kHz or wider will be distorted. Specifically, such spectra will suffer from unacceptable baseline and intensity distortions and from reduced signal to noise, since the early part of the FID, which contains most of the spectral information, is lost. To circumvent this problem, a solid echo burst sequence (10) has been used, consisting of two 90° bursts which are 90° out of phase with each other and separated by a time τ_{echo} . At time τ_{echo} after the second 90° pulse the quadrupolar pairs echo in the rotating frame (11) and digitization of the FID is begun. The

entire sequence is shown in Figure III-1. τ_{echo} can be chosen long enough (of the order of 100 microseconds) so that the coil can ring down completely after the second 90° pulse before data acquisition is begun. In this fashion an undistorted FID can be obtained and as first shown by Davis et al. (12), the transformed spectrum faithfully reproduces the lineshape.

Finally, the need for large spectral widths suggests the use of quadrature phase detection, which has long been suggested in the literature (13,14) but only recently applied to commercial spectrometers. In a spectrometer with a single phase detector, the single FID obtained contains information about the frequency difference between the resonant frequencies and the detector frequency, but only the absolute value of this difference, and not its sign, can be determined. Consider a system with two resonant frequencies, ω_a and ω_B , giving infinitely sharp lines of intensity A and B respectively. By making the lines very sharp we neglect the dispersion mode signal; this oversimplification will not affect our conclusions. Place ω_R halfway between ω_A and ω_B so that $\omega_B - \omega_R = \omega_R - \omega_A = \omega^+$. Consider a frame of reference rotating at frequency ω_R . In this frame of reference we apply a 90° RF burst along x and tip the magnetization ($\vec{M}_A + \vec{M}_B$) to the y axis. Then $\vec{M}_A$ and $\vec{M}_B$ precess about H_0 with equal frequency but opposite sense. We detect this magnetization with a single phase detector which measures

FIGURE III-1

RF burst sequence for solid-echo data acquisition (a), presenting amplitude of transmitter RF voltage on the sample coil (b) and signal induced in RF coil by resulting sample magnetization (c).



the projection of $\vec{M}_{\text{total}}$ on the x axis. The corresponding FID consists of the sum of two sinusoidal components equal in frequency, different in amplitude and 180° out of phase. Fourier transformation of the digitized FID yields a cosine transform which is identically zero and a sine transform with a single resonance line at frequency ω^+ of amplitude $(A - B)$. All of this is diagrammed in Figure III-2a. The resulting spectrum shows a single peak of intensity $B - A$ at frequency ω^+ . One side of the spectrum has "folded over" on the other and there is no way to determine the size of the individual components A and B. To eliminate this confusion, in a spectrometer with a single detector ω_R is always set to be at one end of the spectrum or the other so that all the difference frequencies represented in the FID are of the same sign and no foldover is possible. This resolves the ambiguity but wastes half of the spectral window, whose breadth is set by the intensity of the H_1 field.

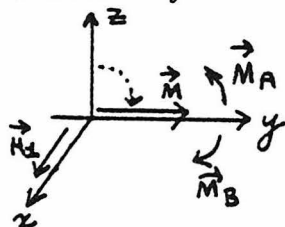
Suppose, however, we add a second phase detector which measures the projection of $\vec{M}$ along the y axis. The resulting spectrum from this detector is shown in Figure III-2b; it also exhibits foldover. However, suppose we rotate the spectrum from the first detector by 90° (interchange sine terms for cosine terms and change the sign of the new sine terms). When we add the spectra from the two detectors, as shown in the figure, we gen-

FIGURE III-2

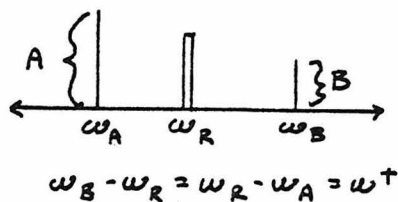
Results of an NMR experiment using quadrature phase detection on a sample whose spectrum contains two infinitely sharp resonance lines, when the frequency of the RF burst is exactly halfway between the two resonant frequencies.

Example of quadrature phase detection:

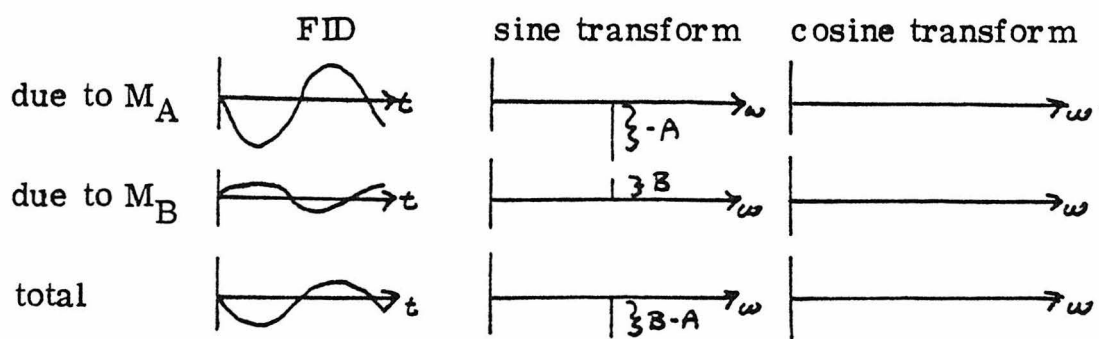
coordinate system:



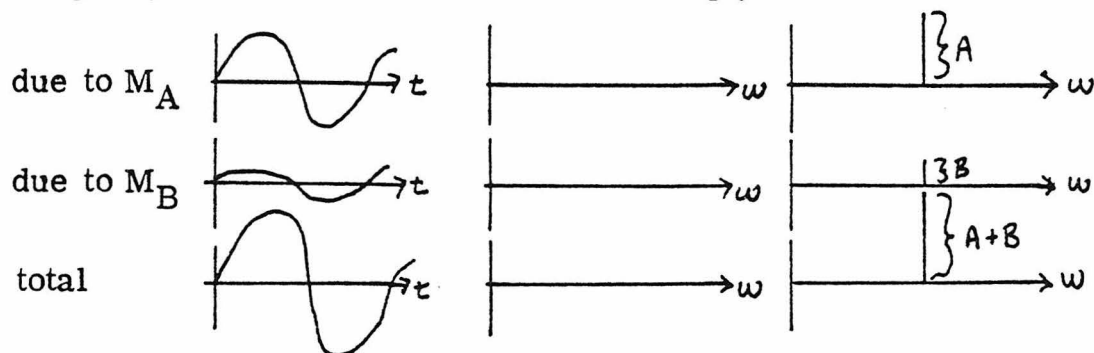
actual spectrum:



a) Single phase detection with detector along x:

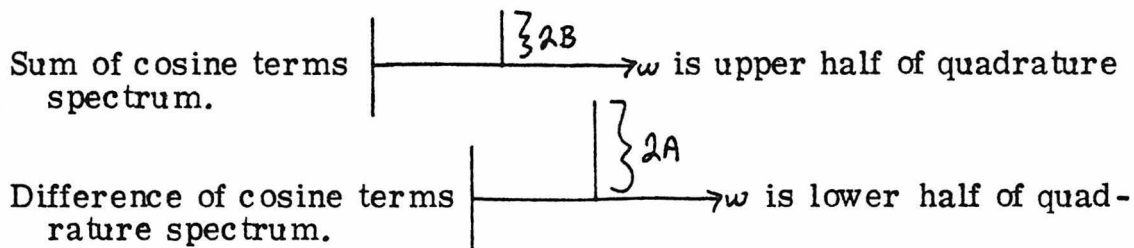


b) Single phase detection with detector along y:



Now rotate results from detector along x by 90° (sine terms become new cosine terms and (-1) cosine terms become new sine terms).

Combine results from the two phase detectors:



erate the $\omega > \omega_R$ portion of the spectrum from the sum. Similarly, from the difference we form the $\omega < \omega_R$ portion. Information from the two detectors allows us to assign both amplitudes and signs of the frequency components in the FID, and thus to make use of the entire spectral window provided by the H_1 field. Moreover, since the two detectors provide independent information, we effectively sample FIDs twice as fast by using two phase detectors simultaneously after each burst sequence; and thus for a given number of bursts, we gain in signal-to-noise by a factor of $\sqrt{2}$.

Finally, consider the application of quadrature phase detection to perfectly symmetric spectra such as are observed for 2H with incomplete motional averaging. If the RF frequency is set exactly at the center of the spectrum, the diagrams in Figure III-2 apply with the further restriction that $A = B$. In this case one of the phase detectors will give no signal at all, while the other one contains the information. In this case it is counterproductive to add the results from the two detectors, since one contains only noise. A double-sided spectrum may be generated for the sake of appearance by the so-called "spectrum reverse" technique (15), but all of the information is contained in the single-sided transform from the y-axis phase detector. As noted, this applies only to the case where ω_R is at the exact center of the spectrum. Quadrature phase detection will produce

a proper spectrum for any offset of the transmitter frequency.

2. Sensitivity

In addition to the sensitivity advantages of quadrature phase detection and of the solid-echo pulse sequence as mentioned above, only two further considerations regarding signal-to-noise require discussion. First, it is obvious that large sample volumes will be useful. This means that larger diameter sample tubes will be used and that longer sample coils will be constructed so as to enclose a large volume. Second, since signal-to-noise is proportional to, among other things, the Q of the coil (8), a fairly high Q is desirable. For the standard sequence of a 90° burst followed by data acquisition a compromise must be reached between signal-to-noise and recovery time in establishing Q . For the solid-echo sequence the recovery time requirement is relaxed, and large Q 's are acceptable.

3. Resolution

For wide-line membrane work (resolution needed ≥ 10 Hz), the homogeneity of the magnetic field needed is intermediate between that required for high resolution work and that usually used for wide-line work with solids. For a high resolution field, the amount of computer memory space may actually be the factor which limits spectral resolution if the desired spectral width is large.

C. Spectrometer hardware

A block diagram of the 9.21 MHz ^2H spectrometer is shown in Figure III-3. Table III-1 lists the description and sources of the various components in the block diagram. The discussion will cover the five main spectrometer systems: (1) the magnet with field/frequency lock, (2) the transmitter, (3) the receiver, (4) the probe, and (5) the data system. The spectrometer is based on three existing units, namely (1) a 14 kGauss wide-gap Varian high resolution electromagnet, (2) a Tomlinson high power 3-75 MHz transmitter, quadrature receiver and pulse programmer and (3) a Varian data system utilizing a 620i minicomputer. The magnet flux stabilizer, spectrometer frequency base, probe, probe transmit/receive switching, wideband preamplifier system, hard-wired solid-echo phase alternating pulse sequence, spectrometer/data system interfacing, and console power supply were all constructed at Caltech. A commercial temperature controller, a frequency synthesizer, a tuned preamplifier and a Varian external reference lock system were also added to the basic spectrometer units. In addition, the Varian computer software for NMR was extensively modified, as described in section D of this chapter.

1. Magnet and field/frequency lock

The ^2H spectrometer is built around a Varian model V4013A 12" electromagnet energized by a model V2100B

FIGURE III-3

Block diagram of the 9.21 MHz ^2H
NMR spectrometer.

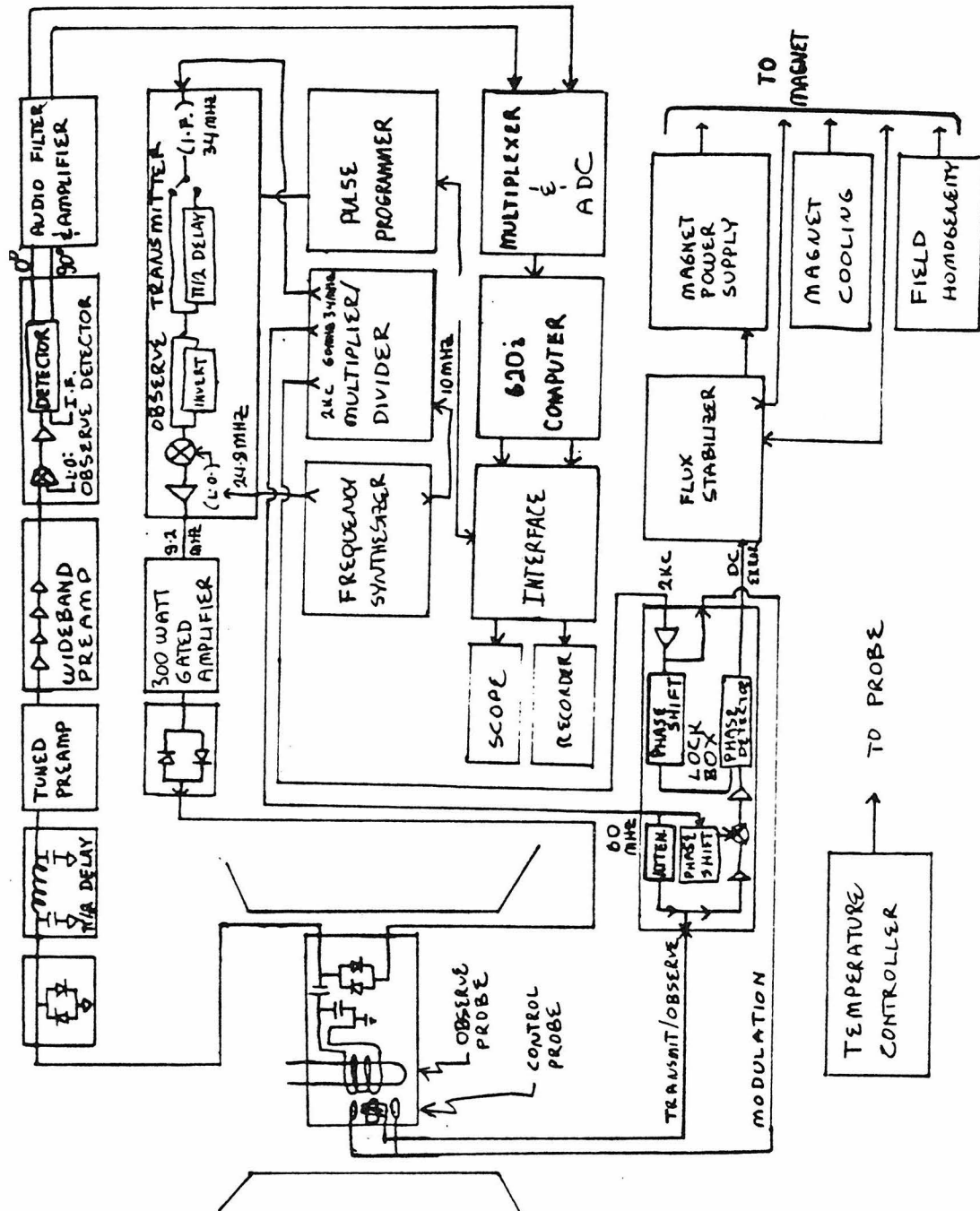


TABLE III-1 COMPONENTS USED IN CONSTRUCTING THE 9.21
MHz ^2H NMR SPECTROMETER

Tuned Preamp	:	Janel Laboratories Model PB Noise Figure: 2.0 dB Gain : 16 dB
Wideband Preamp	:	Avantek Model: GPD 401(1), 402(2), 403(1) Gain : 56 dB Construction: CIT Chem Elec Shop K. Bloch
Observe Transmit/ Receive	:	Tomlinson Research Model BK Superhet Interface
Dual Audio Filter and Amplifier	:	Rockland Model 432
300 Watt Gated Amplifier	:	Tomlinson Research Model BS RF Amplifier
Lock Box	:	Varian Associates Model 4353 External Reference Proton Stabilization Controller
Flux Stabilizer	:	CIT: T. Pietsch
Magnet System	:	Varian Associates Model V4013A 14kG Power Supply: Model V2100B Cooling Unit: Model V3520A Homogeneity Unit: Model V4365A
Temperature Controller	:	Varian Associates Model V4540
Frequency Synthesizer	:	Hewlett-Packard Model 8333A
Spectrometer Frequency Base	:	CIT: N. Singh/K. Bloch
Pulse Programmer	:	Tomlinson Research Model BA Pulse Programmer Echo PABS Pulse Card: CIT: K. Bloch, W.R. Croasmun

TABLE III-1 CON'T

Data System	:	Varian Associates (From existing HR220) Modifications: Hardware: CIT: K. Bloch, W.R. Croasmun 620i Computer, Software: CIT: W.R. Croasmun S-1401 MUX & ADC, Spectrometer Interface, HR220 Bed Recorder, HP Oscilloscope
Probe	:	CIT: K. Bloch, W.R. Croasmun, R. Sanchez, G. Durenburg

magnet power supply, cooled by a model V3520A magnet coolant system and equipped with a model V4365A shim system utilizing four sets of shim coils applied to the magnet pole faces. The magnet provides a 14 kG field of high resolution quality in an air gap of 4.5 cm. Field stabilization is provided by a flux stabilizer and field sweep unit constructed by the Caltech electronics shop. The flux stabilizer regulates the magnet power supply in order to maintain a constant field, and it sweeps the field when desired. It consists of an ac and a dc amplifier to which are input the currents induced in a set of pickup coils on the magnet pole faces by fluctuation in the field. Also input to the amplifiers are current ramps generated by the field sweep unit and control signals from the field/frequency lock system. Output from the dc amplifier is fed back to the magnet power supply. The ac amplifier output goes directly to a set of buckout coils also located on the magnet pole faces.

The frequency sweep unit in the flux stabilizer unit is used to search the field for the reference NMR signal used in the field/frequency lock system. This system consists of a 60 MHz transmitter and receiver and a lock probe containing a sealed water sample surrounded by a single transmit/receive coil and two field modulation coils. (Varian external reference proton stabilization controller model V4353) The lock box supplies 2 kHz to

the modulation coils and 60 MHz to the transmit/receive coil. The upper sideband dispersion mode signal is detected by a 60 MHz amplifier and phase detector followed by a 2 kHz amplifier and phase detector. The resulting DC signal from the dispersion mode is used directly as input to the flux stabilizer. The output of the 2 kHz amplifier is available on an oscilloscope screen and may be monitored while sweeping the field to locate the lock signal. When the correct value of the field is set a 2 kHz sine wave will appear on the screen.

Frequency input to the lock box is, in turn, provided by the spectrometer frequency base, constructed in the Chemistry electronics shop, which produces 2 kHz and 60 MHz signals via a divider and a multiplier from a 10 MHz input supplied by the observe channel frequency synthesizer. This 10 MHz is derived from the synthesizer's 10 MHz temperature controlled crystal oscillator (TCXO) which serves as a high stability frequency standard for the synthesizer. Thus this TCXO serves as the frequency standard for the entire instrument, and the reference and observe channel frequencies are locked together.

2. Transmitter and pulse programmer

The transmitter section of the observe channel consists of a Tomlinson model BK superheterodyne interface, a Tomlinson model BS high-power gated RF amplifier and a Tomlinson model BA pulse programmer. The superhet interface mixes the output of a Hewlett Packard model

8333A frequency synthesizer (24.846 MHz for the ^2H case) with the output from an internal local oscillator (34.057 MHz) to generate an observe frequency between 3 and 75 MHz (9.210 MHz in the ^2H case). This signal is gated and phase shifted under the direction of the pulse programmer, filtered, amplified and input to the RF amplifier. The amplifier generates RF bursts with a power of about 300 watts and nominal rise and fall times of 250 nanoseconds.

The pulse programmer consists of a number of pulse generating and timing circuits interconnected by a printed circuit board called the pulse card, on which the desired pulse sequence may be hard-wired. The programmer provides for a sequence containing an arbitrary number of pulses of constant spacing and at most two different lengths. The pulses may have phase 0° , 90° , 180° or 270° with respect to an arbitrary phase point. Pulse sequences may be initiated either by an internal timer or externally by a TTL pulse supplied to a BNC connector. Pulse lengths and intervals may be adjusted by means of front panel controls. The pulse programmer supplies to the superhet interface and the RF amplifier a TTL compatible keying signal to indicate when the RF is to be gated on and a two-bit phase word used by the superhet interface to set the transmitter RF phase.

The pulse card used for ^2H spectra provides for a solid-echo phase alternating burst sequence. The

standard solid-echo sequence is $90^\circ (0^\circ) - \tau_{\text{echo}} - 90^\circ (90^\circ) - \tau_{\text{echo}} - \text{acquire data} - \text{recover} - \text{repeat}$. In order to average out phase and intensity errors between channels, it is desirable to increment the phase of each of the bursts by 90° after each sequence:

```

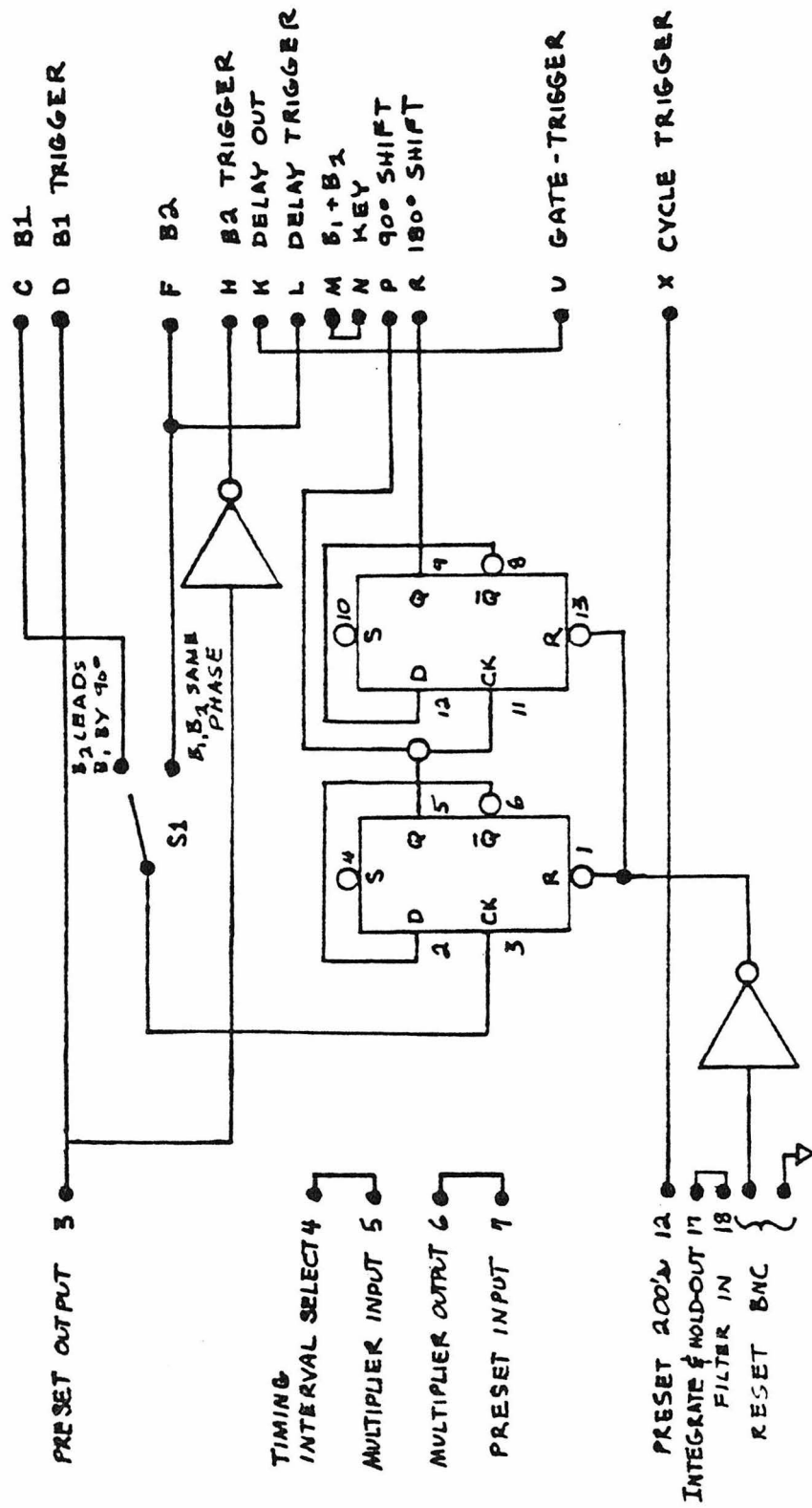
     $90^\circ (0^\circ) - \tau_e - 90^\circ (90^\circ) - \tau_e - \text{acquire-recover}$ 
     $- 90^\circ (90^\circ) - \tau_e - 90^\circ (180^\circ) - \tau_e - \text{acquire-recover}$ 
     $- 90^\circ (180^\circ) - \tau_e - 90^\circ (270^\circ) - \tau_e - \text{acquire-recover}$ 
     $- 90^\circ (270^\circ) - \tau_e - 90^\circ (0^\circ) - \tau_e - \text{acquire-recover}$ 
    -repeat
  
```

(for a complete rationale for this phase alternating burst sequence see the software description which follows this section). The pulse card for the ^2H spectrometer provides two RF bursts, B_1 and B_2 , of variable length (the length of the B_2 is less than or equal to the length of B_1) with a variable interval between them. The two bursts either have the same phase or else B_2 is 90° advanced in phase with respect to B_1 , depending on the position of a toggle switch on the card. Each two-burst sequence has phases 90° advanced with respect to the previous sequence. The phase of B_1 may be reset to zero by supplying a positive-going TTL compatible pulse to the "reset" BNC connector on the card.

The circuit diagram of the pulse card is given in Figure III-4. The entire cycle is triggered either by an external TTL pulse supplied to the "EXT 2" BNC connector (front panel "TRIG" switch set to "ext 2") or by

FIGURE III-4

Circuit diagram for pulse card used in Tomlinson model BA pulse programmer of the ^2H spectrometer to generate a solid-echo phase alternating RF burst sequence.



an internal timer ("TRIG" switch set to "free") running at 200 times the rate set by the front panel timing controls (pin 12 connector to pin X). The front panel timing rate is given by the "time base" times the "multiplier" setting times the "timing" (called "preset" on the pulse card) setting (pin 4 to pin 5, pin 6 to pin 7). When the cycle is triggered, the preset output goes low, triggering B_1 (pin 3 to pin D). The length of B_1 is set by the front panel "pulse source" and " B_1 length" controls. After the timing interval set on the front panel, the preset output goes high. The inverse of the preset output gives a trailing edge at the end of the preset interval, and is used to trigger pulse B_2 (pin 3 through inverter to pin H). The length of pulse B_2 is set as a fraction of the length of pulse B_1 by the front panel " B_2 length" control. The inclusive or of pulses B_1 and B_2 is used as the keying signal to initiate RF bursts (pin M to pin N). Either pulse B_1 or pulse B_2 may be used as the input to a dual edge-triggered flip-flop connected as a two-bit counter (pins C to F to switch 1, switch 1 to 7474 pin 3). The least significant bit of the counter output goes to the 90° shift line (7474 pin 5 to pin P); the most significant bit goes to the 180° shift line (7474 pin 7 to pin R). The transmitter phase is incremented once per two-burst sequence. If the B_1 pulse is connected to the counter (switch 1 up), the phase is incremented at the start of the sequence

and the two bursts have the same phase. If pulse B_2 is connected to the counter (switch 1 down), the phase is incremented between the two bursts and burst B_2 leads burst B_1 in phase by 90° .

3. Probe and transmit/receive switching

The ^2H spectrometer uses a homemade 15 mm single coil high power probe. The body of the probe is a Varian model 4333A probe which is constructed from a solid block of aluminum. It has been entirely reworked for the new probe, including removal of the tuning networks, removal of the transmitter coil and Faraday shield and re-milling of the two cavities provided for installation of electronic components. Modulation coils were left intact but are not used. The top plate and bottom plate are bored out to give a free bore of about 40 mm through the entire probe.

To this shell have been added two machined Delrin bottom plates which support the insert, contain the insert connector, and form a pathway for the admission of variable temperature air. The insert itself is a 12 turn coil wound on a specially machined Lucite coil form. A new aluminum end plate is used to mount the probe tuning network and transmit and receive connectors. A commercial 15 mm spinner is used. A simple passive scheme for switching the probe from transmitter to receiver utilizes a delay circuit contained in a separate aluminum enclosure.

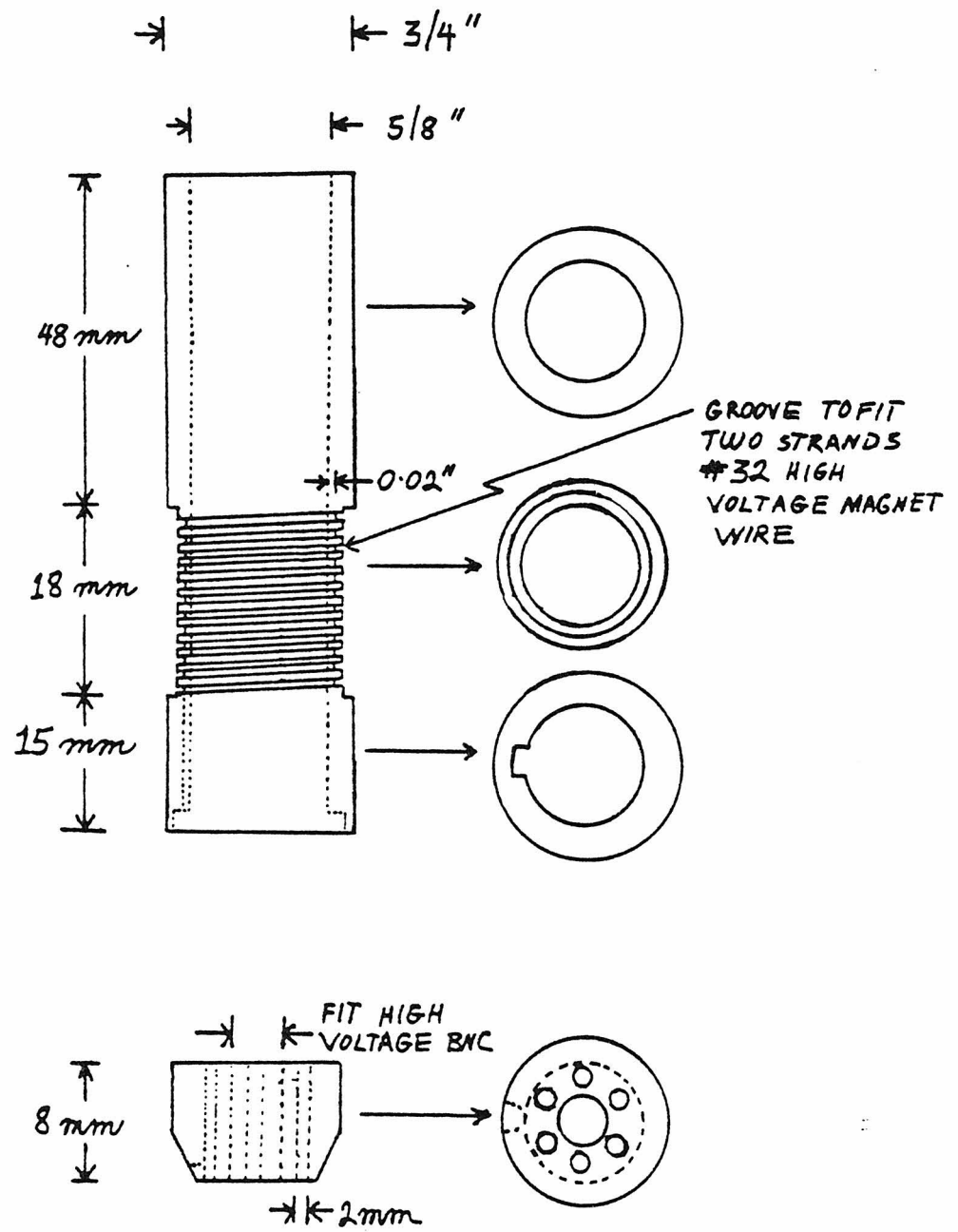
a. Probe electronics and coil insert

The insert coil form consists of two machined Lucite pieces: a tube on which the coil is wound and a bottom plug containing the BNC connector and a set of holes which admit variable temperature air to the sample. The pieces are sketched in Figure III-5. The tube is grooved with a 12 turn spiral at the rate of 18 turns per inch giving a total length of the coil of 18 mm. Thus the useful volume in a 15 mm diameter tube is about 2 ml. The depth of the groove is set so as to leave as thin a wall to the insert as practicable, about 0.02", and thus provides a maximal filling factor while still permitting the coil to be wound on the outside of the form. The coil is wound from two parallel strands of #32 high voltage magnet wire pressed into the groove to give a perfectly regular coil for good H_1 homogeneity as required in echo pulse sequences.

There is a space of about one and one half strand diameters between turns. The resulting coil has an inductance of 3.2 μ Henries and a Q of about 100 at 9.210 MHz. The relatively high Q for this frequency is achieved by using a double strand of wire and spacing the turns. At the lower end of the coil the two strands pass through a hole in the insert and thence down a groove in the inside wall of the insert to the center pin of the high voltage BNC connector in the bottom plug. This connector is cut and turned to fit from a standard high voltage

FIGURE III-5

Insert coil form for 9.21 MHz high-power,
single coil NMR probe.

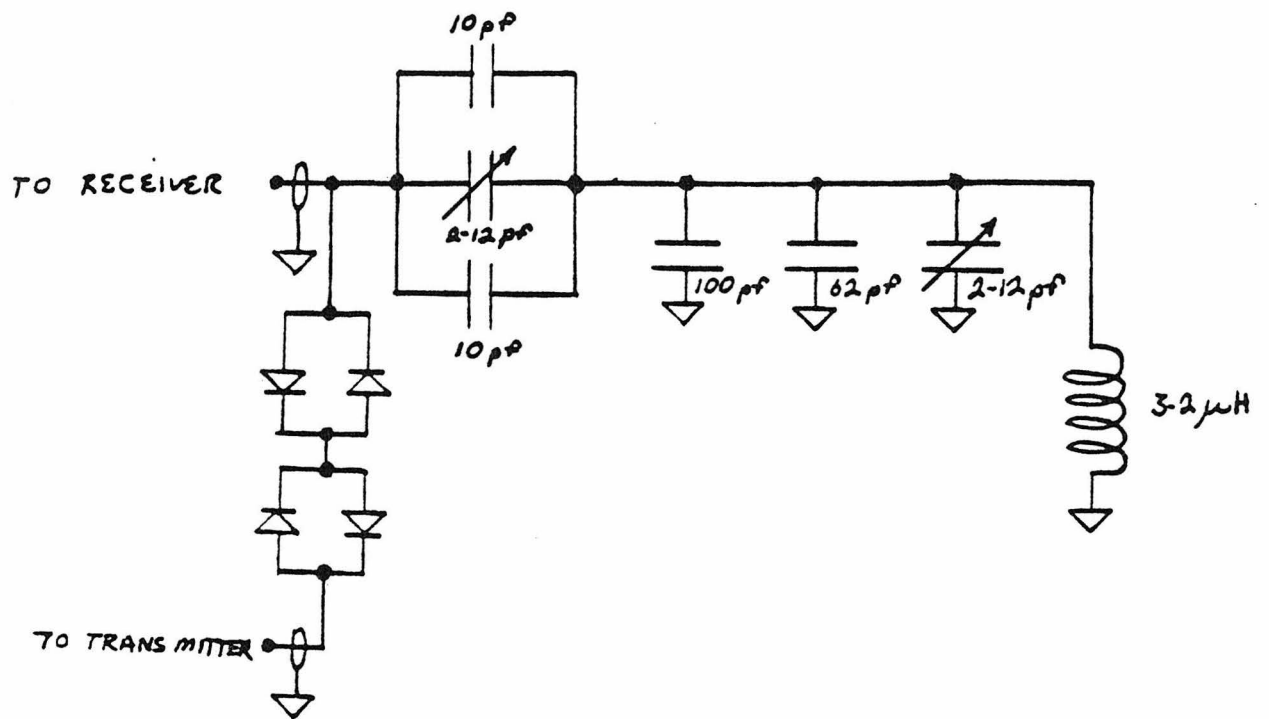


female BNC. At the top of the coil the two strands enter a Teflon tube which runs back down the outside of the insert across the coil to the bottom of the plug, where the wires are soldered to the shield of the BNC connector in a groove provided in the plug. In this fashion the two ends of the coil are kept well insulated from each other since there is a large potential across them. Variable temperature air is admitted to the insert through six holes in the bottom plug.

The Delrin plate which supports the insert contains a high voltage male BNC, also cut to fit, which is connected to the tuning network in the lower tuning network chamber of the probe by a length of miniature coaxial cable. The probe circuit is shown in Figure III-6. The two variable capacitors may be adjusted to give a complex impedance of 50 ohms at 0° phase angle for a frequency of 9.210 MHz. The variable capacitors are Polyflon Corporation high voltage RF trim capacitors variable between 2 and 12 μ H and rated to 6 kV peak working voltage. The fixed value capacitors are Vitramin high voltage porcelain capacitors rated at 2500 V DC. Probe voltages almost certainly exceed these ratings, so a low duty cycle is essential to prevent capacitor breakdown. The variable capacitors are mounted directly in the probe end plate with the connection between them at the interior end. The smaller fixed capacitors are mounted on the variable ones and the entire tuning

FIGURE III-6

Circuit diagram for 9.21 MHz high-power,
single-coil NMR probe.



assembly is covered with several coats of Glyptol high voltage insulating varnish. In addition the chamber containing the tuning components is lined with Teflon sheets. All of these precautions are absolutely necessary to prevent arcing in the probe. The tuning network is connected to the receiver BNC and through two pairs of crossed diodes (1N914) to the transmitter BNC.

b. Probe physical arrangements - variable temperature

The NMR probe consists of an aluminum block with a 40 mm vertical bore near one end and two milled chambers in the top and bottom near the other. The chambers are sealed by three aluminum plates on the top, on the bottom and on the end. The tuning components are mounted on the end plate. This arrangement is sketched in Figure III-7. The top plate supports a spinner base and a commercial 15 mm air turbine spinner. Three aluminum Allen screws secure the spinner to the spinner base and may be adjusted independently to align the spinner with respect to the insert. To the bottom plate are attached two milled Delrin pieces called the coil base and the variable temperature base. The coil base, Figure III-8, contains a specially ground high voltage male BNC which mates with the BNC connector in the insert. A miniature coaxial cable runs from the bottom of the coil base through the base horizontally, then through a channel between the probe body and the bottom plate and into the lower tuning network chamber where the probe matching

FIGURE III-7

9.21 MHz high-power, single-coil NMR probe.

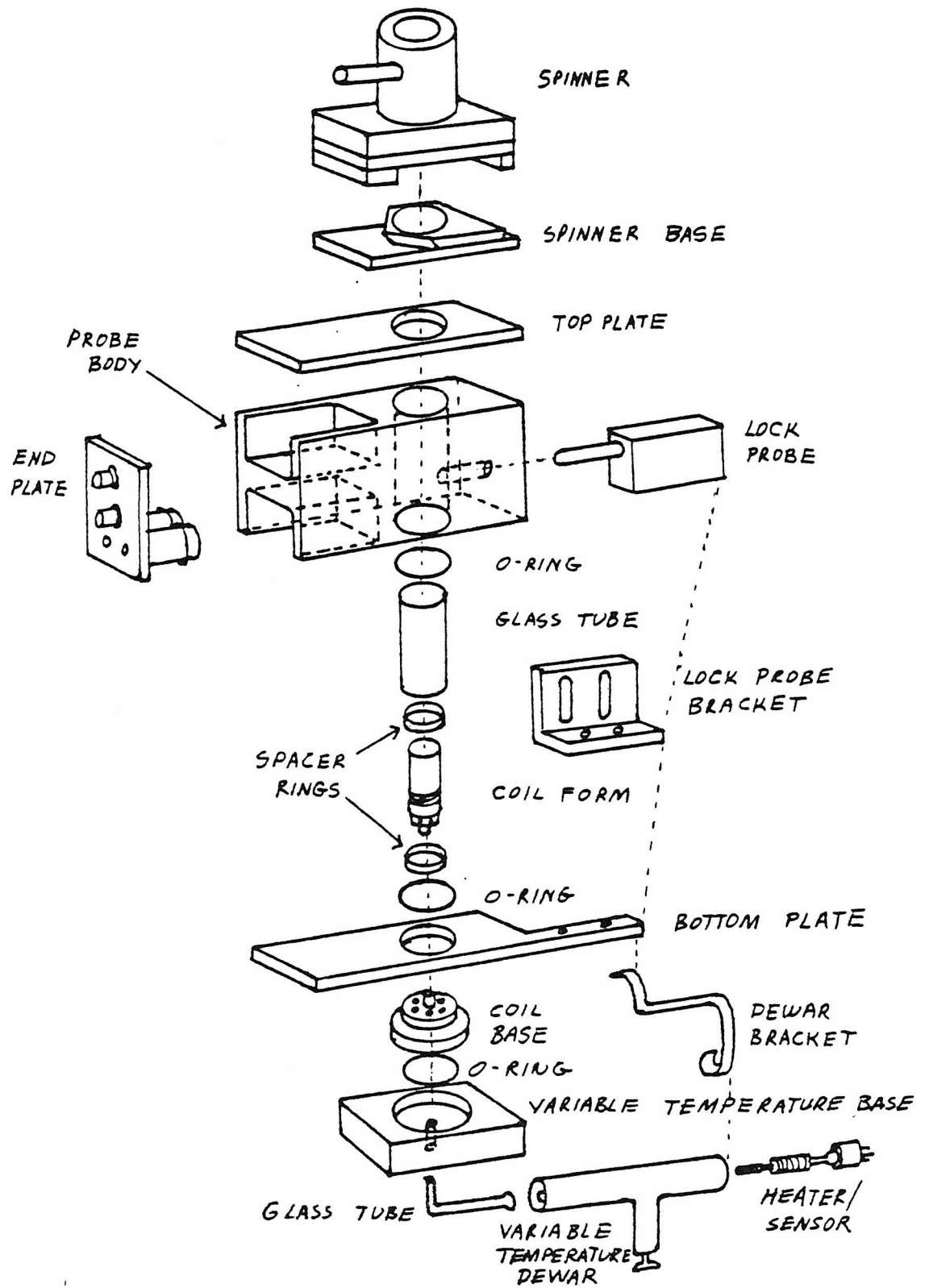
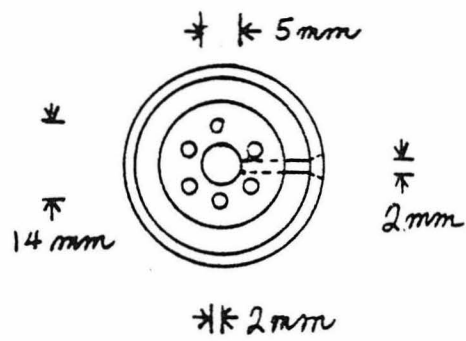
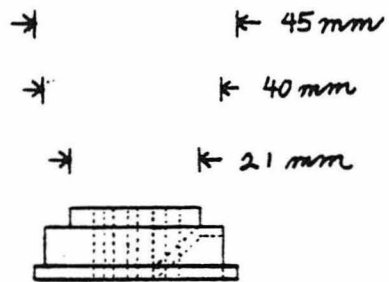


FIGURE III-8

Coil base which supports sample coil in
9.21 MHz NMR probe.



network components are housed. The coaxial cable - BNC connector joint is Glyptol-coated to prevent arcing. Six vertical holes in the coil base admit variable temperature air from below into the probe bore. The coil base is press fit into the bottom plate and secured by the variable temperature base below it. The variable temperature base, Figure III-9, is secured to the bottom of the probe via screws at the corners running through the variable temperature base and the bottom plate and into the probe body. The variable temperature base is countersunk to fit around the coil base, and has a central depression which forms a small chamber for variable temperature air. The air is admitted from below through a glass elbow tube which press fits into the variable temperature base.

The variable temperature air path is indicated on a cross section sketch of the assembled probe in Figure III-10. Air flows through the variable temperature dewar over the heater and sensor, through the glass elbow and into the central opening in the bottom of the variable temperature base, entering a small chamber formed by the variable temperature base and the coil base. This chamber is sealed by an O-ring between the two pieces. The air passes through the coil base in six holes arranged radially around the BNC connector, entering a second small cylindrical chamber which is enclosed on the bottom by the coil base, on the outside by a glass tube sealed to the

FIGURE III-9

Variable temperature base, which admits
variable temperature air to 9.21 MHz
NMR probe.

