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FLUIDITY AND LIPID-PROTEIN INTERACTIONS
IN THE CHLOROPLAST MEMBRANES
: A MAGNETIC RESONANCE STUDY

by

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A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy of Science

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Carleton University
Ottawa, Ontario
April 29, 1980

The undersigned hereby recommend to the Faculty
of Graduate Studies acceptance of this thesis, submitted
by Rakesh Chander YashRoy, M. Sc. Hons., in partial
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ABSTRACT

Membranes from spinach chloroplasts, and model membranes of their lipid-extract, have been studied by spin label electron spin resonance (ESR) and carbon-13 nuclear magnetic resonance (C-13 NMR) techniques. Experiments were designed to examine the fluidity of lipids in these membranes under the influence of temperature and light, as well as manifestations of lipid-protein interaction.

A fluidity gradient was observed in both the natural and model membranes in terms of increasing rates and amplitudes of motions of the fatty-acyl segments from the lipid headgroup to the terminal methyl group. Both ESR spectral characteristics of the spin-labelled membranes (with a nitroxide) label at the 5th, 7th, 9th, 12th, 13th, 14th, or 16th carbon from the carboxyl group of stearic acid) and the C-13 NMR spectral line-widths reveal a fluidity or flexibility gradient in the chloroplast membranes and aqueous dispersions of their lipid-extract. This gradient is similar to that observed for lipid-bilayer structures in

general.

Multilamellar (liposomal) structures were observed when negatively-stained aqueous dispersions of the lipid-extract of the chloroplast membranes were examined by electron microscopy. Upon sonication unilamellar vesicles (diameter 250 to 750 Angstroms) were formed.

A procedure was devised for C-13 enrichment of the chloroplast membranes. This minimizes the complexity of the NMR spectra which may arise from C-13 -- C-13 couplings and yet enhances the sensitivity considerably so as to reduce the possible membrane degeneration during the collection time of the spectrum.

The assignments of C-13 resonances of the fatty-acyl chains in the chloroplast membranes were facilitated greatly in view of the marked abundance of linolenic acid in these membranes.

Increase in temperature of the samples resulted in an overall increase in their lipid fluidity as revealed both by ESR and NMR studies. ESR studies of the chloroplast membranes labelled with 5-doxyl stearic acid and 8-doxyl phosphatidyl choline showed a behaviour suggestive of a gel-liquid crystal phase transition between 15 and 20

deg. C. With 16-doxyl stearic acid as the spin label, this transition was observed around 14 deg. C.

Illumination of the spin-labelled chloroplast membranes did not alter their fluidity. However, light caused an initial transitory oxidation, followed by a rather continuous reduction of the spin label incorporated in these membranes. On lowering the temperature from 37 to 5 deg. C, the initial photo-oxidation process was appreciably slowed down for 5 and 16-doxyl spin labels.

Lipid-protein interactions have been studied by comparing the fluidity of the chloroplast membranes with that of the model membranes made from their lipid extract. With the above-mentioned stearic acid spin probes, the ESR studies revealed that the chloroplast membranes are considerably more ordered than the model lipid membranes. The fatty-acyl segmental motions seemed somewhat slower in the former. These studies suggest that the spin labels used in the present study detect mainly the lipid seemingly 'entrapped' among the intramembraneous proteins.

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*In all thy ways acknowledge Him
and He shall direct thy paths*

The Bible

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LIST OF ABBREVIATIONS

C-13	Carbon-13
CDCl_3	Deuterated chloroform
CD_3OD	Deuterated methanol
D_2O	Deuterated water
DGDG	Digalactosyl diglyceride
EM	Electron microscope
ESR	Electron spin resonance
g	Gauche
G	Gauss
GHz	Gigahertz
MGDG	Monogalactosyl diglyceride
MHz	Megahertz
NMR	Nuclear magnetic resonance
PC	Phosphatidyl choline
PG	Phosphatidyl glycerol
PI	Phosphatidyl inositol
PL	Phospholipid
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
SASL	Stearic acid spin label
SL	Sulpholipid
TGDG	Trigalactosyl diglyceride

His ways became my convictions.
Dedicated to my father, late
Shri Patanjali Prasad.

I N T R O D U C T I O N

This thesis deals primarily with studies on the dynamic state of lipids in the chloroplast membranes and the influence of light, temperature, and the membrane proteins on it. Therefore, the review of literature in the INTRODUCTION will be confined mainly to the membrane lipids with emphasis on the chloroplast membranes. A general reference to the membrane proteins will be made only where necessary (for example, while introducing or discussing the lipid-protein interactions).

I. THE CHLOROPLAST MEMBRANES

In the eukaryotic plant cells, the photosynthetic membranes form a distinct cellular organelle, termed the chloroplast. Figure 1 shows the general arrangement of the chloroplast membranes. On the right hand side, it shows how these membranes appear under the electron microscope and to the left is a diagrammatic representation of their arrangement. There is an outer double membrane, called the chloroplast envelope, and

Figure 1

Arrangement of membranes in the chloroplast.
The right hand side shows their appearance
under an electron microscope, and the left
hand side represents a diagrammatic view.

internal membranes designated the stromal and the granal lamellae. Each granum consists of stacks of flattened membrane disks, the thylakoids.

I-A. LIPIDS OF CHLOROPLAST MEMBRANES

In general, the overall lipid composition of the chloroplast membranes includes the chlorophylls, carotenoids, sterols, quinones, phospholipids, and glycolipids (Benson, 1971). Thus the chloroplast membranes have an unusual lipid composition (see Table 1) when compared with the other cellular membranes in the biological world. The most common phospholipids in biological membranes (for example, see Table 2 for the lipid composition of the mammalian liver subcellular membranes) constitute only a minor fraction (about 10% of the total lipid) of the lipids in the chloroplast membrane-lipids.

In the spinach chloroplast lamellae the glycolipids constitute about four-fifths of the total lipid. (Wintermans, 1960). Of these glycolipids, 90% are the monogalactosyl diglycerides (MGDG) and digalactosyl diglycerides (DGDG) while the sulpholipid (SL) makes up the

Table 1

Composition of spinach chloroplast lamellar lipids

Kind of Molecules	Relative Number per quanto- some (Park & Biggins, 1967)	Weight % of lipid extract (Kates, et al., 1970)
PIGMENTS		
Chlorophyll a	160	21
Chlorophyll b	70	
Carotenoids	48	3
LIPIDS		
Monogalactosyl diglyceride (MGDG)	346	22
Digalactosyl diglyceride (DGDG)	114	15
Phospholipids (PL)*	116	10
Sulphoquinovosyl diglyceride	48	7

* These phospholipids consist of:
 phosphatidyl glycerol (PG)
 phosphatidyl inositol (PI)
 phosphatidyl choline (PC)

Table 2
SUMMARY OF LIPID PROFILES OF ISOLATED SUBCELLULAR
MEMBRANE STRUCTURES OF MAMMALIAN LIVER*
(From van Hoeben and Emmelot, 1972)

Lipid class	Plasma mem- branes	Lysos- omal mem- branes	Nuclear mem- branes	Endo- plasmic reticulum mem- branes	Mito- chondrial mem- branes	Golgi mem- branes
Phosphatidylcholine	18.5	23.0	44.0	48.0	37.5	24.5
Sphingomyelin	12.0	23.0	3.0	5.0	0.0	6.5
Phosphatidylethanolamine	11.5	12.5	16.5	19.0	28.5	9.0
Phosphatidylserine	7.0	6.0	3.5	4.0	0.0	2.5
Phosphatidylinositol	3.0		6.0	7.5	2.5	5.0
Lysophosphatidylcholine	2.5	0.0	1.0	1.5	0.0	3.0
Cardiolipin	0.0	5.0†	1.0	0.0	14.0	0.0
Other phospholipids	2.5†	§	§	§	§	3.5§
Cholesterol	19.5	14.0	10.0	5.5	2.5	7.5
Cholesteryl esters	2.5	8.0	1.0	1.0	9.0	4.5
Free fatty acids	6.0	§	9.0	3.5		18.0
Triglycerides	7.0	2.5	4.0	5.0	6.0	16.0
Other neutral lipids	8.0	6.0	1.5	§		§
Glycolipids		§	§	§	0.0	§

* Values are expressed as weight percentages of total lipids and are average data rounded off to 0.5%.

† Includes phosphatidic acid and lyso compounds other than lysophosphatidylcholine.

‡ "Solvent front", i.e. cardiolipin plus phosphatidic acid.

§ Not mentioned or not present.

|| Mono- and diglycerides.

¶ Lysophosphatidylethanolamine.

Figure 2

Schematic cross section of a thylakoid showing an intrinsic protein extending across the membrane. It is proposed that the light-harvesting chlorophyll molecules are part of the boundary lipids of their specific intrinsic proteins. Additional lipid molecules would be required to complete the layer of boundary lipids surrounding the intrinsic protein (Anderson, 1975).

rest (Benson, 1971). Phosphatidylglycerol (PG), the principal phospholipid (PL) of the chloroplast membranes, and the SL, are concentrated in the lamellae rather than in the envelope membrane (Harwood and Stumpf, 1976; Kates and Marshall, 1975). It has been speculated that the SL may play a role as a fixed boundary lipid -- relatively immobilized lipid molecules (Figure 2) surrounding the intramembraneous proteins -- in the chloroplast lamellae (Anderson, 1975; Harwood and Nicholls, 1979).

The chlorophylls, though extractable with lipid solvents, are also presumably associated with the membrane proteins (Benson, 1971; Ogawa et al., 1966; Kan and Thornberger, 1976; Argyroudi-Akoyunoglou and Castorinis, 1980). The precise organization of chlorophyll in the chloroplast membranes still remains to be known. Poincelot (1976) found that the average amount of lipid in the spinach chloroplasts is 4.3 microgram per microgram of chlorophyll. He also found that the lipids of spinach chloroplast envelope membranes are not qualitatively different from those of the lamellae; stripping off the envelope results only in loss of 17% of the lipid.

Abundance of Poly-cis-unsaturated Fatty Acids:

Table 3 shows the fatty acid composition of spinach chloroplast lamellae. Most abundant of these is linolenic acid, an 18-carbon fatty acid with three double bonds (designated 18:3) in the cis conformation, at 9-10, 12-13 and 15-16 carbons. It has been noted that the cis double bonds (see Figure 3), due to their restricted rotation, tend to create a structural disorder in the membranes containing such unsaturated fatty-acyl chains in the lipids (Quinn and Williams, 1978; Seelig and Waespe-Sarcevic, 1978; Eletér and Keith, 1972; King and Spector, 1978).

II-A. DYNAMIC STUDIES OF MEMBRANE-LIPIDS

A simple idea that needs to be appreciated is that a particular organization of a group of molecules allows different degrees of motional freedom to various segments of these molecules. For instance, a measure of the membrane-lipid segmental motions by magnetic resonance spectroscopy, would yield information about their lipid organization and

Figure 3

Structure of fatty acyl residues associated with plant polar lipids.

(After Quinn and Williams, 1978)

Table 3

Major fatty acids of spinach chloroplast lamellar lipids Allen et al., (1966)

Lipid	18:3*	18:2	18:1	18:0	16:3	16:2	16:1	16:0	14:0
MGDG	72	2	-	-	25	-	-	-	-
DGDG	87	2	2	-	5	-	-	3	-
TGDG**	70	1	1	-	15	1	-	9	1
PG	47	4	2	-	2	-	32	11	1
SL	52	6	-	-	-	-	-	39	-
Lecithin ₃	58	16	9	-	4	-	-	12	-
PI	27	15	7	2	3	-	-	34	4

* Digit after the colon represents the number of double bonds in cis (except) in 16:1 of PG, which is in trans conformation.

** Trigalactosyl diglyceride (other abbreviations as in Table 1).

possibly, lipid interactions with other membrane components, such as the proteins. A brief introduction to these magnetic resonance techniques -- nuclear magnetic resonance (NMR) of carbon-13 and electron spin resonance (ESR) of nitroxide spin-labels -- is given here.

II-A. MAGNETIC RESONANCE IN BIOMEMBRANES

1. General Principles of Magnetic Resonance:

The phenomenon of magnetic resonance can be observed only in those systems which have a net magnetic moment. Nuclei with an odd mass number have magnetic moment(s), as do atoms and molecules containing an odd number of electrons. Much like a bar magnet in an external magnetic field, such electron or nuclear dipoles tend to align themselves along or against the external magnetic field, B_0 . The alignment along B_0 has lower energy than that against it. A change in this alignment from the lower to the higher energy level requires the absorption of a quantum of electromagnetic radiation of energy equal to the

difference between the energy levels. This condition is termed 'resonance absorption' or magnetic resonance.