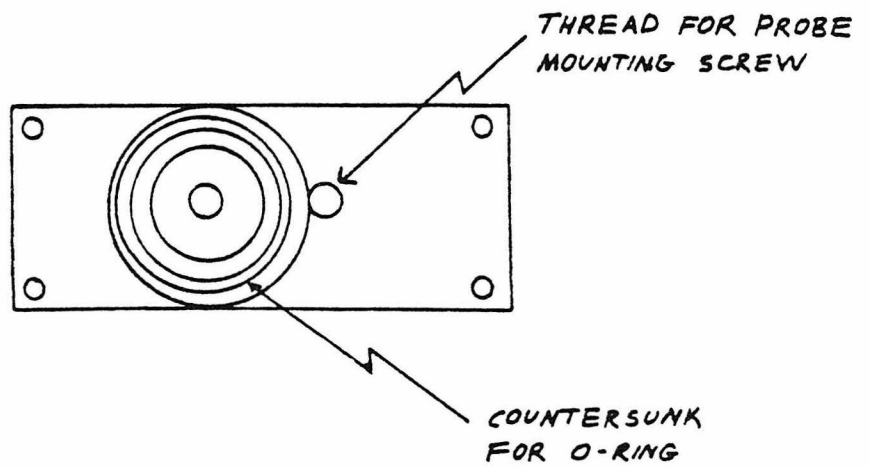
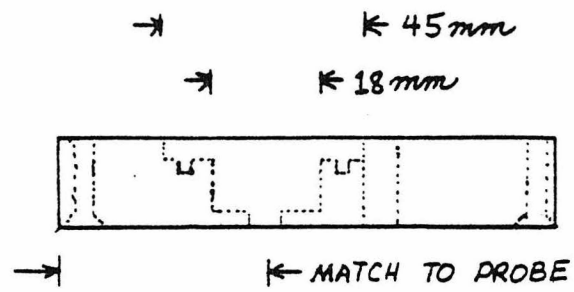
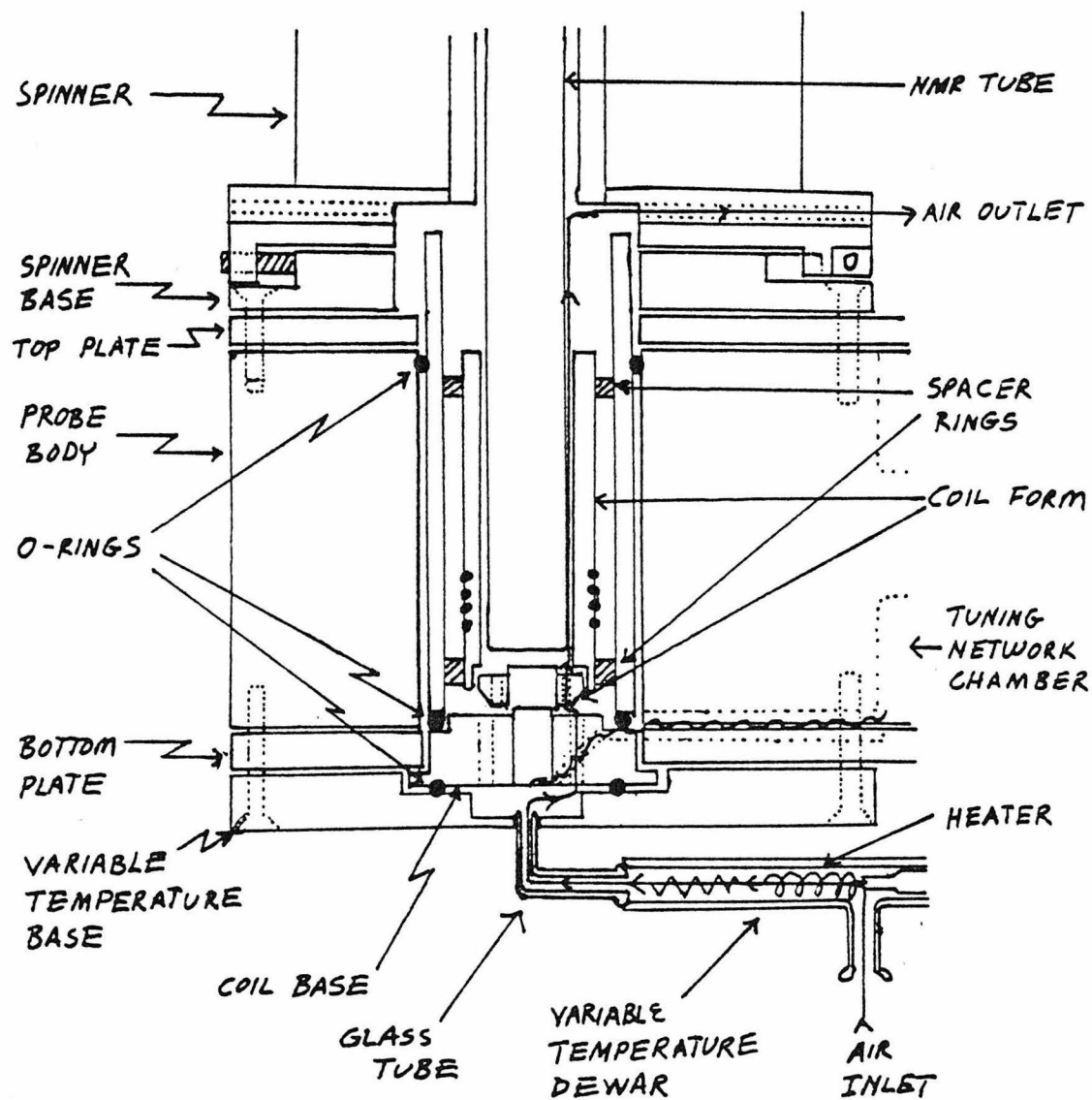


FIGURE III-10

Variable temperature air path, 9.21 MHz
NMR probe.

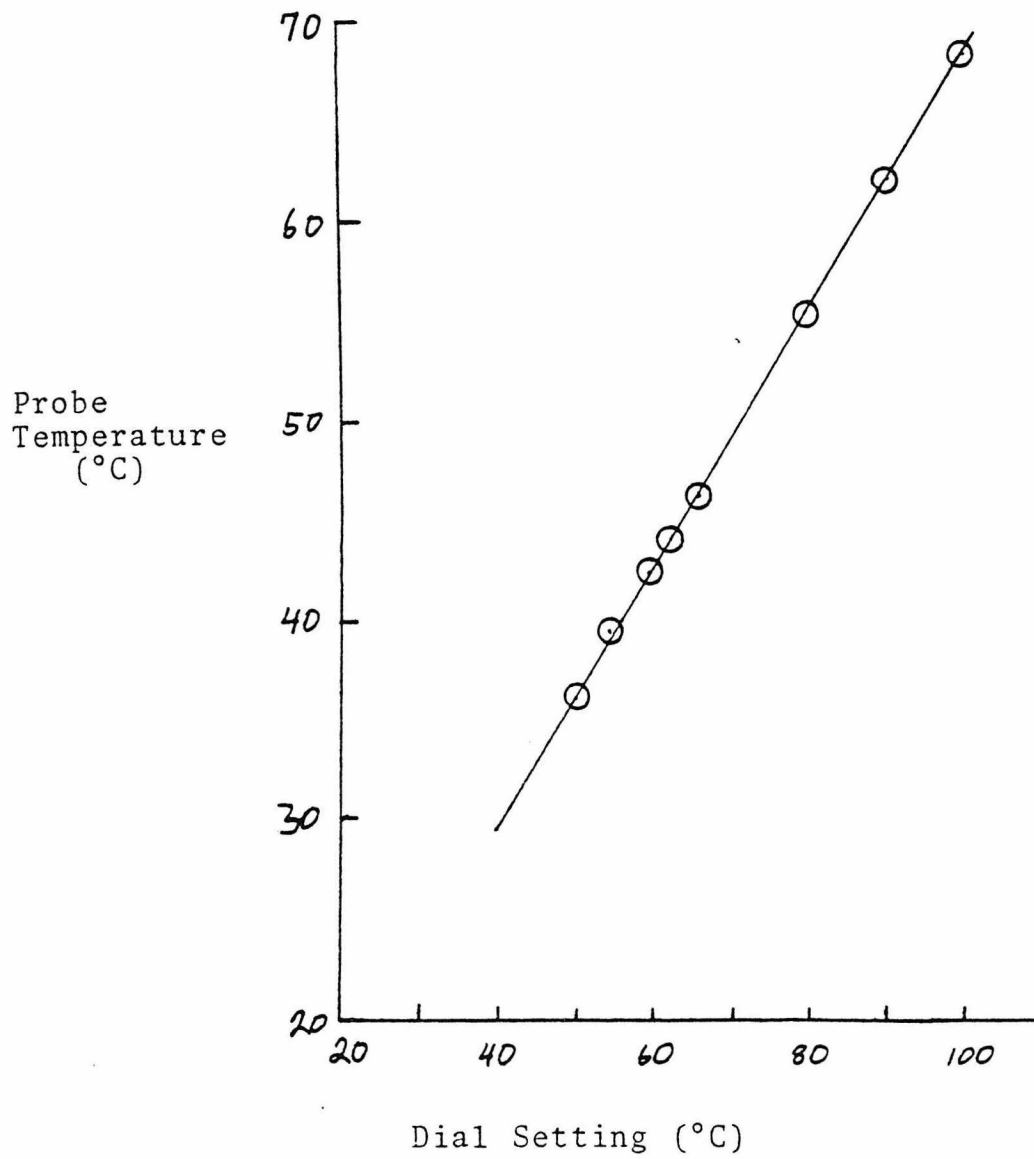


coil base with an O-ring, and on the top by the coil form and a cylindrical Teflon spacer ring which seals the coil form to the glass tube. From this chamber the heated air enters the insert via six more holes radially arranged about the insert BNC connector, and passes upward along the sample tube to the top of the insert, and thence out through two holes in the base of the spinner. The variable temperature pathway is designed for a minimum distance and air path volume between the heater/sensor and the sample tube. The pathway is formed entirely by glass and plastic materials of low thermal conductivity. Variable temperature control is provided by a Varian model 4540 variable temperature controller. The calibration curve, Figure III-11, depends on air flow rate but is linear and highly reproducible. Calibration was done by inserting a digital thermometer probe directly into an NMR tube containing 3-5 ml of H_2O .

The lock probe is in a separate enclosure containing a sealed H_2O sample and coils in a long finger attached to a small aluminum box which holds the tuning network. The long finger is inserted into a hole in the end of the main probe nearest the bore. The lock sample rests against the glass cylinder opposite the observe sample. It is important that the two samples be as close together as possible in order that the lock signal sense the same field experienced by the observe sample.

FIGURE III-11

Calibration curve indicating actual sample temperature ($^{\circ}\text{C}$) for a given temperature setting ($^{\circ}\text{C}$) of the variable temperature controller of the 9.21 MHz ^2H NMR spectrometer.

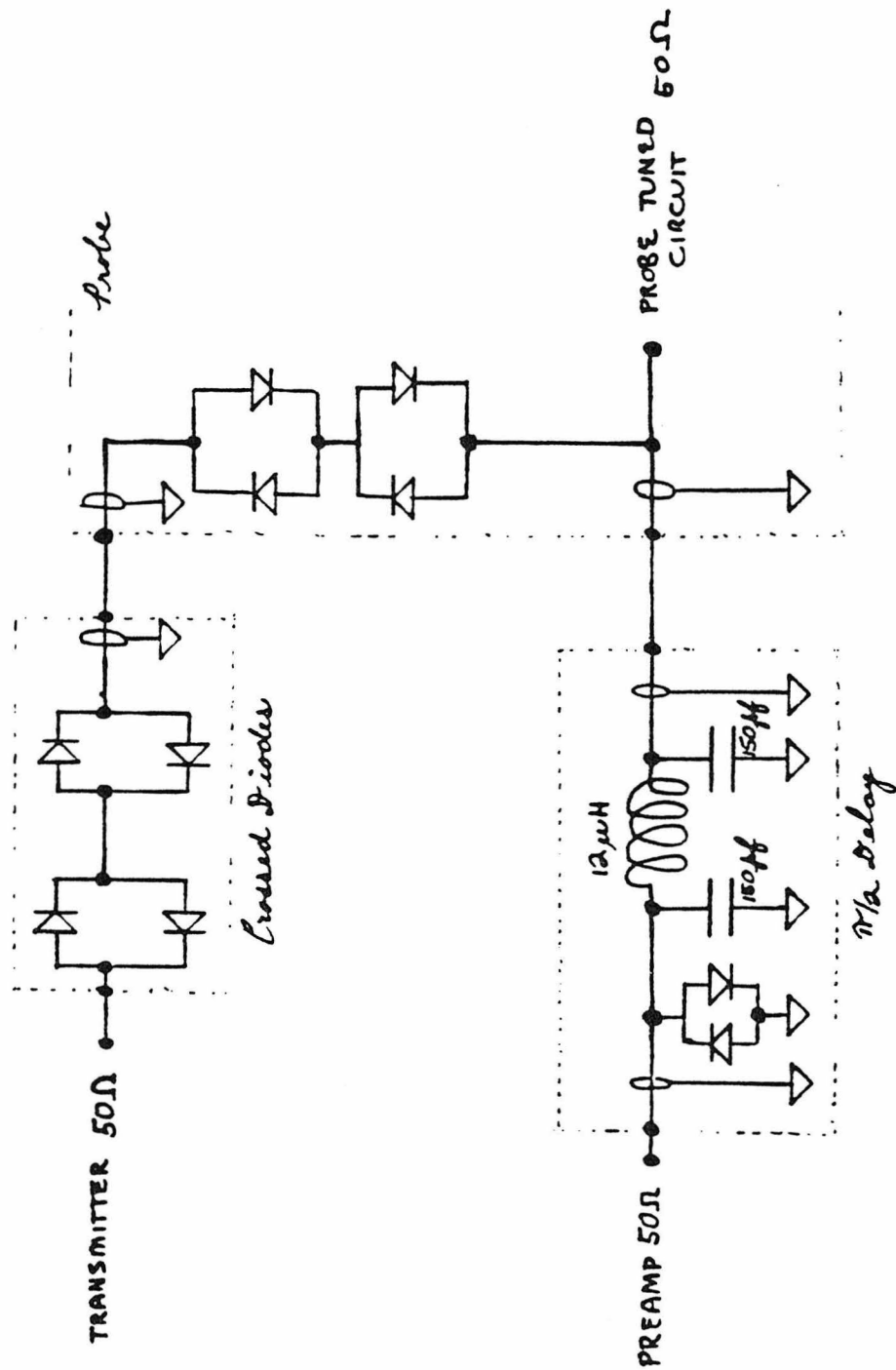


c. Transmit/receive switching

In a single coil probe, during the RF burst the transmitter, with impedance $50\ \Omega$ resistive, must be connected to the probe tuned circuit, which also has a $50\ \Omega$ impedance. The preamplifier must be effectively disconnected from the probe to preserve impedance matching and to protect the delicate preamplifier from the massive transmitter signal. During data acquisition, when the pulse is off, the opposite situation obtains: the preamp must be connected to the probe and the transmitter effectively disconnected to achieve an impedance match. This is accomplished by the simple passive circuit in Figure III-12. This standard design (8) takes advantage of the differing magnitude of the transmitted and received radio frequency signals. The probe is connected to the transmitter through a set of series crossed diodes and to the receiver through a $\pi/2$ delay circuit followed by a set of crossed diodes to ground. When the transmitter is on, the large transmitter RF voltage breaks down the series crossed diodes to reach the probe; signals of lesser intensity at other frequencies are stopped by the diodes. The connection from the probe tuned circuit back to the preamp consists of a $\pi/2$ delay followed by a set of crossed diodes to ground; since the transmitter pulse breaks down the diodes, the transmitter sees a quarter wave delay with a short at the end, which looks like an infinite impedance since

FIGURE III-12

Circuit diagram illustrating how receiver transmitter and probe of the 9.21 MHz ^2H spectrometer are interconnected to achieve passive switching of the sample coil between transmitter and receiver.



there is a node at the end of the quarter wavelength. Thus the transmitter is impedance matched to the probe and no signal reaches the preamplifier. When the burst is off the signal from the probe is too small to break down any of the diodes; thus it sees an infinite impedance in the direction of the transmitter and $50\ \Omega$ impedance in the direction of the preamp. The advantage of this switching method is that it places a relatively small number of low-noise passive components between the probe and the preamplifier and thus produces minimum degradation of the signal-to-noise. It is possible to use a quarter wavelength cable in place of the discrete delay circuit. However, at 9.210 MHz a quarter wave cable is 18 feet long and acts as an admirable antenna for all varieties of noise. A signal-to-noise advantage of almost a factor of three is obtained on our spectrometer by using the discrete delay circuit in place of the quarter wave cable. By the same token it is possible to eliminate the set of series crossed diodes in the probe if one uses a half-wave cable between the transmitter series diodes and the probe. (If this cable is not half wave it looks like a stub to the receiver and ruins the impedance match.) However, the half-wave cable also acts as an antenna for the receiver.

4. Preamplifier and receiver

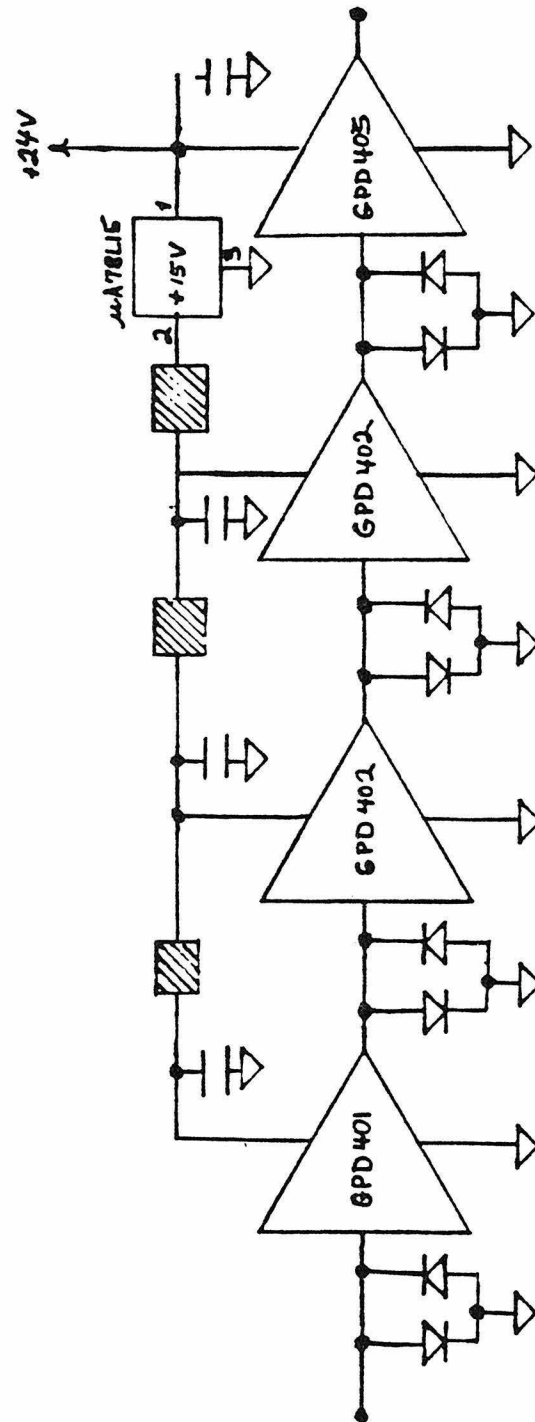
The ^2H spectrometer preamplifier system consists of a tuned 9.210 MHz low noise preamplifier followed by

a wideband high gain preamplifier. The tuned preamp, Janel Laboratories model PB, is rated at 2 dB noise figure and 18 dB gain. As long as the first stage gain is of the order of 20 dB or more, the noise figure of an amplifier cascade is approximately the noise figure of the first stage, so the gain in signal-to-noise in cascading two low-noise amplifiers is negligible. The wideband preamplifier was constructed by Mr. Ken Block from four Avantek GPD series canned RF amplifiers, as shown in Figure III-13. The first stage (model 401) provides a slightly lower noise figure and the last stage (model 403) provides higher power output to drive the receiver. Overall gain of the unit is about 48 dB. Crossed diodes are used between stages to minimize preamplifier recovery time following an RF burst. To minimize noise pickup the probe, delay circuit, and the tuned preamplifier are interconnected directly by adapters without the use of cables. A solid coax cable connects the tuned preamplifier to the wideband preamp. A homemade DC power supply supplies 12 V to the tuned preamps and 24 V to the wideband amplifier (as well as +15 V and -5 V to the lock box).

The receiver is part of the Tomlinson model BK Superhet Interface. In the receiver the preamplified signal is first mixed with the signal from the synthesizer, giving an output at the IF frequency (34.057 MHz). This signal is amplified and applied to a dual phase

FIGURE III-13

Circuit diagram of wideband preamplifier
constructed from Aventek GPD series amplifiers
and used on the 9.21 MHz ^2H NMR spectrometer.



▨ = ferrite bead

detector, which detects against two IF reference signals 90° out of phase with each other. The raw detector outputs, which provide FIDs in phase quadrature, are used directly, bypassing the active filter provided in the BK. Instead the raw signal is applied to the inputs of a Rockland model 432 dual hi/lo filter. This unit provides matched 4-pole active Butterworth low-pass filters with a 20 dB gain and a cutoff frequency adjustable between 1 Hz and 100 kHz. The outputs of the two filter channels are fed to the display oscilloscope and the spectrometer data acquisition system.

5. Data system

The spectrometer data system is borrowed essentially intact from a Varian HR220 NMR spectrometer. It is based on a Varian 620i 16-bit minicomputer which initiates burst sequences, time averages free induction decays and performs Fourier transformation. Data input occurs via a Varian model S-1401 analog to digital converter (ADC). The ADC has four input channels multiplexed to a single converter. Two of the channels are used to input free induction decays from the two detector channels and two are used to input initial and final phase values for phase correction of transformed spectra. Computer control of spectrometer function and data output to the HR220 bed recorder and console oscilloscope occur via a Varian model S-1411 spectrometer interface. The interface contains a digital to analog converter for

data output and logic for setting and resetting control lines in response to computer commands. The control lines operate the bed recorder under computer control, operate a Varian model V-4357 pulse unit module, which generates a TTL pulse for initiating pulse burst sequences, and perform various other computer controlled functions.

Interfacing the data system to the ^2H spectrometer is accomplished fairly simply. In the HR220, free induction decay signals from the final audio phase detector are amplified and filtered in the pulse unit module before being input to the ADC multiplexer. The final element in the pulse unit module is a gain control, which is simply a variable attenuator used to prevent overload of the ADC. In the deuterium spectrometer the output of channel 1 of the audio filter is fed directly to the mux connector provided for input of CW data from the HR220 to channel 1 of the multiplexer. Input occurs via a BNC connector. The channel 2 output is fed to the pulse unit module, through the gain control and thence to the multiplexer channel 0. A front panel switch has been provided on the pulse unit module to switch the gain control input between the ^2H spectrometer channel 2 signal and the HR220 signal. Channel 2 is slightly higher in gain than channel 1, and in this fashion the gain control on the pulse unit module may be used to match the gain of the two channels.

Initiation of burst sequences by the computer is provided for by outputting from the spectrometer interface via a new BNC connector on the back panel the control line which initiates pulses and times their length. This control line is input to the "ext 2" BNC on the Tomlinson pulse programmer of the ^2H spectrometer and used to initiate burst sequences.

Finally, it is also necessary for the computer to be able to reset the RF phase as controlled by the pulse programmer. A control line for this purpose is borrowed from the HR220. The particular control line taken is intended for use on the HR220 for spoiling the field homogeneity; the hardware for this function does not exist anyway. Setting and resetting this control line, which is connected to the pulse card in the pulse programmer, resets the RF phase of the transmitter to zero.

D. Computer Software

In constructing the ^2H spectrometer considerable modification was made to the existing computer software, primarily because of the requirement for large spectral widths. To this end the standard Varian NMR program for the Varian 620i minicomputer called VFT (16) was extensively modified to give a wideband quadrature phase detection program, called QPD. Since no source (unassembled) version of VFT was available, modifications were made directly in machine code without the use of

an assembler program. In this section we describe the changes made to VFT with emphasis on the strategies required to generate a wideband, quadrature phase detection program utilizing the available hardware. For readers seeking further details, Appendix I of this thesis gives a thorough description, a flowchart and an annotated listing for each of the new or modified subroutines of QPD. Appendix II gives a brief description of the 620i minicomputer; for further details see reference (17).

The Varian NMR program VFT uses the spectrometer as a peripheral device to generate free induction decays ("FIDs" or "transients") which are averaged in computer memory to generate a single accumulated FID $F(t)$ occupying the entire 8192 (8K) memory locations devoted to data. After data acquisition is completed, $F(t)$ may be multiplied by an exponential weighting function $e^{\alpha t}$ to enhance signal-to-noise of the transformed spectrum at the expense of resolution ($\alpha < 0$) or to enhance resolution at the expense of signal-to-noise ($\alpha > 0$). $e^{\alpha t}F(t)$ is then Fourier transformed to yield a sine transform and a cosine transform arranged in memory as 4096 (sine, cosine) pairs. VFT uses a version of the Cooley-Tukey algorithm (18) for Fourier transformation. $e^{\alpha t}F(t)$ is first multiplied by an apodization function to compensate for the finite extent of the data in the time domain. The actual Fourier transformation is carried out using integer arithmetic with rescaling of the data after

each level of the algorithm. In the absence of phase errors introduced by the spectrometer electronics the sine transform $A(\omega)$ should yield the absorption mode spectrum while the cosine transform $B(\omega)$ yields the dispersion mode. To correct for phase errors, which are introduced primarily by filtering, the single-sided absorption mode spectrum $S(\omega)$ is generated by combining $A(\omega)$ and $B(\omega)$ using a phase angle $\phi(\omega)$ which varies linearly from $\phi(0)$ to $\phi(\omega_{\max})$:

$$S(\omega) = A(\omega)\cos\phi(\omega)+B(\omega)\sin\phi(\omega). \quad (\text{III-1})$$

VFT has been modified for use with the ^2H spectrometer in two important respects. First, as indicated VFT was originally written for instruments using single-phase detection. Additional routines for handling data from detection in phase quadrature have been added. QPD acquires two time-averaged FIDs from phase detectors 90° out of phase with respect to each other. These are transformed separately to yield two transformed spectra, which are then combined using a phase angle $\phi(\omega)$ to generate a double-sided absorption mode spectrum. Second, VFT as originally written was severely limited in data acquisition rate and thus in spectral width; it has been modified for the maximum data input rate possible with the existing hardware. We will discuss three aspects of spectral acquisition using QPD. First, we consider the acquisition of two separate

FIDs; this discussion focuses on maximizing the data digitization rate. Second, we consider the separate transformation of the two FIDs; and third, we indicate how they are combined to yield the double-sided absorption mode spectrum.

1. Acquisition of two free induction decays

^2H spectra of chemical systems with restricted motion, such as model membranes, show broad peaks requiring spectral widths of up to 300 kHz and resolution of at most 10 Hz. The QPD data acquisition routine provides quadrature phase detection and a maximum data digitization rate for maximum spectral width, but in so doing sacrifices efficient use of data memory space and to a lesser extent overall rate of free induction decay accumulation. Thus resolution and in some cases signal-to-noise are reduced in order to gain spectral width.

The maximum spectral width obtainable on an NMR instrument may be limited by one of two factors. The first is the magnitude of the H_1 field. As indicated earlier, the length t_{90} of a 90° burst is customarily used as a measure of the available spectral width. If Δ is the distance off-resonance in Hz, then for an H_1 producing a t_{90} of length $(4\Delta)^{-1}$, there will be a 2% intensity error at position Δ with respect to the intensity at the spectrometer frequency (7). For the deuterium spectrometer $t_{90}=3.5$ microseconds so Δ could

be as much as 70 kHz with at most 2% intensity distortion, and the effective spectral width is limited to $2\Delta=140$ kHz for a quadrature phase detection experiment.

The spectral width may also be limited by the maximum available data digitization rate. In order to define the frequency of a sine wave it is necessary to sample it at least twice during a single period, so that the sampling rate or Nyquist frequency must be 2Δ or equal to the desired spectral width for a quadrature phase detection experiment. What limits the data digitization rate? In the most current spectrometers data input to the computer is done by direct memory access (DMA) so that the Nyquist frequency is limited to one or two times the computer cycle time, or limited by the maximum rate of the analog-to-digital converter (ADC). In the 620i as currently configured direct memory access is not available. To acquire data under VFT, the data must be input to the central processor before storage. Thus the computer interrogates the ADC to see whether a datum is available, inputs the datum to the next memory storage location, checks whether it has reached the end of the allocated memory space, and repeats. It is desirable to find the minimum program routine to accomplish these functions. It turns out that this routine always takes longer than the minimum ADC cycle time, which is 10 microseconds. Thus it is the length of the data input routine which limits the data

acquisition rate, and thus limits the maximum spectral width.

There are three versions of the QPD acquisition routine which apply a total of four different strategies to minimize the length of the input routine:

(1) First, in acquiring data, some manipulation must be performed before final data storage. For one thing, to prevent memory overflow, scaling of data is required in many long-term experiments. Also, data must be averaged with previously accumulated data before storage. In order to maximize the data input rate, in QPD during the sampling of an FID, the data are input to a temporary storage area. After the FID is finished data are scaled and averaged with previously accumulated data in the permanent storage area. Since two separate FIDs must be accumulated the 8192 (8K) points of memory available for data are divided into four parts: two temporary storage areas and two permanent storage areas. A map of the data area is shown in Figure III-14. By comparison, VFT accumulates a single phase FID 8K points in length. In this case there are 4K points in the transformed spectrum, since the Fourier transform produces a 4K cosine transform and a 4K sine transform, and each (sin, cos) pair generates one output point. In QPD two 2K FIDs are accumulated to produce 2K points in the double-sided quadrature spectrum. Moreover the quadrature spectrum has twice the spectral width. Thus

FIGURE III-14

Data memory area map for NMR computer
program QPD.

<u>MEMORY LOCATION</u>	<u>CONTENTS</u>
011774-015773	AVERAGED FID, 90° SPECTRUM
015774-021773	TRANSIENT FID, 90° SPECTRUM
	WEIGHTED FID, 90° SPECTRUM
	TRANSFORMED 90° SPECTRUM
021774-025773	AVERAGED FID, 0° SPECTRUM
025774-031773	TRANSIENT FID, 0° SPECTRUM
	WEIGHTED FID, 0° SPECTRUM
	TRANSFORMED 0° SPECTRUM

it has only one-fourth the resolution of a single phase spectrum, taken at the same data digitization rate.

(2) The second method for shortening the input routine reduces the rate at which transients may be accumulated, and thus, effectively reduces the signal-to-noise attainable in a given time. In true quadrature phase detection both detectors are sampled after each RF burst and two FIDs are input. This requires either two ADCs or a single ADC and a multiplexer arrangement. In the most recent spectrometers two ADCs and two DMA channels are used for maximum input rate. The 620i system has available one ADC with a multiplexer. Sampling both FIDs after an RF burst halves the data digitization rate for an individual FID since the same number of computer steps is required for each input point of each FID when the multiplexer alternates between detector channels. Even if two ADCs were available two data input and two data storage steps would be required to input one point from each FID; this cuts the data digitization rate by about one-third. Instead, QPD does a "poor-man's" version of quadrature detection in which only one channel is sampled during each transient, and the channel to be sampled is alternated between transients by switching the multiplexer. True quadrature detection results in an improvement in signal-to-noise over single phase detection by a factor of $\sqrt{2}$ since two independent sets of data are sampled during each FID, each with the

signal-to-noise of the single phase FID, and signal-to-noise goes as the square root of the number of transients. In the "poor-man's" version only one FID is recorded per transient and no signal-to-noise advantage over single phase detection is realized. Moreover additional time is required between bursts to scale the transient data and average it with the accumulated FID. Thus signal-to-noise is sacrificed in order to increase the available spectral width.

The use of the term "poor-man's" quadrature detection in discussing the Acquisition routines should be clarified. It is possible to generate double-sided spectra on a machine with a single detector by incrementing the phase of the transmitter RF by 90° between transients. This technique is usually referred to as "poor-man's" quadrature detection. The ^2H spectrometer does a version of "poor-man's" quadrature in that it samples only one channel per transient. However, it is set up with two complete detector channels, both of which are used in QPD, and could easily be programmed for true quadrature detection where both channels are sampled during each transient. However, the resulting program would be limited to 20 kHz spectral widths and would not be useful for motionally restricted ^2H samples.

Used in combination the first two methods of increasing the data digitization rate allow the programmer to generate an input routine which operates

reliably at up to a 40 kHz conversion rate. The spectrometer program based on this version of the input routine is called QPD-40KP.

(3) The third modification for maximizing the data digitization rate involves a change in the way the Nyquist frequency is clocked. In VFT, analog-to-digital conversion by the ADC is triggered by an external timer. The timer frequency is set by the program to correspond to the Nyquist frequency for the spectral width selected by the operator. Data conversion is started by initializing the ADC and then outputting a timer word from memory to start the external timer. Conversion is halted when the computer has input the number of data points set by the operator. It is possible to initiate conversion directly by outputting a command from the computer to the ADC, rather than using the external timer. In this mode the input is performed by a "do-loop" in the computer program. The Nyquist frequency is set directly by the cycle time of this input loop, and care must be taken by the programmer to assure that the cycle time is constant. A time saving is realized because with this method it is not necessary for the computer to interrogate the ADC to see whether data are available in the buffer. The maximum conversion time of the ADC is 10 microseconds, and as long as more than 10 microsecond's worth of commands fall between the "start conversion" and "input data" commands no sense loop is required.

In this type of acquisition the spectral width is varied by changing the length of the input loop. This is conveniently accomplished by including in the routine an otherwise nonfunctional logical rotate command. The execution time of a rotation is one cycle plus one-quarter cycle per step of rotation (e.g., a rotation of five places takes $2 \frac{1}{4}$ cycles). In this fashion the dwell time may be set with a resolution of $\frac{1}{4}$ cycle or 0.45 microseconds. Since there is a maximum number of steps of rotation that may be performed with a single logical rotate command, there is an upper limit as well as a lower limit to the dwell time that may be set in this manner, and thus both an upper and a lower limit to the available spectral width. The version of QPD utilizing this type of output routine is called QPD-68K and is useful for conversion rates between 34 and 68 kHz. The minimum length of the input cycle is achieved by omitting the logical rotate command entirely; in this special version of the program, called QPD-78KP, the spectral width is maximized, but the operator has no control over the spectral width.

(4) The fourth method for maximizing the data digitization rate, used in both the 68K and 78K versions of QPD, may be called the "method of multiple copies" of the input instructions. If there were space in the program to include 2048 consecutive sets of data input instructions, each set of instructions would be shortened

since no counting of data points or looping to the beginning of the input instructions would be required. This is impractical, but there is a timesaving compromise using several sets of input instructions in succession. If the overall input routine must include a loop, then once per loop the routine must test whether it is done taking data and branch accordingly; this test and branch step requires at least a conditional jump command. The last copy of the input instructions in the loop must then do at least the following: start data conversion, input data, store data, and branch. Each of these functions can be done in a single two-cycle command, except that starting conversion requires only one cycle. The total length of the last copy is thus at least seven cycles. Other copies of the input instructions need no branching command, but they must still be seven cycles long in order that the Nyquist frequency be constant. The "extra" time may be used for incrementing the data storage address and counting the number of points that have been input. The counting of input points is simplified by counting them in pairs. At least four copies of the input instructions are required to accomplish the necessary counting and incrementing using seven cycle sets of instructions. Using more than four sets gains nothing since all of the sets must be equal in length and the last set must be at least seven cycles long. Seven cycles corresponds to a dwell time of 12.6

microseconds and a spectral width of 79.4 kHz. This is the maximum spectral width possible with QPD.

It is necessary to mention one more complication, affecting the acquisition of data resulting from quadrature phase detection. In order to obtain satisfactory cancellation of image peaks the two channels of the receiver must be matched in amplitude to about 1% and 90° out of phase with one another to within $1/2^\circ$. Since in practice these criteria are difficult to meet, a burst sequence has been devised which averages out phase and intensity errors (16). In this sequence the transmitter phase is incremented by 90° between transients, producing the pattern 0° , 90° , 180° , 270° ($90^\circ + 180^\circ$), 0° , 90° , etc. Every time a 90° shift is included the two channels of the detector and ADC are effectively exchanged. When a 180° shift is used data must be subtracted from memory rather than added. In "poor-man's" quadrature detection, on a spectrometer equipped for true quadrature detection, two entire cycles of phase are required to run through each channel with each choice of transmitter phase. The pattern of burst phase, ADC channel selection, memory area for data storage and addition or subtraction of transient from accumulated FID is shown in Table III-2. Each channel is both added to and subtracted from each FID, so intensity and phase errors are minimized. In addition DC baseline shifts are averaged out.

TABLE III-2 PATTERN OF RF BURST PHASE, ANALOG TO DIGITAL CONVERTER CHANNEL SELECTION, DATA MEMORY AREA, AND ADDITION OR SUBTRACTION OF DATA FROM MEMORY USED IN PHASE ALTERNATING PULSE SEQUENCE OF 9.21 MHz ²H SPECTROMETER

PULSE	TRANSMITTER PHASE	90° SHIFT	180° SHIFT	CHANNEL SAMPLED	AVERAGE INTO WHICH FID?	ADD OR SUBTRACT TO MEMORY CONTENTS?
1	0°			1	0° (upper half of memory)	A
2	90°	✓		1	90° (lower half of memory)	S
3	180°		✓	1	0°	S
4	270°	✓	✓	1	90°	A
5	0°			2	90°	A
6	90°	✓		2	0°	A
7	180°		✓	2	90°	S
8	270°	✓	✓	2	0°	S

Finally, recall that for perfectly symmetric spectra we need acquire only one of the two FIDs, since it contains all of the spectral information. By changing only three computer program locations the operator of QPD can cause the program to acquire only the 0° FID (in upper memory); this acquisition is performed without use of the phase alternating pulse sequence. The transformed spectrum is then displayed in "spectrum reverse" fashion. Since twice as many 0° FIDs are accumulated in the same time period and no noise from the 90° channel is added into the transformed spectrum, a signal-to-noise advantage of a factor of two over quadrature acquisition is realized by this method. The same computer patches allow quadrature data to be displayed in spectrum reverse fashion.

2. Fourier transformation

In VFT a single FID of 8K data points is transformed to yield an 8K Fourier transform consisting of 4096 (sin,cos) pairs. In QPD two 2K FIDs are stored in the first and third quadrants of memory; after acquisition is complete the second and fourth quadrants contain transient data which are no longer needed. When the operator calls the exponential weighting function the accumulated FIDs are copied from the first quadrant into the second and the third quadrant into the fourth. The new copies are then weighted and the display shows the contents of the entire 8K of memory directly: the

unweighted 90° FID in the first quadrant, the weighted 90° FID in the second quadrant, the unweighted 0° FID in the third quadrant and the weighted 0° FID in the fourth quadrant. This display may be plotted if desired. When FT is called the weighted FIDs are transformed separately and then rotated end for end so that the zero frequency end is uppermost in memory. Memory now contains the unweighted FIDs in the first and third quadrants and the transformed spectra in the second and fourth quadrants. Since the original FIDs are retained in memory the operator can call the exponential weighting function again after transforming, using a new value of the weighting function, then call Fourier transformation again to generate a new transform with the new line broadening applied. In VFT this is possible only by storing the FID on cassette tape before calling the weighting function. Moreover, at any time during acquisition the operator may interrupt, call the weighting and transforming routines, check the progress of the accumulation, and then resume data acquisition, as long as the number of transients originally set has not been reached. Again, in VFT this "peeking" is possible only by storing the FID on tape and recalling it before resuming data acquisition. In VFT if Fourier transformation is called before the data are stored the FID is lost; in QPD the FIDs are never destroyed.

3. Generation of the double-sided spectrum

The Fourier transform routine in VFT generates a sine transform $A(\omega)$ and a cosine transform $B(\omega)$ from a single FID. These are combined using a phase angle ϕ (ϕ varies linearly from $\phi_{\text{initial}}(\omega=0)$ to $\phi_{\text{final}}(\omega=\omega_{\text{max}})$) to generate a phased, single sided spectrum $A(\omega)\cos\phi(\omega)+B(\omega)\sin\phi(\omega)$. In QPD two FIDs from phase detectors in phase quadrature are transformed yielding $A(\omega)$, $B(\omega)$ for the 90° spectrum and $C(\omega)$, $D(\omega)$ for the 0° spectrum. The 90° transform is then generated using phase angle ϕ , giving $A(\omega)\cos(\phi)+B(\omega)\sin(\phi)$, while the 0° spectrum is formed using phase angle $\phi + 90^\circ$, yielding $c(\omega)\cos(\phi+90^\circ)+D(\omega)\sin(\phi+90^\circ) = -C(\omega)\sin(\phi)+D(\omega)\cos(\phi)$. The sum of the two transforms yields the upper half of the two-sided spectrum while the difference of the transforms yields the lower half of the two-sided spectrum.

In VFT the values $\sin\phi(\omega)$ and $\cos\phi(\omega)$ are computed by the phasing routine from $\phi_{\text{initial}} = \phi(\omega_{\text{max}})$ and $\phi_{\text{final}} = \phi(\omega=0)$ and stored in a (sin,cos) table. ϕ_i and ϕ_f are read from the phase knobs on the spectrometer console by the ADC. The scope and plot routines run through the data table and the (sin,cos) table simultaneously from bottom to top, calling another subroutine, PHAV, to generate each output point. PHAV uses the position in the data table, called SAVX, to find $A(\omega)$, $B(\omega)$, $\sin\phi(\omega)$ and $\cos\phi(\omega)$ in the two tables, and then computes the output value. SAVX runs from 0 to 4096 for an 8K trans-

form (which consists of 4096 sin,cos pairs). In a double-sided or quadrature spectrum the phase angle varies from $\phi(-\omega_{\max})$ to $\phi(0)$ to $\phi(\omega_{\max})$ ($=\phi(-\omega_{\max})$). Thus the QPD phase routine generates a double-sided phase table using the value ϕ_{initial} for the phase at the center of the spectrum and the value ϕ_{final} for the phase at either end of the spectrum; the table runs from $\sin\phi(\omega_{\max})$, $\cos\phi(\omega_{\max})$ through $\sin\phi(\omega=0)$, $\cos\phi(\omega=0)$ back to $\sin\phi(\omega_{\max})$, $\cos\phi(\omega_{\max})$. The scope and plot routines work through the (sin,cos) table from bottom to top as usual, but they work through the two transformed spectra in the data table from bottom to top and then back to the bottom again, using a new PHAV routine. This new routine finds the value $\sin\phi$ and $\cos\phi$ from SAVX in the usual way. Then it checks SAVX to see whether the data location is in the lower half of the quadrature spectrum (upward pass through the data table) or the upper half of the quadrature spectrum (downward pass through the data table). SAVX runs from 0 to 1K on the way up (or through the first half of the (sin,cos) table) and 1K to 2K on the way back down (through the second half of the (sin,cos) table). PHAV then finds $A(\omega)$, $B(\omega)$, $C(\omega)$ and $D(\omega)$ and generates the output value.

E. Spectrometer Performance

1. Resolution

The maximum resolution obtainable on the ^2H spectrometer for a D_2O sample at room temperature is about 1.6 Hz (Figure III-15). This is very adequate for partially ordered systems but marginal for high resolution studies. Resolution of the current system is limited by the large sample volume and rudimentary shim system. ^2H chemical shifts are analagous to the corresponding proton shifts, and 1.6 Hz represents some 0.17 parts per million. Moreover the use of an external lock means that the operator must monitor the transient free induction decay of the sample to asses progress during shimming. He should not monitor the lock signal, since the lock signal samples a different portion of the field than the observe channel sample. Since it is not possible to monitor the transient free induction decay for samples of low concentration, it is not possible to shim the field with these samples in the probe, and the resolution for dilute samples is generally about 4 Hz, achieved by shimming with a concentrated sample and then switching to the dilute sample.

2. Sensitivity

A signal-to-noise of 7:1 may be achieved in 32 FIDs for natural abundance HDO in tap water, using a 5 Hz line broadening (Figure III-16). Allowing for differences in sample size and magnetic field, this number is comparable to the sensitivities for commercial instrumentation, indicating that the spectrometer electronics,

FIGURE III-15

High-resolution ^2H NMR spectrum of neat D_2O illustrating 1.6 Hz resolution possible with 9.21 MHz ^2H NMR spectrometer utilizing a 15 mm sample tube.

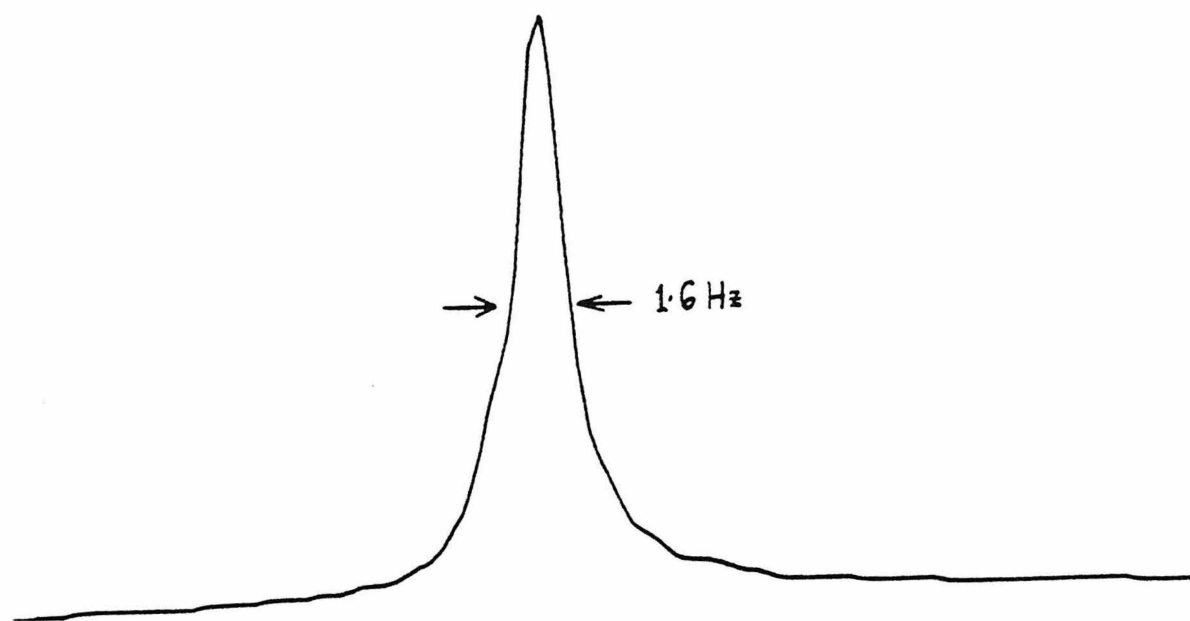
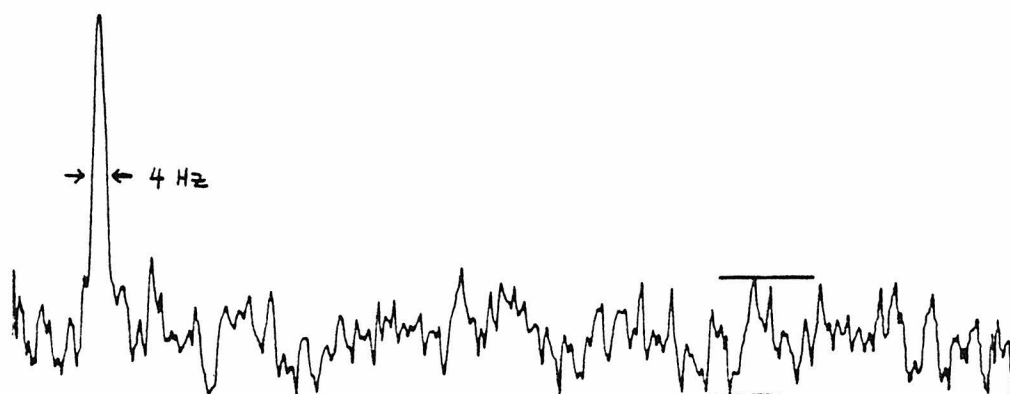


FIGURE III-16

High-resolution ^2H NMR spectrum of natural abundance HDO in tap water, obtained by accumulating 32 free induction decays and using a 5 Hz line-broadening. Signal-to-noise is 7:1, with a linewidth of about 4 Hz.



principally the probe and preamplifier, perform reasonably well. The principal impediment in comparing sensitivity to that of commercial instruments is the 4 Hz linewidth, which is about 8 times that observed for commercial machines operating in high resolution mode. For partially ordered samples the difference in resolution has no effect on the signal-to-noise of the spectra, of course. In absolute terms, the signal-to-noise of the instrument is limited by the 14 kG H_0 field.

3. Quadrature phase detection

Figure III-17 shows the quadrature spectrum of a specifically deuterated cysteine in aqueous solution at low resolution, along with the output of the two phase detectors before addition. The cancellation of image peaks is excellent, and the glitch at the RF frequency at the center of the spectrum is barely noticeable.