The phenomenon of magnetic resonance is due to the spin of the electron or the nucleus -- hence the term electron spin resonance (ESR) or nuclear magnetic resonance (NMR). Electrons have a spin quantum number, S , equal to $\pm\frac{1}{2}$. Nuclei have a spin quantum number, I , equal to or greater than $\frac{1}{2}$.

The spin-system has a magnetic moment as well as an angular momentum. As a result, the electron or the nuclear magnetic moment vectors, designated μ , precess around B_0 (much like the precession of the spinning gyroscopic-top in the earth's gravitational field). Reorientation of the precessing spin (electron or nucleus, as the case may be) with respect to B_0 can be brought about by causing the spin to interact with a second (fluctuating) magnetic field, B which is applied at right angles to B_0 and which is of the same frequency and phase as the precessional motion of the spin. Under these conditions, the precessing spin now changes its angle of precession relative to B_0 ; if it is caused to tilt away from

alignment with B_0 , energy, E is absorbed (magnetic resonance condition in classical description), given as

$$E = u \cdot B_0$$

that is the product of B_0 and the projection of u along B_0 .

According to quantum physics, for a spin with I or S equal to $\pm \frac{1}{2}$ (under consideration here), only two precessional orientations are possible (given by the expression, $2S + 1$ or $2I + 1$). One of these has a component of u parallel to B_0 , and the other antiparallel to it. Quantum energy of $h\nu_0$ (where h is the Planck's constant and ν_0 is the frequency of the electromagnetic radiation absorbed, resulting in the change in the direction of precession of the spin. In quantum mechanics, the magnetic moment u of an electron $u(e)$ or a nucleus $u(n)$ is related to its spin by the relations:

$$u(e) = -g_e M_e S \text{ (for electron) and}$$

$$u(n) = g_n M_n I \text{ (for nucleus)}$$

where, M_e and M_n are the Bohr and the nuclear magnetons, and g_e and g_n are the proportionality constants called electron and nuclear g -values,

respectively.

The negative sign in the expression for the electron shows that the electron-spin and its magnetic moment, μ are in the opposite directions, whereas they are in the same direction for the nucleus. However, this is of little practical significance because in magnetic resonance spectroscopy, one measures the difference between the energy levels. The dependence of μ on the mass of the spinning particle can be realized from the expressions for the Bohr and the nuclear magnetons:

$$\mu_e = eh/4\pi mc \quad \text{and} \quad \mu_n = eh/4\pi m'c$$

where e is the charge of the electron (same charge for the C-13 nucleus under consideration here), c is the speed of light, m is the mass of electron and m' is the mass of the nucleus.

On account of a large difference in masses of the electron and the nuclei (even the simplest nucleus, a proton, of hydrogen has a mass 1836 times that of an electron) $\mu(e)$ is much larger than $\mu(n)$. For example, $\mu(e)$ is about 2000 times larger than nuclear magnetic moments for a free proton. This is the reason why ESR is observed in the gigahertz (GHz) (microwave) but NMR in the

megahertz (MHz) (radiofrequency) regions of the electromagnetic spectrum.

The resonance absorption, in quantum mechanical terms can therefore be given (by combining $E = -u B_0$ and $u = -gMS$) as:

$\Delta E = h\nu = gMB_0$ where M is designated Bohr or nuclear magneton, which ever is applicable.

The g -value specifies the position of the spectral absorption and is characteristic of a particular magnetic species. The position of the absorption (on the frequency or magnetic field scale) also depends on the molecular environment of the magnetic species observed, though in a rather different way for NMR than for ESR. For example, $u(e)$ depends not only on the electron spin, but also, to a certain degree, on its orbital motion in the atom. Dependence of this orbital contribution on the neighbouring bonded atoms renders the electron g -value sensitive to the molecular environment.

The position of the NMR resonance of a particular nuclear species in a certain chemical environment is determined by the modified magnetic field (due to the shielding effect of the

electron-cloud around the nucleus in question) seen by it. This modification by the chemical environment of the nucleus is called the chemical shift (with respect to a standard) which will be discussed later.

The magnetic resonance spectroscopy is not a single-spin process as it appeared from the discussion heretofore (see Figure 4A). It is actually a population phenomenon which depends upon the relative number of spins in the high (N) and low (n) energy states determined by the average thermal energy kT , and the energy gap, $gMBo$ between the energy levels, (Figure 4B), according to the Boltzman distribution:

$$N/n = \exp (-gMBo/kT)$$

where k is the Boltzman constant and T is the absolute temperature.

For the resonance absorption to occur, there must be more spins in the lower than in the higher energy state. In practice, the energy gap $gMBo$, is so small that the ratio N/n is almost unity at room temperature, especially for NMR. When the populations in these two levels are equal, no signal can be seen (saturation); this is usually the case when the radiofrequency (rf) power used

Figure 4

Figure 4A Energy-level splitting and magnetic resonance transition for an electron or nucleus, of a spin of one-half, in a magnetic field

Figure 4B Thermal populations of energy levels and spin-lattice relaxation from the excited energy level

is high. Thus, the system must restore the thermal equilibrium before re-excitation. The time required, T_1 , is determined by the so-called spin-lattice (or longitudinal) relaxation process. In the T_1 process, the spins lose their energy to the lattice.

There is another relaxation process called the spin-spin (or transverse) relaxation, in which the spins of the same species exchange their energy resulting in the loss of phase coherence of magnetization in the transverse plane. This relaxation time is designated T_2 . The T_1 process causes line-broadening and disappearance of the signal if the relaxation time (T_1) is too short, or saturation at high power if it is too long. On the other hand, T_2 normally determines the linewidths. When the lineshape is Lorentzian, the condition that holds approximately, for the half line-width, and T_2 is:

$$\text{half line-width} = 1/\pi T_2$$

This relationship is useful as the line-width measurements are indicative of the mobility of the spin. The smaller the line-width, the greater the mobility.

2. ESR of the Spin-labelled Membranes:

The first paper on the spin-label method appeared in 1965 from the McConnell group (Stone et al., 1965). According to the foregoing discussion, one electron should give only one resonance due to the (Zeeman) splitting of its spin angular momentum in the external magnetic field. In practice, however, a three-line spectrum is observed (see Figure 5A) due to the (hyperfine) interactions of the electron spin with the magnetic moment of (N-14) nucleus of the nitroxide moiety in the spin-labelled (fatty acid) molecule (see Figure 6). Qualitatively, the magnetic moment of the N-14 can be aligned parallel, antiparallel, or perpendicular to the $u(e)$. This is because N-14 has the nuclear spin, I equal to unity, which would split the Zeeman line into $2I + 1$, that is 3 lines (corresponding to 1, 0 and -1 states) of equal intensity separated by the so-called coupling constant.

An immobilized spin label embedded in a crystal oriented in a magnetic field would show the maximum (limiting) hyperfine splittings: separated by T_{zz} of 32 Gauss (G) when B_0 is

Figure 5

Figure 5A. The ESR experiment for a nitroxide spin label. The separations between the resonance lines yield the hyperfine splittings. Note the changes in the line-shape and splittings when the spin label is incorporated in a membrane system.

Figure 5B. A diagrammatic representation of the alignment of the spin label (s) in a lipid (l) bilayer (membrane) system.

parallel to the z-axis that contains a p_z (or $2p_\pi$) orbital (confined largely to the N atom), occupied by the unpaired electron; by $T_{xx} = T_{yy} = 6G$ when B_0 is directed along the x- or the y-axis as shown in Figure 6.

When intercalated in a lipid membrane (see Figure 5B), the spin-label spectra (see lower spectrum, not to scale, in Figure 5A), show changes in splittings, signal - intensity distribution, and the line-shapes. The positions and the amplitudes of the outer lines (arrows) are affected by the anisotropically restricted molecular motion of the nitroxide label. Decrease in this splitting (designated $2T_{max}$) with increasing amplitude of molecular motion is due to the increased extent of the limited motional averaging of the z and x components (shown in Figure 6).

For rapid motional averaging within restricted volumes, the order parameter, S , has been used to describe the degree of motional constraints as:

$$S = (T_{max} - T_{min}) / (T_{zz} - T_{xx})$$

where T_{max} and T_{min} are the observed hyperfine splittings as shown in Figure 5A. This is simply

Figure 6. Maximum (limiting) splittings of the nitroxide spin label.

2

the ratio of the observed hyperfine anisotropy to 25G, the maximum theoretically obtainable $T_{zz}-T_{xx}$ from the rigid crystal orientation. Obviously, S is equal to 1 for perfect order, and S is equal to 0 for 'isotropic' molecular motion. For the intermediate cases, S is directly related to the angular amplitude of the anisotropic molecular motion given as:

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

where β is the instantaneous angle between the molecule and the director-axis (normal to the bilayer surface). The angular brackets represent a 'time' and ensemble average over the molecular motion.

In a critical review, Smith and co-workers (Schreier et al., 1978b), describe the order parameter, S as a measure of the distribution of molecular orientations relative to some reference axis (the director). S is then defined as the ensemble average over all molecules. They caution that "the derivation of the order parameter does not explicitly involve the rate(s) of the motion of the spin probes, but assumes that motions within the restricted space are fast on the EPR

time scale'' i.e. the rotational correlation time (time for rotation through one radian) $\tau < 10^{-9}$ sec.

When a lipoidal substance like a fatty acid or a lipid (or other lipid-soluble materials), with covalently-bound spin label, protected sterically against chemical reactions, is allowed to come in contact with a membrane system, the spin-labelled molecules diffuse into the lipid part of the membrane and, presumably, orient themselves along with the membrane lipids (see Figure 5B). With small amounts (up to about 1% of the membrane lipid molecules) used, almost all the spin labelled molecules seem to be dissolved into the membrane lipid. ESR spectra of such labelled membranes therefore give information about the motional properties (dynamic state) of the membrane lipids. The technique has had a considerable success in elucidating the amplitudes and rates of motion of spin-labelled components in model and biological membranes (Cannon et al., 1975).

3. C-13 NMR of Membranes:

The chemical basis of nuclear magnetic resonance (NMR) can be understood in terms of the differential shielding from the applied field, B_0 by the various chemical groups of the nucleus observed. This shielding is due to the changed (local) field (B_{loc}) actually seen by the nucleus, as a result magnetic fields induced in the opposite direction (B_{opp}) by the electrons circulating around the nucleus (see Figure 7A). Thus, the net magnetic field in different parts of the molecule is expressed in terms of the shielding factor or screening constant (σ) as:

$$B_{loc} = B_0 (1 - \sigma)$$

One consequence of the shielding effect is that the energies of the NMR-transitions are altered according to

$$h\nu_0 = \gamma (h/2\pi) B_0 (1 - \sigma)$$

or

$$\nu_0 = 1/2\pi \cdot \gamma B_0 (1 - \sigma)$$

where γ is the gyromagnetic ratio and is proportional to the ratio of charge to mass of the nucleus in question. It is difficult, and not even necessary to perform the absolute

measurements of frequency. Instead, one can easily relate the frequency and the shielding factor to a standard reference (usually tetramethylsilane, TMS). The range of chemical shift (the shift in resonance position with respect to the reference) in parts per million (ppm) is given in Figure 7B.

The carbon-13 NMR spectra can be complicated due to the coupling of carbons to protons. However, when the protons are de-coupled by irradiating at proton frequencies, each resonance peak corresponds to a single carbon nucleus unless there is an overlap in chemical shifts. When the chemical shifts in parts per million (ppm) have been assigned (Wehrli, and Wirthlin, 1976) to the specific carbons, the resonance line-widths and intensities can be analyzed in terms of the relative mobility of the various groups.

The very fact that we can see the high-resolution NMR spectra of the biological and model lipid membranes is a consequence of their fluidity (Lee et al., 1974; Bocian and Chan, 1978; Smith et al., 1978). Among the components of the biological membranes, the proteins, being relatively rigid, are hard to observe. This

actually proves to be beneficial in the sense that the spectra are fairly simple and give information mainly about the lipids of the membranes. On account of the anchoring effect of the lipid-headgroups in the lipid-water interface of the bilayer, their resonances are considerably broad. On the other hand, the hydrocarbon chains, with their relatively less restricted motion, show up as fairly sharp peaks. When the resonance lines are resolvable, their widths at half the intensities (half-line widths) can be interpreted (relevant details given in DISCUSSION) in terms of the relative mobilities of the corresponding nuclei. To our knowledge, there has been just one study on the C-13 NMR spectra of native chloroplast membranes (Keough et al., 1973) but no attempt was made to interpret this spectrum.

4. Carbon-13 Enriched Chloroplast Membrane:

One bottle-neck for the carbon-13 NMR studies is the low (1.1%) natural abundance of this isotope. With moderate-sensitivity NMR machines, it is useful to enrich the biological membranes with C-13, in order to cut short the

time for which the spectra are run so as to minimize the possible sample-degeneration. Earlier attempts to enrich the photosynthetic membranes with C-13 (Flaumenhaft et al., 1970; Strouse et al., 1972) from carbon dioxide gas were not very fruitful because of the complication of the spectra as a result of C13 - C13 couplings. During the present studies, this problem was practically solved with a new approach to it (see pages 47-49).

II-B. FLEXIBILITY GRADIENT IN BIOMEMBRANES

Employing a lipid or a fatty-acid spin label with the nitroxide ring at different positions along the fatty-acyl chain, one can study the order or the motions in the membrane lipids at different depths. If one plots the order parameter, S , against the number, n , of the fatty-acyl carbon carrying the nitroxide ring, incorporated in a model lipid or some biological membranes (see Figure 8), the slope of this curve indicates a gradient in the degree of order, which decreases with the increase in n . If some motional parameter is measured in a similar

Figure 8

Figure 8. An example of the "Flexibility Gradient" in the fully hydrated multi-bilayers of egg lecithin containing phospholipid spin labels with the nitroxide moiety at positions, n , equal to 2-14, of the fatty-acyl carbons, with respect to the carbonyl-carbon linked to the glycerol backbone of the phospholipid spin label. Modified from McConnell and McFarland, 1972)

experiment, it is found that both the rates and the amplitudes of the motions increase with n . This gradient in order and mobility, which is found in model lipid membranes, believed to form a bilayer structure, is termed the flexibility gradient. This has been explained as due to the anchoring of the lipid headgroup along the outer hydrophilic boundaries of the bilayer structure (Hubbel and McConnell, 1969, 1971; Jost et al., 1971; Seelig and Hasselbach, 1971; McConnell and McFarland, 1972; Seelig and Niederberger, 1974; Seelig et al., 1972; Keith et al., 1968; Waggoner et al., 1969; Rottem et al., 1970., Stockton et al., 1977).

The present project explores if such a flexibility gradient also exists in the chloroplast membranes. Although a major lipid, MGDG, extracted from these membranes, does not form aqueous bilayers (Shipley et al., 1977) and yet when mixed with DGDG and other polar lipids extracted from these membranes, it can form a stable bilayer (Larsson and Puang-Ngern, 1979). These observations will be dealt with, to a greater length, in light of the studies conducted

in the present project and the earlier non-bilayer model of the chloroplast membranes by Weier and Benson (1966).

II-C. LIQUID CRYSTAL - GEL PHASE TRANSITION

1. Phase Transition in Biomembranes in General:

It seems that a 'major' role of lipid in the biological membranes is to provide a suitable environment for the membrane proteins to function optimally. Perhaps lipids tend to 'lubricate' the proteins so that they can move around in the two-dimensional matrix of the lipid 'bilayer'. The lipid matrix can assume a gel (relatively less fluid) or a liquid-crystalline (relatively more fluid) state (see Figure 9A). The transition between these two states (phases) is termed the phase transition. Such a transition is thought to be due to the 'fluidization' of the lipid hydrocarbon chains when the sample is heated through its phase-transition temperature (T_c).

In some instances, there may be a pre-transition a few degrees below the 'main' phase transition (see Figure 9B). Such a pretransition has been explained by Rand et al., (1975) as due

Figure 9

A. Diagrammatic representation of the structure of two lamellar phases of polar lipids in excess water. (Quinn and Williams, 1978)

B. Diagram of a dipalmitoylphosphatidylcholine bilayer, showing structural changes at 35°C and 42°C. Circles represent polar head groups, straight lines the solid, fully extended hydrocarbon chains and the wiggly lines represent melted hydrocarbon chains. The change from a tilted to straight orientation is thought to represent the pre-transition at 35°C. (see Fig. 5), and the solid to fluid hydrocarbon chains the main endothermic melt (from Rand, Chapman and Larsson, 1975).

C. The maximum hyperfine splitting of EPR spectrum, $2T_1$, of 12NS vs the reciprocal of absolute temperature from bush bean chloroplasts and a water dispersion of extracted bush bean lipids. The 12NS was at a concentration of one per 15 chlorophyll in (a) bush bean chloroplasts and (b) a lipid-water dispersion (20 mg chlorophyll; 300 mg water) of lipids extracted from bush bean chloroplasts. The EPR spectrometer settings were: scan time, 4 min; time constant, 1 s; microwave power, 20 mW; and modulation amplitude, 1 G.

P. JURŠINIC and GOVINDJEE (1972)

to a change from "tilted" to "perpendicular" configuration of the hydrocarbon chains of the lipid bilayer, prior to the "melting" or fluidization of these chains. The mechanism of the phase transition is believed to be the temperature-induced changes in the degree of rotational isomerism of the single C-C bonds of the lipid fatty-acyl chains (Hubbel and McConnell, 1971; Seelig and Niederberger, 1974). More details of this mechanism and its significance to the chloroplast membrane lipids is being included in DISCUSSION.

2. Phase Transition in Chloroplast Membranes:

Jursinic and Govindjee (1977), studying the temperature dependence of the delayed-light emission decay-rate of isolated spinach chloroplasts found a T_c at 12-20 deg. C, which was unaffected by glutaraldehyde fixation (that blocks large structural changes). With spin-label ESR spectroscopy, employing 12-doxyl stearate they also determined the T_c for the Bush bean and pea chloroplasts and lipid-water dispersions thereof (see Figure 9C). Measuring the partitioning of

the hydrophobic spin label, 6N11 (2, 2-dimethyl-5, 5-dipentyl-N-oxylloxazolidine), in the spinach chloroplast membranes, following glutaraldehyde-fixation, Torres-Pereira et al., (1974) determined the T_c to be around 18 deg. C. Other reports on the T_c of the chloroplast membranes employed proton movement across the thylakoid membranes (Gräber and Witt, 1974; Yamamoto and Nishimurá, 1976), Hill reaction (Inoué, 1978) and the cytochrome b oxidation (details in Discussion) (Yamamoto and Ke, 1979) studies as a function of temperature.

In this project, the T_c of the spinach chloroplast membranes has been studied by means of the doxyl moiety of the stearic acid spin label at two extreme positions (5 and 16) to explore if the observed phase transition sets in at different temperatures at these 'levels' of their lipid matrix. A phospholipid spin label, 8-doxyl phosphatidyl choline, was also employed to determine if the T_c for a phospholipid spin probe was different, compared to a nonspecific probe such as doxyl-stearic acid. The T_c of membranes is expected to be relatively low when the membrane contain cis-unsaturated double bonds (King and

Spector, 1978). This point has a relevance to abundant poly-cis-unsaturation of the chloroplast membranes and hence will be discussed at length in the DISCUSSION.

II-D.EFFECT OF LIGHT ON FLUIDITY OF THE CHLOROPLAST MEMBRANES

Despite numerous studies in literature (Tzapin et al., 1971; Tsapin et al., 1974; Subchinski et al., 1974; Packer, 1975; Itoh et al., 1963; Crofts et al., 1967; Deamer et al., 1967; Molotkovsky and Dzyubenko, 1967; Tevini, 1977; Staehelin et al., 1978; Vozvyshayeva et al., 1974), there is no clear answer to the question: What is the effect of light on the fluidity of the chloroplast membranes?

Tzapin et al., (1971) studying the ESR spectra of iminoxyl radical probes incorporated into the isolated chloroplasts of Vicia faba, concluded that light induces a sharp decrease in the microviscosity of the hydrophobic parts of their membranes. On the contrary, more recent studies on spinach (Subchinski et al., 1974; Packer, 1975) and bean (Ruuge et al., 1977)

chloroplast membranes labelled with 5-doxyl stearic acid did not show any changes in the microviscosity caused by illumination.

In this project, the effect of light has been studied to explore if it really affected the fluidity of the lipid matrix at different depths and also if the fluidity of the membranes (as a function of temperature) affects the rates of reduction or oxidation of the spin label by light.

II-E. LIPID-PROTEIN INTERACTIONS IN THE CHLOROPLAST MEMBRANES

Spinach chloroplast membranes contain about equal amounts by dry weight of the proteins and the lipids (Lichtenthaler and Park, 1973). Wehrli's freeze-fracture model (see Figure 10A) of the thylakoid membrane (Wehrli, 1975) shows different kinds of proteins penetrating to varying depths into the lipid matrix. The membrane proteins, in general, are termed intrinsic (or integral) and extrinsic (or peripheral or surface) proteins (see Figure 10B) depending on whether they are embedded deep into the hydrophobic fatty-

Figure 10

A. Model of the thylakoid membrane based on freeze-fracture studies. Inner membrane half (IMH), outer membrane half (AMH), inner surface (IS), outer surface (OS), particle (P), inner surface particles (IS-P) small particles (P_1), large particles (P_2) (WEHRLI, 1975)

B. Architectural (chemical and structural) model of a biological membrane

SELWYN and DAWSON (1977)

acyl chains (integral) or are bound at the surface (peripheral) of the membrane.

In this project, the proteins were not studied directly, but their influence on the lipid organizational dynamics was studied indirectly. This was achieved by examining the spin label motions and the order parameters for the chloroplast membranes relative to those of the model membranes of their extracted lipids.

III. THE PROJECT

Despite the fundamental importance of the photosynthetic membranes to life, and their conspicuously different lipid composition with respect to most other biological membranes, relatively little work has been done on the dynamic state of their lipids and lipid-protein interactions. The present project was therefore undertaken to study such important aspects of the chloroplast membranes with emphasis on the following points:

1. To examine if the chloroplast membranes and the model membranes made from their lipid-extract show a flexibility gradient, similar to that found in most other biological and model lipid membranes.
2. To study the effect of light on the fluidity of the chloroplast membranes.
3. To see if the membrane proteins could influence the dynamic behaviour of lipids by comparing the order and mobility of the chloroplast membranes with those of the model membranes made from their lipid-extract.

M A T E R I A L S A N D M E T H O D S

A. SPINACH

Spinach was grown hydroponically in a green house under the conditions of 16 hours of light (75 deg. F) and 8 hours of dark (70 deg. F). The composition of the nutrient medium used to grow spinach is given in Table 4. Bloomsdale spinach seeds were used in these studies. Spinach grown in this manner was found equivalent to that obtained from the local market in respect of C-13-NMR and spin label E.S.R. characteristics of the chloroplast membranes.

B. ISOLATION OF CHLOROPLAST MEMBRANES

Chloroplasts were isolated from the chilled spinach leaves (without mid-ribs) by the method of Arnon et al., (1956) and suspended in 0.2M sodium chloride containing 5mM magnesium chloride. The preparation was sonicated for 5 minutes at 0-5 deg. C under a stream of nitrogen gas and centrifuged at 1000g (0 deg. C) for seven minutes to obtain the membrane-pellet.

In using water as the suspension medium, the above membrane pellet was dispersed with 200 times the volume of ice-chilled double-distilled water

Table 4

Composition of the nutrient medium used for hydroponically growing the spinach plants.

SQUASH NUTRIENTS	VOLUME (ml) PER LITRE
KNO_3 (IM)	5
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (IM)	2
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (IM)	10
KH_2PO_4 (IM)	1
$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (1 gram per litre) containing Versenol (2.1 gram per litre)	7.2
Minor elements*	1

*
Minor Elements

COMPOUND	WEIGHT (g/100 ml)	VOLUME (ml)
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	18.1	10.0
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	22.0	1.0
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1.25	10.0
Molybdic acid	0.70	6.4
Boric acid	0.287	10.0

and centrifuged at 48,000g at 0 deg. C for 15 minutes to concentrate the membranes down to the bottom of the centrifuge tube. The supernate was pipetted away and the membrane fraction was re-dispersed in 100 volumes of pre-chilled double distilled water and recentrifuged to concentrate the membranes.

C. EXTRACTION OF LIPIDS

For the extraction of lipids from the above (B) membrane preparation, the procedure of Nichols (1963) was employed. All solvents used were bubbled with nitrogen gas before use, and routine drying and concentration of lipids was accomplished under a stream of nitrogen gas. Finally, the lipid extract was dissolved in chloroform-methanol (2:1 v/v).

D. MEASUREMENT OF CHLOROPHYLL

The total chlorophyll was determined in 80% acetone in water using the two-wavelength method of Vernon (1960). Chlorophyll-content of the lipid-extract was accomplished in the same way by evaporating the solvent first under nitrogen gas

* Bubbling of solvents with nitrogen gas makes them largely free of dissolved oxygen. This minimizes oxidation of unsaturated lipids during extraction.

and then adding the 80% acetone solution.