4. 90° Burst

The length τ_{90} of a 90° burst is approximately 3.5 microseconds, providing a special window of about 140 kHz. The short 90° burst indicates that the probe is well tuned with a fairly high overall Q and the transmitter system is free of electrical arcing.

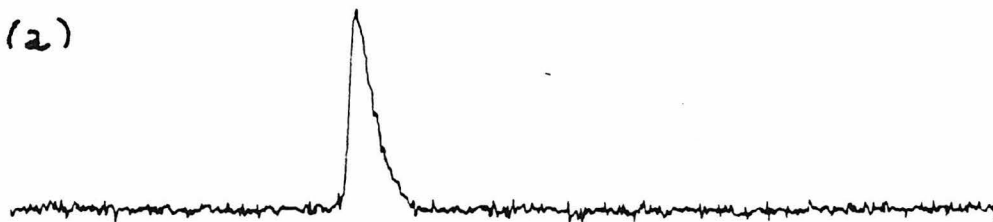
5. Transmitter and receiver phasing

One measure of the accuracy of adjustment possible in the transmitter and receiver phasing is the symmetry of the quadrature phase detection powder pattern spectrum

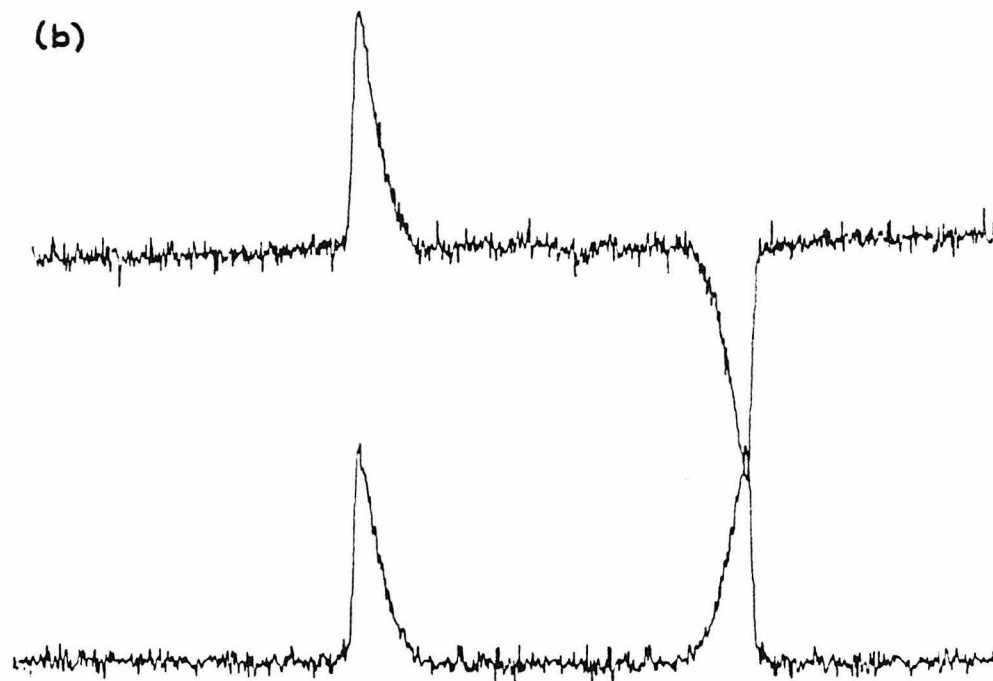
FIGURE III-17

(a) Low-resolution quadrature phase detection ^2H spectrum of a specifically deuterated cysteine in aqueous solution, along with individual output from the two phase detectors (b), illustrating excellent image peak cancellation and absence of distortion at zero frequency due to quadrature phase detection.

(a)



(b)



resulting when a sample showing restricted motion is pulsed at exactly the center frequency. Figure III-18 illustrates a spectrum of d_{22} decane in DPPC multilayers, showing excellent symmetry in all four of the observed quadrupolar splittings.

6. Solid-echo burst sequence

The solid-echo burst sequence should faithfully reproduce the powder pattern predicted by theory with little baseline or intensity distortion. The sample spectrum in Figure III-19 (d_{34} -palmitic acid in DPPC) is presented in spectrum reverse display. The order parameter plateau characteristic of bilayer systems exhibiting a flexibility gradient is accurately reproduced. The 0° discontinuities of the broadest powder pattern at splitting $2\Delta\nu_Q$ are clearly visible, the 90° discontinuity of the broadest powder pattern produces the characteristic vertical edges of the plateau, and the 0° discontinuities of the two narrowest powder patterns are also visible. These features indicate excellent reproduction of the theoretical lineshape(20).

Finally, in the previous chapter we have used this spectrometer to examine several model membrane systems also investigated by others in the literature. As described in that chapter, we find both qualitative and quantitative agreement between their data and ours. The investigation of hydrocarbon-containing bilayers described in chapter II illustrates the utility of this

FIGURE III-18

Quadrature phase detection solid-echo 9.21 MHz, ^2H spectrum of a multilamellar dispersion of d_{22} -decane (10.3 mole %) in DPPC, illustrating good symmetry obtainable with careful adjustment of transmitter phase, receiver phase and burst sequence timing.

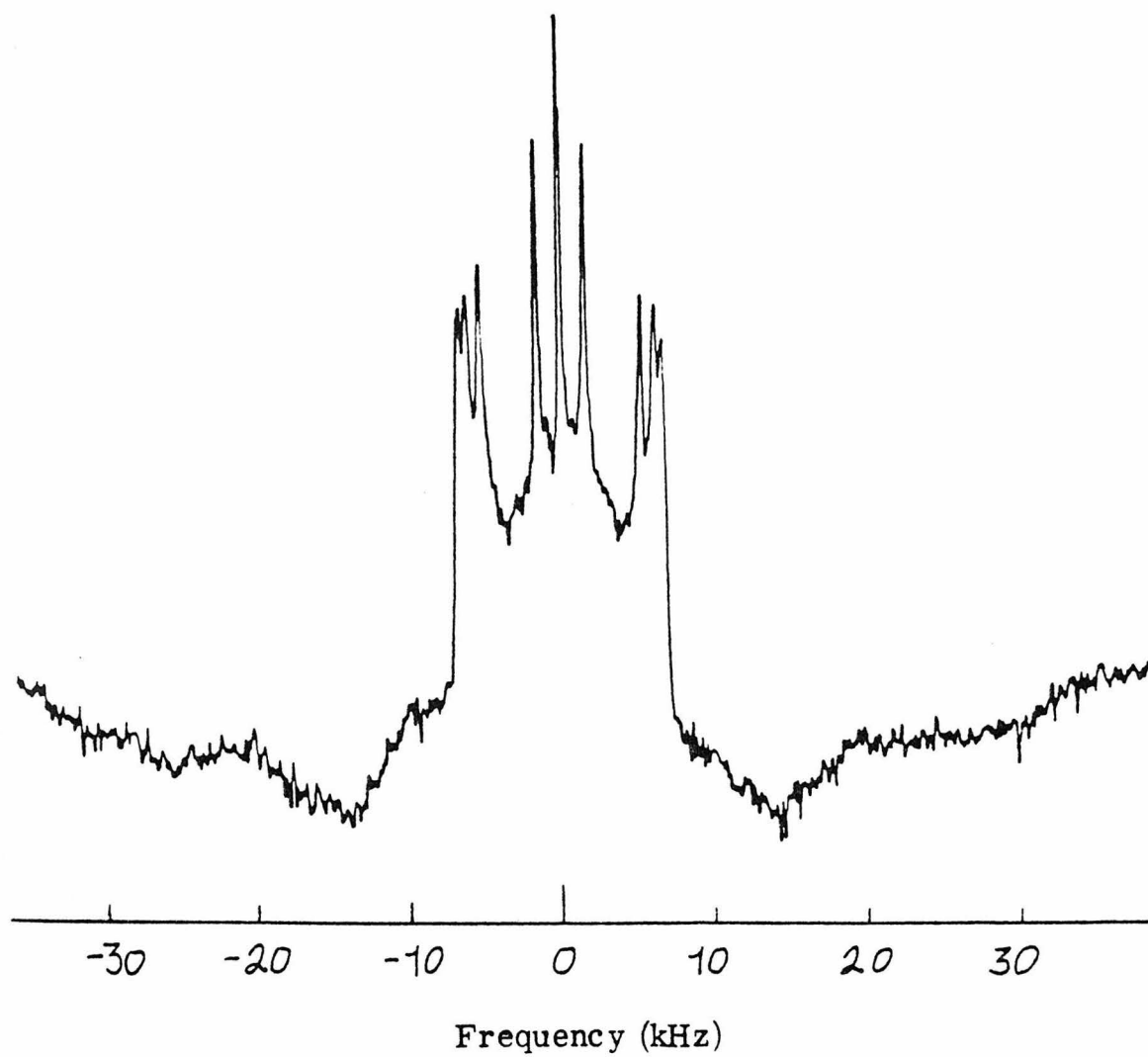
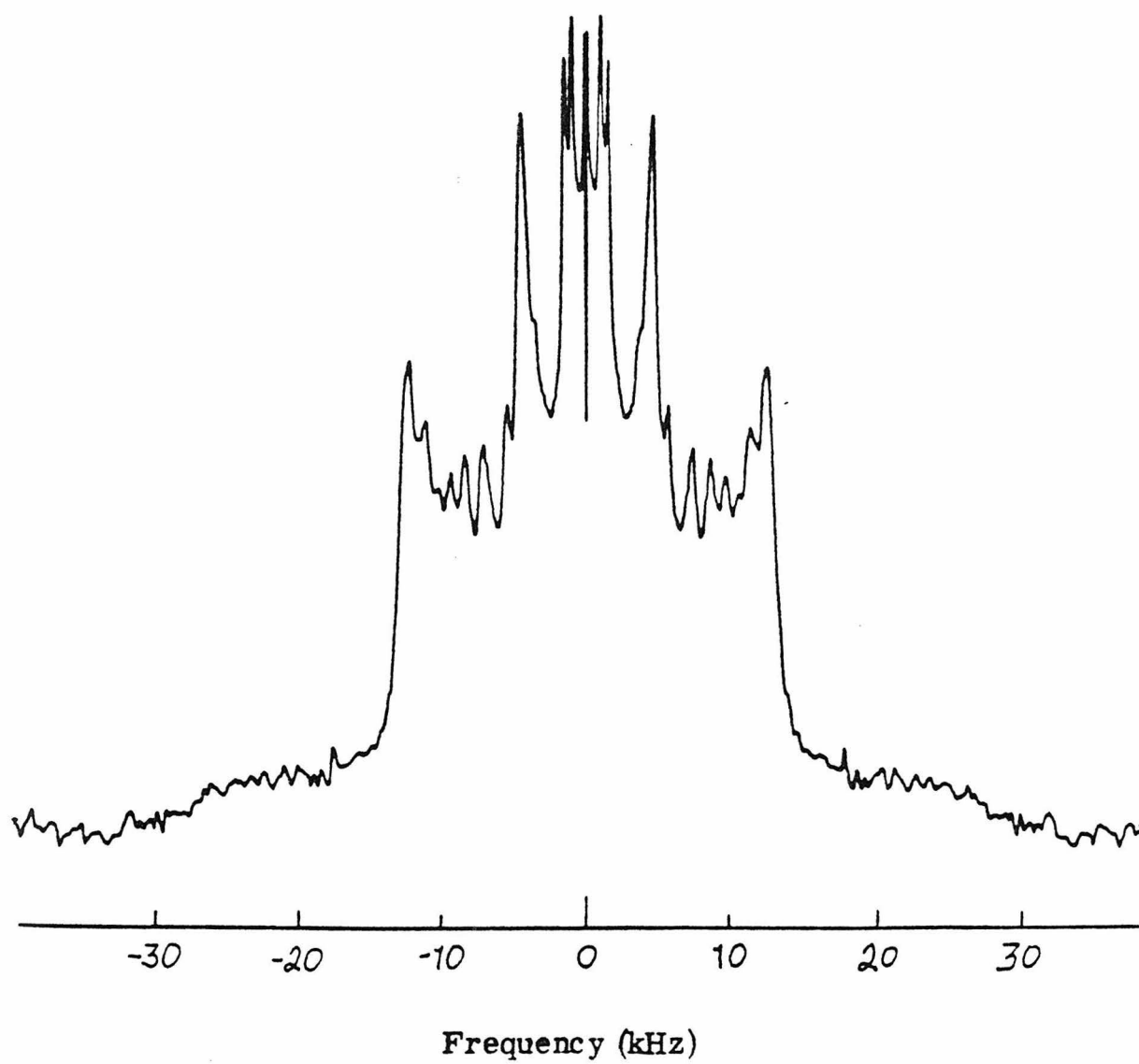


FIGURE III-19

Spectrum reverse solid-echo 9.21 MHz, ^2H
spectrum of a multilamellar dispersion of d_{34} -
palmitic acid in dipalmitoylphosphatidylcholine,
illustrating flat baseline and good reproduction
of powder pattern lineshape possible with
solid-echo burst sequence.



instrument in determining the motional state of various components of model membrane systems.

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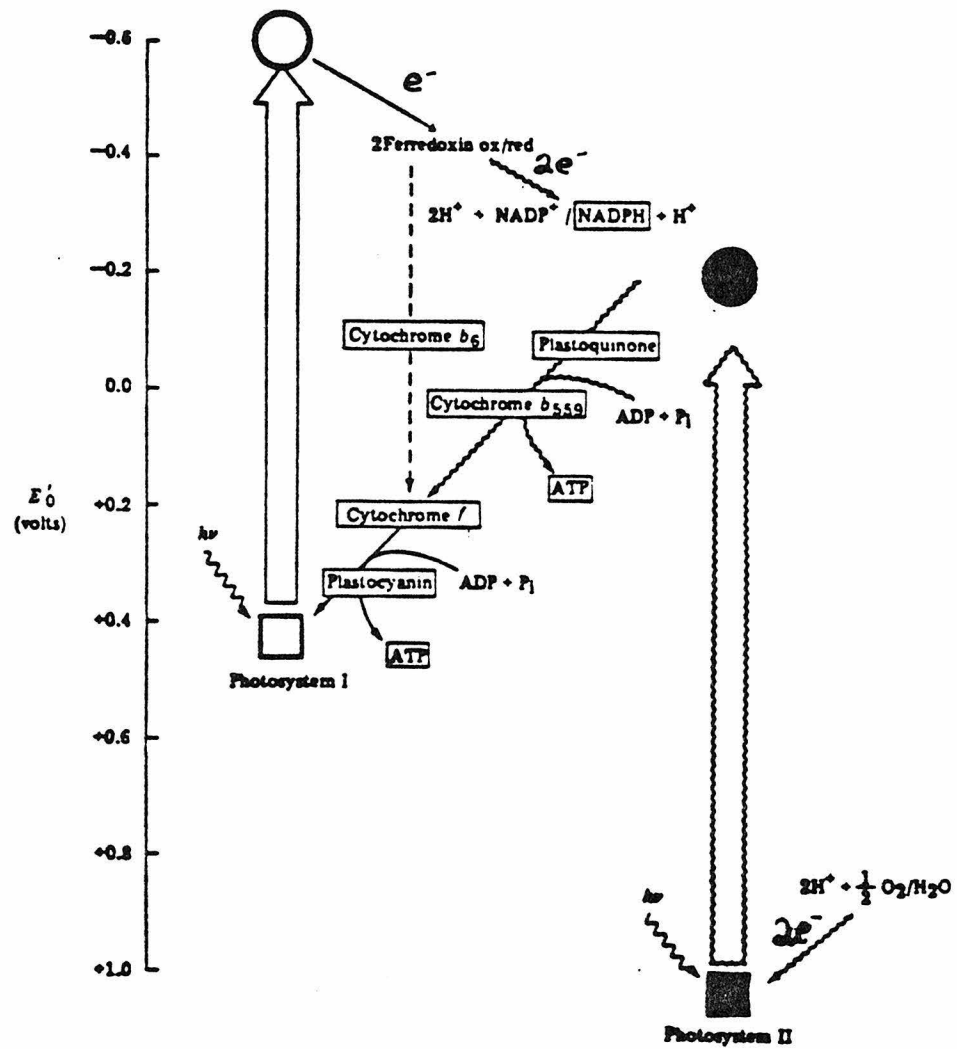
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CHAPTER IV. MODEL MEMBRANES CONTAINING CHLOROPHYLL aA. Introduction

Photosynthesis, the conversion of light energy to useful chemical energy, is the ultimate source of nearly all the energy consumed in the biosphere. In this process light energy absorbed by various photosynthetic pigments is used to raise the electrochemical potential of low energy electrons derived from an inorganic or substrate source. The resulting high energy electrons are funneled through an electron transport chain consisting of electron carriers of progressively lower potential. Part of the electron energy is conserved in the form of ATP via oxidative phosphorylation coupled to the electron transport. Additional energy is conserved in the form of reduced NADPH, the ultimate electron acceptor of the transport chain. In higher plants the electron source is water, which is oxidized to molecular oxygen. Electrons are energized in two stages by two separate photosystems where light energy from photosynthetic pigments is applied to boost the potential of the electrons. There are three electron transport chains, from water to photosystem 2, from photosystem 2 to photosystem 1, and from photosystem 1 to NADP. This so-called Z-scheme (1) is illustrated in Figure IV-1, where the vertical axis indicates the redox potential of the various electron carriers. Energy

FIGURE IV-1

"Z-scheme" for electron transport in photosynthesis. Vertical scale represents the redox potential of the various electron carriers.



conserved as ATP and NADPH is subsequently used to reduce CO_2 in a set of reactions which are not light-requiring.

In higher plants and some algae photosynthesis takes place in specialized plastid organelles called chloroplasts. These organelles are generally football-shaped, several microns in length and one or two microns across (Figure IV-2). They are surrounded by an outer envelope membrane and contain a second, specialized membrane structure called the thylakoid system, which is differentiated into stacked regions of membrane, called grana, and unstacked regions called stroma. Within a granum disk-shaped sacks of membrane are stacked on top of one another, with stroma membrane connecting individual disks with disks in neighboring grana (Figure IV-3). All of the light-requiring events of photosynthesis take place in the thylakoid membrane; photosynthesis is a membrane process.

In nearly all photosynthetic organisms the principal photosynthetic pigments are one or more types of chlorophyll (2) (Figure IV-4). However, while specialized pairs of chlorophylls called reaction center chlorophyll are involved in the primary photochemistry occurring in the two photosystems, most of the chlorophyll serves a different purpose. The function of the majority of the chlorophyll is to absorb light energy and transfer light energy by a non-radiative energy transfer process from

FIGURE IV-2

Sketch of chloroplast illustrating the
principal substructures of the organelle.

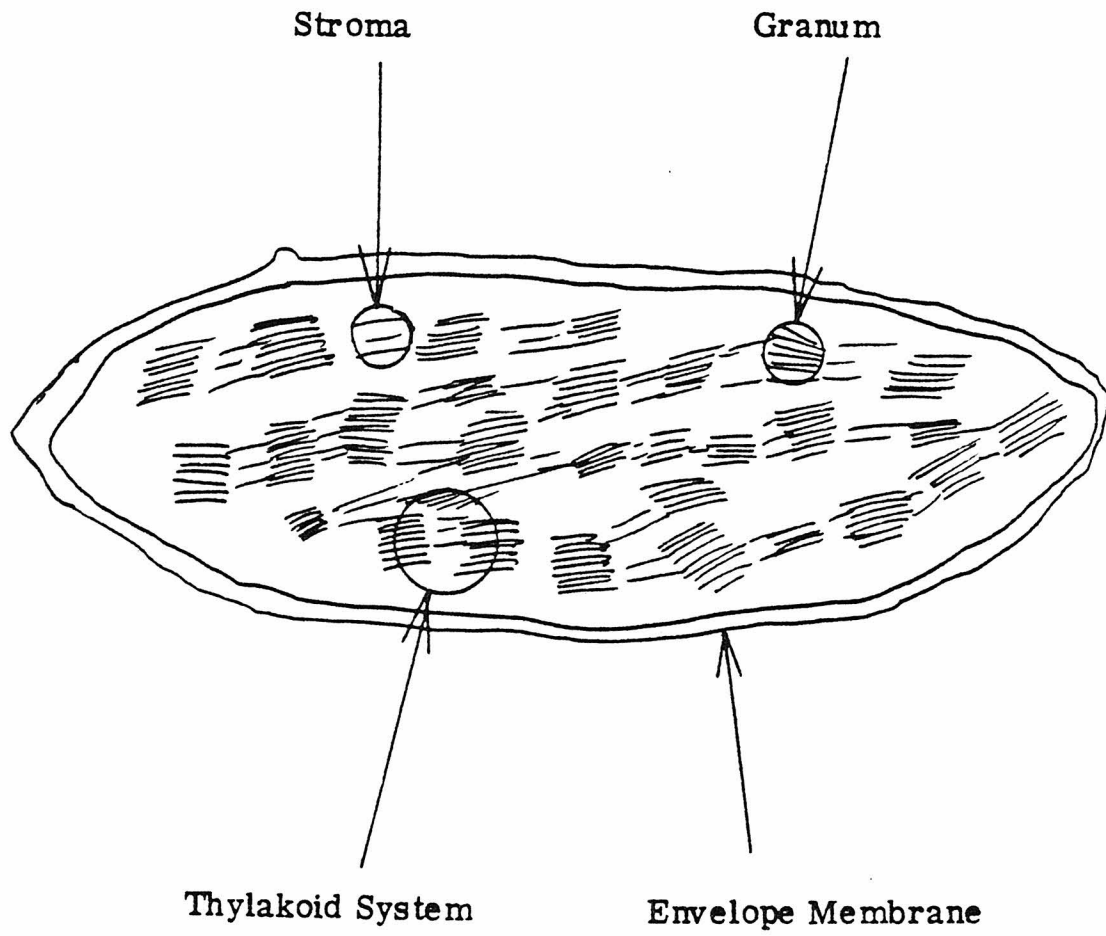


FIGURE IV-3

Artist's conception of way in which grana
and stroma membranes are interconnected.

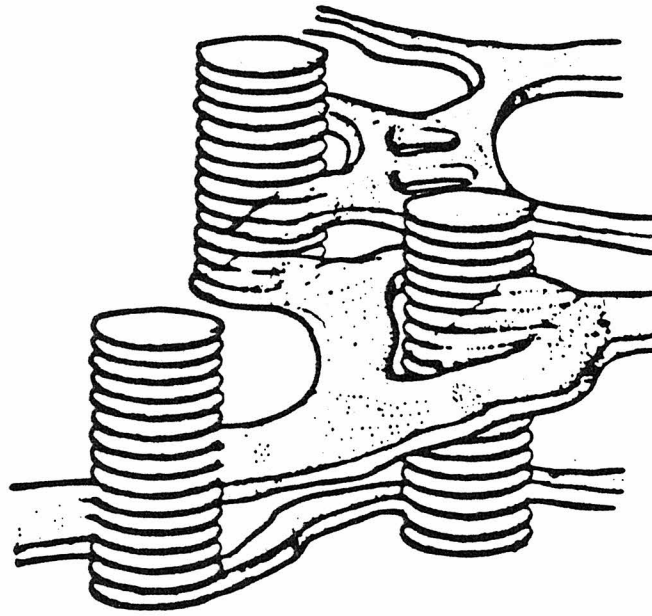
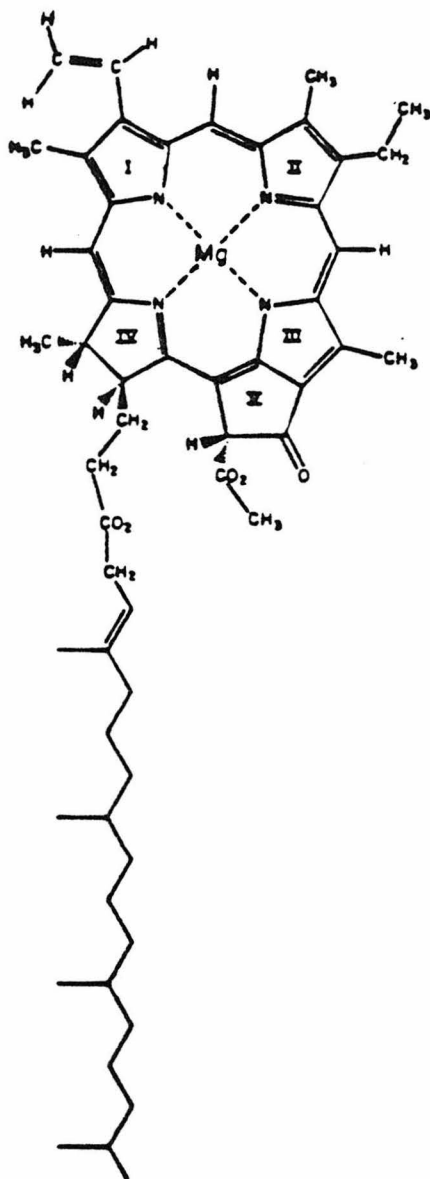


FIGURE IV-4

Structure of chlorophyll a.



chlorophyll to chlorophyll until it reaches the reaction center, where the energy conserving photochemical transduction occurs. This bulk of the chlorophyll is called antenna chlorophyll and it outnumbers reaction center chlorophyll by about 200:1.

While the function of chlorophyll in the photosynthetic process is reasonably well defined, the structural organization of chlorophyll in the thylakoid membrane system remains a matter of dispute (3,4). A number of chlorophyll-containing proteins have been isolated from thylakoid membranes (5). Two of these have been known for several years and are well-characterized. One contains the reaction center of photosystem 1. The other, called a/b protein, contains one chlorophyll a and one chlorophyll b per protein unit and is thought to serve an antenna function for several reasons. First, this protein shows no photosystem activity. Second, it accounts quantitatively for the chlorophyll b content of the membrane and light absorbed by chlorophyll b is known to produce photosynthetic activity. Third, there exists a barley mutant which contains no chlorophyll b and presumably no a/b protein, but still shows normal activity of both photosystems. More recently two more chlorophyll-containing complexes associated with photosystem 2 have been isolated (6). Thus it is fairly well established that the specific chemical environment for chlorophyll in the

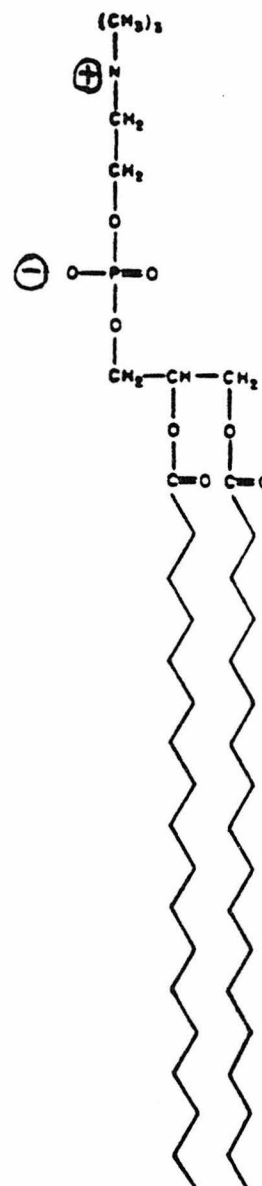
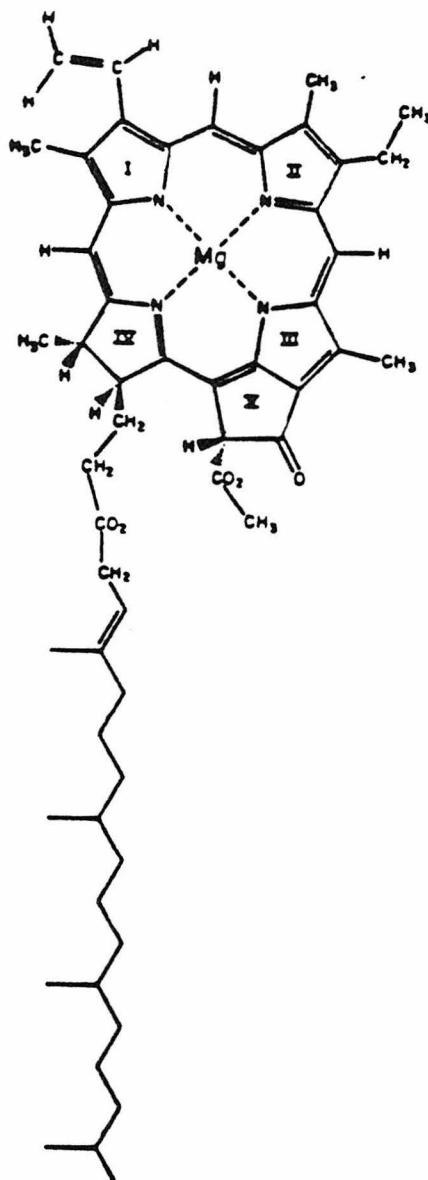
two reaction centers is provided by protein.

However, no specific protein containing chlorophyll a in the absence of chlorophyll b and serving an antenna function has yet been demonstrated. Moreover, in initial fractionations of detergent solubilized thylakoid membranes there is nearly always a "free pigment" fraction which contains chlorophyll and lipids but no protein. By using very mild detergent conditions Markwell et al. have recently been able to fractionate thylakoid membranes in such a way that every chlorophyll-containing fraction also contains protein (7), but this may merely indicate incomplete breakup of the membrane supramolecular structure, rather than the specific association of each chlorophyll molecule with a polypeptide chain. Indeed it is possible that detergent fractionation causes artifactual association of chlorophyll with protein. Thus while there are vocal advocates of the notion that every chlorophyll molecule including antenna chlorophyll is associated with a protein (4), this proposal is by no means proven.

Several authors have proposed an alternate scheme in which some of the antenna chlorophyll is associated with the lipid portion of the thylakoid membrane, forming a chlorophyll-lipid antenna array (3,8,9). Indeed it is chemically quite reasonable that chlorophyll should be associated with membrane lipids. Figure IV-5 compares the structure of chlorophyll with

FIGURE IV-5

Structure of (a) chlorophyll a and (b)
distearoylphosphatidylcholine.



that of distearoylphosphatidylcholine (DSPC), a phospholipid. Notice that the phospholipid molecule may be divided into a relatively hydrophilic headgroup (the glycerol backbone with esterified phosphoryl choline moiety) and two relatively hydrophobic tails (the long chain fatty acids). Chlorophyll also may be divided into headgroup (porphyrin macrocycle) and tail (phytol chain) regions, although for chlorophyll the headgroup is large and the tail relatively narrow. Perhaps for this reason chlorophyll does not form bilayers by itself (10). However, chlorophyll does form monolayers at an air-water interface (11,12), and has been shown to form multilamellar and single-walled vesicular bilayer structures in combination with other lipids (9,13-17). In these bilayer systems the chlorophyll is anchored to the hydrophobic center portion of the bilayer of the phytol chain, while the porphyrin macrocycle protrudes through the headgroup region and into the aqueous medium. In our current investigations we seek to determine whether such mixed-bilayer regions containing chlorophyll with other lipids serve as light-gathering antennae in vivo.

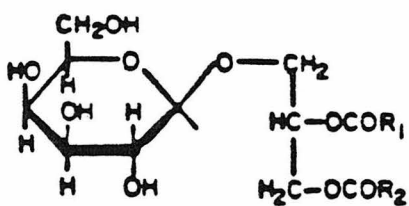
In addition to the hydrophobic phytol chain, which can serve as a lipophilic anchor to membranes, there is a second structural feature of chlorophyll which plays a key role in determining the way in which chlorophyll interacts with other molecules in mixed systems. The central magnesium atom of the chlorophyll macrocycle is coordinatively unsaturated, and is nearly always found

to be complexed by one or sometimes two axial ligands (18-20). Any Lewis base can serve as an axial ligand. In solutions of chlorophyll in nonpolarizable solvents the axial ligand for one chlorophyll is provided by the C-9 ketone group of an adjacent chlorophyll acting as an electron donor. In polar solvents the fifth ligand is provided by the solvent. There are four possible axial ligands available to chlorophyll in vivo, namely water, chlorophyll, protein, and lipid. Preliminary resonance Raman results (21) rule out water or another chlorophyll molecule as electron donors to the magnesium. Thus to understand the organization of antenna chlorophyll in vivo we seek to determine whether the axial ligand to chlorophyll is provided by a specific membrane protein or by membrane lipid.

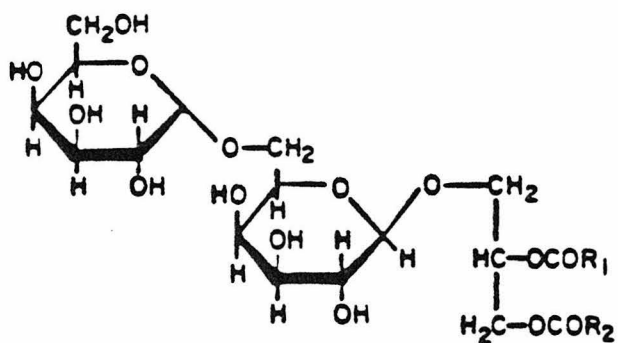
In addition to the chemical rationale for lipid-associated chlorophyll there is independent biochemical evidence to suggest that lipids are necessary for normal photosynthetic function. Figure IV-6 illustrates the principal lipid components of the thylakoid membrane, with the weight percent of the total lipid (excluding pigments) in parentheses (22). (Chlorophyll itself makes up 10% by weight of the total thylakoid membrane.) 75% of the total thylakoid lipid (excluding pigments) is galactolipid, either monogalactosyldiglyceride (MGD), digalactosyldiglyceride (DGD) or sulfoquinovopyranosyldiglyceride (sulfolipid, SL). Present in smaller

FIGURE IV-6

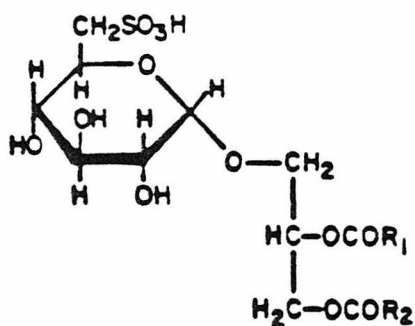
Principal lipid components of the thylakoid membrane. Numbers in parentheses are weight percent of total lipid made up by each component. MGD: monogalactosyldiglyceride; DGD: digalactosyldiglyceride; SL: sulfoquinovopyranosyldiglyceride; PG: phosphatidylglycerol.



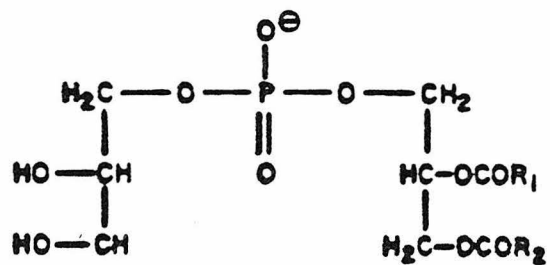
MGD (40%)



DGD (20%)



SL (10%)



PG (20%)

amounts are phospholipids, mainly phosphatidyl glycerols. 80% to 90% of the lipid fatty acid chains are triply unsaturated fatty acids, either 16 or 18 carbons in length (23,24). A variety of evidence suggests that certain lipids are required for photosynthetic function. MGD is found to develop simultaneously with chlorophyll upon greening of *Euglena* (25). Extraction of thylakoid membranes with hexane, which preferentially removes MGD from the membrane, reduces photosystem 1 activity (26). Treatment of the membrane with galactolipase has a similar effect (27). In either case, if MGD is added back to the membrane photosystem 1 activity is restored. These experiments imply that galactolipids are required for normal photosynthetic function.

Thus in our laboratory we have been investigating the hypothesis that lipid-chlorophyll arrays act as light-gathering antennae in vivo. Our approach has been primarily a synthetic one: we have prepared model membrane systems containing chlorophyll a and studied them by a variety of physical techniques to determine whether they duplicate the properties of chlorophyll a in vivo and display additional properties deemed appropriate for a light-gathering system. In addition we have studied thylakoid membranes themselves in an attempt to determine whether there is indeed membrane-associated chlorophyll which is free of protein. In this section we describe primarily calorimetric and magnetic resonance

measurements on model membranes containing chlorophyll a. We interpret the measurements on model systems in light of other physical data on these systems, and point out those features of the model system which make it a particularly attractive candidate for an in vivo antenna.

B. Experimental

1. Preparation of membrane lipids

Chlorophyll a was isolated by powdered sugar chromatography from a methanol extract of fresh spinach leaves by the method of Strain et al. (28). Analysis by optical criteria indicated that the resulting material was consistently 98-99% pure. We believe that the chlorophyll is isolated as the monohydrate, in which case the absorbance data would indicate essentially pure material. Lipids were purchased from Calbiochem and used without further purification. Occasional checks of the distearoylphosphatidylcholine (DSPC), which is the major lipid used in the following studies, indicated that it was pure by thin layer chromatography.

2. Preparation of model membranes

Two types of model membrane systems containing chlorophyll a were prepared. To prepare multilamellar systems for optical measurements chlorophyll a and the lipid of interest were dissolved in dry, spectroquality chloroform. The chloroform was evaporated under a stream of air or nitrogen, and the dried sample was

evacuated overnight to a final vacuum of ten microns or less. The sample was hydrated by adding deionized water and heating (maximum temperature $\sim 80^{\circ}\text{C}$) and shaking until no chunks of undispersed lipid mixture were visible in the suspension or on the side of the container. Multilamellar samples for differential thermal analysis were hydrated by placing ~ 1 mg of dried mixture in the bottom of a capillary tube, adding 1 - 2 mg H_2O , sealing and incubating for at least one hour at 60°C .

To prepare single-walled bilayer vesicles for ^1H or ^{31}P NMR, chlorophyll a and lipid (60 mg total) were mixed and dried from chloroform as described above, usually in a 3 ml centrifuge cone. D_2O (1 ml) was added to the cone and the sample was irradiated with an MSE model 150 sonicator (for experiments performed at Caltech) or a Branson sonifier with stepped microtip (for experiments performed at the Stanford Magnetic Resonance Laboratory). Sonication was performed without sample cooling in order that the sample temperature rise above the gel to liquid crystalline phase transition temperature; intermittent sonication was required to prevent boiling of the sample. Sonication was continued only until a clear suspension (no turbidity) was obtained. This required 2 - 5 minutes sonication time on the Branson instrument and 10 - 20 minutes on the MSE instrument. Clear, stable samples could not be obtained

for concentrations of chlorophyll in lipid beyond 40 mole %. Samples sonicated on the MSE instrument were centrifuged for 2 - 3 minutes at low speed to remove titanium particles released from the sonicator tip; this was not found to be necessary for the Branson-sonicated samples. Centrifugation of the samples is undesirable as it occasionally caused the entire sample to form a dark green gelatin-like mass; this gelatin could sometimes be resuspended by resonation. We have been unable to correlate the appearance of this gelatin with parameters of sonication or sample composition. Finally, it is important to mix lipid and chlorophyll in chloroform. Drying from methanol produces inconsistent results in optical measurements; we believe that methanol is retained as a ligand to the chlorophyll magnesium in spite of exhaustive drying, preventing the interaction of chlorophyll with other molecules.

3. Optical measurements

Optical measurements were made on a Beckman Acta CIII uv-visible spectrophotometer equipped with a temperature programmer. $\lambda_{\max}$ for absorption bands was determined by marking two positions of equal absorption a few nanometers on either side of the absorption maximum and taking the wavelength halfway between them as $\lambda_{\max}$. For temperature profiles a time of at least 5 minutes was allowed for temperature equilibration after

a temperature change.

4. Calorimetry

Calorimetry by differential thermal analysis was performed on a Dupont model 900 differential thermal analyzer. A heating rate of 7°C per minute was utilized; cooling rates were variable, since cooling was done with a stream of ice-cold nitrogen, which swamped the effects of the temperature controller. Traces were reproducible over several heating and cooling cycles.

5. Magnetic resonance

Proton nuclear magnetic resonance spectra at 360 MHz were taken on the Stanford Magnetic Resonance Laboratory Bruker HXS-360 spectrometer utilizing the Fourier transform technique and 5 mm tubes. Temperature control was provided by a Bruker Instruments temperature controller. Probe temperature was calibrated using the splitting of the two resonance lines of ethylene glycol. For spectra at 360 MHz of sonicated vesicles containing chlorophyll a and DSPC, the residual HDO peak was substantially reduced by single frequency irradiation of the HDO resonance just prior to the sampling RF burst. Fourier transform ^{31}P NMR spectra were obtained on a Varian XL 100 spectrometer at 40 MHz in 12 mm tubes utilizing a Varian temperature controller calibrated with a standard thermometer.

C. Results

We will describe in this section results of physical studies on model membranes containing chlorophyll a, and indicate what these results imply about the physical properties of the model membrane system. The discussion section which follows will interpret the results in terms of antenna function and assess the relevance of the model system to the organization of antenna chlorophyll a in vivo.

1. Optical properties of chlorophyll a model membranes

The absorption spectrum of chlorophyll a shows the strong Soret band and x and y polarized visible absorption bands typical of substituted porphyrins which do not retain D_{4h} symmetry (29). The visible spectrum is dominated by a strong Q_y band which appears at 659 nanometers for chlorophyll a dissolved in organic solvents (such as diethyl ether) in which chlorophyll does not aggregate. Chlorophyll in vivo shows several changes in the optical spectrum, the most obvious of which is a change in the position of the red absorption maximum to about 679 nanometers (30), a red shift of some 20 nanometers. While the in vivo band is probably the superposition of several bands from chlorophylls in various chemical environments the largest single contributor must be antenna chlorophyll, which thus shows a substantial Q_y red shift.

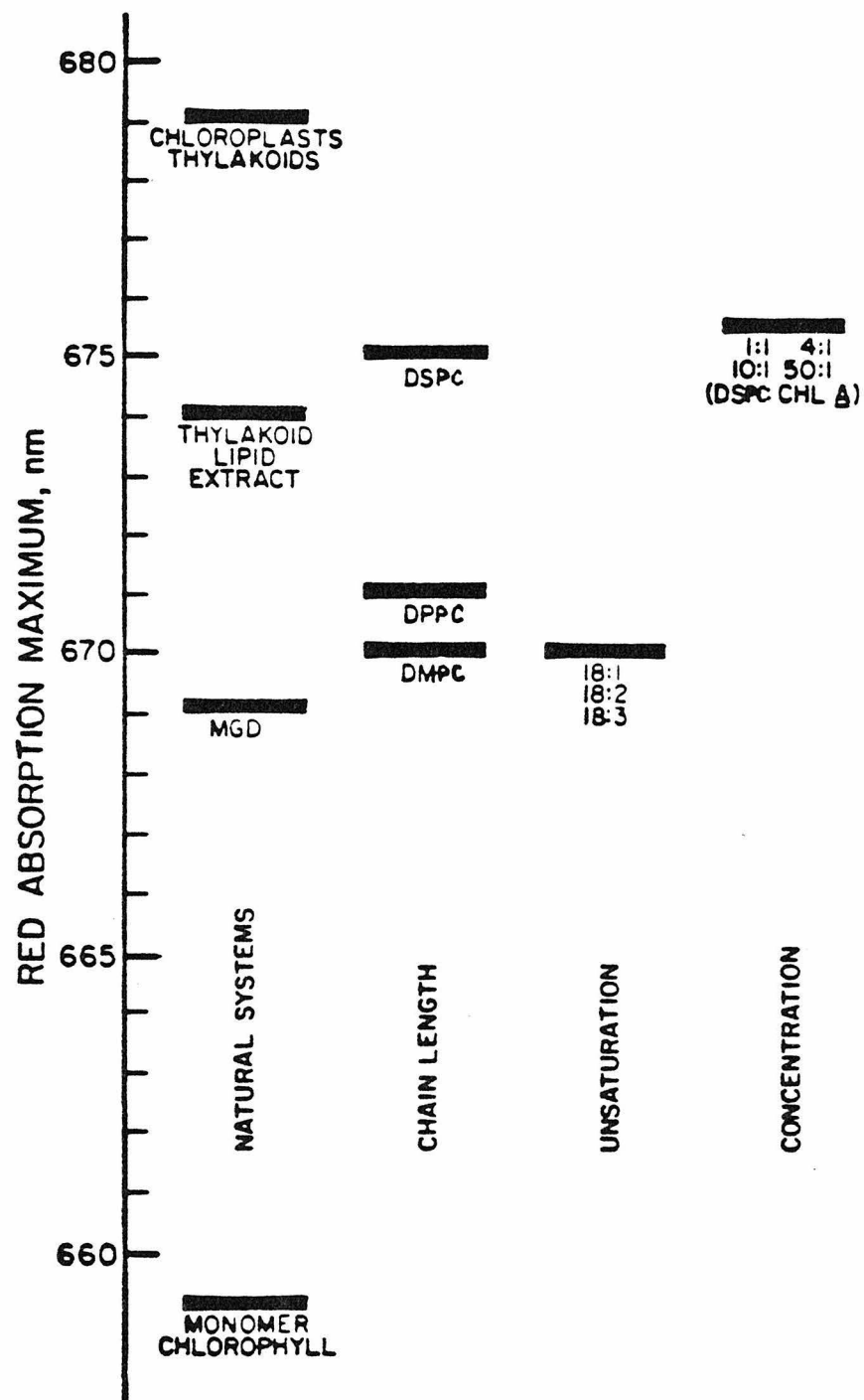
Red shifts are also observed for multilamellar

dispersions of chlorophyll a in various lipids. Figure IV-7 shows the position of the Q_y band at room temperature for chlorophyll a in a number of model and natural membrane systems. As indicated in the left most column of the figure, isolated thylakoid membranes show the same red shift for chlorophyll a as intact chloroplasts. When thylakoid lipids are extracted from the membrane (31), dried from chloroform and rehydrated, the chlorophyll red shift is about 15 nm. Dispersions of chlorophyll a in phospholipid show a red shift which varies with the length of the saturated hydrocarbon chains. For 18 carbon chains a 15 nm shift is observed while shorter chains produce only a 10 nm shift. Unsaturated chains produce only the 10 nm shift regardless of the degree of unsaturation. The galactolipids MGD and SL (not shown), which are native to thylakoid membranes, also show about a 10 nm red shift. Finally, over DSPC:chlorophyll a ratios from 50:1 to 1:1 no effect of concentration on red shift is observed.

The variation of red shift with the identity of the lipid may be easily explained. The only lipid which shows a red shift comparable to that observed for rehydrated thylakoid lipids is DSPC; other lipids give a red shift of about 10 nm. Recall that all of these red shifts were measured at room temperature. Phospholipid model membranes undergo a gel to liquid crystalline phase transition near physiological temperatures (32).