E. PREPARATION OF MODEL MEMBRANES

A known volume of the lipid extract was dried under nitrogen gas and then in vacuum (1-2 hours) and was vortexed with a measured amount of double-distilled water in a glass tube sealed in an atmosphere of nitrogen gas, for about 15 minutes at room temperature.

To prepare sonicated lipid-water dispersions the above preparation was sonicated, under an atmosphere of nitrogen gas, between 15-20 deg. C for about 10 minutes until a clear green solution was obtained.

F. ELECTRON MICROSCOPY

A small drop of aqueous lipid dispersion (vortexed or sonicated as prepared above) containing 1% phosphotungstic acid was placed on a formvar-coated grid for about 15 seconds and then excess liquid was removed by touching the grid with a filter paper. It was air-dried (Lucy and Glauert, 1960) and observed with Siemens Elmiskop I electron microscope at the instrumental

magnification of 40,000.

G. SPIN LABELLING

Figure 11 shows the stearic acid and 8-doxyl phosphatidylcholine spin labels used for the present study. Both for the model and the chloroplast membranes the molar ratio of the chlorophyll/spin label was kept at 20:1. For the model membranes, the spin label was dissolved in the lipid-extract before drying. The chloroplast(B) membranes, dark-adapted for 30 minutes at 10 deg. C were vortexed in the dark for about 15 minutes with a dried film of the spin label in a glass tube, for a satisfactory labelling.(see Appendix I).

H. ELECTRON SPIN RESONANCE (ESR) EXPERIMENTS

The Varian E-9 spectrometer equipped with a variable temperature control unit (± 0.2 deg. C) was employed for the present studies. The magnetic field used was 3232 Gauss (G) with a scan range of 100 G, time constant of 0.3 and 1.0 second, and the scan time of 4 or 8 minutes, microwave power of 5-20 mW and frequency 9.0 Gigahertz (GHz).

doxyl-
Position

Stearic
acid
spin
labels

Figure 11

In many instances, the maxima and the minima of the hyperfine splitting were resolved by increasing the receiver gain about 10-fold.* Illumination of the samples in the cavity of the spectrometer^a was carried out using 300W Tungsten projector lamp (Verma et al., 1972).

I. C-13 NUCLEAR MAGNETIC RESONANCE

(C-13 NMR) EXPERIMENTS

For the C-13 NMR studies, three Fourier transform instruments were used, namely:

XL-100, 25 Megahertz (MHz), 12 mm tubes;

CFT-20, 20 MHz, 10 mm tubes and

CXP-300, 75 MHz, 15 mm tubes.

Because of the relatively low sensitivity, the XL-100 and CFT-20 were used principally for the extracted lipids or the C-13-enriched membrane preparations. The signal-to-noise ratio was greatly improved by using the CXP-300 with the advantages of greater sensitivity, frequency dispersion, and 15 mm sample tubes, which was, therefore, found very useful for the natural abundance C-13 NMR studies of the chloroplast

* The resolution of these maxima and minima can be improved further by multi-scan averaging and increasing the modulation amplitude (see page 56a).

membranes. For both CXP-300 and CFT-20 instruments, the spectra were collected with field-frequency lock on deuterium of the solvent, whereas, with the XL-100 an external fluorine lock was found useful for viscous membrane samples. All spectra were taken at 30 deg. C. In all cases, tetramethyl silane (TMS) was used as the standard reference, internally in the organic solvents and externally for aqueous samples.

The radiofrequency pulse angle used for the chloroplast and sonicated model membranes was 45 degrees, and for the lipid in organic solvents, 22.5 degrees. Acquisition times for the membranes were 0.3-0.5 seconds, and those for lipids in the organic solvents were 0.8-1.0 second.

J. C-13 ENRICHMENT PROCEDURE

Potted spinach plants, about six-week old were placed in a glass chamber with a sealable lid (Vacuum seal) fitted with a rubber-stopper on the top (see Figure 12). Some nutrient medium (200-500 ml) was placed in the chamber before putting the spinach pot in it. Near the top of the chamber a small platform was secured by sticking-tape or a joining gum in order to keep a beaker or

Figure 12. Arrangement for carbon-13 enrichment experiment.

flask (50-100 ml) on it. In this beaker was kept 200 to 500 mg of a mixture of C-13 enriched (80-90%) barium carbonate and isotopically normal barium carbonate (1 : 1.5-2). After sealing the chamber and putting it in the light, 25-50 ml of dilute acid (0.5N to 1N HCl) was injected into the beaker with a large syringe through the rubber-stopper. The plants were left in this atmosphere of C-13 enriched carbon dioxide for about 10 hours in light, after which the lid of the chamber was removed. This procedure was repeated daily for 4 to 10 days depending on the degree of C-13 enrichment desired.

K. ESR PARAMETERS

In the spin label ESR studies, the two parameters measured were the order parameter, S , and the rotational correlation times, τ . The order parameter, S , is given by the relationship shown in Figure 13A. The value of $T_{\max}$ and $T_{\min}$ can be measured from the spectrum (see Figure 13A); the quantities T_{zz} and T_{xx} are the extreme possible values of these splittings, $T_{zz} = 30.8$ G and $T_{xx} = 5.8$ G (Hubbel and McConnell, 1971).

Figure 13 A

$$S = \frac{T_{\max} - T_{\min}}{25}$$

Figure 13 B

$$\tau_1 = 6.5 \times W_O \left[\left(I_O / I_{-1} \right)^{\frac{1}{2}} + \left(I_O / I_{+1} \right)^{\frac{1}{2}} - 2 \right] \times 10^{-10} \text{ sec.}$$

$$\tau_2 = 5.9 \times W_O \left[\left(I_O / I_{-1} \right)^{\frac{1}{2}} - \left(I_O / I_{+1} \right)^{\frac{1}{2}} \right] \times 10^{-10} \text{ sec.}$$

The rotational correlation times were measured according to Cannon et al., (1975) from the equations shown in Figure 13B. where I_0 , I_{+1} and I_{-1} are the intensities of the central, low-field and high-field lines of the spectrum and W_0 is the line-width of the central line in Gauss (see Figure 13 B).

L. NMR PARAMETERS

The chemical shift in parts per million (ppm), as given in Figure 7B is the main parameter measured in these studies. In addition, line-width at half the intensity of the resonance has been utilized qualitatively as an index of the mobility of the nucleus in question.

RESULTS

The results will be presented in two sections:

- I. FLUIDITY OF CHLOROPLAST MEMBRANES and
- II. LIPID-PROTEIN INTERACTIONS IN CHLOROPLAST
MEMBRANES

All observations reported here were made with the samples in dark, except when the effect of light was studied specifically.

Section I on the FLUIDITY OF CHLOROPLAST MEMBRANES comprises observations on the following:

- I-A. MEMBRANE FLEXIBILITY GRADIENT BY SPIN LABEL
ESR
- I-B. EVIDENCE OF A FLEXIBILITY GRADIENT BY
CARBON-13 NMR
- I-C. LIQUID CRYSTAL - GEL PHASE TRANSITION, and
- I-D. EFFECT OF LIGHT ON CHLOROPLAST MEMBRANES BY ESR.

In section II on the LIPID-PROTEIN INTERACTIONS IN CHLOROPLAST MEMBRANES, spin label studies have been reported at sample temperatures of 37 deg. C and 5 deg. C. The choice of these two temperatures was determined by the two-fold advantage: firstly, it allows the measurement of the rotational correlation times (at 37 deg. C), as well as the maximum hyperfine splittings, $2T_{max}$ (at 5 deg. C), and secondly, it allows the study

of lipid-protein interactions both in the 'liquid-crystalline' (at 37 deg. C) and the 'gel' (at 5 deg. C) states of the membranes. Studies at an intermediate temperature (21 deg. C) are also included.

I. FLUIDITY OF CHLOROPLAST MEMBRANES

I-A. MEMBRANE FLEXIBILITY GRADIENT BY SPIN

LABEL ESR

Figure 14 shows the ESR spectra of 5, 7, 9, 12, 13, 14, and 16-doxyl stearic acid spin labels incorporated in the spinach chloroplast membranes suspended in 0.2M sodium chloride solution containing 5 mM magnesium chloride. Qualitative information about the flexibility gradient in these membranes can be acquired from a study of the line-shapes of the spectra for 5 to 16-doxyl stearic acid incorporated in these membranes. By moving the doxyl moiety down the fatty acyl chains, the spectral lines are becoming progressively narrower and the outer and the inner maxima shift so as to give finally a 3 line spectrum (16-doxyl). A comparable fluidity gradient is also depicted by the model membranes

Figure 14

Spin label ESR spectra of (top to bottom) 5, 7, 9, 12, 13, 14 and 16-doxyl stearic acid incorporated in the chloroplast membranes at 37 deg. C in dark. The suspension medium is 0.2M sodium chloride containing 5mM magnesium chloride.

Figure 14. ESR SPECTRA OF CHLOROPLAST MEMBRANES

made by dispersing the extracted lipids from the chloroplast membranes in water (see Figure 15). Freshly water-dispersed, these lipids form multilamellar or liposomal structures as shown by electron microscope studies (see Figure 16).

Actually, both the degree of ordering of the membrane fatty acyl chains, as well as the rates of their anisotropic motions, contribute to the fluidity gradient, mentioned above. A quantitative estimate of this gradient can be made by calculating an ORDER PARAMETER, S . Alternatively, (or in addition to the order parameter, if the spectral line-shapes permit) one can calculate ROTATIONAL CORRELATION TIMES, τ , as a measure of rates of motions of the fatty-acyl segments of the membrane lipids.

Fluidity or Flexibility

Although both the terms "fluidity" and "flexibility" (Seelig and Niederberger, 1974) have been used in the literature to describe the liquid-like properties of the biological and model membrane systems, the term "flexibility" will be preferred here. The reason being that the type of motions measured in these experiments are

Figure 15

Spin label ESR spectra of stearic acid spin label (with the position of the doxyl moiety shown), incorporated in aqueous dispersions of the lipid-extract of the chloroplast membranes at 37 deg. C in dark. Extra lines (see arrows) shown on spectra for 5 and 9-doxyl positions are the amplified regions of the $2T_{\max}$ splittings. It is normally achieved by raising the receiver gain 10 folds. In cases where increase in the noise level may interfere with the measurement of the splittings, the value of the modulation amplitude can be raised from 1 to 2 Gauss. Multi-scan averaging is also useful to resolve these maxima and minima, especially when the signal-to-noise ratio of the spin labelled sample is poor.

Figure 15

Figure 16

Lipid-water dispersions made from the lipid-
extract of the chloroplast membranes.
Electron micrograph after negative staining
with phosphotungstic acid.

anisotropic and not really liquid-like. Also a more versatile expression for dealing with the order and mobility contributions together, as it occurs in the membrane systems, is the "flexibility". The term fluidity has been used here only to refer qualitatively to these LIQUID-crystalline systems.

Order Parameter Measurements

Figure 17 shows a plot of the order parameter (S) versus the nitroxide ring position of stearic acid spin label incorporated into the chloroplast membranes suspended in 0.2M sodium chloride containing 5mM magnesium chloride, and their lipid extract dispersed in water (the term "model membranes" will be used henceforth), both at 37 deg. C. The chloroplast membranes show a mild decrease in S from 5 to 9 doxyl spin labels, whereas the model membranes depict a relatively sharp decrease in S for the same nitroxide spin labelled positions. Both these membrane systems therefore indicate a flexibility gradient increasing down the fatty acyl chains.

The order parameter, S , however cannot be calculated for the ESR spectra arising from 12 to

Figure 17

Variation of order parameter, S with the position of the doxyl moiety of the stearic acid spin label (SASL) incorporated in chloroplast membranes (●) and model membranes (▲) at 37 deg. C in dark (as in Figure 14 and 15).

Figure 17

16-doxyl stearic acid spin labels incorporated in either the chloroplast or the model membranes at this temperature because of the difficulty in resolving the maximum hyperfine splittings (T_{max}). This occurs due to the predominance at these positions of fast motions which average substantially the splittings. Therefore, there is little density in the regions of the spectra where T_{max} hyperfine splittings have to be measured (see MATERIALS AND METHODS for the measurement of $2T_{max}$).

Rotational Correlation Time Measurements

As described above, because of the difficulty of measurement of the order parameter for 12-16 doxyl spin labels, another parameter which is sensitive to mobility should be used. In MATERIALS AND METHODS, the method of calculating the rotational correlation times (τ_1) and (τ_2) has already been given. These times provide a measure of the motions of the spin label in its local membrane micro-environment. As the motions become greater the value of the rotational correlation times become smaller. When these times are of the order of 10^{-9} second, the spectra of the nitroxide

spin labels can be analyzed for mobility using the equations described in the MATERIALS AND METHODS (Cannon et al., 1975). The difference between these two times ($\tau_1 - \tau_2$) is a measure of anisotropy in the motion studied (Schreier et al., 1978a).

Table 5 shows the values of the rotational correlational times for the 12 to 16-doxyl stearic acid spin probes incorporated in the chloroplast and the model membranes at 37 deg. C. Both τ_1 and τ_2 , for the chloroplast and the model membranes, show a flexibility gradient with successively increasing mobility (decreasing correlation times) for 12, 13, 14 and 16-doxyl labels. In addition to this increasing motion towards the methyl terminal of the fatty-acyl chains, there also occurs a gradual decrease in the degree of anisotropy of the motion ($\tau_1 - \tau_2$) down the fatty-acyl chains, which follows quite well for the chloroplast membranes.

I-B. Evidence of a Flexibility Gradient by Carbon-13 NMR:

The very fact that relatively sharp resonances can be seen in an NMR spectrum of a membrane sample is indicative of its fluidity.

Table 5

Variation of the empirical rotational correlation times with the position of the doxyl moiety on the stearic acid spin label incorporated in the chloroplast membranes suspended in 0.2M NaCl with 5mM $MgCl_2$ and their extracted lipid model membranes in water. All the measurements were made at 37°C with the samples in the dark, by ESR.

Position of the doxyl moiety	Rotational Correlation time $\times 10^{10}$ sec.			
	Chloroplast Membranes		Extracted Lipids	
	τ_1	τ_2	τ_1	τ_2
12	39±2*	22±2	29±1	29±1
($\tau_1 - \tau_2$)	17±4		17±2	
13	27±2	18±2	22±1	13±1
($\tau_1 - \tau_2$)	9±4		9±2	
14	21±2	16±2	22±2	15±1
($\tau_1 - \tau_2$)	6±4		7±3	
16	12±1	9±1	10±2	7±2
($\tau_1 - \tau_2$)	3±2		3±4	

* Average of 3 experiments

The relatively short ESR correlation times calculated for the membrane lipid environment have already shown the markedly fluid nature of the chloroplast and the model membranes under study. An attempt was made here to study the C-13 NMR spectra of the chloroplast membranes, with the advantage that this method does not require the addition of any extraneous probe to the membranes, as is required for the spin label studies (Taylor and Smith, 1980). Initial work was conducted on the 25 MHz frequency machine using a 10 mm sample tube diameter. In order to improve in signal-to-noise ratio it was found advantageous to enrich the sample with carbon-13 using C-13 enriched carbon dioxide gas diluted with approximately three parts of the isotopically normal gas. This approach to C-13 enrichment of a plant material, described for the first time in this thesis (see MATERIALS AND METHODS), markedly improved the signal-to-noise ratio of the chloroplast membrane spectra without any detectable complication caused by the C13-C13 couplings. Figure 18A shows the 25 MHz spectrum of the C-13 enriched spinach chloroplast membranes, and Figure 18B, a natural abundance spectrum at the same frequency.

Figure 18

A: 25 MHz C-13 NMR spectrum of the chloroplast membranes enriched with carbon-13. The membranes suspended in 0.2M sodium chloride containing 5mM magnesium chloride in deuterated water at 30 deg. C. Chemical shift in parts per million (ppm) in reference to external tetramethylsilane (TMS).

B: 25 MHz C-13 NMR spectrum of the chloroplast membranes in natural abundance. Suspension medium and temperature same as in Figure 18A.

Figure 18

With the acquisition of a 75 Mhz superconducting NMR machine permitting the use of a 15 mm diameter sample tube, the enrichment of the sample with carbon-13, became unnecessary for the routine chemical shift - measurement studies. Figure 19 gives a 75 MHz natural abundance C-13 NMR spectrum of the chloroplast membranes at 30 deg. C. A comparison of the carbon-13 enriched (Figure 18) and natural abundance (Figure 19) C-13 NMR spectra shows their close resemblance with respect to the chemical shifts and the relative intensities and the line-shape characteristics of the two spectra. Only a few peaks (see the shaded resonances in Figure 18A) in the enriched spectrum (for example, those at 15.9, 39.9, 124.3 and 134.5 ppm) seem to have been selectively enriched. These resonances could not be assigned to any of the chloroplast glycolipids or their fatty acids as studied by Johns et al., (1977a, b and 1978) and these could arise from some other membrane components (including proteins, pigments and other electron transport chain complexes). As these resonances are difficult to observe in the natural abundance C-13 spectra of the chloroplast

2

Figure 19

75 MHz C-13 NMR spectrum of chloroplast membranes in natural abundance. Suspension medium and temperature same as in Figure 18.

Figure 19

D

5

Figure 20

25 MHz C-13 NMR spectrum of sonicated vesicles of lipid extract of the chloroplast membranes in deuterated water 30 deg. C.

Figure 20

membranes or their lipids, their discussion in relation to the membrane fluidity does not seem to be important here.

Before an attempt is made to interpret the lineshapes of the carbon-13 resonances in terms of a flexibility gradient, if any, in these membranes, it must be determined as to which resonances of the chloroplast membranes arise from their constituent lipid fatty-acyl chains. Figure 20 shows the natural abundance carbon-13 NMR spectra at 25 MHz of the sonicated lipid vesicles at 30 deg. C. These sonicated lipid vesicles, made from the lipid-extract of the chloroplast membranes, comprise unilamellar vesicles of size ranging from 250 to 750 Angstroms as shown by electron microscopy (see Figure 21).

Comparing the chemical shifts of model membranes (sonicated lipid vesicles) (Figure 20) with those of the chloroplast membranes (Figure 19), it becomes evident that a major set of carbon-13 resonances in both the membrane systems under comparison are the same. This shows that the principal carbon-13 resonance lines arising from the chloroplast membranes originate from their constituent lipids. In order to get some

Figure 21

Sonicated vesicles of lipid extract of the chloroplast membranes. Electron micrograph of a negatively stained preparation with phosphotungstic acid.

information about the fluidity of the different micro-environments along the different segments of the fatty-acyl chains in these membranes, the immediate problem is to assign the chemical shifts to as many carbons as possible.

The chloroplast membranes, like most other biological membranes, have a complex mixture of lipids in addition to the chlorophylls and other pigments. However, recalling (refer to INTRODUCTION) that the chloroplast lipids have an unusually large content of 9, 12, 15 (all) cis-unsaturated linolenic acid (Benson, 1971), it should be useful to look for the carbon-13 resonances arising from the carbons of the linolenic acid. Table 6 shows that this approach is successful; most of the fatty-acyl C-13 resonances seen in the spectra of the chloroplast membranes (Figure 19), the model membranes made from their extracted lipids (Figure 20), as well as this lipid extract in deuterated chloroform (Figure 22) can be identified with the chemical shifts of linolenic acid as assigned by Johns et al., (1977a) (also see Figure 24 from Johns et al., 1977b).

A major difference in chemical shift is

Table 6

Assignment of the chemical shifts (ppm) of the fatty acyl resonances in C-13-NMR spectra of the chloroplast membranes and lipids.

Chloroplast membranes	sonicated lipid in D ₂ O	Lipid in CDCl ₃	Linolenic acid in CDCl ₃ (Johns et al., 1977a)	Carbon number
	173.75	173.76	180.3	C-1
173.36		173.39		C-1
34.2	34.3	34.17	34.2	C-2
		24.91	24.8	C-3
		29.24	29.1	C-4
		29.24	29.2	C-5
		29.24	29.1	C-6
29.7	29.6	29.71	29.6	C-7
27.3	27.3	27.27	27.3	C-8
130.0	130.1	130.25	130.2	C-9
128.2	128.2	127.85	128.1	C-10
25.67	25.67	25.67	25.7	C-11
25.67	25.67	25.67	25.6	C-14
128.2	128.2	128.35	128.4	C-12
128.2	128.2	128.35	128.4	C-13
127.3	127.3	127.2	127.3	C-15
131.7	131.7	132.02	132.0	C-16
20.64	20.62	20.60	20.60	C-17
14.27	14.29	14.31	14.2	C-18

Figure 22

20 MHz natural abundance C-13 NMR spectrum of
the lipid-extract of the chloroplast
membranes dissolved in denterated chloroform
at 30 deg. C.

Figure 22

noticeable in the carbon at position 1 (see C-1 in Table 6) between the linolenic acid and the chloroplast membranes and lipid-extracts. This resonance arises from the carbonyl group in linolenic acid, but is esterified to the glycerol backbone in the lipids under study.

In general, the carbon-13 resonances from the chloroplast membranes (Figures 19 and 20) fall into five categories:

- a) carbonyls (173.2 and 173.3 ppm),
- (b) olefinic carbons (between 124.5 and 131.7 ppm),
- (c) lipid headgroup carbons (between 61 and 73 ppm),
- (d) methylene group envelope (between 20 and 30 ppm) &
- (e) terminal methyl groups (between 14 and 20 ppm).

A similar categorization can be made for the extracted lipids of the chloroplast membranes, whether in aqueous dispersion (Figure 20), or in organic solvents like chloroform (Figure 22) or methanol (Figure 23).

About 80% of the chloroplast membrane lipids are galactolipids (Benson, 1971); therefore, the major headgroup carbon resonances should arise from the galactose moiety (see Figure 24) as assigned by Johns et al., (1977b). The headgroup

Figure 23

20 MHz natural abundance C-13 NMR spectrum of the chloroplast membrane lipids dissolved in deuterated methanol at 30 deg. C. Chemical shift with respect to internal TMS.

Figure 23

Figure. 24

Assignments of C-13 resonances of monogalactosyl diglyceride (mgg) (upper half) and digalactosyl diglyceride (dgg) (lower half) dissolved in deuterated methanol. Taken from Johns et al 1977b).

Figure 24

Proton-decoupled pulsed Fourier transform ^{13}C n.m.r. spectrum of rgg in CD_3OD .

Johns et al. 1977 b

Proton-decoupled pulsed Fourier transform ^{13}C n.m.r. spectrum of dgg in CD_3OD .

carbon resonances are not well resolved in the NMR spectra of the chloroplast and model membranes (Figures 18-20), as well as in the lipid extract in chloroform (Figure 22). On the other hand, these lipid headgroup carbons give rise to sharp and well-resolved resonances when the lipid-extract is dissolved in methanol (see Figure 23).

C-13 NMR Line-widths

A rather qualitative idea of the flexibility gradient is suggested by the differential line-broadening effect noticeable in the chloroplast and the model membrane spectra (Figures 18-20) compared to the apparently monomeric solution spectra of these lipids in methanol (Figure 23).

Now if one inspects the spectrum of the chloroplast membrane lipid-extract in chloroform (see Figure 22), it is observed that all the resonances except those of the headgroups (including anomeric carbons, not seen here) are fairly uniformly sharp in their line-width. The selective broadening of the headgroup resonances indicates their reduced mobility. This observation seems to hold true as the major

chloroplast membrane lipids are known to form inverted micellar aggregates in chloroform (Johns et al., 1977b and 1978). With the headgroups restricted in their motions and most other segments of the fatty-acyl chains almost equally mobile, this does represent a sharp increase in mobility (decrease in line-widths from the lipid headgroups to the fatty-acyl chains, but not the type of mobility (flexibility) gradient found in bilayer structures.

Finally, a look at the chloroplast membrane (Figures 18 and 19) and the model membrane (Figure 20) spectra, reveals a line-width variation different from those of both the monomeric solution (Figure 23) and the inverted micellar aggregates (Figure 22). In the membrane spectra (Figures 18-20), not only the headgroup resonances are broadened but also the carbonyl, methylene and olefinic carbons, to some extent. There was not much difference between the linewidths of the terminal methyl (about 14 ppm) and the carbonyl (about 173 ppm) in the lipid dissolved in chloroform (Figure 22) or in methanol (Figure 23). On the contrary, in the membrane spectra (Figures 18-20), the carbonyl resonance is appreciably

broader than the terminal methyl resonance. Next in degree of broadening are the methylene resonances at about 29.7 and 29.9 ppm in the membrane spectra, especially marked in the model membrane spectrum (Figure 20). Similarly, the resonance at 128.2 ppm also appears broader than the terminal methyl group resonance in the membrane spectra (Figure 20) in contrast to their respective counterparts in the solution spectra (Figure 23). Table 7 gives the measurable half-line widths of some of the lipid resonances in methanol and in water.

The overall observation that emerges from this qualitative treatment of the degree of broadening is that the line-widths decrease from the headgroup down the fatty-acyl chain in a rough sequence:

- i) headgroup carbons (60-73 ppm).
- ii) carbonyl (C-1, about 173 ppm).
- iii) methylene (C-7, about 29.7 and C-5 about 29.2 ppm)

(It may be useful to recall that 5 and 7-doxyl stearic acid spin labels incorporated in these membranes show a comparable rigid environment by ESR spectroscopy as shown in Figures 14 and 15).