FIGURE IV-7

λ_{max} of the Qy band of chlorophyll a at room temperature for various model and natural membrane systems. MGD: monogalactosyldiglyceride; DSPC: distearoylphosphatidylcholine; DPPC: dipalmitoylphosphatidylcholine; DMPC: dimyristoylphosphatidylcholine; 18-1 - dioleoylphosphatidylcholine; 18-2 - dilinoleoylphosphatidylcholine; 18-3 - dilinolenoylphosphatidylcholine.

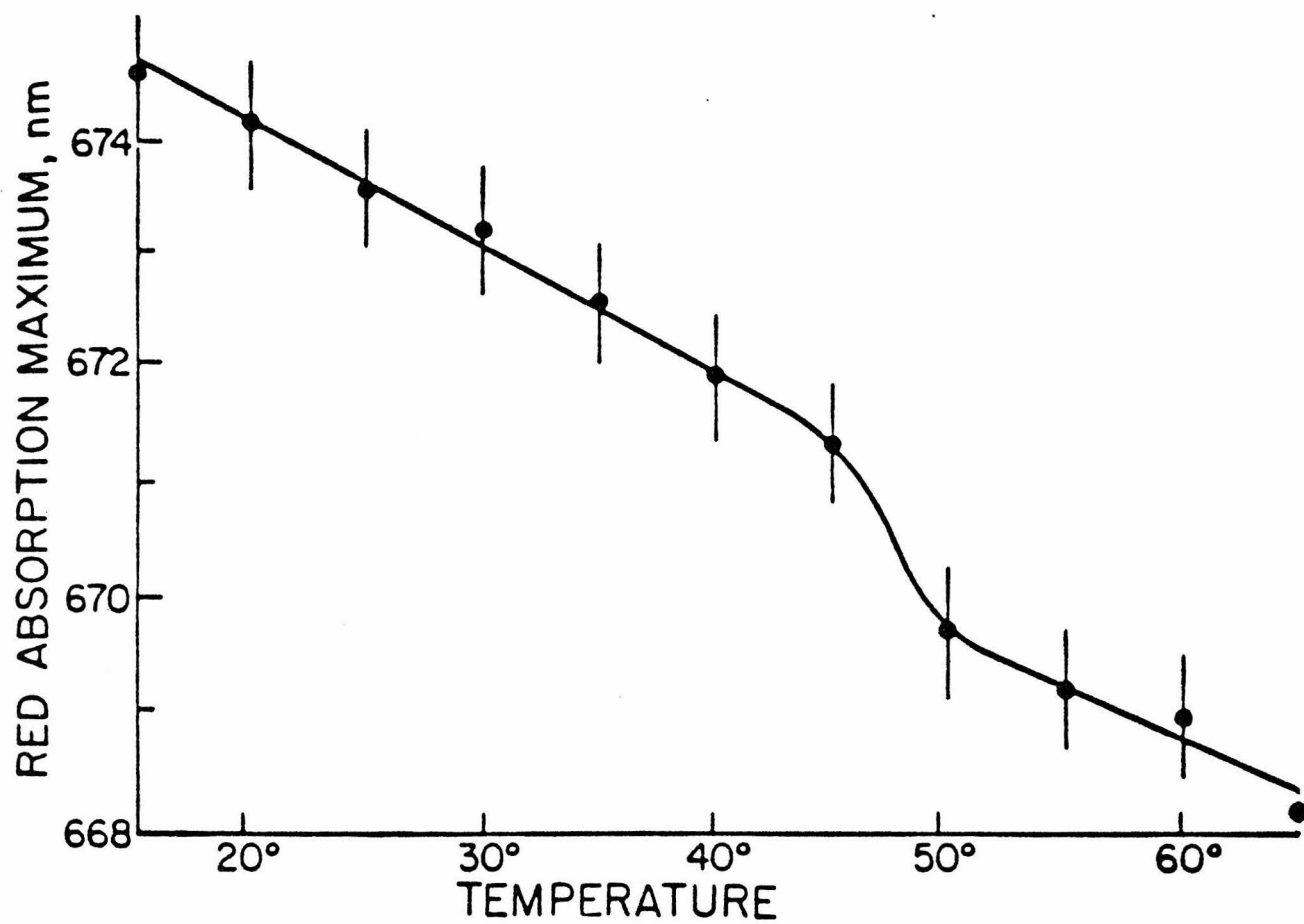


Principal changes in phospholipid bilayers occurring upon heating above the transition temperature T_c are a change in the motional state of hydrocarbon chains of the lipid from the fully extended, all trans conformation to a less ordered state in which there are kinks and jogs in the chain introduced by gauche bonds, an increase in the lateral diffusion rate by about two orders of magnitude, and changes in headgroup conformation (33). In Figure IV-7 DSPC is the only lipid expected to be below the gel to liquid crystalline phase transition temperature (T_c) at room temperature (T_c for DPPC is 41.5°C but addition of chlorophyll lowers this temperature, as indicated below). Thus it is apparently the change in the phase of the system which causes the variation in λ_{max} of the Q_y band.

The importance of the phase transition in determining the red shift may be verified by measuring red shift as a function of temperature for a 20 mole % chl a in DSPC multilamellar suspension (Figure IV-8). There are two components to the temperature dependence of λ_{max} of the Q_y band, a linear drop with increasing temperature and a sharp drop between 50 and 55°C , approximately 5 to 10°C below T_c for pure DSPC multilayers (59°C). The sharp drop may be correlated with the phase transition region for the mixed system, which occurs several degrees below the T_c for pure DSPC (see below). This change in λ_{max} is reversible after a time lag of the

FIGURE IV-8

λ_{max} (nm) versus temperature ($^{\circ}\text{C}$) for a multilamellar dispersion of chlorophyll a (20 mole %) in DSPC.



order of minutes.

Two different mechanisms have been suggested to account for the red shifts observed in vivo. One is the exciton shift (34). Dipole-dipole interactions between chlorophylls which have a fixed geometrical orientation relative to one another produce mixing of the individual transition dipoles to form a band of allowed excited states. Variations in the magnitude of the transition dipole from the low to high energy end of the band produce shifts in the position of the composite λ_{max} either to the blue or to the red, depending on the specific geometry of the interaction. The other suggested explanation is the so-called "environmental" red shift (35) arising from perturbations to the electronic structure of the chlorophyll via interactions with neighboring molecules, particularly those serving as axial ligands to the magnesium. Quantitative explanations using either model are not possible, in the first case because nothing is known about the geometrical relationship of antenna chlorophyll molecules to one another in vivo and in the second case because the theory of the electronic structure of porphyrins is insufficiently developed to predict small spectral changes (36).

The present data may be interpreted in terms of either scheme, and it is probable that each effect plays a role in the total red shift. Consider first the exciton hypothesis. Recall that there is a drop in red shift on

extraction and reconstitution of thylakoid lipids. Interpreted in light of our lipid antenna hypothesis this observation indicates a role of protein in ordering the lipid-chlorophyll portion of the bilayer; thus the protein indirectly modulates an exciton effect. The linear decrease of the red shift with increasing temperature may indicate progressively large disorder-averaging of exciton effects. The jump in red shift through the transition region may be explained in terms of greater ordering of chlorophylls in the gel state, assuming that hydrocarbon chain ordering and a decrease in lateral mobility are accompanied by an increase in ordering of the chlorophyll "headgroups". On the other hand the jump in red shift at T_c may also be explained by a difference in intermolecular interactions of chlorophyll and lipid in the two temperature regions. This also is perfectly consistent with our model, and we will present evidence that such differences in the interaction between chlorophyll and lipid as a function of temperature do indeed occur. Whether the interactions mediate an environmental shift in $\lambda_{\max}$ of the Q_y band cannot at present be determined. Note however that differences in red shift based on the chemical nature of the lipid are not observed (Figure IV-7).

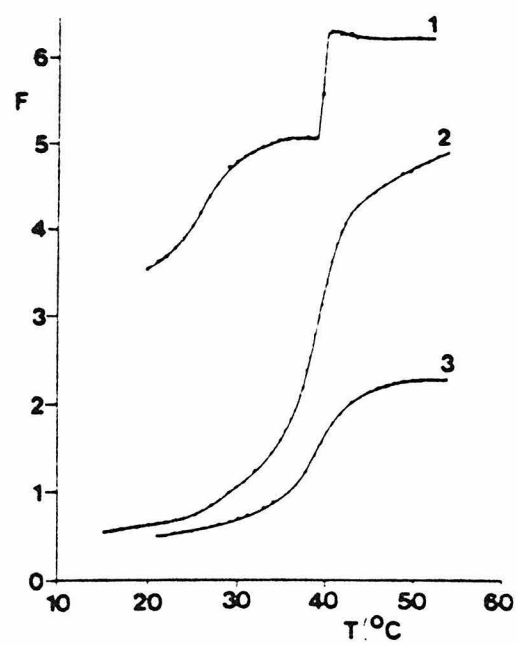
Thus the red shift seen for chlorophyll in vivo also occurs in our model systems. To complete even a cursory examination of the optical properties of model

membranes containing chlorophyll a we should also examine the fluorescence of chlorophyll in bilayers. Chlorophyll a in organic solution is highly fluorescent, but it was long ago observed that chlorophyll in vivo fluoresces only weakly. Chlorophyll a in model membranes also exhibits fluorescence which is markedly reduced in intensity. A. G. Lee has monitored fluorescence as a function of temperature for bilayer systems containing small concentrations of chlorophyll a (15). Temperature profiles for dipalmitoylphosphatidylcholine (DPPC) vesicles containing chl a are reproduced in Figure III-9. The fluorescence decreases abruptly below T_c , and the quantum yield of fluorescence at temperatures below T_c is at most a factor of two or three larger than the fluorescence in vivo. To understand the fluorescence observations, note that there are four possible pathways for the decay of excited singlet antenna chlorophyll in vivo, each with a characteristic rate constant. Energy may be transferred to another antenna chlorophyll (rate constant τ transfer), transferred to the reaction center (τ_{rc}), lost via fluorescence (τ_f), or lost via non-radiative decay (τ_{nr}) to the groundstate. Three of these processes (τ_{rc} , τ_f , τ_{nr}) cause loss of energy from the antenna system, the other (τ transfer) merely shifts the energy around.

On the one hand it might be claimed that a model system which does not fluoresce is not viable since lack

FIGURE IV-9

Fluorescence intensity (arbitrary units) versus temperature for DPPC vesicles containing chlorophyll a in molar ratios of (1) 1:1560, (2) 1:77 (sensitivity increased by a factor of 20), (3) 1:39 (sensitivity increased by a factor of 45). Reproduced from (15).



of fluorescence implies lack of singlet-singlet energy transfer via a Förster mechanism. However it is not true that observation of reduced fluorescence implies absence of energy transfer, for transfer without fluorescence is precisely the situation which obtains in vivo. Since the antenna system is relatively efficient, τ transfer (which is comparable in size to τ_{rc}) must be the shortest timescale. One or more transfers may precede transfer to the reaction center, but the energy must reach the reaction center before it is lost to nonproductive pathways. Thus chlorophyll a in vivo shows reduced fluorescence because energy is lost from the antenna to the reaction center before fluorescence can occur.

On the other hand, while the model system exhibits reduced fluorescence similar in intensity to the fluorescence of chlorophyll a in vivo, this cannot be construed as support for the relevance of our model system. In our model system no reaction center is present, and all of the energy must ultimately be lost via fluorescent or non-radiative mechanisms regardless of the number of preceding transfers. Thus, in comparison to chlorophyll in organic solution, in the model system the number of non-radiative losses must be increased in proportion relative to the number of fluorescent events. Both Lee (15) and Beddard and Porter (9), who also studied chlorophyll fluorescence in model membranes, concluded that intramembraneous aggregation of chlorophyll produces dimers

which show rapid non-fluorescent decay; these "traps" are responsible for the concentration quenching of fluorescence. While the existence of the traps produces reduced fluorescence in the model system, in vivo the traps would compete with the reaction centers for energy, and thus it is important to note that fluorescence in the model system remains two or three times larger than fluorescence in vivo. Quantitative analysis of concentration quenching by Beddard and Porter implied that resonance transfer indeed occurs in the model system and the possibility of trap formation led them to propose that if antenna chlorophyll is not protein associated it must be present in a lipid-chlorophyll array where interspersed lipid molecules keep chlorophylls far enough apart ($\geq 10 \text{ \AA}$ separation) to prevent trap formation. Finally, Lee attributed reduced fluorescence in the gel state relative to the liquid crystalline state as indicating increased aggregation at low temperatures.

Summarizing the optical data, red shifts for model systems mimic the behavior of chlorophyll in vivo. The property of reduced fluorescence is also shared by natural and model membrane systems, but the mechanism of quenching is almost certainly different in the two cases. Both absorbance and fluorescence measurements in model systems show a temperature dependence suggesting that the phase behavior of the system is important in determining its optical (and energy transfer) properties.

2. Calorimetry of model membranes

In view of its importance in understanding the optical data we sought to study the phase behavior of the system in greater detail. Model membrane systems consisting of two kinds of lipid may be viewed as two component thermodynamic systems in the presence of excess water. Since the gel to liquid crystalline phase transition is endothermic a pseudo-two component phase diagram may be determined calorimetrically. We have used differential thermal analysis (DTA) to study phase behavior as a function of chlorophyll a concentration in multilayers containing chlorophyll a and DSPC. In the DTA technique a sample (a hydrated multilamellar dispersion) and a reference (water) are placed in glass capillaries in a heating block, which is heated so that the block temperature increases at a constant rate. The difference in temperature between reference and sample is plotted against the sample temperature. Endothermic or exothermic transitions in the sample produce respectively negative or positive deviations of the trace from the baseline indicating that the sample temperature is lagging behind or running ahead of the reference temperature. We chose to investigate DSPC systems containing chlorophyll because the lipid is easily available in gram quantities, has T_c convenient for calorimetric and magnetic resonance purposes, is relatively stable, and is well understood in terms of the calorimetry and magnetic resonance properties

of the pure material.

DTA traces for chlorophyll a-DSPC multilayers of increasing chlorophyll content (Figure IV-10) show two endothermic transitions which coalesce at a eutectic composition and then reappear at still higher concentrations of chlorophyll. The appearance of two transitions and a eutectic point indicates that phase separation is occurring. The DTA data may be plotted in terms of a phase diagram (Figure IV-II) in which the lower of the two transition temperatures is represented by the open circles, while the filled circles represent the upper transition temperature at a given composition. Transition temperatures are taken as the position of maximum deflection from the baseline of the DTA trace; theoretical treatment of heat exchange in differential thermal analysis indicates that this procedure is justified for our experimental arrangement, in which the sample cells are cylindrical, the thermocouples sit in the center of the sample and the sample temperature is used as the horizontal variable in the data presentation (37). No correction for the width of the transition, which increases with increasing chlorophyll content, has been made. We present data only for chlorophyll concentrations up to 40 mole %. While we have attempted to prepare aqueous dispersions of chlorophyll a and DSPC at chlorophyll concentrations as high as 50 mole % these samples give inconsistent DTA results and do not form stable single-

FIGURE IV-10

DTA thermograms for chlorophyll a - DSPC multi-lamellar dispersions at various mole % chlorophyll. Heating traces were obtained at a heating rate of 7°C per minute.

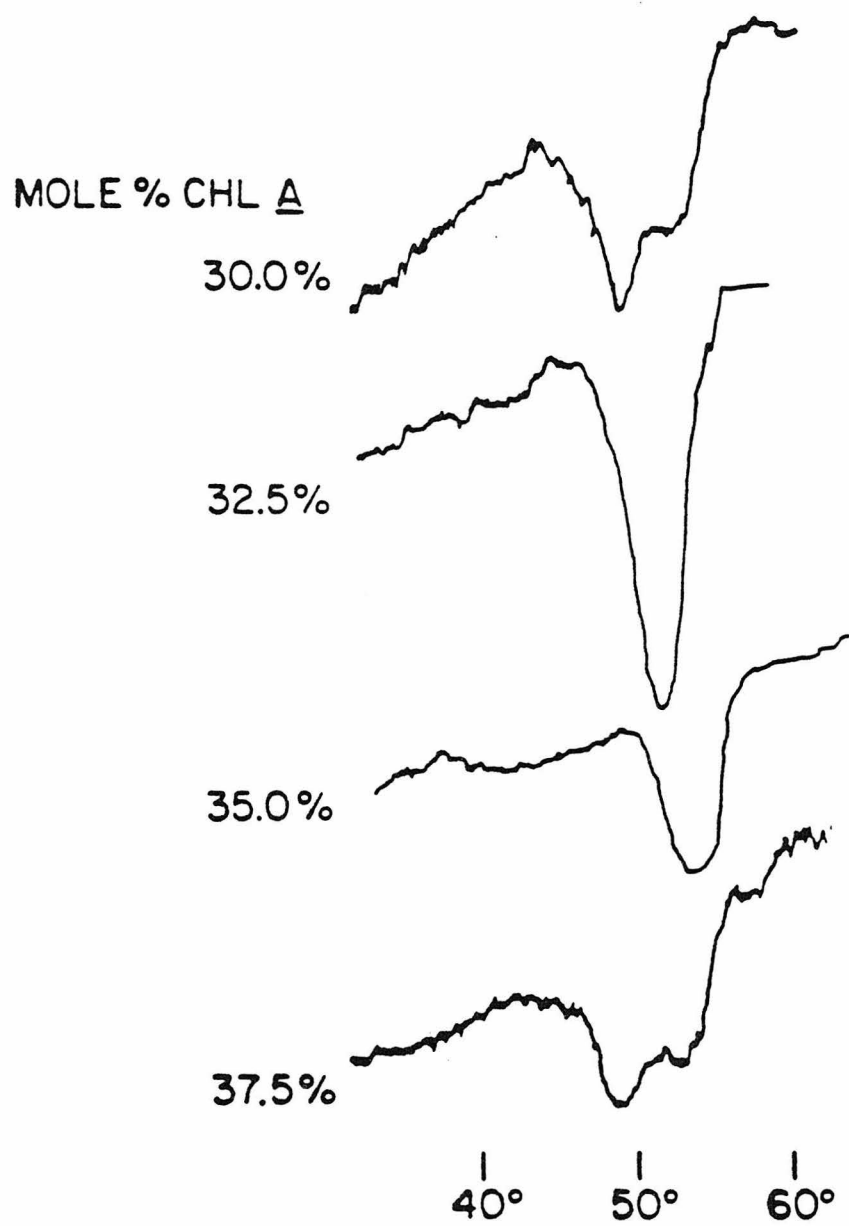
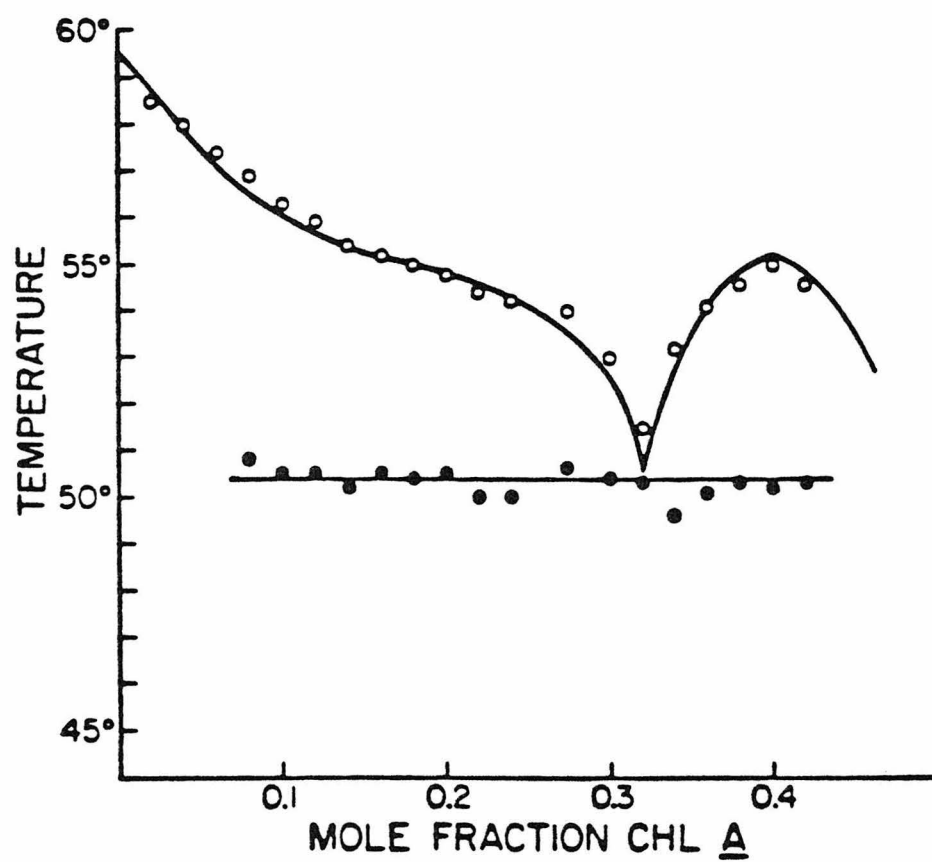


FIGURE IV-11

Phase diagram plotted from DTA data. Open circles represent lower transition temperature observed by DTA while closed circles represent upper transition temperature. Transition temperatures are taken as the point of maximum deviation of the trace from the baseline (see text).



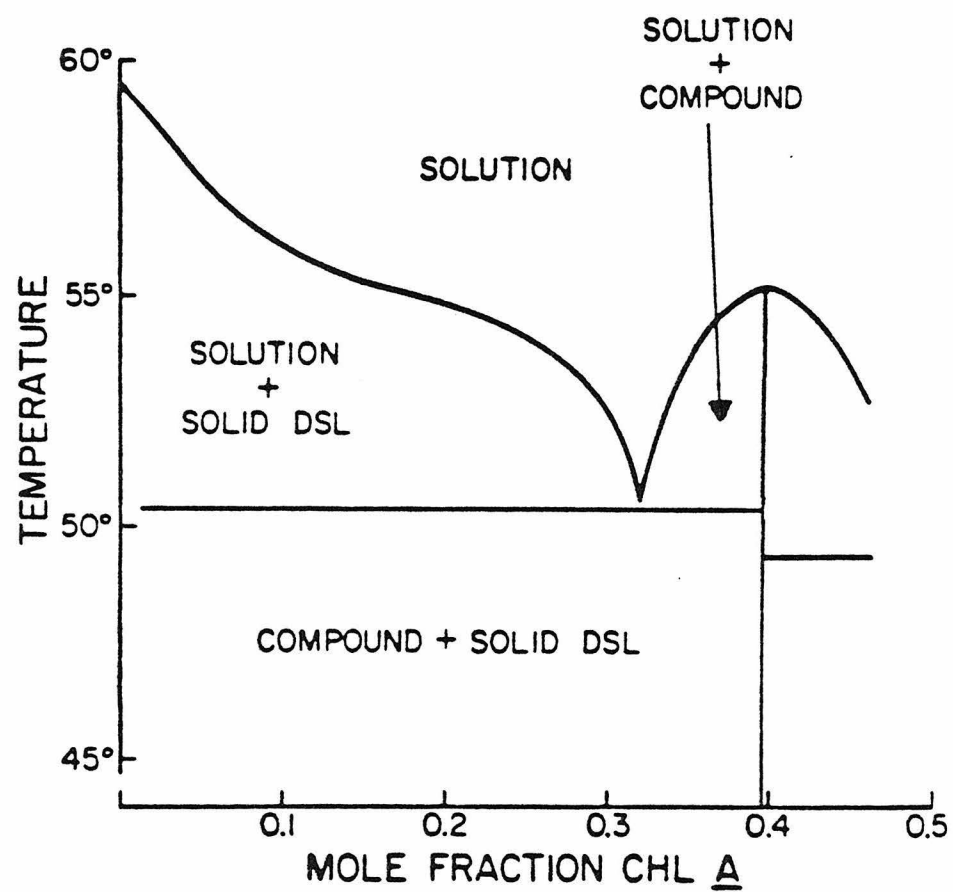
walled vesicles upon sonication. We suspect that bilayers are not the stable phase for these mixtures containing large amounts of chlorophyll.

In spite of the fact that they extend only to 40 mole % chlorophyll a these data suggest a particular type of phase diagram, namely a double eutectic with compound formation. While there are suggestions of this shape in the decrease of the transition temperature at 40 mole % chlorophyll a, the principal justification for this interpretation comes from theoretical fits to the liquidus curve by K. E. Eigenberg in our laboratory. The liquidus line may be fit to high precision using a non-ideality of mixing entropy as the only adjustable parameter, but only with the assumption that the compound is present at around 40 mole %. In particular, simulations based on a single eutectic phase diagram fail to reproduce the liquidus line.

Figure IV-12 reproduces the phase diagram under the assumption of a double eutectic with compound formation and indicates the phase composition of the various regions. At high temperatures a single, fluid bilayer phase is present, consisting of a homogeneous mixture of chlorophyll a and lipid. For compositions to the left of the eutectic, as the temperature is lowered, when the liquidus line is reached a "solid" (gel-like) phase which is essentially pure lipid begins to form. Upon further decrease in temperature additional solid

FIGURE IV-12

Phase diagram for hydrated chlorophyll a - DSPC system illustrating phases present in various regions of the diagram. Location of vertical, compound line is uncertain.



DSPC is formed in equilibrium with the remaining fluid solution, which becomes increasingly enriched in chlorophyll a. When the solidus is reached the remaining solution, now of eutectic composition, goes to "solid" DSL plus "solid" compound--a homogeneous mixture of chlorophyll and lipid of defined composition. For compositions between eutectic composition and compound composition, when the liquidus line is reached upon cooling compound begins to form at the expense of solution, which becomes relatively enriched in DSPC. At the solidus the remaining solution is again of eutectic composition and again goes to solid DSPC plus compound. Below the solidus is a two phase region in which compound is in equilibrium with solid DSPC (neglecting the finite solubility of compound in DSPC). Within this region, if the overall composition of the bilayer is changed, the relative amounts of the two phases will be altered, but the composition of the compound phase remains constant.

3. Magnetic resonance of model membranes

In the low temperature region of the phase diagram, where the largest red shifts occur, all of the chlorophyll is present as compound. While the phase diagram predicts the existence of this compound, it does not provide information about molecular details. In particular we wish to investigate the relationship between chlorophyll a and DSPC in the compound. Magnetic resonance measurements

allow us to verify the existence of the compound, along with other features of the phase diagram, and to investigate properties of the compound phase.

Chlorophyll a has one feature which makes it particularly suited for magnetic resonance studies of molecular interactions in mixed systems. The large conjugated porphyrin pi system generates a large ring current magnetic anisotropy which shifts the resonance position of nearby nuclei (35). The direction and magnitude of the shift depends on the distance of the nucleus from the plane of the macrocycle and on the angle between the nucleus-plane vector and the normal to the plane. The upfield and downfield shifted regions around the macrocycle are sketched qualitatively in Figure IV-13, where a point dipole model for the ring current has been assumed. In point of fact the region of upfield shift directly above the plane is larger than the figure indicates. Finally the figure is intended to suggest how we might deduce the orientation of a neighboring molecule from the ring current shifts at various nuclei in the molecule produced by a neighboring chlorophyll. This idea has been exploited by investigators modelling the chlorophyll dimer thought to be present at the reaction center (39).

a. ^{31}P NMR

Figure IV-14 shows the ^{31}P NMR spectrum of sonicated DSPC vesicles containing 20 mole % chlorophyll a

FIGURE IV-13

Regions of space where upfield and downfield shifts of NMR signals are produced by ring current magnetic anisotropy of chlorophyll a. The sketch underestimates the extent of the upfield shifted region. A lipid molecule is included to illustrate how ring current shifts of nuclei in a nearby molecule may be used to determine the orientation of the molecule with respect to the porphyrin plane of the chlorophyll.

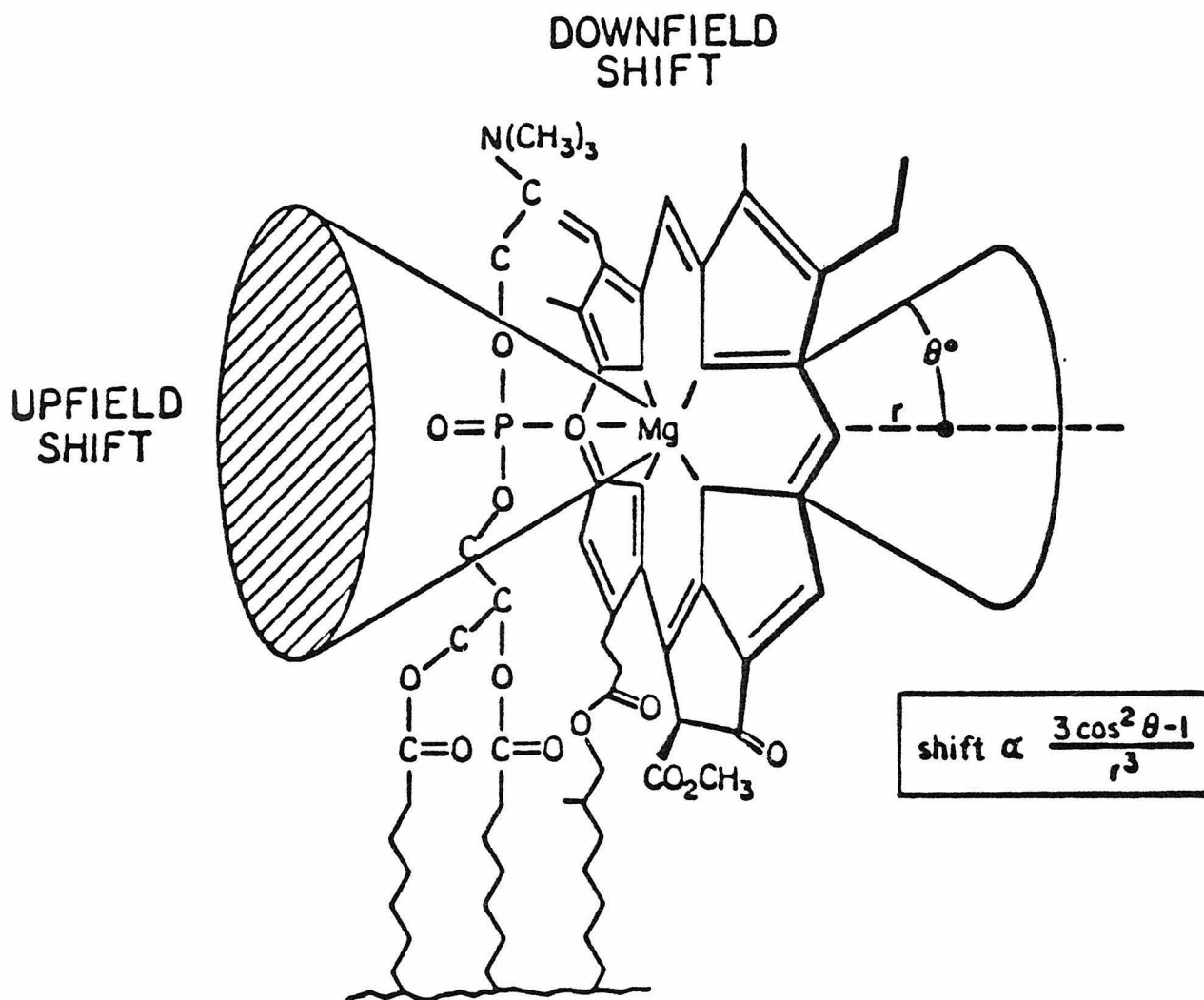
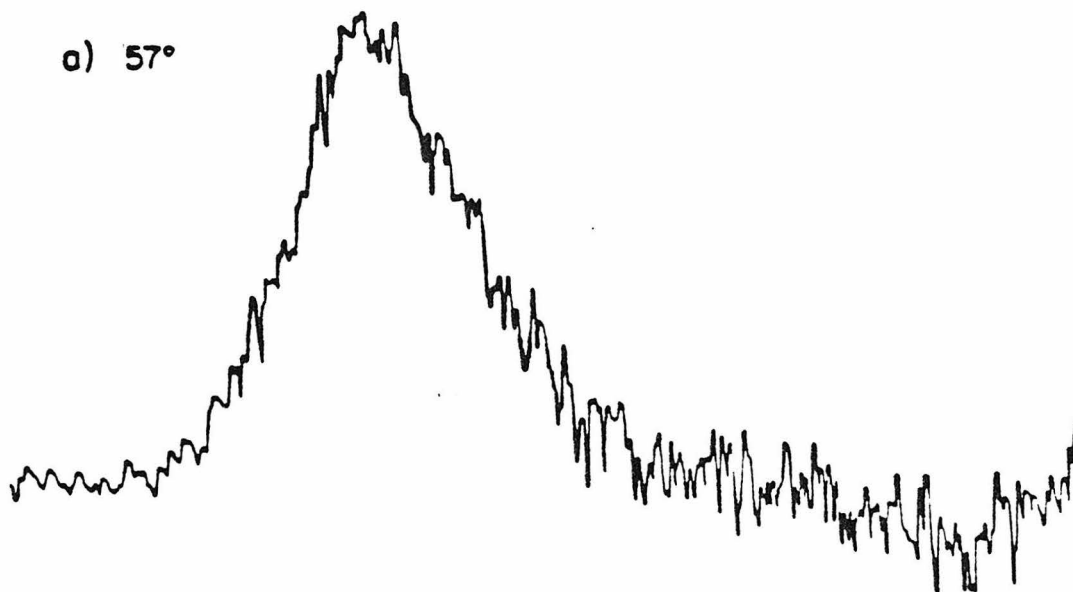


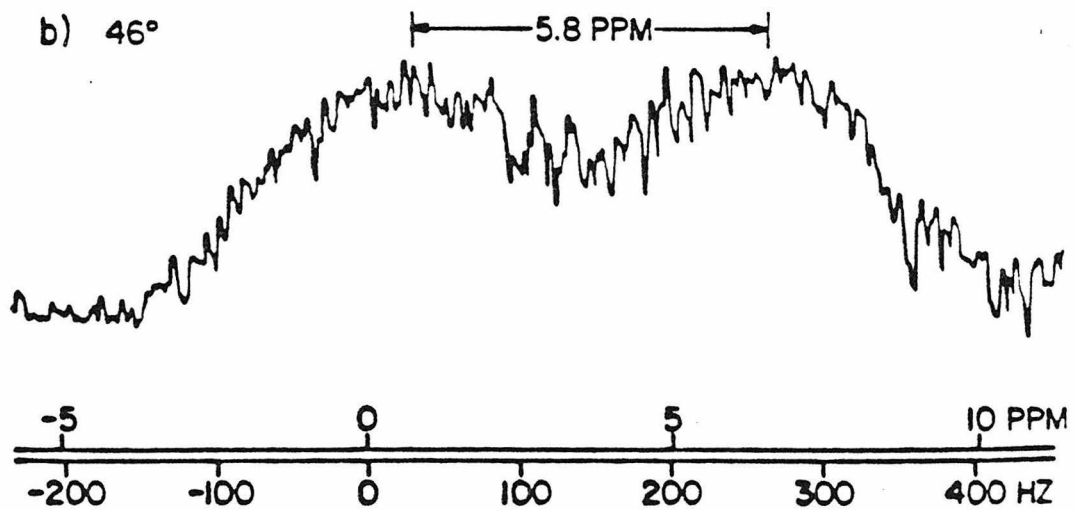
FIGURE IV-14

40 MHz ^{31}P NMR spectra of DSPC vesicles containing 20 mole % chlorophyll at (a) 57°C and (b) 46°C.

a) 57°



b) 46°



at two temperatures. At 57°C a single broad line is observed from the phosphate of the lipid headgroup. The full width at half height of this line is 3.6 ppm. At 46°C two broad lines which are partially resolved are obtained. One has approximately the same chemical shift as the high temperature line; the other is shifted up-field by some 5.8 ppm. Each line is approximately 5.0 ppm wide at half height.

The appearance of two resonances at low temperatures is easily explained in terms of the phase diagram. At temperatures above the liquidus a single homogeneous "liquid" solution is predicted, corresponding to the single peak observed. At temperatures below the solidus the phase diagram predicts an equilibrium between two phases, only one of which contains chlorophyll. We observe two lipid peaks corresponding to the two lipid environments. One peak shows a chemical shift similar to that of the high temperature resonance and may be assigned to the pure DSPC phase. The other peak is shifted up-field, and may be assigned to lipid closely associated with chlorophyll in the compound phase. The areas of the two low field peaks are similar, in agreement with the tie lines one could draw on the phase diagram indicating roughly equal proportions of the two phases at this composition and temperature, and assuming equal NOE's for the two peaks in the proton-decoupled spectrum. Since separate peaks are observed we may conclude that exchange

between phases is slow, the exchange rate being less than 6.8×10^4 /sec.

The upfield shift of the lipid in the compound phase implies that the phosphate is located above the plane of the porphyrin, and the magnitude of the shift suggests that the phosphorous nucleus must be quite near to the central magnesium ion of the porphyrin. Shulman et al. (40) have derived an empirical formula for calculating the magnitude of the ring current shift produced in the resonance of a nucleus at a given orientation with respect to the porphyrin plane. If we assume that the entire shift is due to the ring current effect and in addition assume that the phosphate is centered above the plane of the porphyrin, we calculate a magnesium to phosphorous distance of 5.4 Å, which is a reasonable separation for a Mg to phosphate coordination bond. It is necessary to assume that the phosphate is centered above the plane in order to generate a reasonable distance, since as the angle between the Mg to phosphate vector and the normal to the porphyrin plane becomes larger, smaller and smaller Mg-phosphate distances are required to explain the magnitude of the shift. Thus this result indicates that the lipid phosphate is near the center of the porphyrin plane in the compound, and suggests that the axial ligand to the magnesium in this system may be provided by the phosphate of the lipid headgroup.

The linewidth of the phosphorous resonances in this system also suggests that chlorophyll perturbs the neighboring lipid. In pure phospholipid vesicles linewidths of ^{31}P signals have been measured at 36 MHz (10% lower field than for our data (42)). The linewidth of the phosphorous resonance at temperatures above T_c is of the order of 0.1 ppm. Below T_c the linewidth is of the order of 0.5 ppm. While these linewidths are strongly field dependent owing to the substantial contribution of chemical shift anisotropy to T_2 , an increase in field of 10% is hardly sufficient to explain the increase in linewidth of an order of magnitude upon addition of chlorophyll. Rather we conclude that the presence of chlorophyll in the bilayer produces a change in the motional state of the headgroup. This effect occurs in all phases both above and below T_c .

Thus the ^{31}P experiment is a relatively lucrative one. It permits us to verify two important predictions of the phase diagram, namely that phase separation occurs at low temperatures and that there is an interaction between chlorophyll a and phospholipid. Quantitative interpretation of the ring current shift allows us to suggest a specific molecular interaction between chlorophyll and lipid in the compound phase, while change in ^{31}P linewidths with respect to single-component bilayers indicate a perturbation of chlorophyll on the motional state of the bilayer as a whole. We next

investigate the data available from two other spin probes in the phospholipid headgroup.

b. ^{13}C NMR

If the phosphorous resonance of the lipid in DSL-chlorophyll vesicles is ring shifted upfield because it is located over the center of the porphyrin plane, ring current shifts should also be observed for various carbon atoms in the headgroup of the lipid in ^{13}C spectra of mixed systems. Unfortunately, ^{13}C spectra of phospholipid vesicles show broad lines for both chain and headgroup carbons, and it is not possible to resolve shifts of the order of magnitude of one ppm. Therefore we chose as an alternative to use ^{13}C NMR to look at the interaction of DSL and chlorophyll a in organic solution. Figure IV-15 shows ^{13}C spectra at 25 MHz of DSL, chlorophyll a, and a 1:1 mixture of DSL and chlorophyll a, all in CCl_4 solution. By using the spectra of the individual compounds it is easily possible to identify the lipid resonances in the spectrum of the mixture, and to assign these lipid resonances based on the published spectra (41).

A comparison of the chemical shift of the lipid resonances in the presence and absence of chlorophyll indicates that chlorophyll and lipid do interact in this solvent. Figure IV-16 shows the change in chemical shift for various lipid carbons upon addition of chlorophyll, with plus signs indicating an upfield shift. Clearly the shifts

FIGURE IV-15

25 MHz ^{13}C NMR spectra of (a) a 1:1 mixture of chlorophyll a and DSPC in CCl_4 solution, (b) chlorophyll a in CCl_4 solution and (c) DSPC in CCl_4 solution. Chemical shifts are relative to CCl_4 .

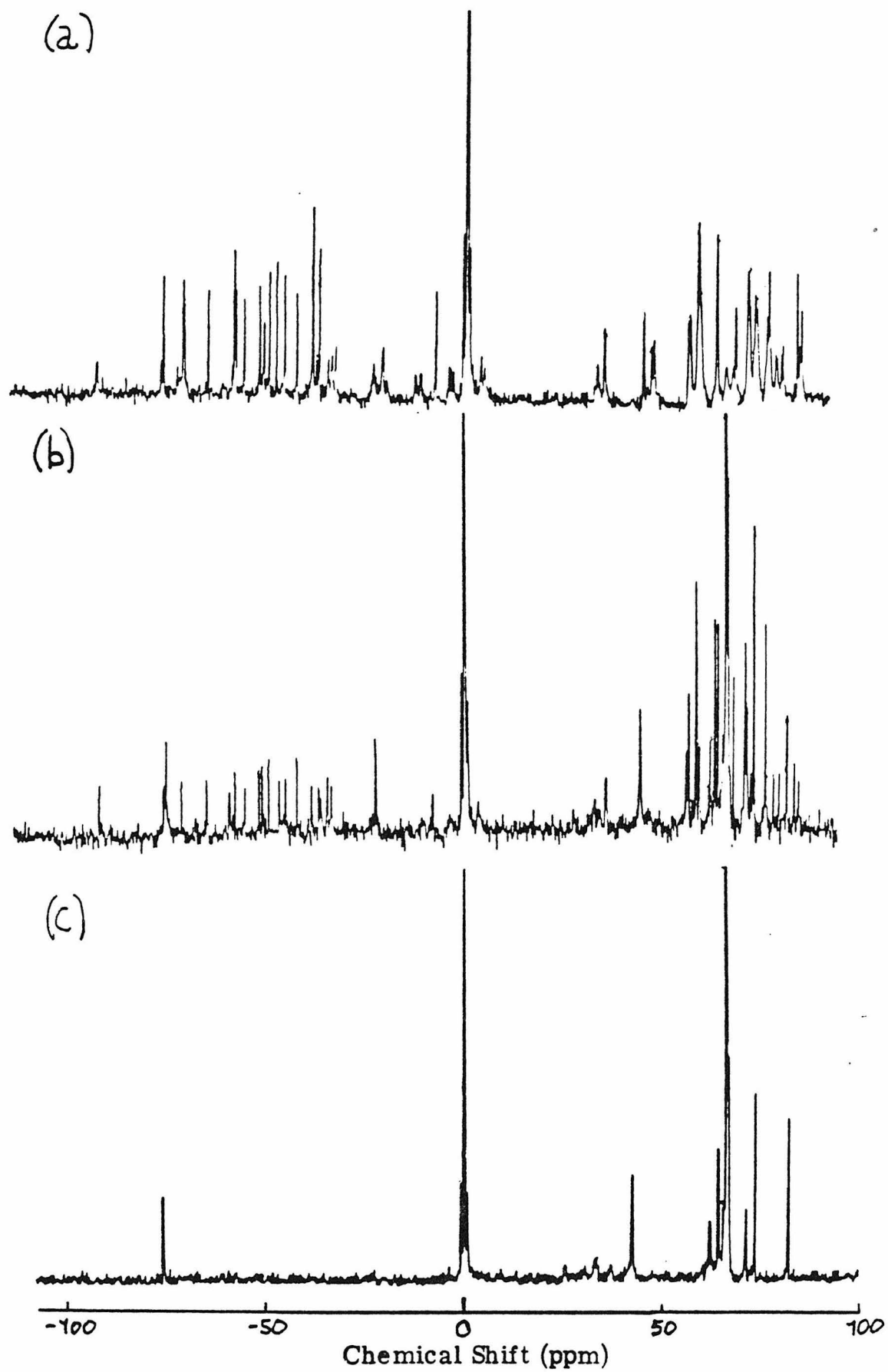
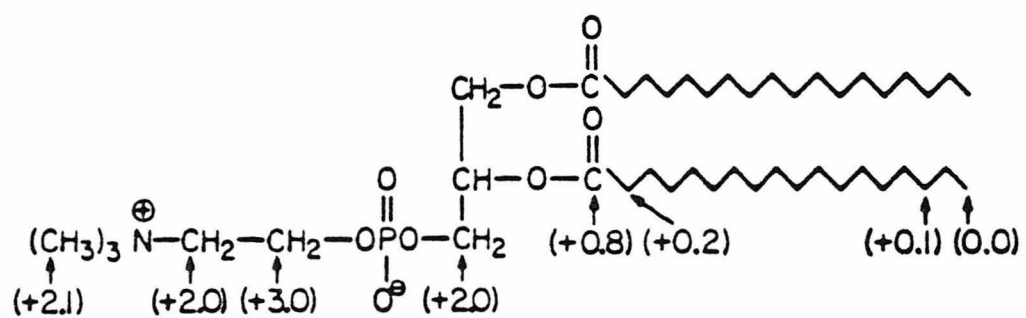


FIGURE IV-16

Differences in chemical shift in parts per million of ^{13}C resonances of DSPC in CCl_4 solution upon addition of chlorophyll a. Positive values indicate an upfield shift on addition of chlorophyll.



are largest in the headgroup near the phosphate, smaller in the glycerol backbone and very small indeed in the chains. While it is not possible to draw quantitative conclusions about the bilayer systems based on the magnitudes of the shift in this solvent, nonetheless the pattern of the shifts clearly implies that chlorophyll a and DSL can interact via their headgroups, probably via coordination of magnesium by phosphate. Moreover, in CCl_4 the predominant form of dissolved chlorophyll is the dimer, while some chlorophyll will be ligated by any water present. Since we took no elaborate precautions to exclude water from our samples we conclude that lipid competes effectively with both water and chlorophyll as a Mg ligand in this solvent.

c. ^1H NMR

We have also looked for ring current shifts in ^1H NMR spectra of chlorophyll a-DSPC vesicles. Figure IV-17 shows a series of 360 MHz ^1H NMR spectra of DSPC vesicles containing 20 mole % chlorophyll a taken as a function of temperature. The principal lipid resonances appear as three broad peaks. These are due to the choline methyl protons at lowest field, the chain methylene protons, and the chain terminal methyl protons at highest field. Four smaller broad resonances are due to protons of the lipid glycerol backbone and choline methylenes. The three sharp lines are (beginning at lowest field) residual CHCl_3 in the external reference

FIGURE IV-17

360 MHz ^1H NMR spectra of DSPC vesicles containing 20 mole % chlorophyll a at various temperatures.



capillary, residual HDO in the aqueous phase and tetramethylsilane, the external chemical shift reference.