Table 7

Line-widths at half the intensity of some carbon-13 NMR resonances in the lipid extract from the chloroplast membranes. A flexibility gradient is noticeable in the model membrane (lipid-water) system compared to the monomeric lipid solution in deuterated methanol.

Carbon group	Chemical shift. ppm	Line-width (Hertz) at half intensity (Half line-width (Hertz))	
		Monomeric lipid	Sonicated lipid vesicles
CH ₃ (terminal) (C-18)	14.3	10	10
CH ₂ (C-14) (C-11)	26	10 10	15 (The two lines appear as a single superimposed)
= CH (C-10)	128.2	12.5	25
C=O (C-1) (two partially resolved lines together)	173	18.0	60±15

iv) olefinic (C-12 and C-13 at 128.2 ppm). (Both these carbons are linked by a cis double bond in linolenic acid). (v) terminal methyl (C-18) at about 14.2 ppm) (Note that C-16, at 131.7 ppm in model membranes, seen in Figure 20, is much sharper than C-12 and C-13 at 128.2 ppm).

The anomeric carbons (about 105 and 100 ppm) could not be seen in the spectra of the membranes (Figures 18-20) or of the inverted micelles (Figure 22), perhaps due to a high degree of line-broadening.

I-C LIQUID CRYSTAL - GEL PHASE TRANSITION

Dependence of membrane fluidity on the temperature is another important aspect of the structure and function of membranes. There may be characteristic temperature(s), at which the biomembranes or model lipid membranes undergo a co-operative transition from the gel to the liquid crystalline phase. The transition should result in a 'sudden' change in the order and mobility parameters measureable by spin labels incorporated in the chloroplast or model membranes.

Figure 25 shows a series of ESR spectra of 5-doxyl stearic acid incorporated in the chloroplast membranes suspended in water. These spectra were taken at a number of temperatures between 5 and 32 deg. C. Figure 26 shows the dependence of $2T_{max}$ on temperature, with a noticeable break in the curve between 15 and 20 deg. C. This break signifies a sudden increase in fluidity of spin label micro-environment in going about 15 to 20 deg. C, which may represent a phase transition temperature (T_c) for the chloroplast membranes.

If this transition is actually due to the fluidity changes in the lipids of these membranes, a similar phase transition should be found in the model membranes prepared from the lipid-extract of the chloroplast membranes. Figure 27 shows the ESR spectra of 5-doxyl stearic acid spin label incorporated into the lipid-water dispersion, taken at different temperatures in the same range as that of Figure 25. A plot of $2T_{max}$ versus the temperature (see Figure 28) also reveals a break between 15 and 20 deg. C.

A similar value of T_c is obtained from the ESR spectra (Figure 29 and 30) of a different spin labelled molecule, 8-doxyl phosphatidyl choline. (a

Figure 25

Spin label ESR spectra of the chloroplast membranes in water as a function of temperature. The spin label used is 5-doxyl stearic acid.

Figure 25

Figure 26

Variation of the maximum hyperfine splitting ($2T_{\max}$) with temperature measured from spectra in Figure 25. A break in the graph is observable between 15 and 20 deg. C. The spin label employed was 5-doxyl stearic acid.

Figure 26

Figure 27

ESR spectra at varied temperatures (5-37 deg.
C) of 5-doxyl stearic acid incorporated in
lipid-water dispersions.

Figure 27

Figure 28

Variation of $2T_{max}$ with temperature, measured from Figure 27. A break between 15 and 20 deg. C. The spin label employed was 5-doxyl stearic acid.

Figure 28

Figure 29

ESR spectra of 8-doxyl phosphatidyl choline in chloroplast membranes as a function of temperature. The membranes suspended in 0.2M sodium chloride containing 5mM magnesium chloride.

86b

Figure 29

°C

Figure 30

Variations of $2T_{\max}$ with temperature as measured from spectra in Figure 29. A break is noted around 16 deg. C.

phospholipid) (see Figure 11), incorporated into the chloroplast membranes. The value of T_c obtained is around 16 deg. C. The phase transition temperature can also be measured by studying the temperature dependence of the rotational correlation time, of a probe such as 16-doxyl stearic acid incorporated in the chloroplast membranes (see Figure 31). This should also give information about the variation, if any, in the T_c with the change in the position of the doxyl moiety of the spin label, that is at different segmental positions along the fatty-acyl chains in the membrane system.

Figure 32 shows a plot of the rotational correlation time, versus the sample temperature, for 16-doxyl stearic acid label incorporated in the chloroplast membranes suspended in water. The plot is indicative of a break around 13 deg. C, which is 3-6 deg. C lower than that for 5-doxyl stearic acid spin label under similar experimental conditions. The possible significance of this point will be discussed later in the DISCUSSION.

Further support of the T_c observations of the chloroplast membranes can also be acquired qualitatively from the carbon-13 NMR studies of

Figure 31

ESR spectra of 16-doxyl stearic acid incorporated in the chloroplast membranes suspended in water as a function of temperature.

Figure 31

Figure 32

Variation of rotational correlation time (τ_1)
with temperature calculated for spectra from
Figure 31.

Figure 32.

the chloroplast membranes. Figure 33 shows three spectra of the spinach chloroplast membranes taken in natural abundance at 25 MHz, at the sample temperatures of 5 deg. C (top spectrum), 16.5 deg. C (middle spectrum) and 20.5 deg. C (bottom spectrum). Observing the methylene group carbon resonance at 29.7 ppm (see arrow in Figure 33), assigned to C-7 of the linolenic fatty-acyl chains of the constituent lipids of the chloroplast membranes, one finds little increase in the intensity of this resonance in going from 5 to 16.5 deg. C. On the other hand, there occurs a sharp increase in its intensity in further raising the sample temperature from 16.5 to 20.5 deg. C, which is the same temperature range as determined for the T_c of the chloroplast membranes by the spin-label (5-doxyl stearic acid) ESR technique, as shown above.

The diminished line intensity of this methylene carbon resonance is apparently due to the differential line-broadening effect possibly caused by the increased immobilization of this position at low temperatures, for example, 5 and 16.5 deg. C for the chloroplast membranes (see top and middle spectra of Figure 33). A sudden

Figure 33

25 MHz natural abundance C-13 NMR spectra of chloroplast membranes suspended in 0.2M sodium chloride containing 5mM magnesium chloride as a function of temperature. Methylene peak at 19.7 ppm shows marked enhancement in intensity when the sample temperature is raised from 16.5 to 20.5 deg. C. Each spectrum was obtained from the accumulation of 5000 transients.

Figure 33

increase in mobility of this methylene group in going from 16.5 to 20.5 deg. C (seemingly resulting from the gel - liquid crystalline phase transition of the membrane lipids) causes this resonance to sharpen and hence become visible above the noise of the spectrum (see Figure 33, bottom spectrum).

One advantage of studying these membranes with NMR (despite the qualitative nature of the information in the present experimental conditions) is that we do not have to add any external probe (however small in quantity) as is required for the spin label ESR studies. Therefore, both types of techniques used together as done in the present project, complement each other.

I-D. EFFECT OF LIGHT ON CHLOROPLAST MEMBRANES

In any study of the photosynthetic membranes, one pertinent question is: what is the effect of light on the parameters under study? Chloroplast membranes were kept in dark for 1 hour, spin labelled (in dark), and their ESR spectra were taken in the dark and subsequently under

illumination. Figures 34 to 38 show the ESR spectra for the chloroplast membranes in 0.2 M sodium chloride containing 5 mM magnesium chloride at 37 deg. C for the stearic acid spin labels with the doxyl moiety at 5 (Figure 34A & B), 9 (Figure 35A & B), 13 (Figure 36A & B), 14 (Figure 37A & B) or 16 (Figure 38A & B) position with respect to the carbonyl group. In Figures 34A-38A, the upper spectra are taken in the dark and the lower ones in light (first spectra in light). In Figures 34B-38B, the consecutive spectra (from top to bottom) are taken after extended periods of time in light.

One consistent feature of all the spectra in Figures 34 to 38 is that at no position of the doxyl moiety of the fatty acid probes, nor for the 8-doxyl phosphatidyl choline label (Figures 39A & B) any effect of light on the hyperfine splittings (or order parameter) or the rotational correlation times is observable. Thus, it seems that the fluidity of the chloroplast membranes under the present experimental conditions is not affected by light. The signal intensity is, however, markedly affected in light. There is an initial increase in the signal intensity, and

Figure 34 A and B

ESR spectra of 5-doxyl steric acid in the chloroplast membranes at 37 deg. C in 0.2M sodium chloride containing 5mM magnesium chloride. First spectrum is taken in the dark and the subsequent spectra are all taken in continuous light shined on the ESR cavity. Each consecutive spectrum is at an interval of 4 minute.

Figure 34 A

10 G

Figure 34 B

Figure 35 A and B

ESR spectra of 9-doxyl stearic acid spin label under conditions as in Figure 34.

Figure 35 A

Figure 35 B

Figure 36 A and B.

ESR spectra of 13-doxyl stearic acid spin
label under conditions as in Figure 34.

Figure 36 A

Figure 36 B

Figure 37 A and B

ESR spectra of 14-doxyl stearic acid spin
label under conditions as in Figure 34.

Figure 37 A

Figure 37 B

Figure 38 A and B

ESR spectra of 16-doxyl stearic acid under
conditions as in Figure 34.

Figure 38 A

Figure 38 B

Figure 39 A and B

ESR spectra of 8-doxyl phosphatidyl choline
under conditions as in Figure 34.

Figure 39 A

10 G

Figure 39 B

thereafter a continuous reduction in light, but seemingly to different extents for the different positions of the nitroxide group of the spin label incorporated.

As the primary aim of this study is to enquire into the change in fluidity, if any, caused by light at different depths in the membrane (using various positions of doxyl moiety of the spin label), it is beyond the scope of this project to go into any kinetic studies of the oxidation and reduction of the spin label under illumination. Such analyses, however, may be found in the literature (Subchinski, et al., 1975; Ruuge et al., 1977) for a variety of spin labels, as well as discussion of their possible mechanisms.

The effect of light for two extreme positions (5 and 16-doxyl) of the nitroxide ring in the spin label was studied both at 37 (figure 34 and 38) and at 5 deg. C (see Figures 40 and 41). These Figures reveal that the rate of increase in signal intensity at 5 deg. C is much slower than that at 37 deg. C for both 5- and 16-doxyl stearic acid incorporated in the chloroplast membranes. It shows that the effect of light on the signal

Figure 40 A and B

ESR spectra of 5-doxyl stearic acid in the chloroplast membranes at 5 deg. C. Other conditions same as in Figure 34.

Figure 40 A

Figure 40 B

Figure 41 A and B

ESR spectra of 16-doxyl stearic acid in the chloroplast membranes at 5 deg. C. Other conditions same as in Figure 34.

Figure 41 A

Figure 41 B

intensity is affected by the fluidity of the membrane lipids. The possible implications of this observation will be discussed later.

II. LIPID-PROTEIN INTERACTIONS IN CHLOROPLAST MEMBRANES

The presence of a similar flexibility gradient in both the chloroplast and model membranes has already been noted in section I-A. This presumably means that the lipid organization in both these membrane systems is similar. The subtle differences in the order and mobility of these systems may be attributed to lipid-protein interactions. Many such studies are reported in the literature (Cannon et al., 1975; Hiller and Raison, 1978; Marsh and Barrantes, 1978; Gally et al., 1979; Kang et al., 1979).

Figure 42 shows the ESR spectra of the chloroplast membranes suspended in water with 5-16 doxyl stearic acid spin labels incorporated in them. All the spectra were taken in the dark at

Figure 42

ESR spectra of 5-16 doxyl stearic acid spin labels incorporated in chloroplast membranes in water at 37 deg. C, in dark.

Figure 42

37 deg. C. When these spectra are compared with those of lipid-water dispersions at 37 deg. C (see Figure 15), some subtle differences in the line shape may be detected. The model lipid membranes show spectra with relatively narrow lines reflecting their relatively greater fluidity with respect to the chloroplast membranes. Quantitative estimates of these differences can be made comparing their respective values of S (see Figure 49), or the rotational correlation times (see Figure 43), whichever parameter is measurable at this temperature. A study of Figure 49 shows that although for the 5-doxyl position, there is a negligible difference between the chloroplast and the model membranes, yet at the 7- and 9-doxyl positions the probe environment is considerably more ordered in the chloroplast membranes compared to the model membranes at 37 deg. C.

Further down the fatty acyl chain, S cannot be measured at 37 deg. C because of the poor resolution of the $2T_{max}$ splittings. However, the measurement of the rotational correlation times is possible, and a study of Figure 43 tends to

Figure 43

Variation of rotational correlation time with positions (12-16) of the doxyl moiety at 37 deg. C for the chloroplast (O) and model lipid (Δ) membranes in water.

Figure 43

Position of the doxyl moiety
in the stearic acid spin label.

suggest that the lipids in the chloroplast membranes are somewhat less mobile than those in the model membranes at 12-16 doxyl positions.

The $2T_{\max}$ hyperfine splittings can be resolved even better if the sample temperature is lowered. At 5 deg. C (see Figures 44 and 45), one can compare this splitting for the chloroplast and the model membranes (see Figure 46). Figure 46 reveals that at 5 deg. C too, the chloroplast membranes are considerably more ordered (or immobilized) than their extracted lipid model membranes. The advantage of 5 deg. C is that an estimate of the 'degree of order' can be made for a larger number of doxyl positions. Figure 46 suggests that further down the fatty-acyl chains, the chloroplast membranes become increasingly more ordered compared to the model membranes of their extracted lipids.

It is also possible to measure S at an intermediate temperature, e.g. 21 deg. C (Figures 47 and 48). As revealed by Figure 49, the chloroplast membranes are more ordered than the model membranes both at 37 and 21 deg. C. The overall range of S for these two membrane systems is considerably higher at 21 deg. C than that at

Figure 44

ESR spectra for 5-16 doxyl stearic acid spin labels incorporated in the chloroplast membranes in water at 5 deg. C in dark.

Figure 44

Figure 45

ESR spectra for 5-16 doxyl stearic acid spin
label incorporated in model lipid membranes
in water at 5 deg. C in dark.

Figure 45

Figure 46

Variations of $2T_{max}$ with the position (5-16) of the doxyl moiety of the stearic acid spin label incorporated in chloroplast (●) and model (■) membranes in dark at 5 deg. C.

Error bar indicates that the $2T_{max}$ splitting for the 12-doxyl position of the label in the chloroplast membranes shows a significant variability (average of 5 experiments) at 5° C., compared to the other labelled positions.

Figure 46

Position of the doxyl moiety of the
stearic acid spin label.

Figure 47

ESR spectra of 5-16 doxyl stearic acid in the
chloroplast membranes in water at 21 deg. C
in dark.

Figure 47

Figure 48

ESR spectra of 5-16 doxyl stearic acid in
model lipid membranes in water at 21 deg. C
in dark.

Figure 48

10 G

Figure 49

Variation of order parameter S with position of the doxyl moiety in the chloroplast (O) and model (Δ) membranes in water at 21 deg. C (—) and 37 deg. C (---) in dark.

Order parameter

122b

Position of the doxyl moiety

Figure 49

37 deg. C.

Figure 50 shows the rotational correlation times (τ_1) for 12, 14 and 16-doxyl stearic acid spin labels incorporated in the chloroplast and their extracted lipid membranes at 21 deg. C. It can be seen that at this temperature, the correlation times are significantly longer for the chloroplast membranes than those for the model membranes. The difference is marked for the 12-doxyl positions (see Figure 50). It must be mentioned that at 37 deg. C (see Figure 43) the differences in the correlation times between the natural and the model membranes were not very significant. Hence by lowering the temperature, it is possible to observe the immobilization (longer correlation times) effect of the membrane proteins. In this regard the fatty-acyl segments corresponding to the level of 12-doxyl moiety seem to be more sensitive to detect this effect, at least when the temperature is decreased somewhat (see Figure 50).

Figure 50

Variation of rotation correlation time (τ_1) with the position (12-16) of the doxyl moiety of the stearic acid spin label in the chloroplast (●) and model (▲) membranes in water at 21 deg. C in dark. Note that for 12-doxyl position, the difference in the two curves is marked.

Figure 50

DISCUSSION

DISCUSSION will be presented in two main sections:

- I. DISCUSSION OF FLUIDITY and
- II. DISCUSSION OF LIPID-PROTEIN INTERACTIONS

I. DISCUSSION OF FLUIDITY

THE FLEXIBILITY GRADIENT AND LIPOSOMAL STRUCTURES

1. Spin Label Studies:

The pattern of flexibility gradient as noted in Figures 14, 15 & 17 and Table 5, in the hydrocarbon chains of the membrane lipids (in natural and the model membranes) is similar to that has been observed in lipid-bilayer structures in general (Hubbel and McConnell, 1969, 1971; Jost et al., 1971; Seelig and Hasselbach, 1971; Seelig and Niederberger, 1968; Waggoner et al., 1969; Rottem et al., 1970). This is assumed to be due

to the anchoring of the lipid polar headgroups in the lipid-water interfaces, and thus showing only a restricted mobility. The fatty-acyl chains show increasing degrees of fluidity or segmental motions from near the headgroup down to the methyl-terminal (flexibility gradient). This gradient is observable both in the order parameter as well as in the rotational correlation times.

The observation that the lipids extracted from the chloroplast membranes, form multilamellar liposomal structures (see Figure 16) and that upon sonication these multilamellar structures break into unilamellar microvesicles ranging from 250-750 Angstroms in diameter (see Figure 21) indicates that the chloroplast membrane lipids may form bilayer structures (Kimelberg, 1977; Huang, 1969; Bangham et al., 1974). These structures show a flexibility gradient closely resembling that shown by the chloroplast membranes.

In a recent report, Bishop et al., (1978) studied the motion of 5, 12 and 16-doxyl stearic acid spin labels in the aqueous multibilayers of DGDG, purified from the maize mesophyll chloroplasts. They found a flexibility gradient, which is similar to that seen in bilayer

structures. Study of such 'simplified' systems, although useful, is difficult to extrapolate to the chloroplast membranes. However, together with the studies on the chloroplast and their extracted-lipid model membranes reported in this thesis, it lends support to the possibility that a considerable amount of the lipids in the chloroplast membranes may form a bilayer structure.

Studying the phase diagram of the largest single lipid component of the chloroplast membranes, monogalactosyl diglyceride (MGDG) in mixtures with DGDG, dispersed in water, by X-ray diffraction, Larsson and Puang-Ngern (1978) showed that MGDG, which alone forms a reversed hexagonal liquid crystalline phase, does form a lamellar (bilayer) phase depending on the amount of DGDG added to it (see Figure 51 A). However, the ratio of MGDG/(DGDG plus phospholipid plus sulpholipid) of approximately 1.4, as found in spinach and bean chloroplasts is high enough to form a bilayer structure which would be stabilized by the presence of an amphiphile such as chlorophyll (Figure 51B).

It should be mentioned that a flexibility

Figure 51

(After Larsson and Puang-Ngern, 1979)

B.

Schematic illustration of the geometric conditions favouring lamellar and hexagonal structures of DGDG and MGDG, respectively. The water associated to the polar head group is indicated by broken lines. The effect of a strongly polar amphiphile on MGDG is shown below (bottom).

gradient such as found in the chloroplast membranes and their extracted lipid model membranes by spin label technique has also been observed in other biological membranes for example, the mitochondrial (Vignais et al., 1975), sarcoplasmic reticulum (Seelig and Hasselbach, 1971) and plasma membranes (Rottem et al., 1970) by spin label ESR studies.

2. C-13 NMR Studies

Figure 19 shows how closely the C-13 NMR spectrum of the chloroplast membranes compares with that of the sonicated microvesicles (Figure 20) prepared from the lipid extract of these membranes. This points out that what we observe in the chloroplast membranes by NMR is the lipid part of these membranes. This is no surprise as the high-resolution NMR spectroscopy shows best the mobile (fluid) components of the observed system, because such a mobility is important for the relaxation of the magnetic nuclei observed. As the mobility of the segment carrying the observed nucleus is reduced, the corresponding line tends to be broadened, with an apparent decrease in the line intensity. Therefore, as

3 3

OF / DE

expected for the bilayer organization of lipids, the lines corresponding to the sugar headgroups and glycerol backbone (between 61 and 71 ppm in Figure 20) are quite broad, apparently markedly immobilized due to their anchoring in the lipid-water interfaces. On the other hand, the lines corresponding to the methyl, methylene and methyne groups (see Table 7) of the lipid fatty-acyl chains show much sharper peaks as is expected from their segmental motions in a bilayer.

The marked similarity in the pattern of the line-widths between the chloroplast membranes (Figure 19) and the unilamellar sonicated microvesicles (Figure 20) (model membranes that are thought to be lipid-bilayers from the EM and the spin-label ESR flexibility gradient studies) formed from their extracted lipids, favour the possibility of lipid-bilayer structure in the chloroplast membranes. The relation of line-width to mobility and its relevance to the aggregation of the chloroplast lipids is discussed as follows:

In the fast motional range (when the rotational correlation times are shorter than 10^{-9} second), the two spin relaxation times -- the spin-lattice (T_1) and the spin-spin (T_2) --

should be indicative of the mobility of the spin giving rise to it. Furthermore, highly pronounced differences in line-width of 'comparably' similar samples in separate spectra, may also suggest mobility differences, for example, the chloroplast membrane lipid-extract in chloroform, methanol or water.

In the monomeric solution of this lipid-extract (according to Johns et al., 1977 b and 1978, the major components of the chloroplast-membrane lipids are in the monomeric form in methanol solution) (see Figure 23), the different resonances representing the carbonyl, the olefinic, headgroup, methylene, and terminal methyl groups do not show any significant differences in the apparent line-widths. This indicates that all parts of the lipid molecule are in roughly the same motional state. As the lipid molecules do not have a spherical shape, they are expected to have a preferred axis of motion, which is likely to be the rotation around the longitudinal axis. Various chemical groups in the different segments of individual molecules, should give rise to fairly uniform NMR line-widths. Two resonances at 105.2 and 100.55 ppm in Figure 23,

increase equally with the increase in the motions of the spin in question and therefore, the mobility of such a spin is related to these relaxation times by the expressions:

$$NT_1 \propto \text{mobility} \dots\dots\dots D-1 \text{ and}$$

$$T_2 = 1/\pi\Delta\nu_{1/2} \text{ (where } \Delta\nu_{1/2} \text{ is half line-width)} \dots D-2$$

where N is the number of protons directly attached to the carbon-13 in question. In the fast motion range, as both T_1 and T_2 increase equally with the increase in motions, from the relations D-1 and D-2, one can see the inverse relationship between mobility and the half line-width, with the assumption that the line shape in question is Lorentzian.

For qualitative studies, therefore, one can use the logic that smaller the line-width the greater the mobility of the spin (carbon-13 nucleus here). However, it must be kept in view that line-broadening may be caused also by inhomogeneities in the magnetic field. Nevertheless, in the same spectrum, where line-broadening effect due to the magnetic field inhomogeneities is presumably the same on all the resonance lines in the spectra, the differential line-broadening of a certain specific resonance

arising from the anomeric carbons of the galactolipid headgroups, could not be seen in the chloroplast or the model membranes, or even the lipid-extract in chloroform (Figures 19-22). Anomeric resonance at 105.2 ppm arises from monogalactosyl diglyceride and digalactosyl diglyceride lipids, whereas that at 100.55 ppm comes from digalactosyl diglyceride and perhaps also from sulphoquinovosyl diglyceride (Johns et al., 1977b and 1978).

Direct support to this kind of selective line-broadening approach as an evidence for a possible lipid-bilayer structure can be obtained from Figure 52 (Bishop et al., 1978). Figure 52A is the C-13 NMR spectrum of DGDG (extracted from the silver beet chloroplasts) as multibilayers and Figure 52B of DGDG in the monomeric solution in methanol. Changes in the line shape in these two spectra (Figures 52A and B) compare well with those observed for the lipid-extract of the spinach chloroplast membranes as water dispersions (Figure 23). More references pertaining to the C-13 NMR line-width analysis for membranes may be found in the studies of Godici and Landsberger (1975), Lancée-Hermkens

Figure 52

A. DGDG-multibilayers.

and De Kruijff (1977) and Keough et al., (1973).

Carbon-13 NMR studies of Johns et al., (1977b) on pure digalactosyl diglyceride (DGDG)-water system, which forms a bilayer structure (known from the X-ray studies of Shipley et al., 1973), also reveal a flexibility gradient similar to that observed here by spin labelling and C-13 NMR line-width studies. Measuring the spin-lattice relaxation times, these authors (Johns et al., 1977b, 1978) observed an increasing segmental motion from the headgroup to the terminal methyl group in this chloroplast membrane-lipid (DGDG)-water dispersion. Their studies (Johns et al., 1978) on another chloroplast membrane-lipid, sulphoquinovosyl diglyceride-water dispersions revealed that the mobility of the terminal carbon atoms is greater for unsaturated than for saturated fatty acids. Therefore, they explained the longer correlation times (slower motions) of the fatty-acyl chain carbons in SQDG water system with respect to those of DGDG water system as due to the presence of saturated fatty-acyl chains in SQDG.