The HDO peak has been substantially attenuated by continuous single-frequency irradiation at the HDO frequency between RF bursts.

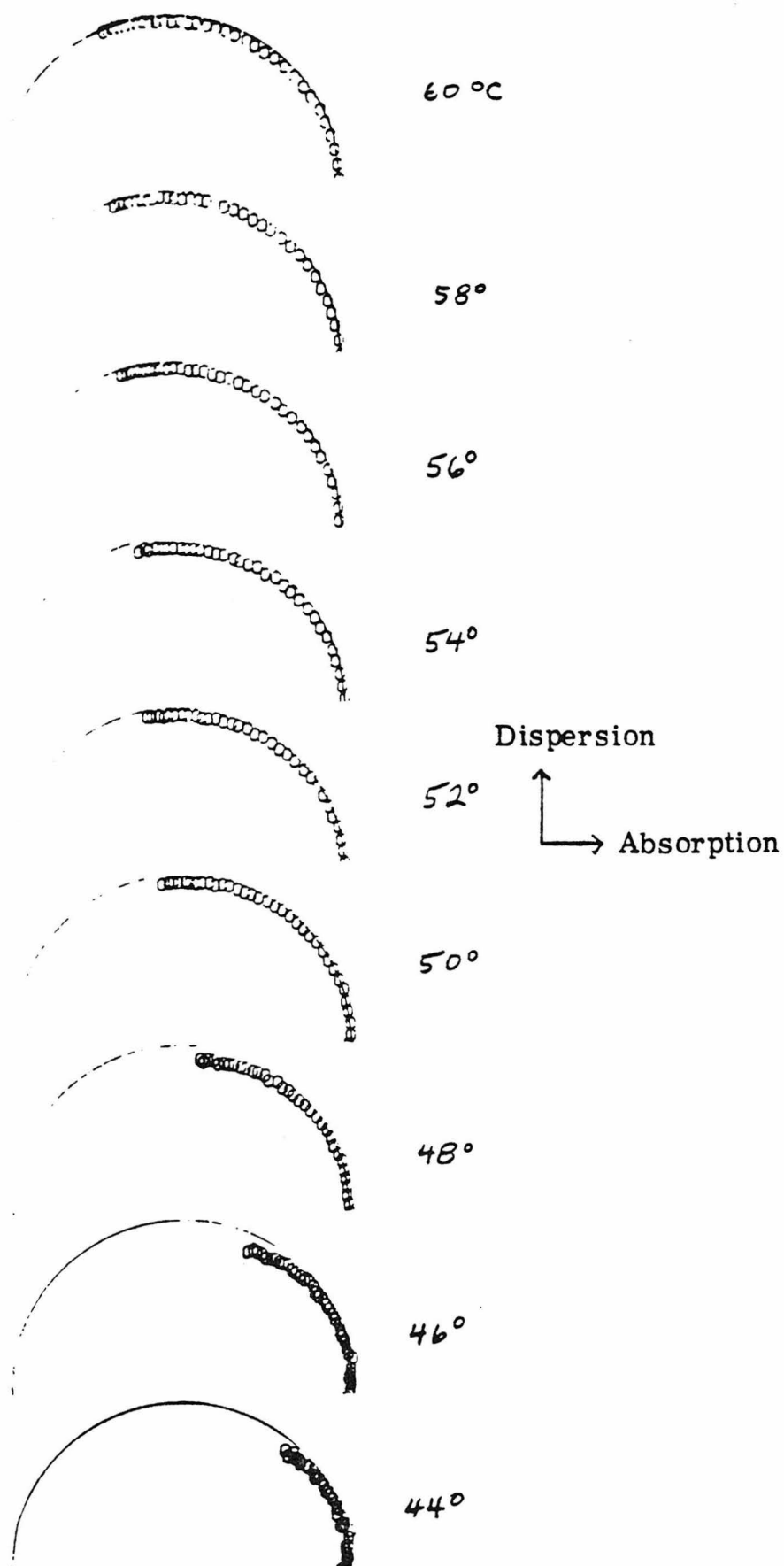
Note first that chlorophyll a proton resonances are substantially absent from the spectrum. The ratio of the area of the chain methyl (or methylene) peak to the area of the choline methyl peak is higher than is observed for pure DSPC vesicles, indicating that there is a contribution to the chain methyl and methylene peaks from the phytol chain of the chlorophyll. However, resonances from the porphyrin ring are not observed. We believe that this results from the anchoring of the chlorophyll in the membrane. Since the porphyrin plane is relatively rigid, motional averaging of dipolar interactions in solution occurs via tumbling of the molecule as a whole. When the chlorophyll is anchored in the membrane, overall tumbling is restricted, resulting in efficient relaxation and broad resonances which we fail to resolve in the spectrum. This interpretation presumes that overall vesicle tumbling is ineffective in narrowing the lines, and also suggests that lateral diffusion of chlorophylls, another potential reorientation mechanism, is no more rapid in this system than it is in pure phospholipid vesicles.

Second, note that both linewidth and chemical shift

of the various lipid resonances vary as a function of temperature. Of the protons in the lipid headgroup, only the choline methyl protons may be followed all the way to the lowest temperatures for which we have obtained data. In this case, as for the other headgroup resonances, we fail to resolve more than one signal even at low temperatures. Since the line becomes relatively broad at temperatures around the solidus, it is unclear from this presentation of the data whether additional unresolved components might be present. As an aid in answering this question, K. E. Eigenberg of our laboratory has prepared dispersion versus absorption (DISPA) plots of the choline methyl resonance using a computer routine provided by Nicolet Instrument Corporation to the Stanford Magnetic Resonance Laboratory. DISPA plots as a function of temperature are shown in Figure IV-18. At high temperatures the semicircle characteristic of a single Lorentzian line is obtained. As we lower the temperature the semicircle is displaced downward at the left. According to Marshall and Roe (43), this pattern is characteristic of an "unresolved superposition of two Lorentzians of equal integrated area and resonant frequency, but different linewidth". (If the two lines are in certain regions of exchange a similar pattern is observed. Exchange is unlikely, based on the ^{31}P result, but cannot be ruled out.) The pattern persists to the lowest temperatures at which we have made measurements.

FIGURE IV-18

Dispersion versus absorption plots for the choline methyl proton resonance at 360 MHz of DSPC in 20 mole % chlorophyll a - DSPC vesicles at various temperatures.



Thus the DISPA format of data presentation indicates that two (or more) relaxation times are contributing to the choline lineshape at low temperatures. This is again consistent with the phase diagram, which predicts phase separation at all temperatures below the liquidus. Our interpretation of the DISPA assumed that the two components have equal chemical shift. In the region between liquidus and solidus where the two phases in equilibrium are pure lipid and solution, this assumption is reasonable. The linewidth is of the order of one hundred hertz while the ring current shifts are only a few hertz. Below the solidus, however, the chemical shift difference between pure lipid and compound may not be negligible. In the case of two Lorentzians of equal width but different resonant frequencies, the DISPA plot is displaced upward and to the right of the reference semicircle. Where unresolved peaks differ in both shift and width, Professor Marshall (44) indicates that for nearly all simulated cases the width difference dominates and the leftward and downward shift is observed. DISPA plots are thus consistent with the presence of two non-exchanging resonances from chlorophyll in two separate phases for temperatures below the liquidus.

Thus magnetic resonance measurements serve to confirm the main features of the phase diagram and to extend our interpretation of the various regions. ^{31}P NMR clearly indicates that phase separation does occur

at low temperatures; DISPA treatment of ^1H choline lineshapes confirms this observation. Ring current shifts indicate that there is indeed interaction between chlorophyll a and lipid. Quantitative interpretation of ^{31}P shifts strongly suggests a coordination interaction between lipid phosphate and chlorophyll magnesium in the compound; this interpretation is reinforced by the pattern of chlorophyll-induced shifts of lipid ^{13}C resonances in CCl_4 solution. ^{31}P linewidths reflect the differences in motional state of the lipid produced by chlorophyll.

D. Discussion

We have emphasized three properties of the compound phase of the model system which relate to its ability to serve as a light-gathering antenna in vivo:

1. Interaction of chlorophyll and lipid

Magnetic resonance data on model systems strongly suggest an interaction between the phosphorous of the lipid headgroup and the magnesium of the chlorophyll in the compound phase. Thus it is indeed plausible that lipid, rather than protein, serves as the axial ligand to antenna chlorophyll in the thylakoid as well. Since ^{13}C magnetic resonance experiments with isolated thylakoids performed in our laboratory indicate the presence of chlorophyll not associated with protein (45), while on the other hand it is well established that proteins contain the two types of reaction centers, we believe

that there are both protein associated and lipid associated chlorophylls in the thylakoid membrane. Indeed, independent biochemical evidence suggests the presence of two separate pools of chlorophyll in the membrane. The two pools have different turnover rates, with the faster pool being also more accessible to solvent extraction (46).

The interaction between chlorophyll and lipid in the model system is important not only to ensure proper ligation of protein-free chlorophyll, but also because it is necessary to maintain a minimum separation between chlorophylls to prevent the formation of non-fluorescent dimers which trap and waste singlet excitation energy. Beddard and Porter (9) calculate that the minimum separation of chlorophylls to prevent trap formation is about 10 Ångstroms. In Corey-Pauling-Koltum (CPK) models of two dimensional arrays of interspersed chlorophyll and lipid, the distances between nearest neighbor chlorophylls are of the order of 10 Ångstroms.

2. Ordering of chlorophyll

A singlet-singlet resonance energy transfer mechanism works most efficiently when the transferring molecules form an ordered system with fixed geometrical relationships to one another. Order with respect to one axis is provided by anchoring chlorophylls in a membrane so that their phytol chains lie roughly parallel. This order is reflected in the failure to observe chlorophyll

headgroup resonances in ^1H NMR spectra of model membranes. Additional ordering may occur below the lower phase transition temperature where lateral diffusion of single component membranes is reduced and the hydrocarbon chains are more highly ordered, provided this additional order extends to the headgroup region. As suggested above, in the model system the larger red shift below the solidus could reflect ordering of the porphyrin planes in the headgroup region below the solidus.

We may speculate in this context about the difference between DSPC and the natural membrane lipid MGD. Suppose we construct as a model for an antenna array a sodium chloride type two-dimensional crystal where each lipid has as its nearest neighbors four chlorophylls and each chlorophyll has as its nearest neighbors four lipids. To provide order and stability to the array, we wish each lipid to interact with at least three of the neighboring chlorophylls. Chlorophyll is "multi-valent" since it can have two axial ligands and interact with neighbors via the C-9 ketone. However, the lipid DSPC is univalent, having a single phosphate capable of interacting with only one chlorophyll. On the other hand, consider MGD, which as indicated above is known to be important in maintaining normal photosynthetic function. The galactolipid headgroup, which contains several hydroxyl groups capable of serving as electron donors to magnesium or participating in hydrogen bonding,

is multivalent. Thus MGD could, along with chlorophyll, form arrays of greater stability and order than formed with phospholipids, due to the greater extent of cross-linking possible with a multivalent lipid. Thus MGD may be the lipid of choice for constructing a model system.

Finally, a number of observations suggest that thylakoid proteins play a role in ordering the lipid bilayer portion of the thylakoid membranes. First, we have observed that natural thylakoid membranes also show a temperature dependence of the red shift. λ_{max} for thylakoids decreases with temperature with a break in the curve around 50°C, but the effect is irreversible. Second, reconstituted thylakoid lipids show a red shift smaller than that observed for intact thylakoids. Third, the high degree of unsaturation in thylakoid lipids would by itself imply that the lipid bilayer should be relatively disordered, rather than ordered, at physiological temperatures. Additional order may be provided by effects due to membrane protein (47).

3. Constant composition

Segregation of all of the chlorophyll into a single compound phase of defined composition is important for two reasons. First, while it is necessary in engineering a light-gathering antenna to maintain a certain minimum separation between chlorophylls it is equally important to minimize insofar as possible the intermolecular

separation so as to maximize the energy transfer rate. In the thylakoid chlorophyll makes up at most 20% of the total membrane lipid. Compound formation in the model system indicates a thermodynamic tendency for lipid-associated chlorophyll to separate into a chlorophyll-rich phase where the chlorophyll to chlorophyll distances will be smaller. Second, in order to maintain an ordered structure, it is important to maintain a constant lipid to chlorophyll ratio in the antenna array. As noted earlier, in the model system below the solidus changes in composition cause changes in the relative amount of the compound and free lipid phases, but the composition of the compound remains constant. In this way the system is thermodynamically buffered against concentration fluctuations. Optimum transfer rates may be maintained and trap formation in regions of high chlorophyll concentration avoided.

4. Further experiments

There are three principal directions in which this study may be extended. First, the model system of chlorophyll a in MGD may show properties different from those of chlorophyll a-DSPC, since interaction with the chlorophyll magnesium could occur through a sugar hydroxyl, rather than phosphate as in the DSPC case. In any event using the naturally occurring lipid MGD will generate results more directly relevant to the thylakoid membrane itself. This study is complicated by the fact that

neither chlorophyll a nor MGD form bilayers by themselves. A more systematic approach to the study of their mixed lipid hydrates than has been taken here would be required since no assumptions could be made concerning the presence of bilayer structure in any region of the phase diagram. Use of a ternary system containing chlorophyll and MGD dissolved in small amounts in a well-behaved lipid is a possible alternate approach.

Second, magnetic resonance studies of the natural membrane, currently in progress (45), hold great promise. In particular if it is possible to improve resolution and signal-to-noise in the ^{13}C experiments, it is important to determine whether particular lipid resonances are ring-current shifted by chlorophylls. Introduction of ^{13}C labelled lipids may be necessary to generate the required signal-to-noise. Alternatively, extracted and rehydrated thylakoid lipid membranes which are free of protein may be investigated as a bridge between model and natural systems.

Finally, we have made inferences throughout about the motional state of various portions of the lipid and chlorophyll molecules. These motions may be quantified by studies of model systems using deuterated lipids or deuterated chlorophylls. In particular we wish to investigate the changes in motional state of the lipid headgroup associated with the various phases, and to see how much motional freedom is available to the chlorophyll porphyrin

ring when the chlorophyll is anchored in the membrane. Deuterium measurements for various lipids specifically labelled in the headgroup could provide a detailed picture of the relative geometry of the lipid headgroup and porphyrin plane. Finally, deuterium labels in the linolenic and phytol chains could be used to investigate the motional state of the hydrocarbon chains in mixed MGD-chlorophyll systems, with a view towards understanding whether the predominance of triply unsaturated fatty acids in thylakoids is the result of a particular interaction of these chains with the phytol of chlorophyll. The work described in this section provides a good basis on which to begin a ^2H NMR investigation, which seeks to understand the motional state of the bilayer components in detail. The ^2H spectrometer described in chapter III of this thesis will be used to undertake these experiments.

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APPENDIX I: SUBROUTINE DESCRIPTIONS FOR NMR COMPUTER PROGRAM QPD

A. Introduction

This section describes in detail QPD, the NMR computer program for the ^2H spectrometer. A general outline of the program and the strategy behind it is given in Chapter III, section D.

The 620i computer controls data acquisition, performs data manipulation including Fourier transformation, and outputs data to the oscilloscope, chart recorder, cassette tape drive or teletype. As indicated above, the software used is a modification of VFT, the Varian Fourier Transform NMR computer program.(1) This program may be divided conceptually into five principal areas, along with some general purpose and accessory routines as shown in Table A-1. Data acquisition routines are those in which the computer makes use of the NMR spectrometer to acquire free induction decays (FIDs) in memory. Data manipulation routines are those which convert FIDs to useful spectral information. The most important ones are the routine for exponential weighting of FIDs and the Fourier transform routine. Data output routines are used to output spectra to the oscilloscope or recorder using the phasing routine to generate an absorption mode spectrum from the sine and cosine transforms and to correct for first order phase errors introduced into the data by the spectrometer receiver and audio filter. Parameter, command, and

TABLE A-1: PRINCIPAL AREAS OF VFT, THE VARIAN FOURIER
TRANSFORM PROGRAM

- (1) Data acquisition: standard sequence (command
NT or NK)
block averaging (command NB)
- (2) Data manipulation: exponential weighting
Fourier transformation
- (3) Data output: phasing
scope display
plotting
- (4) Parameter, Flag and Command input
- (5) Parameter output

General purpose routines: math routines, routine for
clearing data table, decimal
number input routine, etc.

flag input routines allow the operator to control data acquisition and manipulation. Parameter output routines allow the operator to record the conditions under which spectral data were obtained.

In the description of the software which follows, three levels of documentation are provided:

(1) The discussion describes in general outline the workings of the new routines. Occasionally description at the level of individual machine language commands is required to explain clearly how a new programming strategy was implemented. The discussion necessarily assumes some familiarity with assembly level programming and with the architecture of the 620i. 620i instruction mnemonics are used; the meaning of most of these is self-evident (e.g., "LDA" for "load A register"). A list of the mnemonics used in QPD and a brief description of the 620i sufficient for understanding the following discussion are included in Appendix II. For further details see reference (2).

(2) In addition to the discussion flowcharts of the various QPD subroutines are provided; these describe the workings of QPD in detail.

(3) Finally, complete listings of each of the subroutines in machine code are given. The five columns of information in the listings include the memory location (column one), the machine code instruction (column two), and the instruction mnemonic (column three). The fourth

column gives the effective address for single and double word addressing instructions. Indirect addressing is used only to return from a subroutine to the calling routine; in this case column four contains "RETU". Indexed addressing is indicated in column four by a notation of the form "R,S", where R=0 specifies addressing relative to the X register, R=1 addressing relative to the B register and S the number to be added to the contents of X or B to generate the effective address. When transfers are made between registers with incrementing or decrementing of the value transferred, this is indicated in column four by "+1" or "-1". Column five contains comments. Where the effective address of the command is a data location or subroutine with a four-character name in the assembly listing of VFT(3), this four letter name is given first in the comments, usually with a translation. Figure A-21 is a list of data locations referred to in QPD with a description of each.

B. Data acquisition routines

The three versions of QPD are based on three different data acquisition routines; they are essentially identical in all other respects. All three of the QPD acquisition routines consist of three subroutines. The main acquisition routine, which actually inputs data, replaces a portion of the acquisition routine in VFT. The Acquisition Change routine insures that FIDs from

the appropriate channel are added to or subtracted from the appropriate memory area in the appropriate sequence. The Initialization routine insures that the pattern of the computer data handling remains in phase with the burst cycle generated by the program card in the pulse programmer of the spectrometer.

1. Acquisition routine

Version one of the main acquisition routine is listed in Figure A-1. Several points should be noted:

(a) The steady state routine, which caused several RF bursts to be given before the start of data accumulation, has been eliminated. See the VFT listing(3), locations 1212-1221.

(b) The transient is stored in either the second or the fourth quadrant of the data table depending on the contents of the memory area word, location 47, which is set by the acquisition change routine.

(c) The ADC control word, location 41, specifies 13 bit conversion mode, external timing and data input from either channel 1 (control word 001000) or channel 2 (control word 001001)(4). The ADC control word is set by the acquisition change routine.

(d) In the original VFT program the pulse timer word, location 420, specified the length of the RF burst, which was controlled by the length of the TTL compatible pulse generated by the "logic" card of the V-4357 pulse unit. The TTL pulse began at a "pulse on" command (100633)

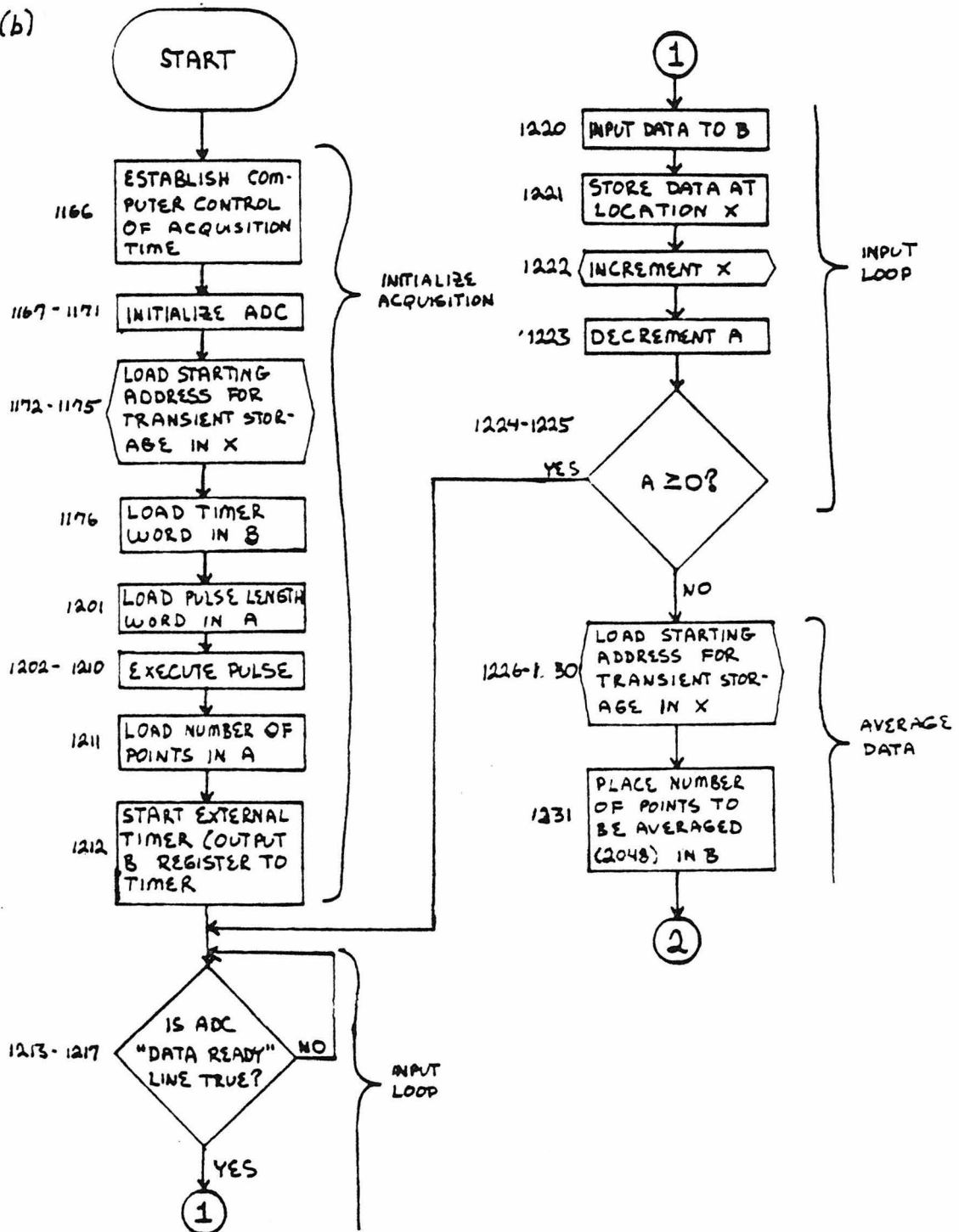
FIGURE A-1

Program listing (a) and flowchart (b) for
QPD subroutine Acquisition, version 1 used in
QPD-40KP.

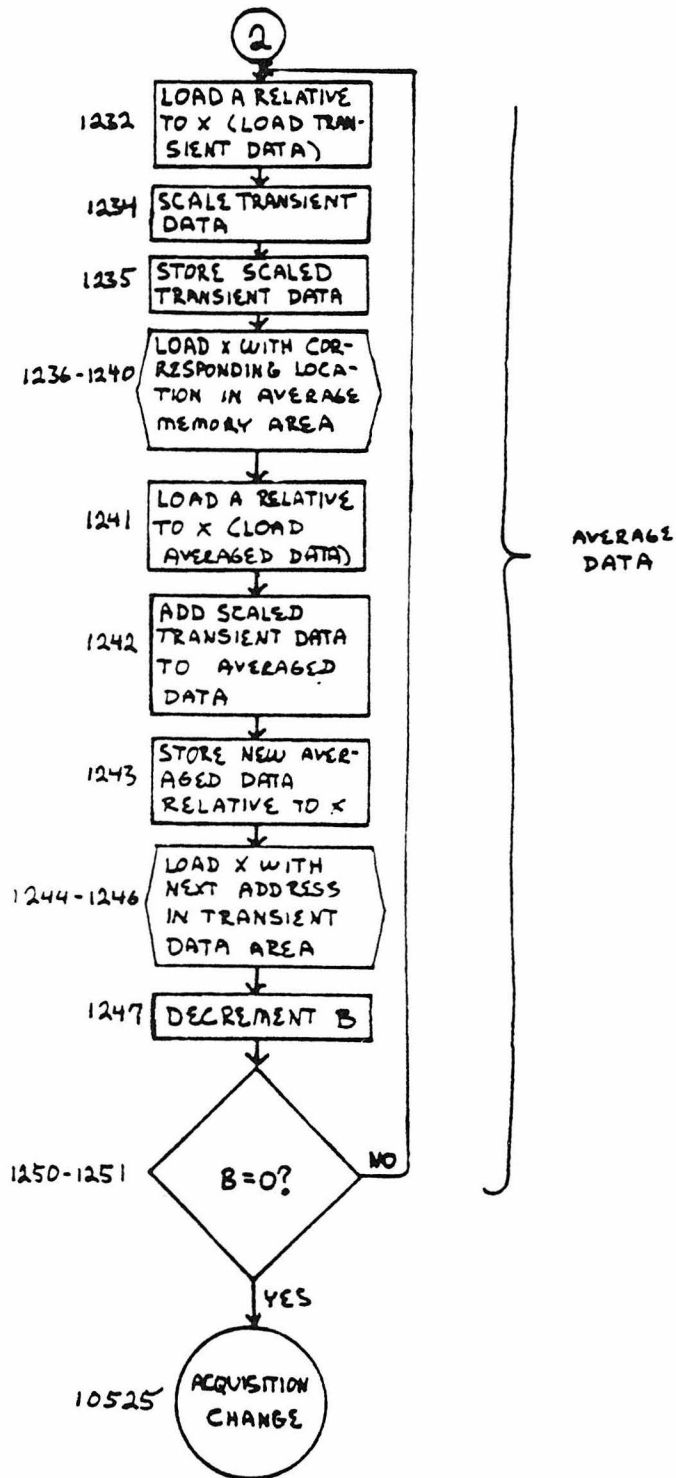
(2) Acquisition routine, replaces original

001166	100435	EXC		Computer control of acquisition time
001167	100266	EXC		Initialize ADC
001170	103066	OME	41	Output ADC control word
001171	000041	-		
001172	010473	LDA	473	TABL, first word address, data table
001173	120044	ADD	44	Quarter table length
001174	120047	ADD	47	Memory area word
001175	005014	TAX		Set starting address for transient storage
001176	020403	LDB	403	VTIM, external timer frequency word
001177	005000	NOP		Holes for computer
001200	005000	NOP		control of pulse phase
001201	010420	LDA	420	PUTM, pulse timer word
001202	100633	EXC		Turn on pulse
001203	005311	DAR		Pulse length timing:
001204	005000	NOP		
001205	001002	JAP	1203	
001206	001203	-		
001207	004300	ASRA		Fine timer word
001210	100733	EXC		Turn off pulse
001211	010400	LDA	400	NPTS, number of points to acquire
001212	103227	OBR	27	Start external timer
001213	101066	SEN	66	ADC ready?
001214	001220	-		If so, input data
001215	005000	NOP		
001216	001000	JMP	1213	If not, look again
001217	001213	-		
001220	102666	CIB	66	Input data
001221	065000	STB	0,1	Store data at location given in X
001222	005144	IXR		Shift to next location
001223	005311	DAR		Count number of data points left
001224	001002	JAP	1213	If not finished, continue
001225	001213	-		
001226	005041	TXA		Reset index to beginning of transient
001227	140400	SUB	400	NPTS
001230	005014	TAX		
001231	020044	LDB	44	Quarter table
001232	015000	LDA	0,1	Load transient data
001233	005000	NOP		Filler
001234	004300	ASRA		Scale transient data
001235	051254	STA	1254	Store transient data temporarily
001236	005041	TXA		Switch index to average FID area
001237	140044	SUB	44	
001240	005014	TAX		
001241	015000	LDA	0,1	Load averaged data
001242	121254	ADD	1254	Add (or subtract as needed) scaled transient
001243	055000	STA	0,1	Store new averaged data
001244	005041	TXA		Reset index to transient memory area
001245	120044	ADD		
001246	005114	TAX	+1	
001247	005322	DBR		Count number of data points left
001250	001020	JBZ	10525	If finished, call acquisition change
001251	010525	-		
001252	001000	JMP	1232	If not, continue
001253	001232	-		
001254	000000	-		Scratch location
001274	060344	LDB	344	Moved to make room for Initialization
003421	051207	STA	1207	Pulse fine timer word
000751	051234	STA	1254	Scaling in data averaging

(b)



(b)



and ended at a pulse off command (100733) from the 620i. In the ^2H spectrometer the leading edge of this TTL pulse triggers whatever burst sequence is hard-wired on the program card in the pulse programmer. The number, timing, phase and duration of the RF bursts in this sequence is not under computer control. These parameters are controlled by the program card and front panel controls of the pulse programmer. The duration of this TTL trigger pulse therefore merely sets the delay between the RF burst or bursts and the beginning of data acquisition. The duration of this delay was not under operator control in VFT. In QPD it may be set simply by entering a "pulse width" in microseconds. In order that the "pulse width" entered by the operator correspond closely to this delay time, the number of program steps between the "pulse off" command and the beginning of data acquisition has been minimized. In high resolution work the "pulse width" is set to equal the dwell time. For the solid echo sequence "pulse width" must be set to twice the length of τ_{echo} .

(e) Locations 001213 to 001225 represent the minimum possible single loop data input routine utilizing external timing of the data digitization rate. All of the instructions require two computer cycle times except IXR, DAR and NOP, which require one cycle, and SEN, which requires $2 \frac{1}{4}$ cycles. Thus the minimum cycle time for the routine is $10 \frac{1}{4}$ computer cycles or 18.5 microseconds and the Nyquist frequency is at most about 50 kHz. In

practice the program will operate reliably only up to about a 40 kHz spectral width since faster rates cause the sense loop timing to become a significant fraction of the conversion time, with the result that occasionally a point is skipped and the program stalls in the sense loop.

The first version of QPD is useful for spectral widths between 100 Hz and 40 kHz. Two additional modifications of the acquisition routine produce a second version of QPD for use with 35 kHz to 68 kHz spectral widths. A special fixed spectral width version of this second program operates at a spectral width of 79.4 kHz. This third version generates the maximum spectral width available on the deuterium spectrometer.

The second version of the QPD Acquisition routine is shown in Figure A-2. Note the following:

- (a) Since the input loop must appear in consecutive memory locations and since it is nearly as long as the entire original acquisition routine, to make room for the new input loop the end of the WAIT subroutine and the preliminary portions of the Acquisition routine have been moved to locations previously occupied by RFRQ, the routine for finding the tallest line in a portion of the transformed spectrum.
- (b) The number of data points to be accumulated, NPTS, is first loaded into B, then after the pulse is generated and conversion is started it is transferred to A with a one-cycle command. In this roundabout fashion the program

FIGURE A-2

Program listing (a) and flowchart (b) for
QPD subroutine Acquisition, version 2 used
in QPD-68KP.

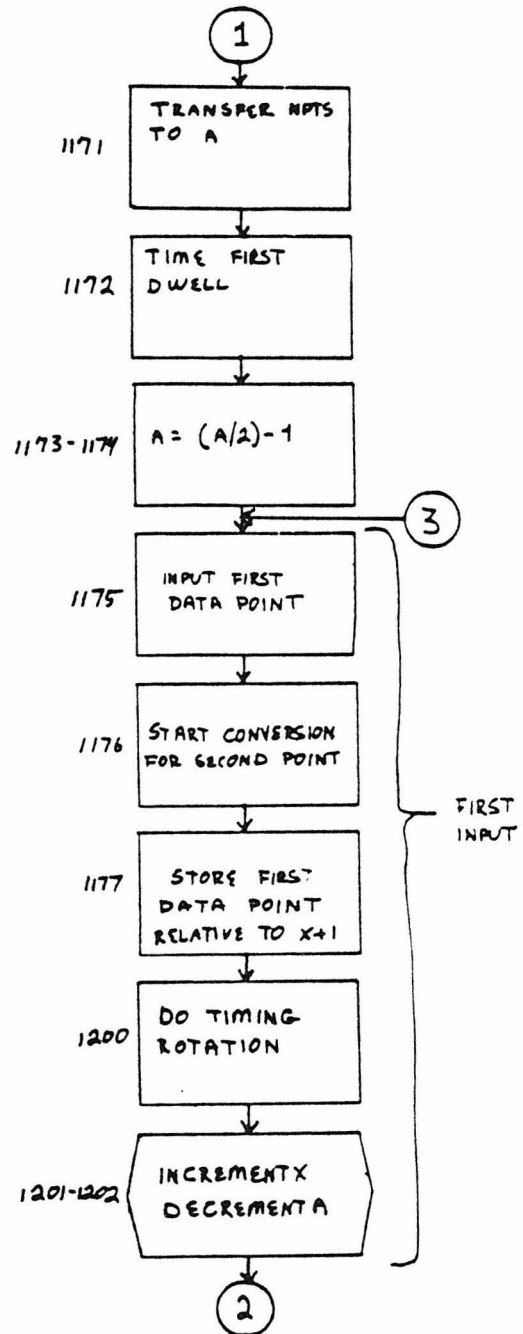
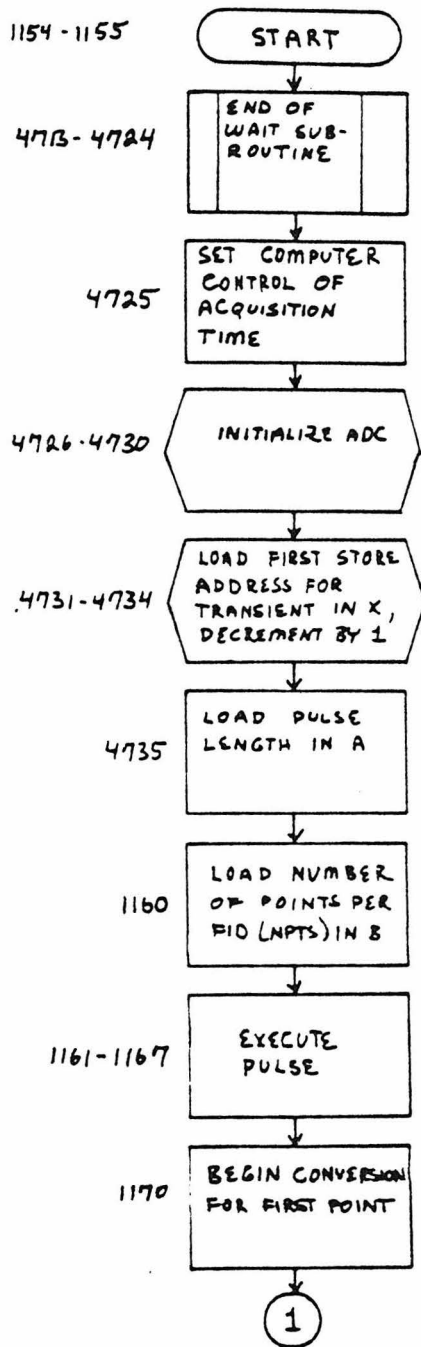
(a) Acquisition routine, replaces original

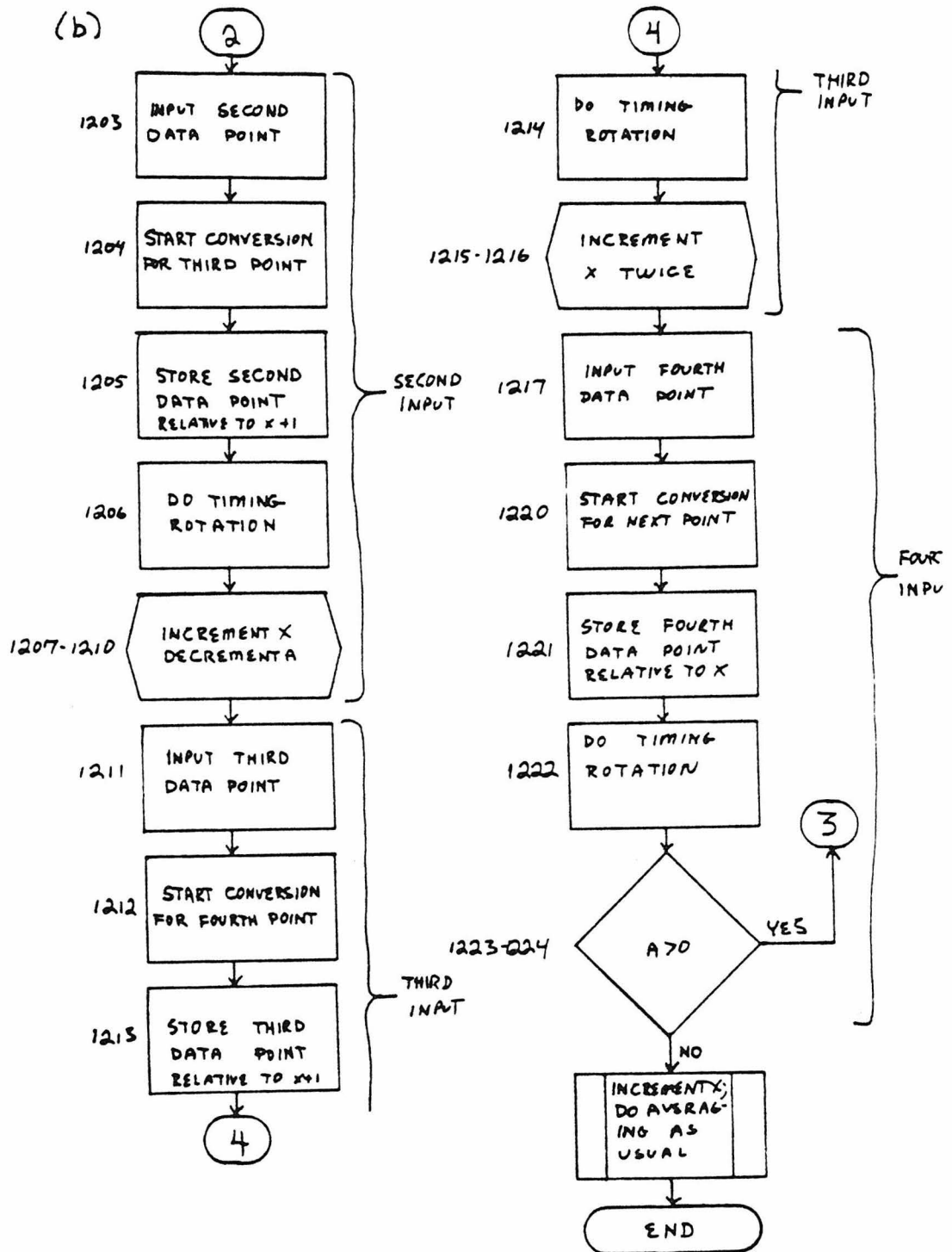
001154	001000	JMP	4713	Prepare for pulse and data input
001155	004713	-		
001156	005000	NOP		Unused locations
001157	005000	NOP		
001160	020400	LDB	400	NPTS (transfer to A later)
001161	100633	EXC		Pulse on
001162	005311	DAR		Coarse pulse timing:
001163	005000	NOP		
001164	001002	JAP	1162	
001165	001162	-		
001166	004300	ASRA		Fine pulse timing
001167	100733	EXC		Pulse off
001170	100366	EXC	66	ADC start, take data point number one
001171	005021	TBA		Count points in A register
001172	004043	LRLB		Timing for first dwell
001173	004301	ASRA	1	Count points by twos
001174	005311	DAR		Now number of points will turn out right
001175	102666	CIB	66	Input data point number one
001176	100366	EXC	66	Take data point number two
001177	065001	STB	1,1	Store data point number one
001200	004040	LRLB		Timing word
001201	005144	IXR		Increment store address
001202	005311	DAR		Count first pair of points
001203	102666	CIB	66	Input data point number two
001204	100366	EXC	66	Take data point number three
001205	065001	STB	1,1	Store data point number two
001206	004040	LRLB		Timing word
001207	005144	IXR		Increment store address
001210	005311	DAR		Count second pair of points
001211	102666	CIB	66	Input data point number three
001212	100366	EXC	66	Take data point number four
001213	065001	STB	1,1	Store data point number three
001214	004040	LRLB		Timing word
001215	005144	IXR		Increment store address
001216	005144	IXR		Increment store address
001217	102666	CIB	66	Input data point number four
001220	100366	EXC	66	Take next data point
001221	065000	STB	0,1	Store data point number four
001222	004040	LRLB		Timing word
001223	001002	JAP	1175	Check if last set of points
001224	001175	-		If not, continue
001225	005144	IXR		If finished increment store address once more and continue

(a) Acquisition routine, replaces part of RFRQ (find tallest line), locations
004677 - 004744

004713	001040	JBZ	101126	End of WAIT routine :
004714	101126	-		
004715	002000	JMPM	010504	
004716	010504	-		
004717	010215	LDA	215	
004720	002000	JMPM	3022	
004721	003022	-		
004722	005344	DXR		
004723	001000	JMP	4713	
004724	004713	-		
004725	100435	EXC		Computer control of acquisition time
004726	100266	EXC	66	Initialize ADC
004727	103066	OME	66	Send ADC control word from 000041
004730	000041	-		
004731	010473	LDA	473	TABL, first word address, data table
004732	120044	ADD	44	Quarter data table
004733	120047	ADD	47	Memory area word
004734	005314	TAX	-1	Set first store address
004735	010420	LDA	420	PUTM, pulse time
004736	001000	JMP	1160	Continue
004737	001160	-		
003421	051166	STA	1166	Pulse fine timer word
001116	101432	SEN	432	Sense loop for test pulse has new address
001117	004725		4725	

(B) 1154-1155





may be written to include no delay between the "pulse off" command and the initiation of data conversion. After transfer NPTS is divided by two so that data points may be counted in pairs. Since the number of points completed is checked only after each set of four points, the number of data points actually taken is always a multiple of four. After the last point is taken the program subtracts NPTS from the location in the X register in order to return the index register to the beginning of the FID. In order for this to work, the operator must ensure that the number of points ("DATA LENGTH" in the parameter printout) is a multiple of four.

(c) LRLB commands at locations 1172, 1200, 1206, 1214, and 1222 are timer words. The number of steps in each rotate command is set by the Spectral Width routine (vide infra) so that the total time between CIB ("take data point") steps (the dwell time) corresponds to the spectral width entered by the operator. The LRLB at 1172 always has three more steps than the others; this makes the total number of cycles between the first two points the same as for subsequent intervals. The four input sections all have an identical length of eight computer cycles plus one-quarter times the number of steps in the LRLB commands. The minimum dwell time is 8×1.8 microseconds = 14.4 microseconds and the maximum spectral width is about 68 kHz.

(d) The index register is always one less than the actual storage address for the first three STB commands of the input loop; for the fourth STB the index register corresponds to the storage address. The index register is incremented twice after the third STB but not incremented after the fourth STB.

By omitting the logical rotate commands entirely it is possible to generate a fixed spectral width version of the second Acquisition routine with a dwell time of 7 computer cycles, or 12.6 microseconds, and hence a 79.4 kHz spectral width. This third Acquisition routine is shown in Figure A-3, where only the changes from the previous routine are noted. In order to shorten the interval between the first two points to seven cycles it is necessary to place one command between the "pulse off" command and the command to initiate conversion.

2. Acquisition Change routine

There are two other acquisition subroutines which are common to all three input routines. Acquisition Change is a subroutine which is called by the main Acquisition routine after each transient. This routine, listed in Figure A-4, determines which channel will be monitored, which memory area the data will be averaged into, and whether the transient will be added to or subtracted from the accumulated FID. As indicated above, the pattern of these data manipulations repeats after every eight transients. To produce this pattern, shown

FIGURE A-3

Program listing for QPD subroutine Acquisition,
version 3 used in QPD-78KP. Only the changes
from version 2 are noted.

Acquisition routine, replaces original

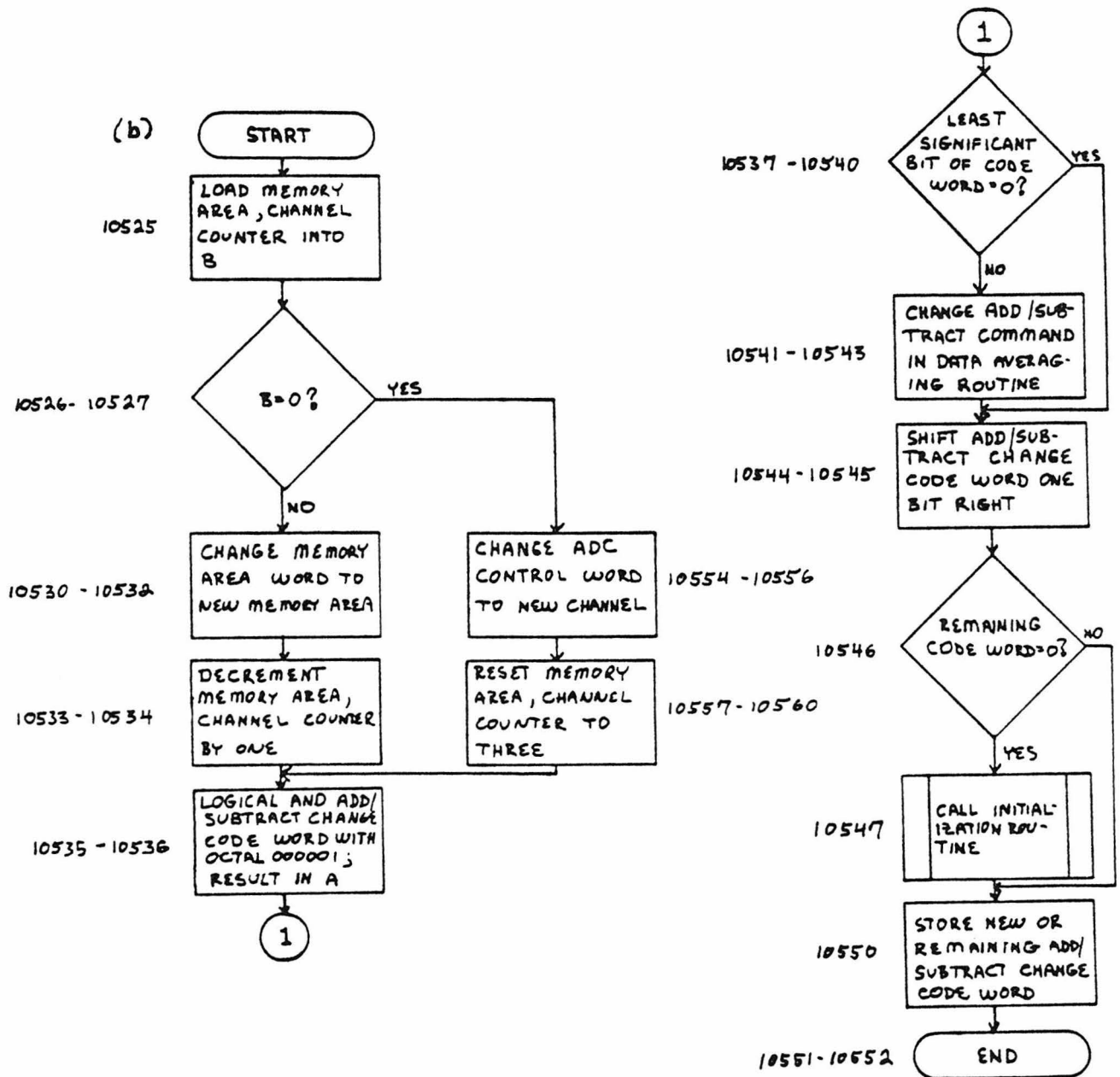
001154	001000	JMP	4713	
001155	004713	-		
001156	005000	NOP		
001157	005000	NOP		
001160	020400	LDB	400	
001161	100633	EXC		
001162	005311	DAR		
001163	005000	NOP		
001164	001002	JAP	1162	
001165	001162	-		
001166	004316	ASRA		
001167	100733	EXC		
001170	005021	TBA		
001171	100366	EXC	66	Reverse these two commands so that
001172	004043	LRLB		first interval is of correct length
001173	004301	ASRA	1	
001174	005311	DAR		
001175	102666	CIB	66	
001176	100366	EXC	66	
001177	065001	STB	1,1	Omit rotate timing words to shorten
001200	005144	IXR		dwell time to minimum
001201	005311	DAR		
001202	102666	CIB	66	
001203	100366	EXC	66	
001204	065001	STB	1,1	
001205	005144	IXR		
001206	005311	DAR		
001207	102666	CIB	66	
001210	100366	EXC	66	
001211	065001	STB	1,1	
001212	005144	IXR		
001213	005144	IXR		
001214	102666	CIB	66	
001215	100366	EXC	66	
001216	065000	STB	0,1	
001217	001002	JAP	1175	
001220	001175	-		
001221	005144	IXR		
001222	005000	NOP		Insert NOPs to fill space before
001223	005000	NOP		averaging routine
001224	005000	NOP		
001225	005000	NOP		

FIGURE A-4

Program listing (a) and flowchart (b)
for QPD subroutine Acquisition Change.

(2) Acquisition change, replaces RSTR (power fail restart), locations
010525 - 010562

010525	020042	LDB	42	Counter for memory area, channel
010526	001020	JBZ	10554	If zero, change channel sampled
010527	010554	-		Otherwise, change memory area
010530	010045	LDA	45	Memory area mask
010531	130047	ERA	47	Memory area word
010532	050047	STA	47	
010533	005322	DBR		Decrement memory area, channel counter
010534	060042	STB	42	
010535	005101	TZA(I)		Transfer 1 to A register
010536	150043	ANA	43	And with add/subtract change code word
010537	001010	JAZ	10544	If least significant bit of code word is zero,
010540	010544	-		no add/subtract change; otherwise do it.
010541	011242	LDA	1242	Add/subtract command in data averaging
010542	130050	ERA	50	Add/subtract mask
010543	051242	STA	1242	
010544	010043	LDA	43	Add/subtract change code word
010545	004301	ASRA	1	Throw out least significant bit
010546	002010	JAZM	1255	If none of the code word remains, call
010547	001255	-		initialization routine.
010550	050043	STA	43	Store new code word
010551	001000	JMP	1274	Done, jump to next transient
010552	001274	-		
010553	010051	LDA	51	Unused code
010554	010054	LDA	54	Decimal one
010555	130041	ERA	41	ADC control word
010556	050041	STA	41	Store new ADC control word
010557	010056	LDA	56	Decimal three
010560	050042	STA	42	Initialize counter for memory area, channel
010561	001000	JMP	10535	Continue with acquisition change
010562	010535	-		



in Figure A-5, the channel sampled is changed after every fourth transient. After the other transients, the memory area into which the data are averaged must be switched. Acquisition Change uses a counter to govern channel and memory area selection. The counter starts at three, the memory area at upper and the channel at zero. After each transient if the counter value is nonzero the memory area is toggled and the counter decremented and replaced. If the counter is zero the channel is toggled and the counter is reset to three. Changes between addition and subtraction occur after the first, third, sixth and eighth transients. This pattern is coded by the last eight bits of a code word. After each FID the program checks the least significant bit of the code word; it changes the add (or subtract) command in the data averaging portion of the main acquisition routine to a subtract (or an add) if this bit is set. Then it shifts the code word right one place, the least significant bit being lost. If the remaining code word is nonzero it is stored and the next FID is obtained. If the remaining code word is zero (after every eight transients) the Initialization routine is called and the code word is reset to the original eight bit pattern.

3. Initialization routine

The Initialization routine, shown in Figure A-6, is called at the beginning of acquisition and after

FIGURE A-5

Pattern of data handling for eight
transient phase alternating burst
sequence for accumulation of data in
phase quadrature using QPD.

PULSE	TRANSMITTER PHASE	90° SHIFT	180° SHIFT	CHANNEL SAMPLED	AVERAGE INTO WHICH FID?	ADD OR SUBTRACT TO MEMORY CONTENTS?
1	0°			1	0° (upper half of memory)	A
2	90°	✓		1	90° (lower half of memory)	S
3	180°		✓	1	0°	S
4	270°	✓	✓	1	90°	A
5	0°			2	90°	A
6	90°	✓		2	0°	A
7	180°		✓	2	90°	S
8	270°	✓	✓	2	0°	S

every eighth FID during accumulation. It assures that each accumulation has the 0° FID in upper memory, the 90° FID in lower memory and the same sign for the two FIDs. It also prevents the spectrometer's transmitter phase from getting out of synchronization with the data manipulations of the computer. Initialization is called prior to the start of data acquisition by a new subroutine, also listed in Figure A-6, which replaces RSGO, the data accumulation initializing routine of VFT. The new subroutine is called in place of RSGO, and in turn calls both RSGO and Initialization. During acquisition Initialization is called after every eighth FID by the Acquisition Change routine. Initialization sets and clears a TTL pulse supplied to the program card of the spectrometer pulse programmer. This TTL pulse resets the transmitter phase to 0° . The subroutine also sets the memory area to upper, the channel to 0, the memory area/channel counter to 3, the add/subtract change code word to 000245, the add/subtract command in the data averaging portion of the main acquisition routine to an add command, and the SHFT range word to quarter memory size (vide infra).

Because two FIDs are accumulated in QPD instead of one, two other data acquisition routines from VFT require modification.

4. MXBT routine

During data acquisition VFT automatically rescales the accumulated FID when necessary to prevent computer

FIGURE A-6

Program listing (a) and flowchart (b) for
QPD subroutine Initialization and subroutine
RSGO.

(a) Initialization, replaces portion of acquisition routine

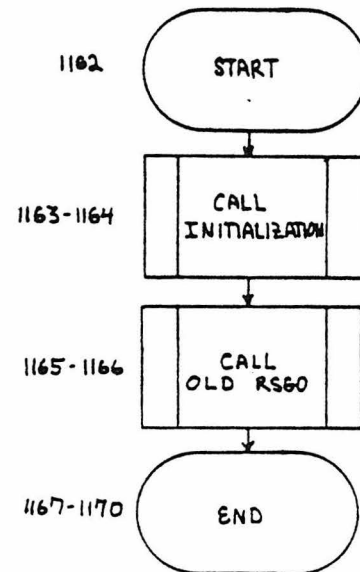
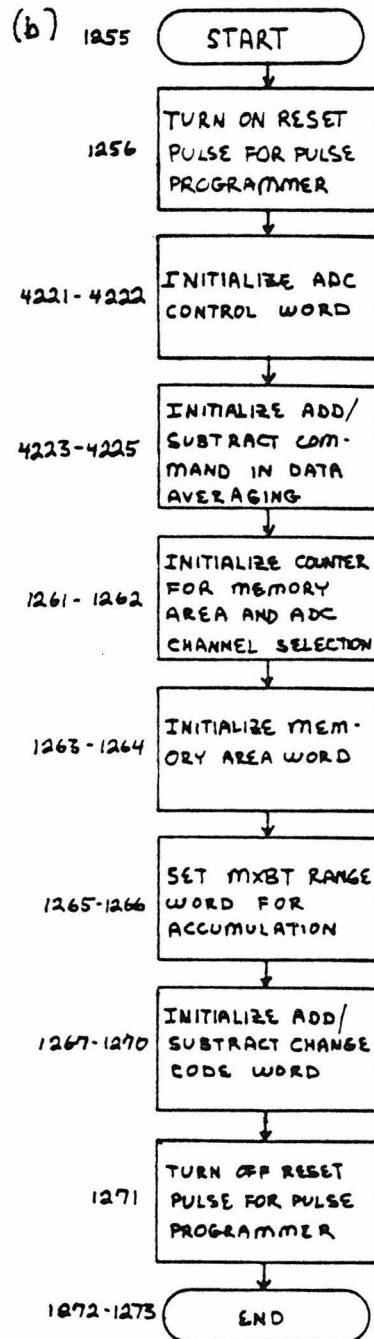
001255	000000	-	RETU	
001256	100235	EXC		Reset pulse for pulse card on
001257	001000	JMP	4221	Continue
001260	004221	-		
001261	010056	LDA	56	Decimal three
001262	050042	STA	42	Initialize counter for memory area, channel
001263	010045	LDA	45	Memory area mask
001264	050047	STA	47	Memory area word initialization
001265	010111	LDA	111	Octal 020000
001266	050277	STA	277	SHFT range word set for accumulation
001267	010051	LDA	51	Octal 000245
001270	050043	STA	43	Initialize add/subtract change code word
001271	100335	EXC		Reset pulse for pulse card off
001272	001000	JMP	RETU	Done
001273	101255	-		

Replaces part of VACB (set drift correction constants), locations

004177 - 004241				
004221	011252	LDA	1252	Octal 001000 (for QPD-40KP)
004221	005001	TZA		
004222	050041	STA	41	Initialize ADC control word
004223	006010	LDAI		
004224	121254	-		
004225	051242	STA		Initialize add/subtract command in data aver.
004226	001000	JMP	1261	Continue
004227	001261	-		

New RSGO routine, replaces part of PABS (absolute value data retrieval), locations 011605 - 011701

011662	000000	-	RETU	
011663	002000	JMPM	1255	Initialization called
011664	001255	-		
011665	002000	JMPM	3614	Call RSGO - clears data table and temporary locations
011666	003614	-		
011667	001000	JMP	RETU	Done
011670	111662	-		
003707	011662	-		Change call address for RSGO to new routine which calls initialization as well
003720	011662	-		Change call address for RSGO to new routine



memory overflow. To accomplish this, after every thousand scans the entire data memory is searched for the data point largest in absolute value using the MXBT routine. If this data point has a 1 in the most significant bit (excluding sign) the data are rescaled downward by a factor of two. The program keeps track of this scaling in a normalization counter (NMRC), which starts at zero and is decremented each time the data are scaled. For two reasons this routine must be altered for QPD. First, to insure correct image peak cancellation the two FIDs must be scaled equally during accumulation and transformation. This is most easily accomplished during acquisition by finding the maximum in both FIDs and scaling both FIDs if either needs scaling. Second, since half of the memory contains transient data which are unscaled and thus large, it is not possible to search the entire memory for a maximum value; only the accumulated FIDs should be searched. In QPD the scaling routine calls a new MXBT, listed in Figure A-7, which finds the maximum of each FID individually and then finds the larger of these two values. The original scaling routine then uses this overall maximum to scale the entire data space.

5. TRAV routine

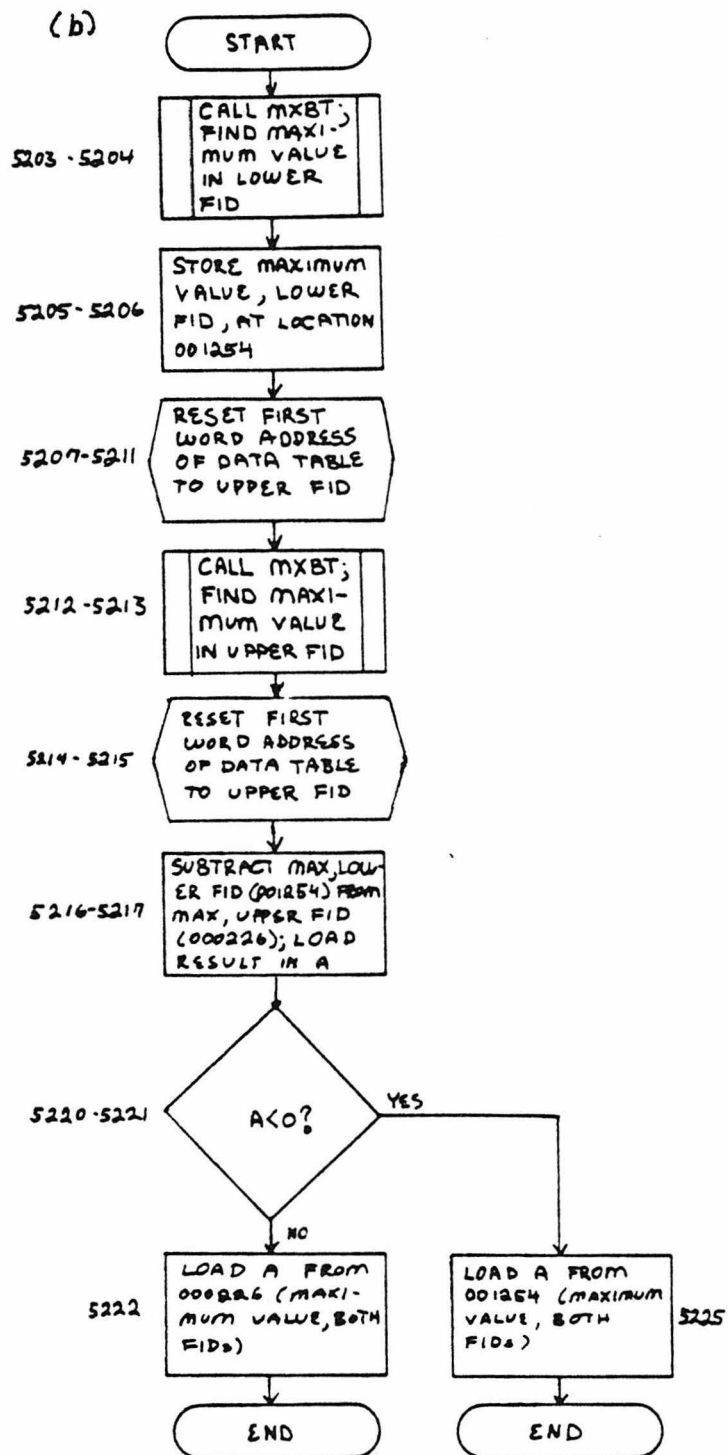
When accumulation is terminated in VFT, either by teletype interrupt or by reaching the end of the set number of transients, the subroutine TRAV is called,

FIGURE A-7

Program listing (a) and flowchart (b) for
QPD subroutine MXBT.

(a) Alternate MXBT for use during acquisition, replaces part of the time total experiment portion of CLER (the clear memory routine), locations 005165 - 005264

005203	002000	JMPM	2517	MXBT, find maximum value in list
005204	002517	-		
005205	010226	LDA	226	Maximum value stored by MXBT
005206	051254	STA	1254	Temporary storage
005207	010473	LDA	473	TABL
005210	120045	ADD	45	Half data table
005211	050473	STA	473	TABL, now set to upper (0°) FID
005212	002000	JMPM	2517	Call MXBT
005213	002517	-		
005214	010052	LDA	52	Starting address, data area
005215	050473	STA	473	TABL, reset to lower (90°) FID
005216	010226	LDA	226	Maximum value in upper FID
005217	141254	SUB	1254	Maximum value in lower FID
005220	001004	JAN	5225	If negative maximum for both FIDs is at
005221	005225	-		1254
005222	010226	LDA	226	Otherwise maximum is at 226
005223	001000	JMP	670	Return to KTRN scaling routine
005224	000670	-		
005225	011254	LDA	1254	Maximum value
005226	001000	JMP	670	Return to KTRN
005227	000670	-		
002570	140277	SUB	277	Use data length word stored at 277 to decide
				whether all data has been scaled
000665	001000	JMP	5203	Call new MXBT routine
000660	005203	-		



which removes DC offset from the accumulated FID. In QPD TRAV is replaced by a new routine, listed in Figure A-8, which calls the old TRAV as a subroutine twice, once for each accumulated FID. Thus DC offset is removed from the two FIDs separately.

To operate on the two FIDs individually the new TRAV uses a method used by many of the subroutines of QPD. There is a data word in VFT called TABL, stored at 000473, which gives the address of the first word of the data table. To operate on the lower averaged FID, TABL is set to 011774; for the upper averaged FID, TABL is set to 021774. QPD subroutines (except display routines) always operate on 2K data points, that is, if TABL is 011774 the first quadrant of memory is taken as data space; if TABL is 21774, the third quadrant of memory is taken as data space.

C. Data manipulation routines

After two FIDs have been accumulated in memory the data manipulation routines are used to apply an exponential weighting function to each of the FIDs and to produce a single-sided Fourier transform from each weighted FID. The data output routines then combine the two transforms using a phasing routine to generate the double-sided (quadrature) spectrum.

The modifications made to VFT in the data manipulation areas consist of five principal subroutines. A

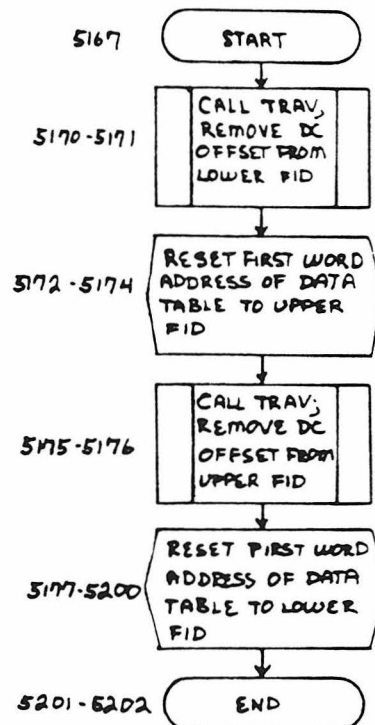
FIGURE A-8

Program listing (a) and flowchart (b)
for QPD subroutine TRAV.

- (a) New TRAV routine, replaces part of the time total experiment portion of CLER (the clear memory routine), locations 005165 - 005264

005167	000000	-	RTU	
005170	002000	JMPM	1375	Call TRAV, removes DC offset from transient data (lower FID)
005171	001375	-		
005172	010473	LDA	473	TABL, first word address of FID
005173	120045	ADD	45	Half data table
005174	050473	STA	473	TABL, now reset to upper FID
005175	002000	JMPM	1375	Call TRAV for upper FID
005176	001375	-		
005177	010052	LDA	52	Starting address, data area
005200	050473	STA	473	TABL now reset to lower FID
005201	001000	JMP	RTU	Finished
005202	105167	-		
001433	030400	LDX	400	NPTS; TRAV now operates only on the number of points actually in a single FID
001311	005167	-		Change call address of TRAV to new routine
001347	005167	-		Change call address of TRAV
005165	001000	JMP	5264	Continue CLER, omitting calculation of total experiment time
005166	005264			

(b)



new Weighting routine acts as a sort of master routine, shifting the unweighted FIDs and calling the old Weighting routine individually for the two FIDs. To shift FIDs a new subroutine, Shift, is called. The new Fourier Transform (FT) routine, which calls the old FT routine for the two FIDs individually, insures that the two resulting transforms are equally scaled and performs various bookkeeping functions. Scaling is accomplished by calling a new subroutine, Scaling Check and Correct. Finally, in order to call the Weighting routine or resume accumulation after transforming data further bookkeeping is necessary. This is performed by the subroutine Reset.

1. Weighting routine

The new Weighting routine is listed in Figure A-9. It first calls Reset for necessary bookkeeping, then calls Shift to move the 90° FID from the first memory quadrant to the second. TABL is set to the second quadrant and WTFU, the old Weighting routine, is called. Then TABL is set to the third quadrant and Shift is called to copy the 0° FID from the third quadrant to the fourth. TABL is set to the fourth quadrant, WTFU is called once more, and TABL is reset to the first quadrant before exiting the subroutine. Weighting also sets a "weight applied" flag indicating that the routine has been called since the last time FT was called.

FIGURE A-9

Program listing (a) and flowchart (b)
for QPD subroutine Weighting.

(a) Weighting routine, replaces part of PNOK(pen OK?), locations

007616-007661

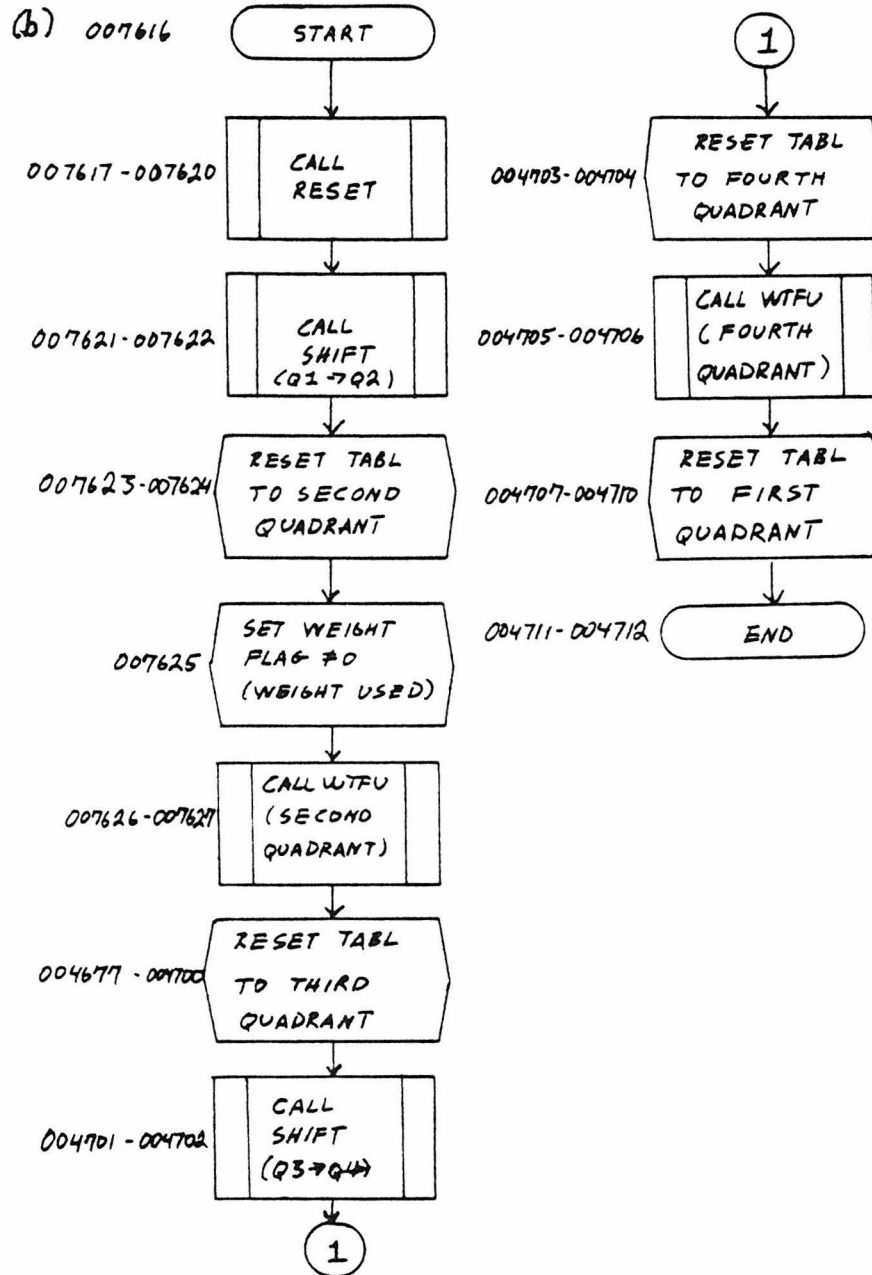
007616	000000	RETU	
007617	002000	JMPM 620	Call reset; reset LOG (FT flag), if needed
007620	000620	-	reset table length for FIDs
007621	002000	JMPM 6432	Call shift data; move lower FID from first
007622	006432	-	to second quadrant of data table
007623	010462	LDA 462	Reset TABL, first word address, data
007624	050473	STA 473	table, to second quadrant
007625	050455	STA 455	Set weight flag nonzero, indicates WT use
007626	002000	JMPM 1501	Call WTFU, apply exponential weighting
007627	001501	-	function to lower FID in second quadrant
007630	001000	JMP 4677	Continue
007631	004677	-	

Replaces part of RFRQ (find tallest line), locations 004677-004744

004677	010474	LDA 474	Reset TABL, first word address, data
004700	050473	STA 473	table, to third quadrant of table
004701	002000	JMPM 6432	Call shift data; move upper FID from
004702	006432	-	third to fourth quadrant of data table
004703	010463	LDA 463	Reset TABL, first word address, data
004704	050473	STA 473	table, to fourth quadrant of table
004705	002000	JMPM 1501	Call WTFU; apply exponential weighting
004706	001501	-	function to upper FID in fourth quadrant
004707	010052	LDA 52	Reset TABL, first word address, data table
004710	050473	STA 473	to first quadrant of data table
004711	001000	JMP RETU	Finished
004712	107616	-	

Modifications to original weighting routine

001503	010350	LDA 350	WTON: weight on flag
001504	005311	DAR	
001505	001002	JAP RETU	If WTON = 1, finished
001506	101501	-	
001507	005002	TZB	Prepare to set WUSD flag (weight used)
001510	005122	IBR	
003727	002000	JMPM 7616	Call weight unconditionally in AU mode
000540	007616	-	Call new weighting routine
003730	007616	-	Call new weighting routine



A minor modification to WTFU itself is also necessary. As originally written WTFU exits to ERPR (the subroutine which generates error messages) if the weighting function (WT) is called when the weighting constant is set to zero. In QPD Weighting must be called before transformation in order that the FIDs be shifted, even if no exponential weighting function is to be applied. Thus WTFU is rewritten so that it checks the flag WTON to see whether an exponential weighting is to be applied; if none is used it returns to the calling routine rather than to ERPR. Finally, in VFT the AU command executes data acquisition, weighting if WTON is set, transformation, and data display automatically upon a single command. In QPD the AU routine calls the new Weighting routine before calling for Fourier transformation regardless of the condition of WTON.

2. Shift subroutine

The subroutine Shift is listed in Figure A-10. When called it shifts 2048 data points, beginning at location TABL, to the 2048 locations immediately above them in memory.

3. Fourier Transform routine

The new Fourier Transform routine is listed in Figure A-11. When the operator types the command FT the routine saves the normalization counter (NMRC), which keeps track of data scaling, since the value of NMRC appropriate to the FIDs will be needed later. Then

FIGURE A-10

Program listing (a) and flowchart (b)
for QPD subroutine Shift.

(2) Shift data, replaces part of INTG (integration routine), locations 006432 - 006460

006432	000000	-	RETU	
006433	030473	LDX	473	TABL, first address of data to be shifted
006434	020044	LDB	44	Quarter data table, length of data to be shifted
006435	015000	LDA	0,1	Load data from lower quarter of memory
006436	051254	STA	1254	Store temporarily
006437	005041	TXA		Shift to upper quarter of memory
006440	120044	ADD	44	Quarter data table
006441	005014	TAX		
006442	011254	LDA	1254	Recover data stored temporarily
006443	055000	STA	0,1	Store data in upper memory quarter
006444	005041	TXA		Shift to lower quarter of memory
006445	140044	SUB		
006446	005114	TAX	+1	And increment
006447	005322	DBR		Count number of points transferred
006450	001020	JBZ	RETU	Return if finished
006451	106432	-		
006452	001000	JMP	6435	Otherwise, continue
006453	006435	-		

(b)

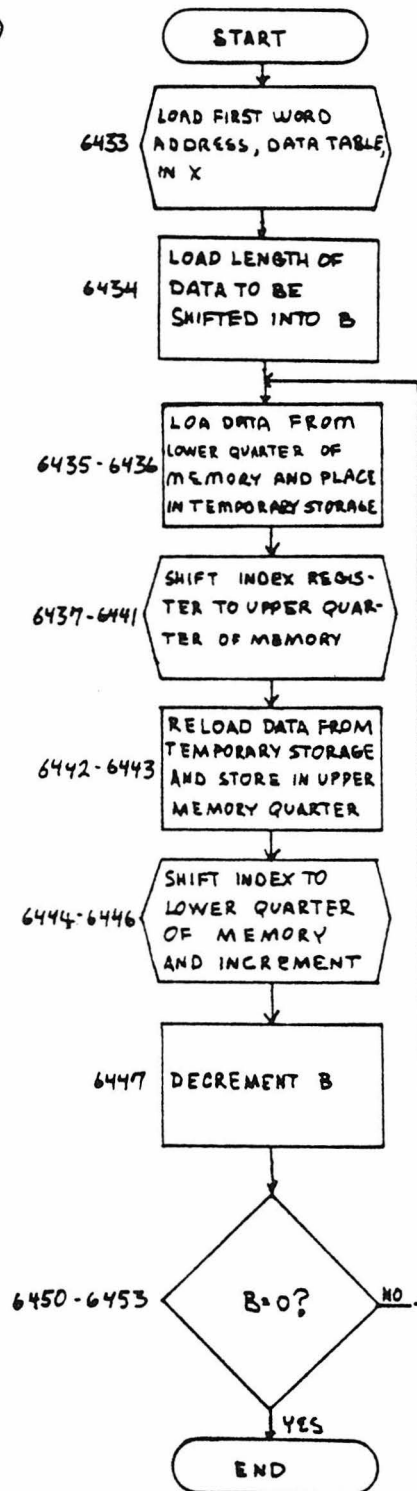


FIGURE A-11

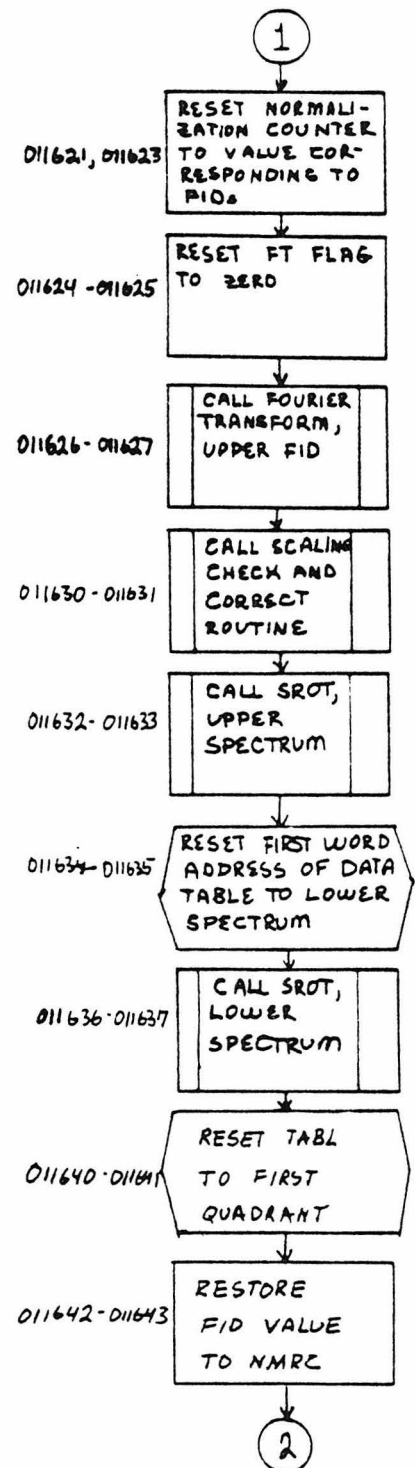
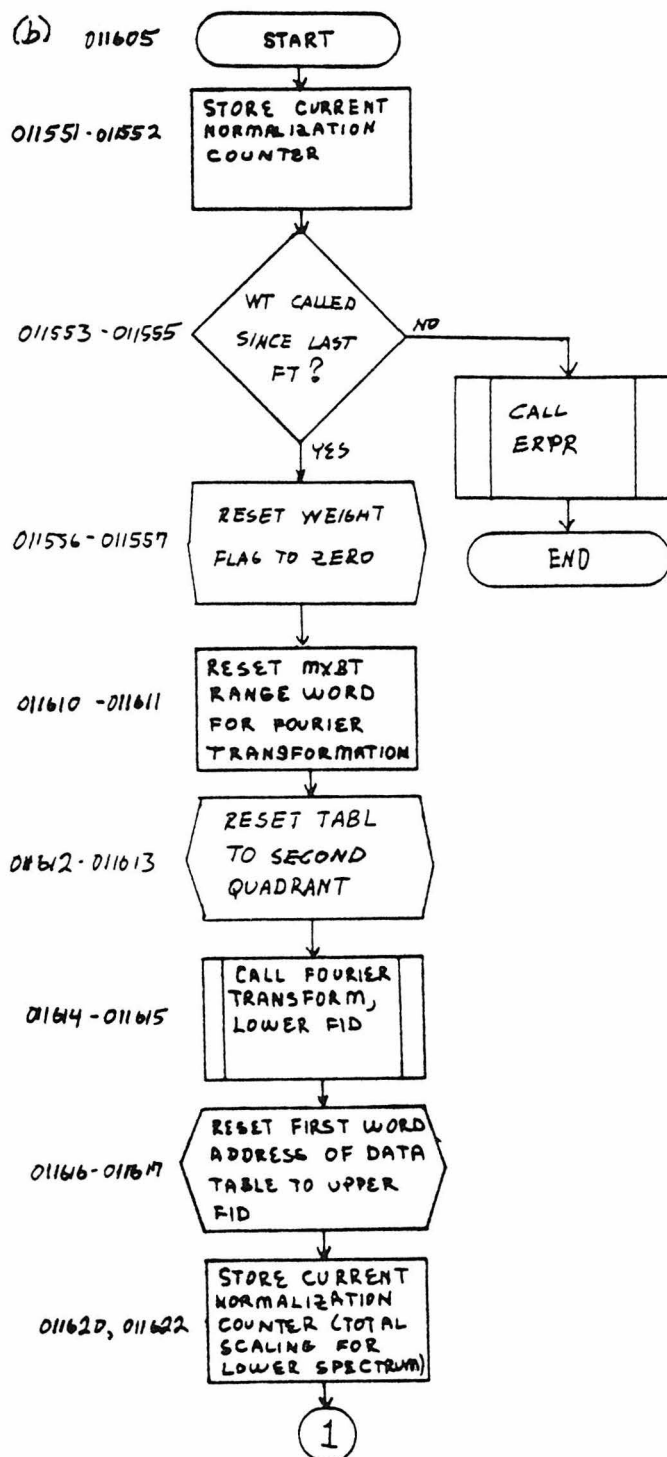
Program listing (a) and flowchart (b)
for QPD subroutine Fourier Transform.

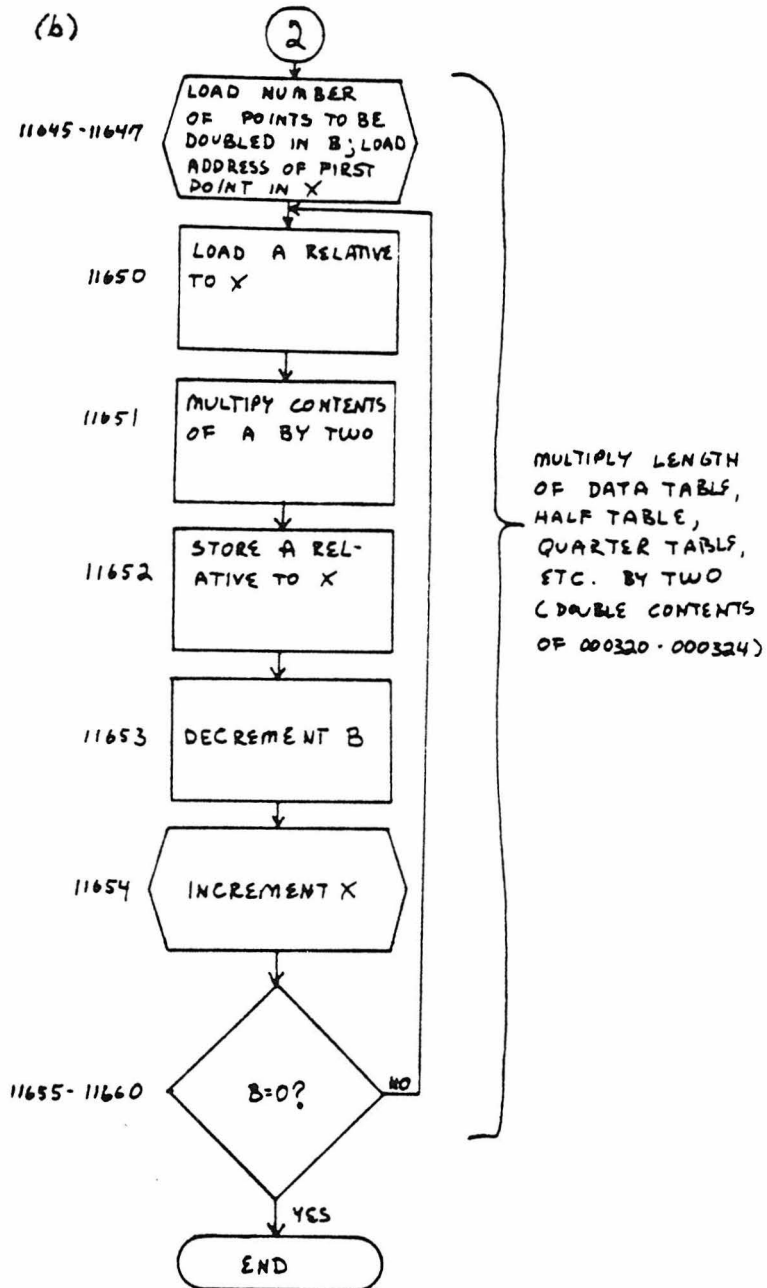
(a) New Fourier transform routine, replaces part of PABS (absolute value data retrieval), locations 011605 - 011701

011605	010673	-	RETU	
011606	001000	JMP	11551	Continue
011607	011551	-		
011610	010324	LDA	324	Octal 010000
011611	050277	STA	277	SHFT range word set for Fourier transform
011612	010462	LDA	462	Reset TABL, first word address, data
011613	050473	STA	473	table, to second quadrant of table
011614	002000	JMPM	1650	Call fourier transform, lower (90°) FID
011615	001650	-		
011616	010463	LDA	463	Reset TABL, first word address, data
011617	050473	STA	473	table, to fourth quadrant of table
011620	010416	LDA	416	Current normalization counter
011621	020470	LDB	470	Normalization counter storage
011622	060416	STR	416	Current normalization counter
011623	050470	STA	457	Normalization counter storage
011624	005001	TZA		
011625	050316	STA	316	LOG; reset "FT completed" flag
011626	002000	JMPM	1650	Call Fourier transform, upper (0°) FID
011627	001650	-		
011630	002000	JMPM	5230	Call scaling check and correct
011631	005230	-		
011632	002000	JMPM	1613	Call SROT, upper spectrum
011633	001613	-		
011634	010462	LDA	462	Reset TABL, first word address, data
011635	050473	STA	473	table, to second quadrant of data
011636	002000	JMPM	1613	Call SROT, lower spectrum
011637	001613	-		
011640	010052	LDA	52	Reset TABL, first word address, data
011641	050473	STA	473	table, to first quadrant of table
011642	010470	LDA	470	Restore value from FIDs to NMRC
011643	050416	STA	416	
011644	005000	NOP		Continue
011645	020060	LDB	60	Decimal five
011646	006030	LDXI	320	DT16; first address, data length table
011647	000320	-		
011650	015000	LDA	0,1	Multiply data table length by two:
011651	004201	ASLA	1	
011652	055000	STA	0,1	
011653	005322	DBR		Count number of words multiplied
011654	005144	IXR		Move to next word
011655	001020	JBZ	RETU	If done, Fourier transform is finished
011656	111605	-		
011657	001000	JMP	11650	If not, continue
011660	011650	-		

New Fourier transform routine, replaces part of ABPL (set plot flags for absolute value mode), locations 011551 - 011562

011551	010416	LDA	416	Normalization counter
011552	050470	STA	470	Normalization counter storage
011553	010455	LDA	455	Weight flag
011554	002010	JAZM	3154	ERPR; if weight not called since last fourier
011555	003154	-		transformation, illegal to fourier transform
011556	005001	TZA		Reset weight flag to zero
011557	050455	STA	455	
011560	001000	JMP	011610	Continue
011561	011610	-		
000542	011605	-		Call new FT routine
003732	011605	-		Call new FT routine





it checks to see whether the "weight applied" flag is set. If Weighting has not been called since the last FT an error message is generated; otherwise the flag is reset and the routine continues. The MXBT range word is set to 2K, TABL is set to the second quadrant, and the old FT routine is called to transform the 90° spectrum. Since integer arithmetic is used, at each level of the Cooley-Tukey algorithm the data are checked to see that they are nearly full scale; otherwise they are scaled up and the normalization counter is incremented to keep track of the amount of scaling. After the transformation the current value of the normalization counter is saved; this value represents the total scaling of the 90° spectrum. The value appropriate to the FID is restored to the normalization counter, TABL is reset to the fourth quadrant and the old FT routine is called again. After transformation of the 0° FID the value of the normalization counter represents the total scaling of the 0° transformed spectrum. The Scaling Check and Correct routine is then called. This subroutine compares the scaling of the two transforms and scales the one which is scaled largest down to match the other. TABL remains at the fourth quadrant. The SROT (spectrum rotate) subroutine is called, which rotates the 0° transform end for end (and also inverts the data), TABL is reset to the second quadrant, and SROT is called again for the 90° spectrum. The end for end shift puts

the zero frequency end of the data uppermost in memory, in preparation for the data output routines. TABL is reset to the first quadrant and the value appropriate to an FID is restored to the normalization counter, in case further data accumulation or transformation is called for. Finally, locations 000320 to 000324, which store the data table length, half table length, quarter table length, etc., are changed from a 2K data table to a 4K data table, also in preparation for the data output routines.

4. Scaling Check and Correct

Scaling Check and Correct is listed in Figure A-12. It subtracts the two normalization counter values to determine which FID must be scaled, places the difference (which is arranged to be less than zero) in the A register, sets TABL to the appropriate quadrant and calls SHFT, the VFT subroutine which scales data according to the value in A beginning at TABL and scaling 2K points. TABL is reset to the fourth quadrant before exit.

5. Reset

Before the Weighting routine is executed or data acquisition is resumed, the subroutine Reset, listed in Figure A-13, is called. This subroutine resets the LOG flag to indicate the Fourier transform routine has not yet been called and checks the stored data table length to see whether it is set to 2K or 4K. If data length is set to 4K, the table length locations (000320-000324) are divided by two to set the table length to 2K.

FIGURE A-12

Program listing (a) and flowchart (b)
for QPD subroutine Scaling Check and
Correct.

(2) Scaling check and correct after FT, replaces part of time total experiment portion of CLER (clear data table routine), locations 005165 - 005264

005230	000000	-	RETU	
005231	010457	LDA	470	Normalization counter, lower spectrum
005232	140416	SUB	416	Normalization counter, upper spectrum
005233	001004	JAN	5252	If upper scaled larger than lower, rescale
005234	005252	-		upper
005235	001010	JAZ	RETU	If scaling equal, finished; otherwise rescale
005236	105230	-		lower
005237	010462	LDA	52	Starting address, data area
005240	050473	STA	473	TABL, now reset to lower FID
005241	010416	LDA	416	Normalization counter, upper spectrum
005242	140457	SUB	470	Normalization counter, lower spectrum
005243	002000	JMPM	2544	Call SHFT, scales data according to value in A
005244	002544	-		register
005245	010463	LDA	52	Starting address, data area
005246	005000	ADD	45	Half data table
005247	050473	STA	473	TABL, once again set to upper FID
005250	001000	JMP	RETU	Finished
005251	105230	-		
005252	002000	JMPM	2544	Call SHFT, scales data according to value in A
005253	002544	-		register
005254	001000	JMP	RETU	Finished
005255	105230	-		

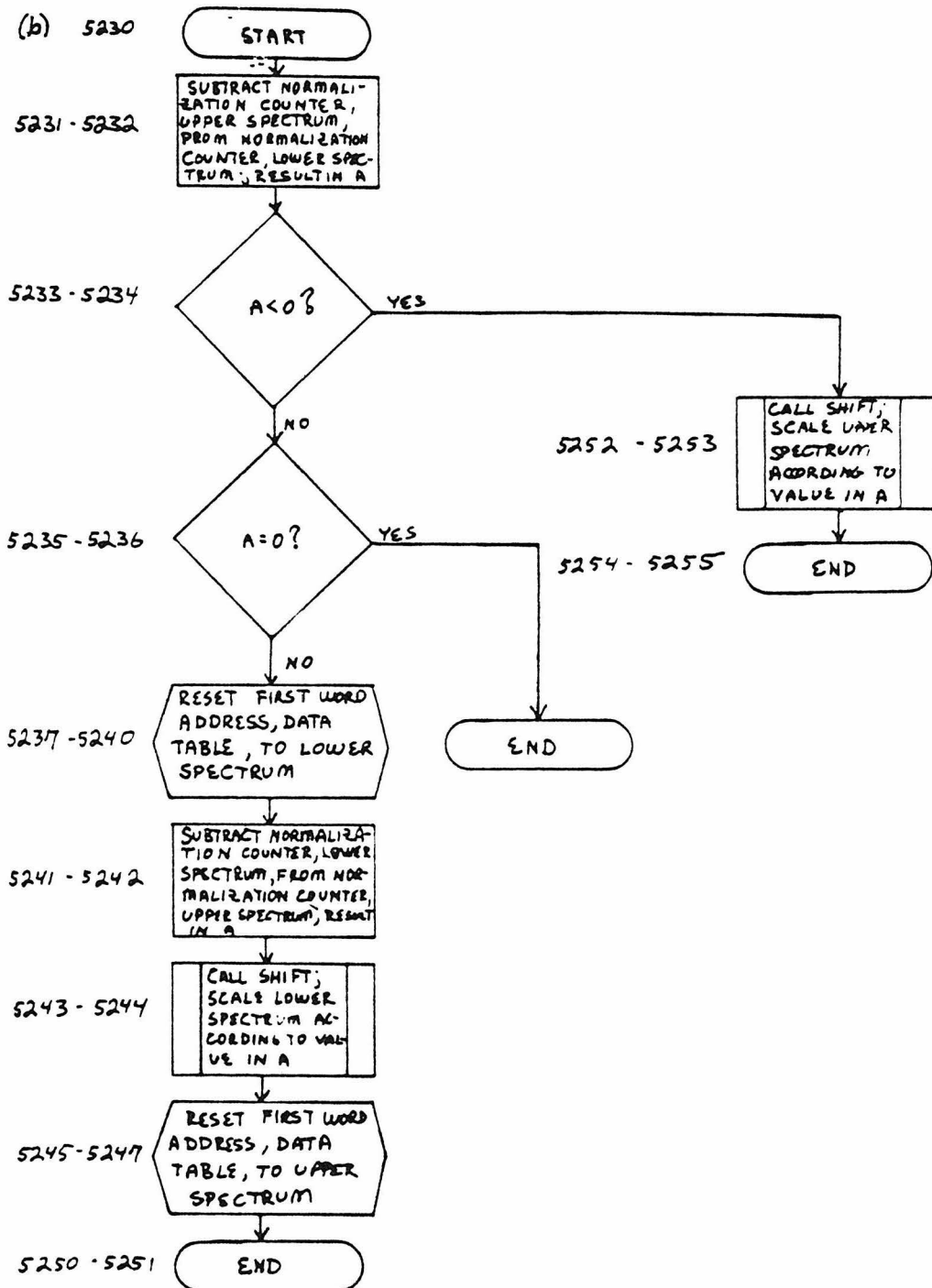
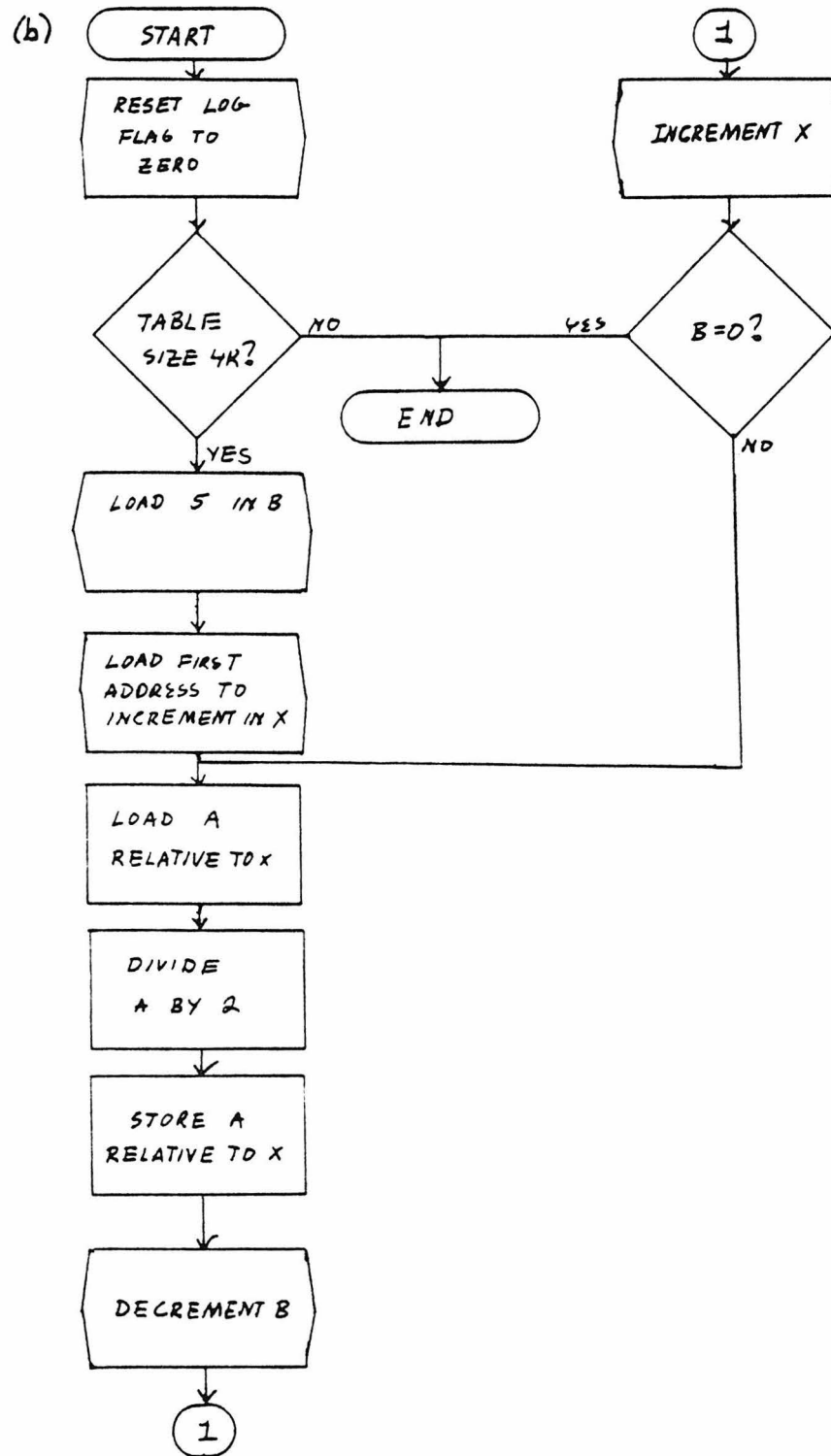


FIGURE A-13

Program listing (a) and flowchart (b)
for QPD subroutine Reset.

(2) Reset, replaces part of time-averaging, locations 000573-000562

000620	000000	RETU		
000621	005001	TZA		Rest LOG (FT flag) to zero
000622	050316	STA	316	
000623	010322	LDA	322	DT04; check to see whether data table size
000624	140656	SUB	656	needs reset
000625	001010	JAZ	631	If so, reset it
000626	00631	-		
000627	001000	JMP	RETU	If not, finished
000630	100620	-		
000631	020060	LDB	60	Decimal five, number of locations to reset
000632	006030	LDXI	320	First address of data to be reset
000633	000320	-		
000634	015000	LDA	0,1	Load contents
000635	004301	ASRA	1	Divide by two
000636	055000	STA	0,1	And replace
000637	005322	DBR		Count locations completed
000640	005144	IXR		Move to next location
000641	001020	JBZ	RETU	If last location, finished
000642	100620	-		
000643	001000	JMP	634	Otherwise, continue
000644	000634	-		
000501	002000	JMPM	620	Call reset before continuing accumulation
000502	000620	-		
000503	005000	NOP		Space



D. Data output routines

Data output routines generate a double-sided absorption mode spectrum from the two individual Fourier transforms. First the phasing routine generates a double-sided phase table. Then for each value of the frequency, the phased value data retrieval routine (PHAV) locates the sine and cosine of the phase angle and the sine and cosine term of each of the two Fourier transforms and combines their six values to generate the output point. The smoothing routine is used to average neighboring values together to improve spectral appearance when spectral features are broad.

1. Phasing routing

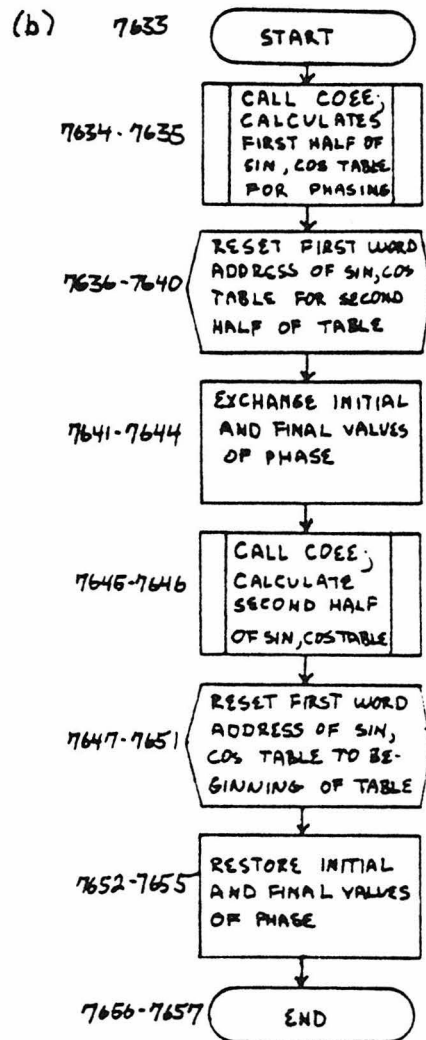
In VFT the (sin, cos) table is calculated by a subroutine COEE from the initial and final values read from the phase knobs. The double-sided (sin, cos) table for QPD is calculated by another "master" routine, listed in Figure A-14, which calls COEE twice as a subroutine. COEE is called first to calculate the lower half of the table. (COEE is modified to calculate 512 ($\sin\phi$, $\cos\phi$) pairs between ϕ_{initial} and ϕ_{final} . Both VFT and QPD use a table of 1024 (sin, cos) pairs for any length of data table.) The first word address of the (sin, cos) table is incremented by 1024, the initial and final values of the phase are interchanged and COEE is called again to compute the upper half of the table. The first word address of the (sin, cos) table and the initial and

FIGURE A-14

Program listing (a) and flowchart (b)
for QPD subroutine Phasing.

(2) Addition to phasing routine, replaces part of PNOK (pen OK?), locations
007616 - 007661

007633	000000	-	RETU	
007634	002000	JMPM	5051	COEF, calculates sin, cos table for phasing
007635	005051	-		Here, we calculate the first half of the table
007636	010475	LDA	475	First word address of sin, cos table
007637	120114	ADD	114	Octal 002000, half the length of sin, cos table
007640	050475	STA	475	Now first word of second half of sin, cos table
007641	010270	LDA	270	PHAF, final value of the phase
007642	020267	LDB	267	PHAI, initial value of the phase
007643	050267	STA	267	Reverse the initial and final values of the
007644	060270	STB	270	phase to calculate second half of table
007645	002000	JMPM	5051	Call COEF, calculate second half of table
007646	005051	-		
007647	010475	LDA	475	
007650	140114	SUB	114	
007651	050475	STA	475	Restore first word address of sin, cos table
007652	010270	LDA	270	
007653	020267	LDB	267	
007654	050267	STA	267	Restore initial and final values of phase so
007655	060270	STB	270	that they will be correct when checked by ADC
007656	001000	JMP	RETU	Finished
007657	107633	-		
005077	170004	DIV	4	Octal 001000; new table length is effectively
				half the old table length (calculate new argument
005145	140004	SUB	4	Octal 001000; new table length (check whether
				table is completed)
004635	007633	-		Call new phase routine instead of COEF directly



final values of the phase are restored before exit.

2. PHAV routine

The new PHAV is listed in Figure A-15; it consists of additions to the original routine. In the new scheme the original routine uses SAVX to locate $\sin\phi$ and $\cos\phi$ as usual. It then jumps to a patch which checks whether SAVX is in the upper or lower half of the spectrum. If SAVX is in the lower half, the add/subtract command used for combining values from the two transforms at the end of the PHAV routine is set to a subtract and $(2 \cdot \text{SAVX}) + 2048 + \text{TABL}$ is computed as an index to locate the values $A(\omega)$ and $B(\omega)$ in the data table. If SAVX is in the upper half of the spectrum, the add/subtract command is set to an add and $((2048 - \text{SAVX}) \cdot 2) + 2048 + \text{TABL}$ is computed as the data table index. The old routine then computes the value for the 90° (lower) spectrum, $A(\omega)\cos\phi + B(\omega)\sin\phi$. A second patch then adds 4096 to the index, locates $C(\omega)$ and $D(\omega)$, and computes the value for the 0° (upper) spectrum, $-C(\omega)\sin\phi + D(\omega)\cos\phi$. A third patch adds or subtracts the upper value from the lower one according to the command set previously and returns to the original PHAV routine to continue data output.

3. Smoothing

At low signal-to-noise deuterium resonances of systems with restricted motion appear as very broad humps. In plotting these spectra it is useful to employ

FIGURE A-15

Program listing (a) and flowchart (b)
for QPD subroutine PHAV.

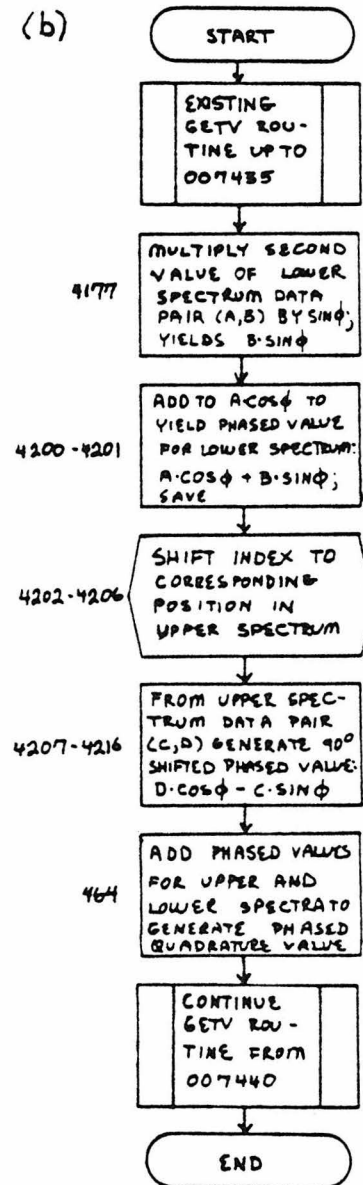
- (2) Addition to PHAV routine (phased data retrieval, replaces VACB (set drift correction constants), locations 004177 - 004241

004177	160237	MUL	237	Yields imaginary value, lower spectrum
004200	120467	ADD	467	Sum of real, imaginary values, lower spectrum
004201	050467	STA	467	Save lower spectrum value
004202	010461	LDA	461	Use corrected value stored earlier
004203	005000	NOP		Space
004204	120473	ADD	473	And add TABL
004205	120045	ADD	45	And add half data table
004206	005014	TAX		To yield corresponding position in upper spectrum
004207	025000	LDB	0,1	Raw data from table
004210	005001	TZA		Prepare to multiply
004211	160237	MUL	237	By temporary value of sin
004212	050237	STA	237	Now finished with value of sin
004213	025001	LDB	1,1	Second value of raw data pair from table
004214	005001	TZA		Prepare to multiply
004215	160236	MUL	236	By temporary value of cos
004216	140237	SUB	237	Difference of real and imaginary values, upper
004217	001000	JMP	4270	Continue
004220	004270	-		
004270	020467	LDB	467	Interchange results for upper and lower
004281	050467	STA	467	spectrum in order to subtract upper
004282	005021	TBA		value from lower value
004283	001000	JMP	464	Continue
004284	000464	-		
007433	050467	STA	467	Use 467 as temporary storage to preserve
				temporary value of cos
007436	001000	JMP	4177	Add above routine to GETV routine for quadrature
007437	004177			phased value data retrieval
000464	120467	ADD	467	Sum of upper and lower spectrum values
000465	001000	JMP	7440	Continue with GETV
000466	007440			

Replaces part of time averaging routine for long term averaging,
locations 00573-00652

000573	010224	LDA	224	SAVX, location in data table
000574	140322	SUB	322	DT04, quarter table length
000575	001002	JAP	605	If positive, location is in upper half
000576	000605	-		of quadrature spectrum
000577	006010	LDAI	140467	Otherwise, location is in lower half so
000600	140467	-		subtract upper spectrum from lower
000601	050464	STA	464	spectrum
000602	010224	LDA	224	SAVX, correct table location
000603	001000	JMP	612	Continue
000604	000612	-		
000605	006010	LDAI	120467	In upper half of quadrature spectrum the
000606	120467	-		two spectra must be added
000607	050464	STA	464	
000610	010323	LDA	323	DT04-SAVX is correct position in data
000611	140224	SUB	224	table
000612	004201	ASLA	1	Multiply by two and add quarter table
000613	120044	ADD	44	to generate index for data retrieval
000614	050461	STA	461	Save for later use in PHAV
000615	001000	JMP	7426	Continue
000616	007426	-		
007424	001000	JMP	573	Continue
007425	000573	-		

(b)



a smoothing function to improve the appearance of the data. VFT has available a weighted three-point smoothing in which the output value $S(\omega_n)$, is computed as $1/4(S(\omega_{n-1}))+1/2(S(\omega_n))+1/4(S(\omega_{n+1}))$. The 40 kHz version of QPD uses instead an unweighted multipoint smoothing in which the number of points averaged to generate each output point is controlled by the operator. When the smoothing parameter is set to m , a $2m+1$ point smoothing is employed. The VFT and QPD smoothing routines act as master routines which call GETV ("get value", a subroutine which in turn calls PHAV) as a subroutine several times. The new smoothing routine is listed in Figure A-16. For an m point smoothing the routine adds the values $S(\omega_{n-m}), S(\omega_{n-m+1}), \dots, S(\omega_n), S(\omega_{n+1}), \dots, S(\omega_{n+m})$ to generate $S(\omega)$. GETV is called $2m+1$ times per output point. To avoid searching for values beyond the edges of the data table, for the first m points and the last m points the output value is computed as $(2m+1)S(\omega_n)$. To use smoothing in VFT the operator sets a flag, SM, to 1. To use smoothing in QPD the operator enters a parameter $SM=m$. The fast acquisition versions of QPD use the original smoothing routine, since they require additional space for their longer acquisition routine.

FIGURE A-16

Program listing (a) and flowchart (b)
for QPD subroutine Smoothing (used in
QPD-40KP only).

(2) Smoothing, replaces original

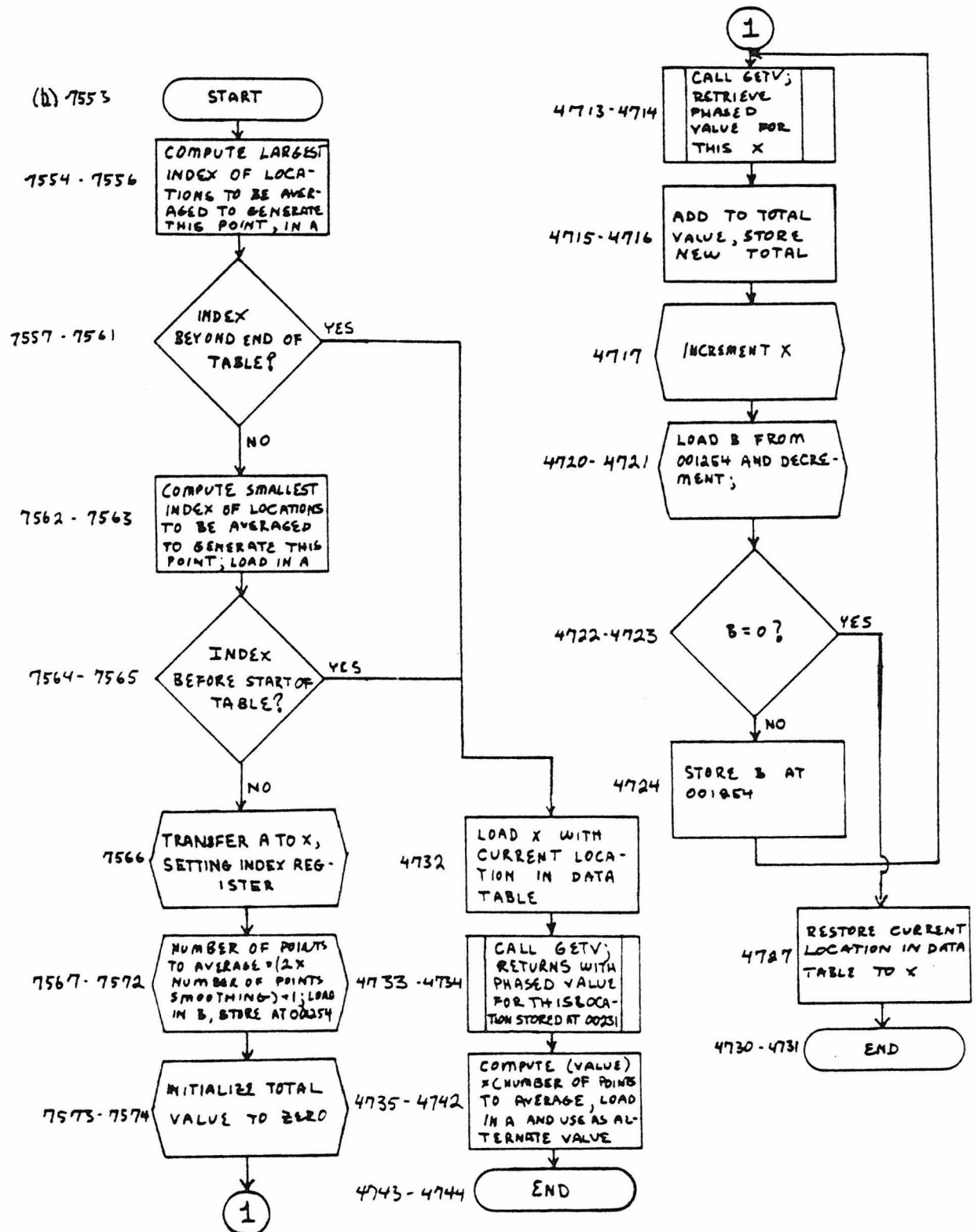
007553	000000	-	RETU	
007554	070224	LDX	224	SAVX, position in data table
007555	005041	TXA		
007556	120471	ADD	471	Number of points of smoothing
007557	140323	SUB	323	Half table length
007560	001002	JAP	4730	If positive, smoothing by this many points
007561	004732	-		goes beyond data table end, use alternate
007562	005041	TXA		
007563	140471	SUB	471	Number of points of smoothing
007564	001004	JAN	4730	If negative, smoothing by this many points
007565	004732	-		starts before table, use alternate routine
007566	005014	TAX		Tests passed, set up to get value:
007567	010471	LDA	471	Number of points of smoothing
007570	004201	ASLA	2	Multiply by two
007571	005112	TAB	+1	Add one, place in B
007572	061254	STB	1254	And store
007573	005001	TZA		
007574	050231	STA	231	Initialize total value
007575	001000	JMP	4713	Continue
007576	004713	-		

Smoothing, replaces part of RFRQ (find tallest line), 004677 - 004744

004713	002000	JMPM	7370	GETV, retrieve value for this X
004714	007370	-		
004715	120231	ADD	231	Add to accumulated value
004716	050231	STA	231	And store
004717	005144	DXR		Move to next X
004720	021254	LDB	1254	Count number of points completed
004721	005322	DBR		
004722	001020	JBZ	4/27	If last point, finish up
004723	004727	-		
004724	061254	STB	1254	Otherwise store counter
004725	001000	JMP	4713	And continue
004726	004713	-		
004727	030224	LDX	224	SAVX, restore X register
004730	001000	JMP	RETU	Finished
004731	107553	-		
004732	030224	LDX	224	SAVX, current location in data table
004733	002000	JMPM	7370	Call GETV, find table value for location X
004734	007370	-		
004735	010231	LDA	231	Place value for location X in A
004736	005012	TAB		Prepare
004737	005001	TZA		To multiply
004740	160471	MUL	471	Multiply by number of points of smoothing,
004741	004201	ASLA	1	Double this value
004742	120231	ADD	231	And add the value once more
004743	001000	JMP	RETU	Use this value for points near the edge of the
004744	107553	-		table; finished

Smoothing, replaces part of VARM (mark frequency), locations 004366 - 004377

004366	002000	JMPM	10236	Call ISND, input decimal number and fraction
004367	010236	-		
004370	050471	STA	471	Store number of points of smoothing
004371	001000	JMP	10563	Return to CODE, the teletype monitor
004372	010563	-		
011013	004366	-		Call input routine after TTY "SM"



E. Parameter Input, Command Input, and Flag Input

1. Spectral Width

In VFT the "spectral width" entered by the operator is used to compute the external timer frequency which controls the rate of analog to digital conversion during accumulation. The program automatically rounds the entered "spectral width" up to the next highest spectral width actually available as a discrete timer frequency. For the fast acquisition version of QPD, the entered "spectral width" is used to compute the number of steps in a set of logical rotate commands in the acquisition routine. Again the program rounds up to the next higher spectral width actually available as a corresponding dwell time. The new spectral width input routine is shown in Figure A-17. Since the maximum spectral width of 68,000 Hz exceeds the largest number the 620i can store in a single word, the operator enters the desired "spectral width" divided by 10. The routine computes the number of steps in the LRLB command, checks to see whether this number is in the allowed range, and if not exits with an error message. If the "spectral width" is in the allowed range the LRLB commands are formed and stored and the actual spectral width is computed and stored before exit.

2. Data Length

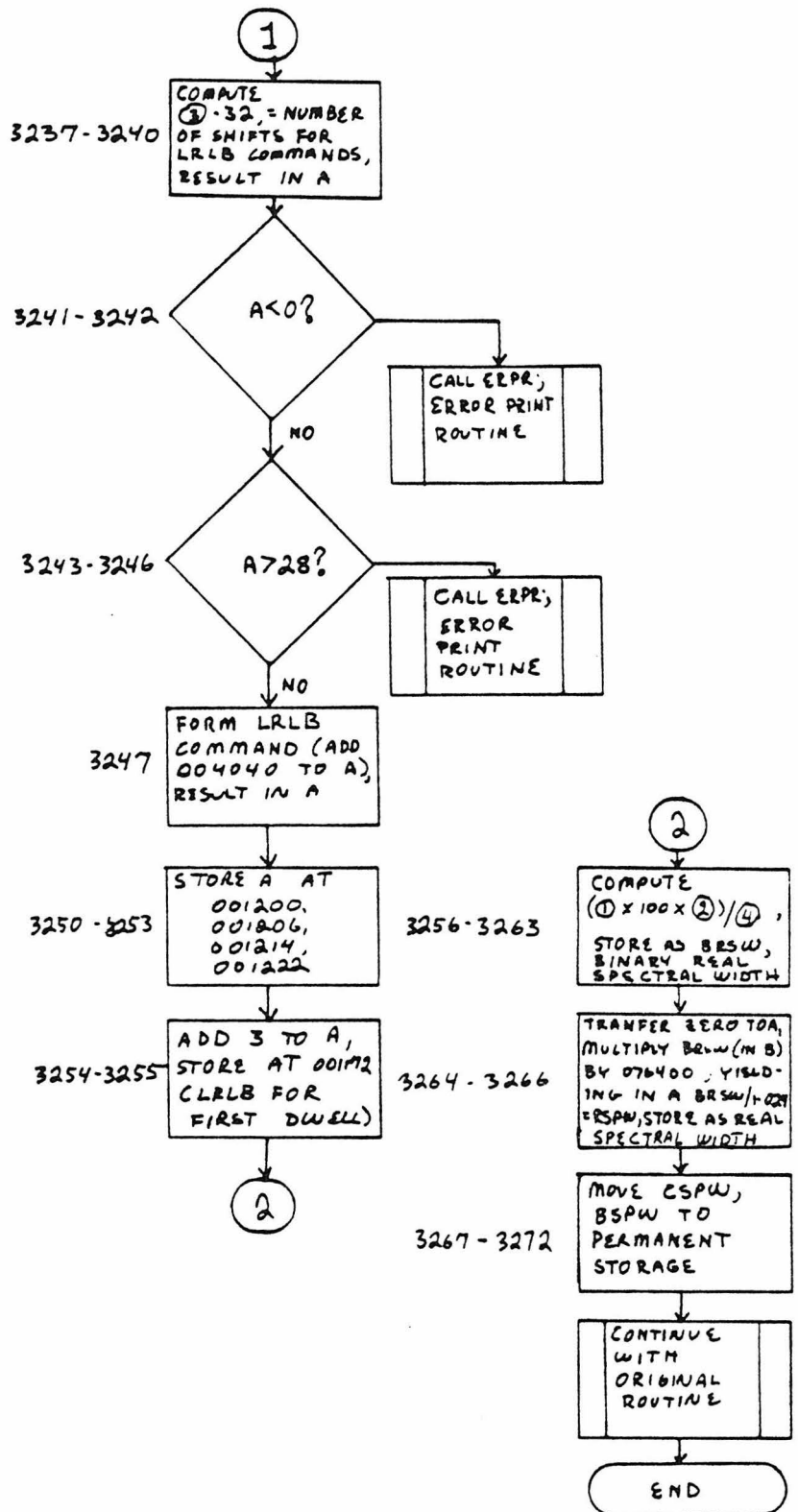
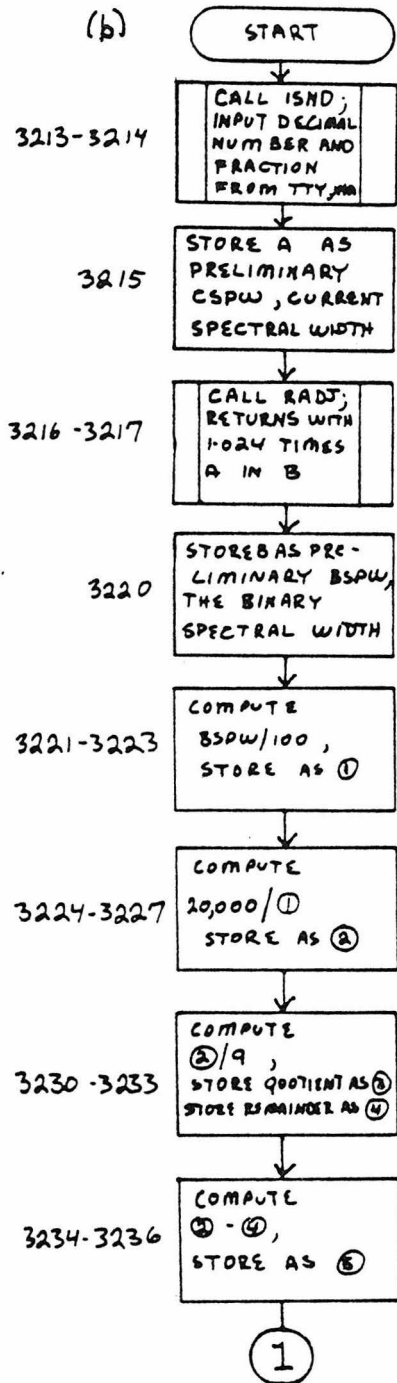
When the operator enters either a "spectral width" (SW) or an "acquisition time" (AT) the program checks to

FIGURE A-17

Program listing (a) and flowchart (b)
for QPD subroutine Spectral Width.

(a) Spectral width routine, 003213-003274, replaces original

003213	002000	JMPM 10236	Call ISND, inputs decimal number and fraction
003214	010236	-	from teletype
003215	050225	STA 225	CSPW, current spectral width
003216	002000	JMPM 3315	Call RADJ, returns with 1.024 times A in B
003217	003315	-	
003220	060226	STB 226	BSPW, binary spectral width
003221	005001	TZA	Prepare to divide
003222	170103	DIV 103	By decimal 100, yields rounded BSPW/100
003223	060237	STB 237	T + 13, temporary storage
003224	020174	LDB 174	Decimal 20,000
003225	005001	TZA	Prepare to divide
003226	170237	DIV 237	By BSPW/100, yields dwell in μ sec times 20
003227	060175	STB 175	Temporary storage
003230	005001	TZA	Prepare to divide
003231	170064	DIV 64	By decimal 9, yields dwell in quarter cycles
003232	060176	STB 176	Temporary storage, quotient
003233	050177	STA 177	Temporary storage, remainder
003234	010175	LDA 175	Dwell, μ sec times 20
003235	140177	SUB 177	Actual dwell in μ sec times 20
003236	050177	STA 177	Save for use in calculating actual spectral width
003237	010176	LDA 176	Dwell in quarter cycles
003240	140075	SUB 75	Decimal 32, minimum number of quarter cycles
003241	002004	JANM 3154	If negative, SW entered is too large, go to
003242	003154	-	error print routine, ERPR
003243	140200	SUB 200	Decimal 29, maximum number of rotate steps
003244	002002	JAPM 3154	If positive, SW entered is too small, go to ERP
003245	003154	-	
003246	120200	ADD 200	Restore number of steps of rotate to A
003247	120201	ADD 201	LRLB command
003250	051200	STA 1200	Store LRLB in its proper place in each
003251	051206	STA 1206	of the four sets of input instructions
003252	051214	STA 1214	
003253	051222	STA 1222	
003254	120056	ADD 56	Decimal three, generates LRLB for first dwell
003255	051172	STA 1172	Store in first dwell
003256	020237	LDB 237	Rounded BSPW divided by 100
003257	005001	TZA	Prepare to multiply
003260	160103	MUL 103	By decimal 100, yields BSPW, rounded
003261	160175	MUL 175	Then by dwell in μ sec times 20
003262	170177	DIV 177	And then divide by actual dwell in μ sec times 20
003263	060363	STB 363	Yielding BRSW, the binary real spectral width
003264	005001	TZA	Prepare to multiply
003265	160134	MUL 134	By R1K, octal 076400, yielding in A
003266	050362	STA 362	RSPW, the real spectral width
003267	010226	LDA 226	BSPW in temporary storage
003270	050361	STA 361	BSPW permanent storage
003271	010225	LDA 225	CSPW in temporary storage
003272	050360	STA 360	CSPW in permanent storage
003273	001000	JMP 3276	Continue
003274	003276	-	
000174	047040	-	Decimal 20,000
000200	000035	-	Decimal 29
000201	004040	-	LRLB



see whether the maximum number of data points is exceeded by the requested AT and SW. If either is too large, the acquisition time for the maximum number of points is computed, displayed and set automatically. Figure A-18 lists several patches which cause the maximum number of points for QPD to be 2048 rather than 8092 as in VFT.

3. Eliminated routines

To make room for the added routines in QPD, a number of subroutines and corresponding functions have been deleted from VFT. These were either inappropriate for quadrature data or deemed less useful for wide-line spectra. The corresponding parameters and commands have been removed from the program vocabulary since their use would be destructive to the program (see Figure A-19). These include the two letter commands AI (absolute intensity plot), AV (absolute value plot), BL (baseling flattening), DC (drift correction), IP (integral plot), IS (integral scale), OK (chart paper position OK?), SR (spectrum rotate), and SS (steady state) and the one letter Zoom commands B (baseline or blocks), control B (baseline drift correction), control D (decrease LTA multiplier), control I (increase LTA multiplier, control L (left baseline correct), M (mark spectral line), O (set offset), control P (plot integrals) and V (LTA V-mode display). Major functions deleted from the program are the long-term averaging or block-averaging acquisition mode, integration, absolute intensity and

FIGURE A-18

Patches used in QPD to change maximum value
of data length for a single FID to 2048
data points.

Limit acquisition time to 2048 points:

003331	020044	LDB	44	Use 2K as length limit
003604	140044	SUB	44	Use 2K as length limit
003607	120044	ADD	44	Use 2K as length limit

FIGURE A-19

Patches used to delete various commands
from VFT program vocabulary in new
program QPD.

Code table: set the following to 010563

010701	(AI deleted from program vocabulary)
010707	(AV)
010711	(BL)
010715	(DC)
010735	(IP)
010737	(IS)
010741	(LH)
010745	(NB)
010753	(OK)
011017	(SR)
011021	(SS)

New ZoomTable:

004117	033703
004120	042104
004121	056505
004122	063511
004123	073513
004124	072413
004125	073133
004126	000274
004127	077514
004130	113516
004131	112416
004132	113136
004133	131720
004134	005676
004135	137122
004136	144123
004137	156326
004140	157615
004141	12670
004142	000000

Characters deleted from Zoom vocabulary:

B,C/B, C/D, C/I, C/L, M,O,C/P, V

absolute value plotting and baseline flattening. Long-term averaging (LTA) would need substantial modification to be compatible with QPD's method of handling data (although in principle QPD already incorporates many features needed for LTA). Integration is not useful for motionally restricted ^2H spectra since all resonances necessarily overlap. Baseline correction is inappropriate for spectra containing very broad lines. Absolute value data retrieval would need substantial rewriting for quadrature spectra, and absolute intensity (constant scaling) data retrieval is seldom used in practice. In principle it would be possible to use a single-sided phase table and use the space saved to write a new long-term-averaging routine--this is the only potentially useful routine omitted from QPD.

F. Parameter output

For the slower version of QPD the spectral width entered is half the quadrature width. The computer lists spectral width with the message "SPCWDT(+/-)". For the fast versions the spectral width entered is one-tenth the quadrature width and is listed with the message "SPEC WDT/10=". In the fast versions the calculated or entered acquisition time is five times larger than the actual acquisition time and is listed as "ACQTIM(S*5)". Similarly, the sensitivity enhancement (exponential weighting) must be entered as desired

number of seconds times five and so is listed as
"SENEH(S*5)". The message changes are listed in
Figure A-20.

FIGURE A-20

Patches to change teletype messages for
parameter output in QPD.

New messages:

011111	151705	"SE"	"SE NE NH(S*5) ="
011112	147305	"NE"	
011113	147310	"NH"	
011114	124323	"(S"	
011115	125265	"*5"	
011116	124675	") ="	
011117	000000	Stopper	
011135	151720	"SP"	"SPC WDT(+/-) ="
011136	141727	"CW"	
011137	142324	"DT"	
011140	124253	"(+"	
011141	127655	"/ -"	
011142	124675	") ="	
011143	000000	Stopper	
011055	140703	"AC"	"ACQ TIM(S*5) ="
011056	120324	"QT"	
011057	144715	"IM"	
011060	124323	"(S"	
011061	125265	"*5"	
011062	124675	") ="	
011063	000000	Stopper	
011135	151720	"SP"	"SPC WDT/10 ="
011136	141640	"C "	
011137	153704	"WD"	
011140	142257	"T/"	
011141	130660	"10"	
011142	120275	" ="	
011143	000000	Stopper	

FIGURE A-21

Data locations cited in QPD subroutine
program listings.

000041	000000	ADC control word
000042	000000	Memory area/channel counter
000043	000000	Add/subtract change code word
000044	004000	Quarter data table length
000045	010000	Half data table length, memory area mask
000047	000000	Memory area word
000050	060000	Add/subtract change mask
000051	000245	Add/subtract code word storage
000052	011774	First word address, first quadrant
000054	000001	Decimal 1
000056	000003	Decimal 3
000060	000005	Decimal 5
000064	000011	Decimal 9
000075	000035	Decimal 29
000103	000144	Decimal 100
000111	020000	Full data table
000134	076400	R1K, 1/1024
000174	000000	Spectral width routine temporary storage
000175	000000	Spectral width routine temporary storage
000176	000000	Spectral width routine temporary storage
000177	047040	Decimal 20,000
000200	000032	Decimal 26
000201	004040	LRLB command
000224	000000	SAVX, T+2, position in data table
000225	000000	T+3, temporary storage CSPW
000226	000000	T+4, temporary storage, BSPW
		Temporary storage, MXBT
000231	000000	T+7, temporary storage, smoothed value
000236	000000	T+12, temporary storage, cos ϕ
000237	000000	T+13, temporary storage, sin ϕ
		Temporary storage, spectral width routine
000267	000000	PHAI, initial value of phase
000270	000000	PHAF, final value of phase
000277	000000	SHFT range word
000316	000000	LOG, FT completed flag
000321	000000	DT08
000322	000000	DT04
000323	000000	DT02
000324	000000	DT01
000350	000000	WT0N, weight on flag
000360	000000	CSPW, current spectral width
000361	000000	BSPW, binary spectral width
000362	000000	RSPW, real spectral width
000363	000000	BRSW, binary real spectral width
000400	000000	NPTS, number of points
000403	000000	VTIM, timer frequency
000416	000000	NMRC, normalization counter
000420	000000	PUTM, pulse time
000455	000000	Weight flag, indicates WT called
000457	000000	NMRC storage, 90° transform
000461	000000	Data table index for PHAV
000462	015774	First word address, second quadrant
000463	025774	First word address, fourth quadrant
000467	000000	Temporary storage, PHAV
000470	000000	NMRC storage, FID
000471	000000	Number of points of smoothing
000473	000000	TABL, first word address, data table
000474	021774	First word address, third quadrant
000475	031776	First word address, sin/cos table
000656	002000	Octal 002000
001252	001000	Octal 001000
001254	000000	Temporary storage, data averaging, new MXBT, Shift Data, Smoothing

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3. VFT/16, Listing Part Number: 994100-02-C (66), Varian Associates, Palo Alto, California.
4. Spectrologic S-1401 Analog to Digital Converter, Preliminary User's Guide, Publication No. 87-300-917, Varian Associates, Palo Alto, California.

APPENDIX II. THE VARIAN 620i MINICOMPUTER

The Varian 620i is a 16 bit minicomputer with 16 kilobytes of memory, a 1.8 microsecond cycle time, and hardware multiply and divide. There are five operator-accessible registers, namely the instruction counter P, instruction register U, accumulator upper half A, accumulator lower half B, and index register X. Addition and subtraction operate on the contents of A and the effective memory address of the instruction. Multiplication operates on the total A+B and the contents of the effective memory location and gives a double precision product in A and B. Division operates on the contents of A and B taken as a double precision dividend and the contents of the effective memory location as divisor and yields quotient in B and remainder in A.

The commands in the 620i instruction set which are used in QPD may be summarized as follows (the form of each command is given in octal format along with the instruction mnemonic in capital letters and a description of the command):

A. Single word, addressing commands:

General form: Z Z Y X X X

address field a

addressing mode:

0-3 direct addressing
 5 index, add a to X
 6 index, add a to B

instruction

1. load/store instructions

01YXXX	LDA	load A from effective address
02YXXX	LDB	load B
03YXXX	LDX	load X
05YXXX	STA	store A at effective address
06YXXX	STB	store B
07YXXX	STX	store X

2. arithmetic instructions

04YXXX	INR	increment and replace
12YXXX	ADD	add contents of effective address to A
14YXXX	SUB	subtract contents of effective address from A
16YXXX	MUL	multiply
17YXXX	DIV	divide

3. logical instructions

13YXXX	ERA	exclusive or, effective address and A
15YXXX	AND	and, effective address and A

B. Single word, nonaddressing commands

1. shift commands: 004XXX

004040+n	LRLB	logical rotate left B n places
004100+n	ASRB	arithmetic shift right B n places
004200+n	ASLA	arithmetic shift left A n places
004300+n	ASRA	arithmetic shift right A n places

(arithmetic shift: sign bit retained
 logical shift: sign bit altered)

2. register change

General form: 005ZYX

Z gives operation: 0 for transfer
 1 for transfer incremented
 2 for transfer complemented
 3 for transfer decremented

Y and X give source and destination of
 transfer respectively:

1 for A register
 2 for B register
 4 for X register

Combinations of registers may be used as
 destinations, e.g., 5 for X and A register,
 6 for B and X

Examples:

005111	IAR	increment A register
005021	TBA	transfer B to A
005142	TXB+1	transfer X to B and then increment B
005326	MERG	"merge", decrement B and place this value in B and X
005000	NOP	no operation

C. Double word, addressing commands

1. Jump command: 001XXX (command word)
ZYYYYY (address word)
2. Jump and mark command: 002XXX (command word)
ZYYYYY (address word)

Jump or jump and mark to effective address if all conditions set in bits 0 to 8 are met:

bit	condition
0	overflow set
1	$A > 0$
2	$A < 0$
3	$A = 0$
4	$B = 0$
5	$X = 0$
6	sense switch 1 set
7	sense switch 2 set
8	sense switch 3 set

If $Z = 0$, effective address is 0YYYYY; if $Z = 1$, address is stored at location 0YYYYY

Examples:

location	contents	effect
001000	002010	if $A = 0$, store 001002 at location 003435, execute instructions beginning at 003436; otherwise execute instruction at 001002
001001	003435	
002000	001011	if $A = 0$ and overflow is set, next instruction at 003435, otherwise next instruction at 002002
002001	003435	
001000	001000	next instruction at 003435
001001	003435	

D. Double word non-addressing

006010	LDAI	load contents of next memory location in A
006020	LDBI	load contents of next memory location in B
006030	LDXI	load contents of next memory location in X

E. Input/Output

100XY	EXC	external control function, device YY, line X
101XY	SEN	sense device YY, line X
1025YY	CIA	clear and input to A register from device YY
1026YY	CIB	clear and input to B register from device YY
1032YY	OBR	output B register to device YY
1031YY	OAR	output A register to device YY
1033YY	OME	output contents of memory location XXXXXX to device YY
XXXXXX		

PROPOSITIONS

A. EFFECT OF PROTEIN ON ORDERING OF LIPID HYDROCARBON CHAINS STUDIED BY SAMPLE SPINNING ^2H NMR

The observation of specific lipid requirements for normal function of some intrinsic membrane proteins (1) and theories concerning lipid mediation of protein-protein interactions (2) suggest an interaction between membrane proteins and membrane lipids. It has been suggested that lipids whose hydrocarbon chains interact with the hydrophobic surface of intrinsic membrane proteins should have relatively more ordered chains than lipids in the bilayer portion of the membrane. Indeed, electron spin resonance measurements exploiting the anisotropy of the electronic Zeeman and nuclear hyperfine tensors to investigate ordering of the hydrocarbon chains may be interpreted in terms of two lipid components, one of which has an order parameter similar to that for lipids in protein-free bilayers while the other shows greater ordering of the chains (3). Quantitative examination of the data indicates that the protein perturbs the motional state of a monolayer of lipid molecules immediately adjacent to its surface, which are in slow exchange with the bulk lipid on the ESR timescale.

On the other hand results of ^2H nuclear magnetic resonance measurements on artificial bilayers containing protein have been difficult to reconcile with the ESR data. At first ^2H experiments appeared to indicate

a disordering effect of protein on neighboring lipid (4), but a closer examination revealed that this effect was due to residual cholate in the membrane system left over from the protein reconstitution (5). When cholate was carefully excluded from the system, ^2H results for protein-containing multilayers were nearly equivalent to those for protein-free multilayers. Thus the ^2H technique has failed to confirm the presence of boundary lipid.

What is the origin of this apparent discrepancy in the magnetic resonance data? The ESR data are relatively straightforward and reproducible. Thus we seek to understand the ^2H data in terms of the existence of boundary lipid. We must first reckon with the fact that the timescales of the two experiments are different. The two order parameters may sample a different set of motions (6) and the timescale for exchange will be different in the two systems. Even if there is rapid exchange of boundary lipid for bulk lipid on the ^2H NMR timescale so that a single ^2H order parameter is observed, the ratio of boundary lipid to bulk lipid is large enough that an alteration of the quadrupolar splitting due to the presence of boundary lipid is expected. Moreover, the relatively rapid motions responsible for reducing the ESR order parameter are also effective in narrowing ^2H quadrupolar splittings, so that any change in motional state which produces an ESR spectrum indicating greater order should affect the ^2H spectrum in the same way. Thus arguments

based on timescale differences cannot resolve the discrepancy.

An alternate explanation which could account for the data is as follows: Suppose that exchange between boundary lipid and bulk lipid is slow on the timescale of the ^2H experiment. Suppose further that boundary lipid has a ^2H order parameter approaching unity, so that the quadrupolar powder pattern for this component has a quadrupole splitting $\Delta\nu_Q$ of the order of 130 kHz. In order to observe this component of the spectrum without distortion or attenuation a very large H_1 field is required. Specifically, to obtain a spectral width of 150 kHz we require sufficient H_1 field to produce a 90° RF burst of no longer than 3.3 microseconds (7). Spectrometers used for experiments on protein-containing model membranes generally have a 90° burst length of 6-7 microseconds (8). On such an instrument spectral components as wide as 130 kHz will be severely attenuated relative to narrower components. Thus it is possible that the widest components in the spectrum have been overlooked.

We therefore require an improved experimental technique to obtain conclusive ^2H NMR data on protein-containing bilayers. There are several possible approaches. One is to use brute force, i.e., to build a spectrometer with a very large H_1 field. This is technically feasible but difficult, especially since ideally we wish to

reproduce the entire powder pattern of the broadest components, which is about 260 kHz wide. A second approach is to use "magic angle" sample spinning to narrow the powder pattern so that it may be observed more conveniently. This is impractical since to be effective in narrowing the spectrum the spinner would need to rotate at about a 250 kHz rate, while conventional sample spinners operate at rates on the order of 5 kHz or less.

Finally one could prepare oriented multilayers and orient them in the externally applied magnetic field so that the membrane normal is nearly at the "magic angle" with respect to H_0 and the splittings are reduced. However, this too may be difficult since the most reliable means of producing oriented samples (9) are incompatible with incorporation of protein into the bilayer.

I propose to study protein-containing model membranes by ^2H NMR using a technique for narrowing the powder pattern which was recently developed in the laboratory of Pines. When a deuterated solid sample is spun at the magic angle at a very constant rate of 1 or 2 kHz, a spin echo is produced after each rotation (10). By digitizing only a single data point at the beginning of each echo, a free induction decay is obtained which contains no contribution from the quadrupolar term of the Hamiltonian. The Fourier transform of this free induction decay is a "high resolution" spectrum of the

material, exhibiting chemical shifts without quadrupolar effects. If the sample is spun at an angle slightly different from the magic angle a narrowed quadrupolar powder pattern is obtained (11), whose breadth depends on both the motional state of the sample and the inclination of the spinner axis with respect to H_0 . By spinning protein-containing multilayers at angles slightly different from the magic angle, we may narrow the powder pattern spectrum into a spectral width range which is easily observed using an H_1 field of moderate intensity. In this fashion we can search for effects due to boundary lipid without the complication of a restricted spectral window.

The principal potential difficulty with this approach is that lipid bilayers are not really a solid system; to obtain the spin-echo it is necessary that the entire bilayer not reorient rapidly on a timescale of several kilohertz. (Motions of lipid molecules within the bilayer do not produce complete reorientation of the C-D axis.) Since a spinning rate of 60,000 rpm or more is required, a force of some 10,000 xg will be produced in a 5 mm sample, and there will be sedimentation of the multilayers toward the perimeter of the rotor. This effect produces a non-random distribution of bilayer normals, which is not critical to the experiment. More important, once an equilibrium is reached, the force on the bilayers perpendicular to the rotor axis may help

prevent overall bilayer reorientation. Thus the experiment applied to solids shows potential for bilayers as well.

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B. FLUORESCENCE MODULATION BY SURFACE COMPRESSION IN CHLOROPHYLL-LIPID MONOLAYERS

It has been proposed (1,2) that a protein-free lipid-chlorophyll array in the thylakoid membrane of chloroplast-containing photosynthetic organisms serves as a light gathering antenna. In this model light energy absorbed by chlorophyll within the array is shuttled from chlorophyll to chlorophyll via a singlet-singlet resonance energy transfer mechanism until it reaches a photosynthetic reaction center, where energy-conserving photochemical transduction occurs. Thus the energy transfer properties of chlorophyll-lipid arrays are of considerable interest.

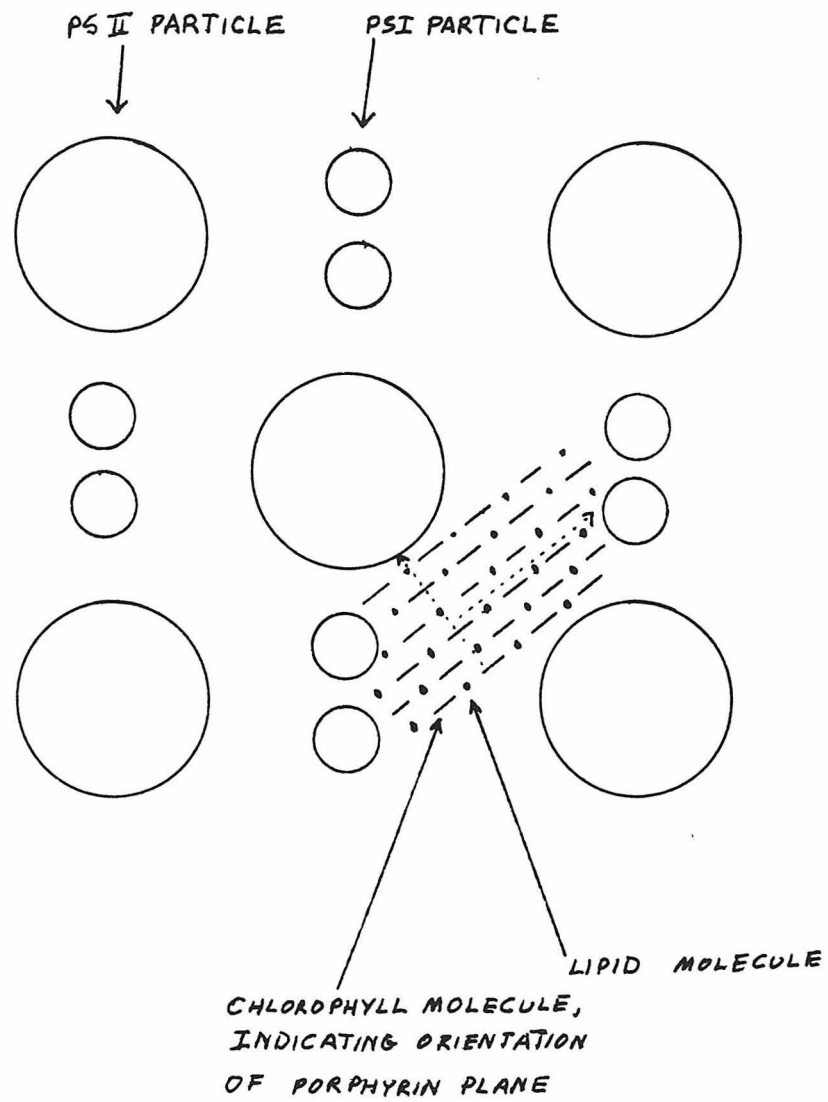
One extension of the chlorophyll-lipid antenna array model (3) serves to indicate how the specific energy transfer properties of the array could be important to the function of the photosynthetic apparatus. Higher photosynthetic organisms have two photosystems. At least some of the antenna apparatus is common to the two photosystems (1), and the proportion of light energy delivered to the two systems is variable (4). Changes in the partitioning of energy between the two photosystems are accompanied by changes in the Mg concentration within the droplet and by swelling of the grana stacks (5). Finally, freeze fracture electron microscopy of thylakoid membranes indicates a regular arrangement within the membrane of particles associated with photosystem I (PSI) and photo-

system II (PSII) (6). All of these observations may be related in terms of our lipid-chlorophyll antenna array model. Suppose we have a two-dimensional sodium chloride type lattice of lipid and chlorophyll serving as an antenna. Because of the geometry and distance dependence of resonance energy transfer (7), we expect the rate of energy transfer in this two-dimensional array to be anisotropic. Suppose further that this type of array occupies the interstitial space between PSI and PSII particles in their regular array in the thylakoid membrane. We may arrange the antenna array in such a fashion that transfer parallel to the chlorophyll planes results in energy reaching one type of photosystem, while energy transfer perpendicular to the chlorophyll plane directs energy to the other type of photosystem (Figure 1). With such an arrangement partitioning of energy between the two photosystems may be varied by controlling the relative rate of energy transfer in the two perpendicular directions in the chlorophyll-lipid array. Since the energy transfer rate depends on the sixth power of the chlorophyll-chlorophyll spacing, small changes in spacing will be effective in altering the rate. Such changes may be affected by shrinking or swelling of the grana due to uptake and loss of magnesium; this shrinking and swelling stretches the membrane preferentially along one direction due to the prolate shape of the granum (8).

I propose to use fluorescence measurements on

FIGURE 1

Chlorophyll-lipid antenna array located in interstitial space in regular array of photosystem particles. Energy transported parallel to porphyrin planes reaches primarily photosystem II particles, while energy transported perpendicular to planes reaches photosystem I.



chlorophyll-lipid monolayers to examine the effect of chlorophyll-chlorophyll spacing on energy transfer rates. Chlorophyll-lipid monolayers at an air water interface have been prepared (9). In this system the porphyrin plane of the chlorophyll projects into the hydrophilic layer with the phytol tail in the hydrophobic layer. If the chlorophyll orientation in this system is similar to that in bilayers, the transition moment Q_y for the intense chlorophyll visible absorption in the red makes an angle of about 40° with respect to the monolayer plane, the transition moment for the Q_x band lies perpendicular to Q_y and the porphyrin plane lies at an angle of 48° with respect to the monolayer plane (10,13). For present purposes we spread the monolayers in a Langmuir trough with a movable Teflon barrier across one end to allow compression of the monolayer as usual (14). We excite the chlorophyll molecules with plane polarized light whose direction of propagation is parallel to the monolayer plane and whose plane of polarization is at an angle of 40° with respect to the membrane plane. Thus we selectively excite a subset of chlorophylls which have Q_y aligned parallel to the direction of polarization. We observe fluorescence by monitoring light emitted perpendicular to the monolayer plane. We monitor depolarization of the fluorescence by detecting separately light emitted which is polarized perpendicular to the direction of propagation of the incident beam and light polarized

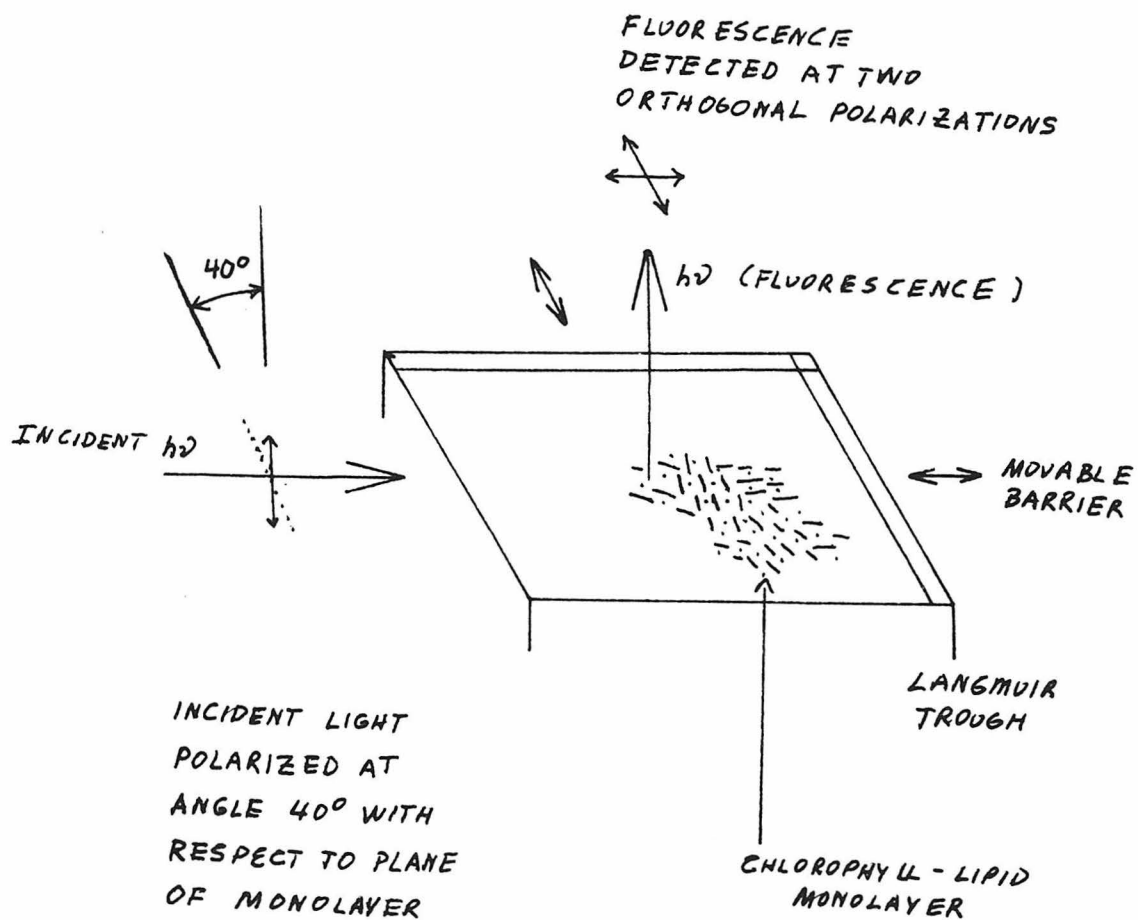
parallel this direction. To look for effects of compression of the array on fluorescence, we may alternately stretch and compress the monolayer by moving the barrier back and forth at a frequency on the order of tens of hertz. We monitor the fluorescence intensity (of either polarization) with a photomultiplier tube and detect the resulting voltage with a lock-in amplifier operating at the frequency of motion of the barrier. By this method we can isolate fluctuations in intensity due to surface pressure changes in the monolayer and greatly improve the sensitivity of the experiment. The geometry of the experiment is illustrated in Figure 2.

We may answer a number of questions using data from such an experiment:

1) Is there a surface pressure effect on the transfer rate? Such a variation should manifest itself in a change in fluorescence intensity. Three processes cause deexcitation of an individual chlorophyll in the array, namely transfer of the energy to another molecule, fluorescence, or non-radiative decay at a chlorophyll dimer non-fluorescent trap formed by close juxtaposition of two chlorophyll molecules. An increase in transfer rate implies that the energy will have a greater opportunity to reach a non-fluorescent trap since it visits a larger number of chlorophylls prior to fluorescence. Thus an increased transfer rate implies reduced fluorescence. Detection at the barrier vibration frequency should give

FIGURE 2

Geometry for fluorescence modulation experiment on monolayers. Two possible orientations of movable barrier are indicated.



a non-zero result. The magnitude of the effect may be compared with various lipids to compare the spacing and compressibility of the resultant arrays.

2) What is the domain size of chlorophyll-lipid arrays? We have assumed that chlorophyll and lipid form ordered arrays (10); in all likelihood this order is of microscopic extent. Transfer of energy between domains results in fluorescence depolarization. By making assumptions regarding array geometry and transfer rates, we may estimate domain size. Once again we may compare various lipids with respect to the extent of ordered structure which they form in association with chlorophyll.

3) Is there a difference in transfer rate parallel to Q_y and perpendicular to Q_y ? We may arrange our apparatus so that the axis along which the monolayer is compressed is either parallel to the axis of propagation of the incident light or perpendicular to this axis. Recall that the use of polarized incident light selects those domains in which Q_y lies perpendicular to the axis of propagation of incident light (10). Thus we may compress either along Q_y or perpendicular to it, and the compression should modulate preferentially one transfer rate or the other. A difference in magnitude of the modulation of fluorescence or in the amount of fluorescence depolarization indicates anisotropy in the transfer rate.

The principal difficulty in this experiment is one of sensitivity since the number of chlorophyll

molecules in the monolayer is relatively small. Optical studies of chlorophyll-containing monolayers or black lipid films have been reported; these have employed either multiple passes of the incident radiation through the film (9,14) or modulation of the angle of polarization of the incident light with subsequent phase-sensitive detection (13,15). The alternate expansion and contraction of the film serves to modulate the output of the photomultiplier tube in the present experiment, and phase sensitive detection rejects noise with an incorrect frequency or phase with respect to the barrier motion. For maximum sensitivity it would be possible to do a double modulation experiment by also rotating the polarizer for the incident beam at a second frequency and then using a double detector; such an apparatus would be highly sensitive to fluctuations in fluorescence.

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C. A NOVEL METHOD OF MOUNTING NATURAL MEMBRANES
FOR ELECTROPHYSIOLOGICAL STUDY

A variety of fundamental and important cellular processes depend on the development and control of electrical and ionic concentration gradients between the cytoplasm of living cells and the extracellular space. Integration, transmission and inhibition of nerve impulses rely on transient changes in these gradients (1). Photoreception (2) and photochemical energy transduction (3) are processes in which incident light triggers changes in ionic gradients, which in turn drive either nerve signals or energy conserving anabolic reactions. Electron transport in mitochondria is coupled to oxidative phosphorylation via generation of an electrochemical gradient between the interior of the organelle and the cytoplasm (4).

Studies of these processes rely on either electrical recordings from inside functioning cells or radioactive tracer measurements recording the flow of ions between the cytoplasm and the exterior. While tremendous progress has been made in this fashion, such experiments are fundamentally limited by the difficulty of access to the interior of the cell. This limitation is felt in at least three important ways. First, in many studies it is useful to have independent control of the ionic composition on both the inside and the outside of the

cell or organelle. This is possible only with very large cells such as the squid giant axon, where the intracellular fluid may be replaced by perfusing the cell with buffer of the experimentalist's choice. Second, electrical recording from inside the cell requires puncturing the cell membrane with one or more micro-electrodes, with the result that the cell membrane is damaged and normal cellular function slowly deteriorates. Third, proteins associated with the interior surface of the cell membrane may be important in modulating the action of intrinsic membrane proteins; these interior proteins are not readily accessible to the experimentalist. Jacobson and Branton have recently reported a method of membrane isolation which suggests a way to improve procedures for studying natural membrane fragments (5). These authors prepared polylysine-coated polyacrylamide beads 30 micrometers in diameter. Negatively charged erythrocyte cells adhered tightly to the positively charged beads, so that disruption of the cells by sonication or vigorous vortexing left portions of the erythrocyte membranes stuck to the beads with their cytoplasmic surfaces exposed. The membranes could subsequently be removed by exposure to buffers of high ionic strength. Subsequently these authors were able to describe optimum conditions to maximize the efficiency of membrane isolation (6), to show that glass beads covalently coated with polylysine could also be used

for erythrocyte membrane isolation with improved efficiency (7), and to verify that the isolation left the exterior surface of the membrane adhering to the bead with the cytoplasmic surface exposed (8).

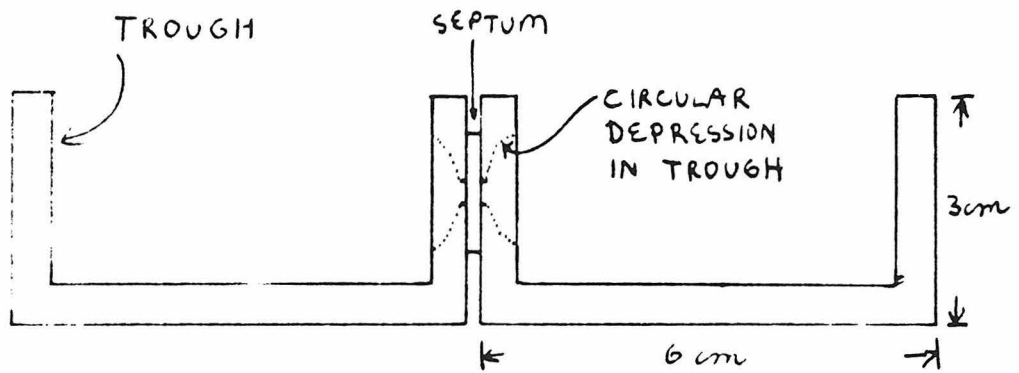
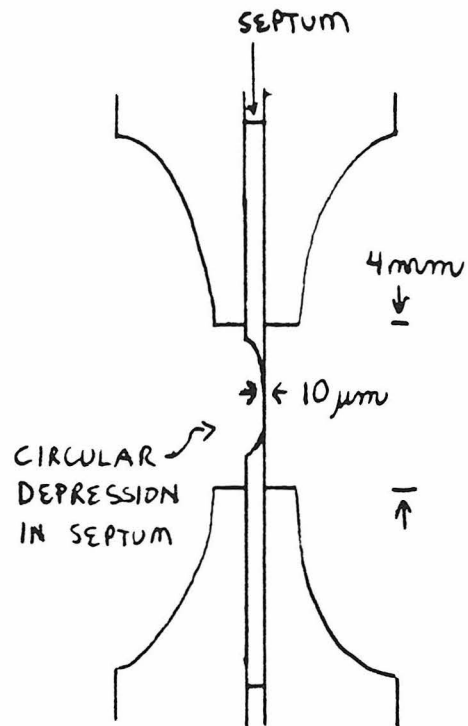
Jacobson and Branton's technique has been used to study the movement of cholesterol between the inside and outside monolayer of membranes (9) and to verify the asymmetry of lipid biosynthesis in microsomes (10).

This method for isolating cell membranes suggests a technique for mounting natural cell membranes for electrophysiological study in such a way that ready access is provided to both sides of the membrane surface and the corresponding solutions. I propose to use a modification of the Jacobson-Branton technique to mount natural membrane fragments over a small hole in a thin septum between two chambers filled with aqueous solutions. This arrangement, similar to that used with black lipid films (11) or the "bilayer lipid membranes" described by Montal and Mueller (12), provides ready access to both sides of the membrane.

Mounting of a membrane fragment may be accomplished as follows: Prepare a thin septum to be sandwiched between two Teflon chambers in an experimental setup similar to the one employed by Montal (13). The chambers contain the solution to be placed on either side of the membrane and the electrode to be used for electrophysiological measurements (Figure 1). The septum is about

FIGURE 1

Chamber for mounting membranes which are
affixed to septum by ionic interactions.



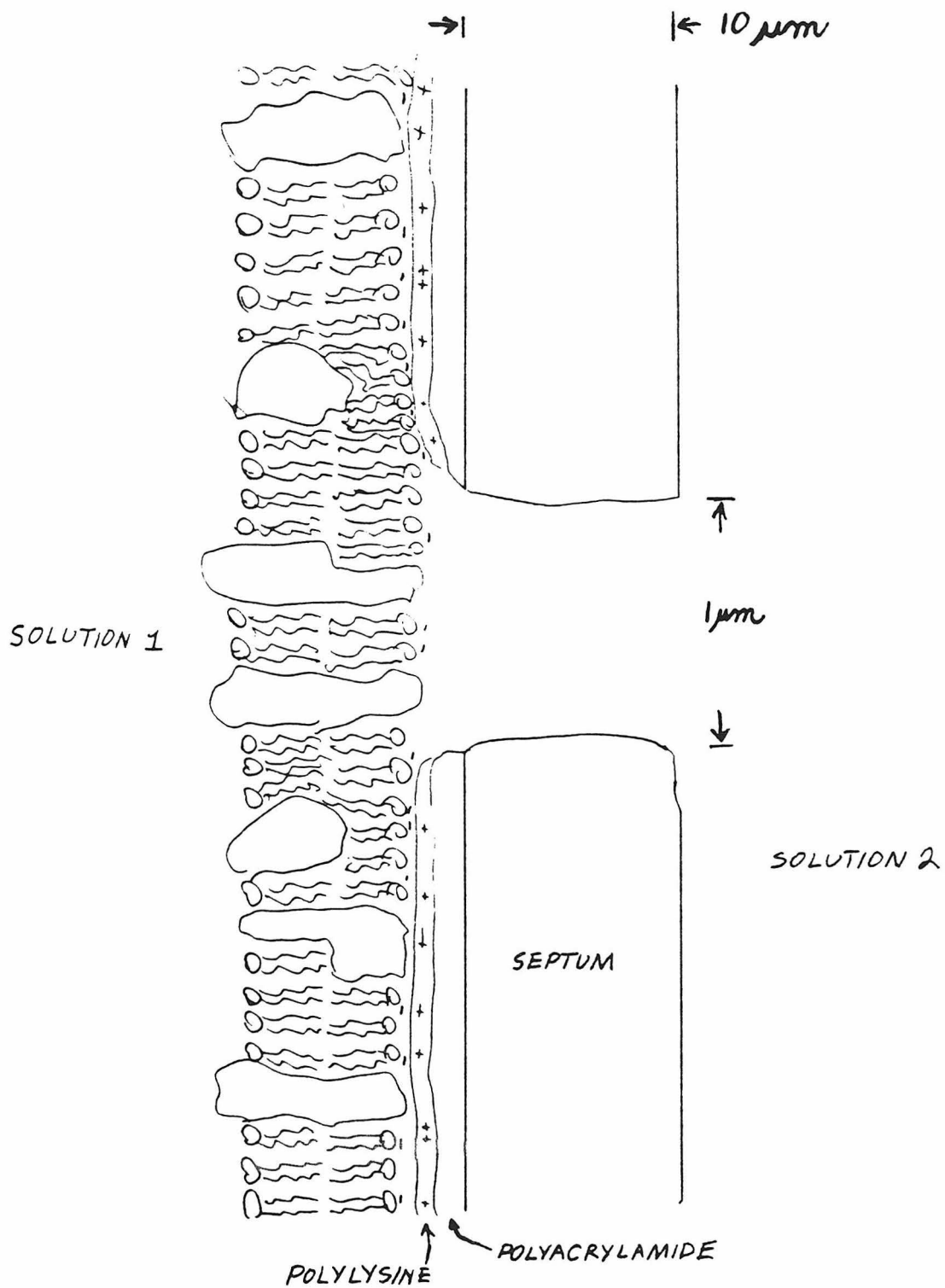
ten micrometers thick. Use a Teflon septum and coat a small circular area (radius on the order of 3 mm) with polyacrylamide. Or, make the entire septum of polyacrylamide if such coating proves impractical. The Teflon has the advantage that the technical problem of sealing the Teflon-Teflon joints between chamber and septum has been overcome. Coat the polyacrylamide with polylysine. After all of the coatings have been completed, bore a hole, using a finely focused laser, through the polylysine, the polyacrylamide and the Teflon. The diameter of the hole must be large compared to the diameter of any molecules used in the experiments but small compared to the size of the cells whose membranes we wish to study. For instance if a nascent myotube, which is on the order of several hundred microns long and maybe thirty microns wide, were to be studied, a hole of approximately one micron would be in order. Such a burning out will leave carbon fragments but these should be electrophysiologically inactive, and can be ignored after the bulk debris in the hole has been removed by washing. Next the septum is incubated with the negatively charged cells or membrane fragments to be used. (Positively charged membranes will require a negatively charged coat, e.g. polyglutamic acid.) Evidence from the electron micrographs presented by Jacobson and Branton indicates that the cells, which have a high affinity for the polylysine-coated

polyacrylamide, will be very close to one another as they adhere to the polylysine-coated surface. Then disrupt the cells by osmotic shock or by using an ultrasonicator if necessary. (Sonication in the present system might alter or destroy the portion of the membrane spanning the hole, which is not stabilized by an interaction with polylysine.) Wash the septum several times to remove any debris remaining from the cell disruption. The septum can be examined under the electron microscope to insure that the hole in the septum is completely covered by a single layer of membrane (Figure 2). The septum may then be inserted in between the two chambers to be used for electrophysiological measurements. We may place an electrode on either side of the cell membrane, we can choose any composition for the solution to be placed on either side of the membrane, and we have access to the inner surface of the membrane so that we can treat this surface in various ways, probing the role of intracellular components in the function of the membrane.

Two other types of experiments may be performed with minor modifications to the present system. First, in an attempt to measure light-induced gating currents associated with the opening and closing of ion channels, capacitative currents have been measured across rhodopsin-containing lipid monolayers adhering to a Teflon septum (14). The present approach permits us to make

FIGURE 2

Natural membrane affixed to polysine-coated septum.

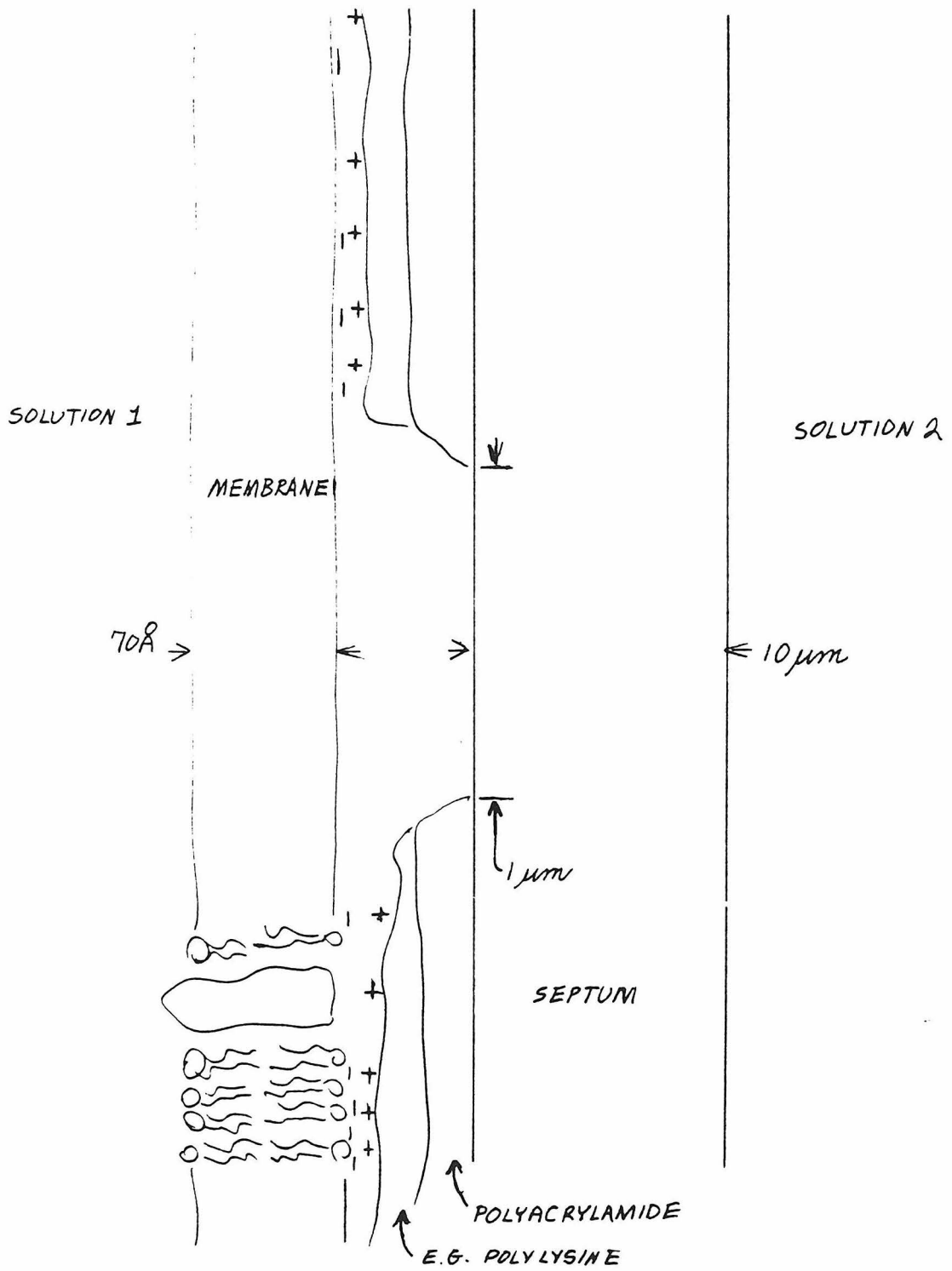


these measurements in a natural bilayer system. We begin by boring the hole in the septum through the polyacrylamide but not completely through the Teflon so that only a capacitative electrical connection between the two chambers is made. We protect (physically or chemically) a micron-size portion of the polyacrylamide so that it is not coated with polylysine. We cover the septum with membranes without boring a hole. In the region where there is no polylysine there is no membrane-septum interaction. By doing the entire septum preparation in situ we arrange to have our stimulating laser directly focused on this region without polylysine. We have thus duplicated the monolayer setup, in that we have only a capacitative connection between the two chambers (Figure 3). We can compare this experiment on a natural system with the experiment on the model system, in which the protein may not have a native conformation.

Second, the present setup might also be useful for the study of proteins reconstituted into model membranes. Large single-walled protein-containing bilayer vesicles, such as those recently reported by Montal (15) will be used in place of erythrocytes. The septum is prepared as above except that the polyacrylamide, instead of being coated with polylysine, is coated with a reagent which binds specifically the protein being incorporated into the membrane. It might be

FIGURE 3

Rhodopsin membrane affixed to septum for measurement of gating current. Only a capacitative electrical connection is made between chambers.



possible to use polylysine-coated polyacrylamide with the model membranes if a certain amount of negatively charged lipid were to be used in the bilayers. It is not clear, however, that the negatively charged lipid bound to the polylysine would result in a stable artificial bilayer membrane, so that the approach of specifically binding the protein seems to be the simplest for a first effort. Otherwise, the experiment may be carried out exactly as described above.

The biggest difficulty to overcome in this experiment as well as in the black lipid film experiments and the planar bilayer membrane experiments recently performed by Montal is the possibility that leaks may develop around the edges of the hole in which or across which the bilayer membranes have been positioned. It remains to be seen exactly how large a problem this will be in the present experiments but there is reason for confidence that this problem could be overcome. Because the size of the hole is small compared to the size of the cell membranes being studied, it is probable that the hole over which the membrane is exposed will be surrounded in all directions by an area of membrane comparable in size which is tightly fastened to the surface. The path from one compartment around the membrane, through the hole and thus into the other compartment without passing through the membrane would involve passage between the membrane and the polyacrylamide

surface over a considerable distance compared with molecular dimensions.

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D. RECEPTOR-RECEPTOR INTERACTIONS IN ACETYLCHOLINE
RECEPTOR MEMBRANES - AN APPROACH UTILIZING
MEMBRANE FUSION.

A number of observations suggest the possibility that receptor-receptor interactions may be important to the function of acetylcholine receptor in the postsynaptic membrane. First, postsynaptic membrane fragments from Torpedo electroplaque observed by freeze-fracture electron microscopy show ordered arrays of particles presumably corresponding to acetylcholine receptor (1). Since the ordered array is disfavored due to its low entropy, there must be a corresponding energy term which favors its formation, probably a term involving favorable protein-protein interactions (2). While it is possible that the ordered array merely represents a means of achieving a high receptor density (3), it is also possible that the protein-protein interactions involved are necessary to normal receptor function. In particular it is possible that an ion channel is associated not with a particular receptor molecule but with an aggregate of receptors. It has been suggested that ion channels associated with membrane receptors might be formed in the interstices between receptor molecules arranged in a regular two-dimensional lattice (4,5). Second, both Lester (6) and Stevens (7) have concluded on the basis of voltage-jump relaxation experiments that the minimum kinetic scheme for channel opening involves at least two (non-cooperative)

agonist binding steps. Such a scheme produces the sigmoid shape of the dose-response curve for acetylcholine at low dose. While the receptor does have two high affinity and two lower affinity agonist binding sites (8), the kinetic evidence does not distinguish whether the two agonist molecules bind to the same receptor molecule or to different molecules. In an interstitial model for the ion channel binding to more than one receptor might be required for channel opening, and the kinetics would be more involved due to the involvement of one receptor in more than one channel. While dose-response curves generally indicate a Hill coefficient slightly less than two (9), a Hill coefficient nearer three is found for frog endplates (10), and Stevens has suggested that a single binding occasionally opens a channel. These observations indicate that the kinetics may involve more than a simple two-step process. Third, a negative cooperativity between receptor sites has also been proposed (4). This type of cooperativity is compatible with positive cooperativity if the positive cooperativity is between subunits of a single receptor and the negative cooperativity is between receptors or if positive cooperativity prevails at low acetylcholine concentration and negative cooperativity prevails at high concentration. This concept was originally invoked to explain the existence of spare receptor since the maximal postsynaptic response of a frog endplate involves only 10% of the avail-

able ion channels (11). More recent experimental data (12,13) suggest that release of a quantum of acetylcholine activates about half of the nearby channels, and suggest that the small number of channels activated during a postsynaptic current results from a large number of inactive release sites. Such a model still leaves as much as half of the receptor at an active release site as "spare", so the concept of a negative cooperativity limiting the magnitude and/or time course of the response may still be useful.

I propose to look for effects of receptor-receptor interactions on the function of the acetylcholine receptor membrane by altering the receptor content of the membrane. The experiment involves two types of acetylcholine-containing membranes. Cultured myotubes represent a convenient preparation of cells expressing acetylcholine receptor which may be studied by electrophysiological techniques (14). Mature myotubes contain extensive patches of receptor, as well as receptor outside of patches which is approximately ten times less dense (15). Patched receptor has relatively low mobility, while both relatively mobile and relatively immobile receptor is found outside patches (16). Both receptor in patches and diffuse receptor are sensitive to acetylcholine, with some differences (17) in response. It is not known what is the extent of protein-protein order in either class of receptor.

I propose to alter the receptor composition of cultured myotubes by fusing to the myotubes acetylcholine-receptor-rich membrane vesicles called microsacs which may be isolated from electroplax (18-21). Hess and co-workers (22,23) have been able to isolate a fraction of microsacs which are evidently closed vesicles, and studies indicate that these vesicles have functional, vectorially oriented acetylcholine receptor. Hess has argued that the efficiency of the ion channel (ions passed per channel per unit time) is similar in the microsacs to that observed in the electroplex (24). In addition to the tight, right-side-out microsacs isolated by Hess, I propose to isolate a population of tight inside-out microsacs by using variations on Hess' procedure of filling the tight microsacs with cesium ion and separating them by density gradient centrifugation.

Either right-side-out or inside-out microsacs may be fused to the myotubes by adapting techniques for liposome fusion to cultured cells (25-29). Some effort and care will be required to define ideal conditions for membrane fusion. Progress of the fusion may be monitored in the light microscope, while interdiffusion of receptors can be followed by using fluorescent-labelled α -bunguotoxin to visualize receptors on the surface of the microsacs and on the surface of the myotube. Two types of perturbation to existing myotube acetylcholine receptor may result from fusion of the microsacs.

First, addition of the right-side-out receptor-rich microsacs will increase receptor concentration in the area of the myotube where fusion occurs. Thus kinetic data from electrophysiological recording may be obtained not only as a function of agonist concentration but also under conditions of varying receptor concentration. In this fashion the number of receptors involved in a channel opening may be estimated providing that variations in receptor concentration can be estimated. The jump in receptor concentration may also perturb the equilibrium between diffuse receptor and patched receptor, so that the fusion experiment will permit study of this equilibrium. Since fusion can proceed either in the area of a patch or in an area of diffuse receptor, and since the spatial resolution of electrophysiological techniques is good enough to study these areas independently, effects of fusion on the two types of receptor may be compared. Fairly simple studies, such as iontophoretic application of acetylcholine for determination of a dose-response curve, or determination of the reversal potential for postsynaptic currents, may be undertaken at first to assess the effects of fusion on receptor function.

Second, receptor function may be perturbed by fusion if the addition of new receptor disrupts receptor-receptor interactions. Since the receptors are from two different species, it may be that fusion of right-side-out microsacs, with the foreign receptor added to the

membrane in the correct vectorial orientation, will be sufficient to disturb receptor-receptor interactions by addition of a similar but incompatible receptor which competes for interprotein contacts. If the two receptors are sufficiently similar that no perturbations results, fusion of inside-out microsacs, with the added receptor vectorially oriented antiparallel to the existing receptor, will assure that the added receptor does not function in a fashion equivalent to existing receptor. Inside-out receptor may interdiffuse but will not be able to participate in specific receptor-receptor interactions with existing receptor. Finally, it is possible that regardless of the orientation of the new receptor the two populations of receptor will not interact. At any rate, monitoring of the effects of changes in receptor-receptor interactions should begin with relatively simple electrophysiological characterizations, correlated if possible with the extent of receptor interdiffusion. In particular, the dose-response curve should be determined at both small and large agonist concentrations to look for effects on the apparent positive cooperativity and postulated negative cooperativity.

While we have described in general terms the considerations which apply to such a fusion experiment and indicated several possible approaches and outcomes, it is not possible to indicate in advance the detailed interpretations of the experimental data since the

number of possible outcomes is large. Nonetheless, the suggested experiments provide a new and general approach to the study of receptor-receptor interactions and effects of receptor concentrations by perturbation of the receptor population in vivo.

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E. CONFORMATIONAL CHANGES IN SUGAR BINDING PROTEINS OF BACTERIA STUDIED BY CONCENTRATION CORRELATION SPECTROSCOPY.

Sugar binding proteins are readily isolated in milligram quantities from the osmotic shock fluid of bacteria (1-3). They are thought to be the primary receptor proteins for sugar transport and chemotaxis (4,5). The galactose binding protein isolated from Escherichia coli and galactose and ribose binding proteins from Salmonella typhimurium have molecular weights on the order of 30,000 Daltons and bind substrate in a 1:1 mole ratio with apparent dissociation constants on the order of 1 micromolar (2,3,6,7). They are soluble periplasmic proteins of known amino acid composition (6,8). The amino terminal amino acid sequences of the Salmonella proteins show a small but statistically significant number of homologies. In spite of the limited sequence similarity in this region the two proteins, when bound to substrate, have been shown to compete for the same binding site at the (hypothetical) first component of the sensory signalling system (9). This observation indicates that the two sugars share a "focused pathway" in the sensory system of bacterial chemotaxis. Such a mechanism implies that upon substrate binding each protein undergoes a conformational change which permits it to bind the first sensory component.

Evidence for such a conformational change was first obtained for the E. coli galactose binding protein by

Boos and co-workers (10,11) who found heterogeneous substrate binding behavior with two apparent K_D s, a change in the electrophoretic mobility of the protein in the presence of saturating concentrations of galactose, and an increase in the fluorescence emitted in the 310-350 nm region (290 nm excitation) in the presence of substrate. Changes in UV absorbance upon binding, along with the fluorescence data, suggested that the substrate interacts with a specific tryptophan residue. Alterations in fluorescence quenching by KI upon binding were attributed to a change in surface charge accompanying binding, also reflected in the altered electrophoretic mobility. Partly because changes in ORD, CD or IR spectra on binding were not observed, Boos et. al. suggested that the conformational change was confined to the active site. Koshland et. al. have used similar methods to discover evidence for a conformational change upon substrate binding in the Salmonella galactose and ribose receptors (3,12). Again fluorescence changes imply a conformational change at the lone tryptophan of the galactose receptor. An extrinsic fluorophore, 5-iodoacetamidofluorescein, attached to a methionine and shown by resonance energy transfer methods to be at least 30 Ångstroms distant from the tryptophan also showed fluorescence changes, indicating that the conformational change was delocalized over at least 30 Ångstroms. In the ribose receptor CD changes in the near UV upon binding suggested alterations in the

environment of at least one phenylalanine and one tyrosine. The ribose receptor contains no tryptophan, but once again the extrinsic fluorophore attached to a methionine showed fluorescence changes when substrate was bound. Since at least three residues report the conformational change, Koshland et. al. concluded that it is delocalized in this molecule as well.

I propose to study the kinetics of binding and conformational changes in these molecules via concentration correlation spectroscopy (13). Concentration correlation spectroscopy is a generic term applied to techniques which exploit the fact that fluctuations about the equilibrium value in the number of molecules of a particular solute in a specified volume relax toward the equilibrium value according to the same processes and rate parameters which govern relaxation from a non-equilibrium state produced by a gross macroscopic perturbation. By determining the correlation function of the fluctuation in occupation number of the various species and interpreting the data in terms of a mechanism for the fluctuations, rate constants for the various processes may be estimated. The advantage of such an approach is that the system is observed at equilibrium, without the need for gross perturbations which may obscure the phenomena of interest. The difficulty in such experiments is that in order to produce resolvable fluctuations in whatever observable

is used to monitor occupation numbers the number of molecules in the volume of interest must be severely restricted; thus the observable must be quite sensitive. Commonly observed quantities are fluorescence, light-scattering, conductance of an ionic solution and polarized light-scattering or fluorescence. If an open system is used, in which diffusion in and out of the volume of interest is permitted, diffusion coefficients may be determined by this method (14,15). If in addition to diffusion chemical reactions can occur which affect occupation numbers, kinetic parameters can also be obtained. In an open system reaction kinetics data and diffusion constants may be estimated simultaneously, although the kinetic analysis is more complicated than for a closed system where diffusion need not be considered. Two simple cases for an open system, namely a unimolecular reaction and binding of a small molecule to a large one, have been worked out in detail (16).

Elson and Webb (13) have suggested that concentration correlation spectroscopy might be profitably applied to the study of conformational changes in proteins. The sugar binding proteins of bacteria may be investigated in such a fashion. The proteins are readily available, fairly well characterized biochemically, and known to undergo conformational changes. These conformational changes are known to be accompanied by changes in the fluorescence of both intrinsic and extrinsic fluorophores.

In addition, the galactose binding protein of E. coli undergoes a change in surface charge upon binding. Thus both fluorescence correlation spectroscopy and correlation utilizing fluctuations in conductance may be undertaken. The kinetic schemes necessary for a preliminary analysis of correlation data from these systems are precisely the simple ones originally worked out in the literature. The original apparatus presented by Elson and Webb required a fluorophore which could be excited below 500 nm and observed above 500 nm; this criterion is met by the fluorophore used by Koshland et. al. Thus this system has many advantages for such a study.

Specifically we may monitor fluorescence from tryptophan of E. coli galactose receptor and Salmonella galactose receptor, fluorescence of the extrinsic fluorophore 5-iodoacetamidofluorescein attached to methionine of the Salmonella galactose and ribose receptors and conductance of solutions containing the Salmonella ribose receptor in the presence of ribose. The conductance measurements are made in an essentially closed system (17,18). A closed system may also be used for the fluorescence measurements if the photostability of the fluorophore permits this approach (13). Kinetic parameters from such an experiment can help to answer the following questions:

(i) What are the kinetic parameters for binding to the

receptors?

(ii) Is there a difference between kinetic parameters for the two fluorescent probes on Salmonella galactose receptor? (Can we resolve the kinetics of binding from the kinetics of the conformational change?)

(iii) Do the simple kinetic schemes fit the data, or are there additional steps in the process of the conformational change which are resolvable by this method?

(iv) Do the conductance and fluorescence probes for the E. coli galactose receptor give consistent results?

(v) Are data similar for the three molecules? In particular, do the data support a local conformational change in E. coli galactose receptor and a delocalized change in the Salmonella receptors, as the literature indicates?

Thus this approach permits us to investigate the receptors individually, to compare them to each other, and to compare the results of various correlation probes on the same molecule. With such data we may reach more detailed conclusions concerning the nature of the conformational change while expanding the range of application of a relatively new spectroscopic tool.

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