The present studies along with the evidence from literature discussed above, therefore,

contradict the earlier model of the chloroplast membranes (see Figure 53) proposed by Weier and Benson (1966). According to these authors, this was "a model of the chloroplast membranes quite opposite of the bimolecular lipid leaflet model membranes of Davson and Danielli (1935)".

According to this model, the chloroplast membranes should not show the kind of flexibility gradient in their lipid organization which the present results clearly demonstrate (see Figures 14, 15, 17, and Table 5). In this model, the fatty-acyl chains of the lipids should be restricted in their motion as they are shown to associate with the hydrophobic regions of the coiled membrane proteins.* In addition, it may be mentioned that the electron microscope studies supporting such a granular (quantosomal) membrane structure are now regarded as artifactual (Branton, 1968). This model may therefore be set aside in view of all these considerations.

*In the "Fluid-mosaic" model of membranes, the hydrophobic interaction between lipid fatty-acyl chains and the intramembraneous proteins is confined largely to the hydrophobic surface of the proteins. Therefore, only a fraction of the membrane lipids may be directly interacting with the proteins.

Figure 53

Fig. 53 Proposed schematic model for lamellar lipoprotein organization (Weira and Branson, 1966)

3. Possible Significance of Poly-cis Unsaturation

A point that is worthy of some discussion here is the possible significance of the predominant polyunsaturation in the fatty-acyl chains of the lipids of the chloroplast membranes. Seelig and Waespe-Sarcevic (1978) have shown that despite the presence of a single cis-double bond, the "C = C bond vector" is still found to be aligned almost perpendicular to the bilayer surface. No such studies exist at present to indicate how the three cis double bonds between 9 & 10, 12 & 13, and 15 & 16 carbon atoms of linolenic acyl-chain would be oriented with respect to the bilayer plane. It may be, at least, partly bent upon itself as seen in Figure 3 (Quinn and Williams, 1978) or that the three cis olefinic double bonds may assume a helical orientation (Benson, 1966). In general, a cis-bond in a fatty-acyl chain is expected to create a considerable disorder in the packing of the fatty-acyl chains in a lipid bilayer (see Figure 54).

According to Seelig and Niederberger (1974), the model used to interpret the flexibility

Figure 54. Schematic representation of a phospholipid bilayer. Circles represent the polar head groups and the wiggly lines the fatty acid chains. Disordering effect of cis double bonds (from: Hubbel and McConnell, 1971)

Figure 55. Palmitic acid with *a*, a single gauche configuration; *b*, two gauche configurations separated by six carbon atoms.
(from: Horwitz et al., 1973)

gradient (Seelig, 1970, 1971; Hubbel and McConnell, 1971): "Each carbon-carbon bond is assumed to have a certain intrinsic flexibility, due to rotational isomerizations between trans (t) and gauche (g⁺, g⁻) conformations. One end of the chains is anchored in the lipid-water interface and thus fixed. The other end is free to move. The overall motion is determined by the superimpositions of segmental motions. Near the polar surface the motional freedom is rather restricted since only a few chain segments are involved. The motion becomes increasingly more random toward the central part of the bilayer due to the increasing number of flexible CH₂ groups. The lipid bilayer has therefore been described as ordered near the polar region and disordered in the interior."

Such segmental motions are primarily responsible for the observed flexibility gradient because it is known that the rate of the anisotropic rotation near the polar region is almost equal to that in the central part of the bilayer (Schindler and Seelig, 1973).

The conceptual difficulty with the above spin label model is that it describes the properties of the lipid bilayer as individual hydrocarbon chains

and tells little about how the lipid molecules are packed in the bilayer. The chains are considered as completely independent of one another except for an apparent increase in the energy for gauche conformation.

Isolated-gauche and Kink Models:

An isolated gauche conformation, for example, tg^+t sequence entails the collective reorientation of all segments between the g element and the terminal group (see Figure 55a). With the assumption of a constant probability for the occurrence of this gauche conformation throughout the chain, this model explains the exponential decrease in order parameter from first carbon (carbonyl) to the terminal methyl group as observed by the spin label technique (Seelig and Niederberger, 1974).

The kink (defined as g^+tg^- or g^-tg^+ sequence) (see Figure 55b and 56) model has been invoked to explain the relatively constant order parameter of 1 to 7 carbon groups of the bilayer fatty-alkyl chains observed by deuterium NMR. The segments before and after the kink are trans and have the same order parameters. At the kink

A. SATURATED
ALKYL
CHAINS

Modified from Lagaly et al., (1977)

B. CIS-DOUBLE
BONDED
ALKYL CHAINS

Figure 56 The Kink model for the saturated and the unsaturated fatty alkyl chains in a lipid bilayer.

itself, two methylene units are bent by 60° with respect to the fatty-chain axis, thereby reducing the order parameters of the 'kinked' segments (Seelig and Niederberger, 1974). Assuming that the kink is fluctuating up and down the chain, with equal probability anywhere in the chain, this model predicts a constant order parameter for all the fatty chain segments. However, at the interface of the two half-layers (in a bilayer), the segmental motion shows an increasing disorder (Seelig and Niederberger, 1974).

The reason why these models have been discussed here is to question their applicability to the poly-cis-unsaturated lipids dominating the lipid composition of chloroplast membranes. Lagaly et al., (1977) have put forward the view that isolated cis double bonds can form a kink-like (gauche-trans-cis) structure (see Figure 56B) but it is not yet known how this view can be translated to 3 cis double-bonds separated from each other by one single bond such as those found in the linolenic acid.

In the chloroplast membranes, however, the abundant 9, 12, 15, cis-double-bonded linolenic acid is likely to assume a helical conformation

Benson, 1971) by rotations around the C-C single bonds. Such a helical structure although 'intrinsically disordered' (due to the helical shape) may not cause the kind of disorder as may be expected from a (kink or an isolated gauche conformation. Therefore, the ideas developed by Dr. Joachim Seelig's group at Basel, Switzerland (Seelig, et al., 1973; Seelig and Seelig, 1974; Seelig and Niederberger, 1974) for saturated and singly-unsaturated lipids may not as such apply to the polyunsaturated lipids under discussion here.

A helical conformation of the linolenic fatty acyl chains may be favoured by the intermolecular interactions among the fatty-acyl chains. This argument agrees with the ideas of Seelig and Seelig (1974): "Only those conformations are probable which do not distort the parallel packing of the chains too much and thus do not create too large a defect volume." Whatever, may be the conformation of these chains in the chloroplast membranes and whatever be the mechanism of the flexibility gradient, the present spin label studies show a flexibility profile similar to that observed in most other biological and model membranes.

4. C-13 Enrichment

Chapman and associates (Keough et al., 1973) published a natural abundance C-13 NMR spectrum of lettuce chloroplast membranes (see Figure 57A). The discussion they gave on this spectrum was:

"The chloroplast membrane spectrum is obviously extremely complicated in the hydrocarbon chain region, possibly because of resonance contributions from the chlorophyll molecules in this region, but no signal appears at 55 p.p.m." (for $-N(CH_3)_3$ groups). Because of the poor signal-to-noise ratio in the natural abundance C-13 NMR spectrum observed by these workers (Keough et al., 1973) it is not possible to say whether the resonances due to the sugar headgroup carbons and the carbonyl group carbon existed or not. These resonances, however, are clearly seen in the C-13-enriched NMR spectrum of the spinach chloroplast membranes taken during the present studies (Figure 57B for the direct comparison and Figure 18A for details). Table 8 shows the pattern of incorporations of carbon-14 labelled (radioactive) carbon dioxide gas in the fatty

57A. 25 MHz Natural abundance C-13 NMR spectrum of lettuce chloroplast membranes by Keough, Oldfield and Chapman (1973).

57B. 25 MHz C-13 enriched C-13 NMR spectrum of spinach chloroplast membranes, reduced in size from Figure 18 A, for direct comparison with Figure 57A.

Table 8

(McKee and Hawke, 1978)

A comparison of the fatty acid labelling pattern and the composition of the constituent fatty acids of monogalactosyldiacylglycerol (MGDG) in leaves of spinach plants grown in $^{14}\text{CO}_2$

	Fatty acid constituent							
	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
Composition of constituent fatty acids (mol %)	1.4	1.0	1.4	18.6	0.5	tr	2.8	74.4
Distribution of radioactivity (% total)	3.8	tr	4.2	26.2	tr	1.1	5.4	59.3

acids of MGDG in spinach chloroplasts. It is likely that a similar pattern of C-13 enrichment may be occurring in the experiments reported here.

In the C-13 enrichment experiment, complexity of the spectrum due to C13-C13 couplings was practically not encountered. Such couplings however, are normally observed with the C-13 enrichment techniques reported by Strouse et al., (1972) and Flaumenhaft et al., (1970). The improved method of C-13 enrichment described here should prove to be useful for the future studies requiring C-13 enrichment of the plants.

In this work, the majority of the resonances in the chloroplast C-13 NMR spectrum have been assigned despite their earlier description as "extremely complicated" Kough et al., (1973). The simple idea that was used for these assignments was the dominance of linolenic acid in these membranes. Table 6 shows that most of the resonances due to the hydrocarbon groups in the chloroplast and their extracted-lipid model membrane spectra can be easily assigned to the carbons of linolenic acid. However, contribution to the intensities of these resonances due to the superimposition of lines from other carbon-13

nuclei of the membrane components resonating at approximately the same frequencies cannot be ruled out in the spectra of such complex systems. The approximate assignments for the sugar headgroups and glycerol backbone have been referred according to the studies on pure MGDG, DGPg, and SQDG lipid fractions in the literature (Johns et al., 1977b, 1978; Bishop et al., 1978).

I-B. LIQUID CRYSTAL - GEL PHASE TRANSITION

Figures 26 and 28 show that the plot of $2T_{max}$ of 5-doxyl stearic acid versus the temperature of measurement has a break between 15 and 20 deg. C both for the chloroplast and the model membranes.

Such a break is thought to be due to the phase transition in the membrane lipids, as already explained in the INTRODUCTION. The observed comparable values of the phase transition temperature (T_c) in the two systems studied, are suggestive of a similar lipid organization in the model as well as in the native chloroplast membranes.

These closely comparable values of T_c for the model and the chloroplast membranes suggest that

the observed phase transition corresponds to the lipids of these membranes, on which the membrane proteins do not appear to exert sufficiently great an effect to alter the T_c value. However, difference in slopes of lines in Figures 26 and 28 could be attributed to the lipid-protein interactions in the chloroplast membranes. The value of T_c around 18 deg. C obtained with 5-doxyl stearic acid in the chloroplast membranes agrees with the qualitative observation of T_c of these membranes employing carbon-13 NMR studies (see Figure 33). This was indicated by the sharp increase in the apparent mobility of the methylene peak at 29.7 ppm in raising the temperature from 16.5° to 20.5 deg. C.

1. Controversy over T_c in Chloroplast Membranes:

In view of the presence of large amounts of poly-cis-unsaturated fatty-acyl chains in the lipids of the chloroplast membranes, one may argue that the T_c of these membranes should be below 0 deg. C. Such a viewpoint gets added support from the T_c measurements made on the chloroplasts by studying the dependence of chlorophyll-fluorescence on temperature. Murata and Fork

(1977), making such measurements on spinach and lettuce chloroplasts found their T_c at -11 and -20 deg. C. respectively. King and Spector (1978) showed that enrichment (6%) with linolenic acid of the cell plasma membrane brings down its T_c from approximately 24 to 20 deg. C. (see Figure 58).

Theoretically, it is not unexpected to get such subzero values of T_c for the chloroplast membranes because many of their individual lipid components, in pure state, show quite low values of T_c in their hydrated forms (see Table 9) (Shipley et al., 1973). Yet, it may be argued that the component lipids may not exist in separate domains (phase-separated forms) in these membrane systems. Whereas a lipid-bilayer organization may exist in these membranes (as the present studies suggest), the major lipid, MGDG, in pure form, forms reversed hexagonal cylinders on hydration. Therefore, it is not justifiable to compare the phase transition temperatures of the individual isolated (hydrated) lipid components with that of the chloroplast membranes.

It is possible that in the chloroplast membranes and the model membranes made from their extracted lipid-mixture, intermolecular

Figure 58

Figure 58 Temperature dependence of τ_0 for I(5,10) in plasma membranes enriched with 18:3. The procedure was identical to that described in Figs. 2 and 3. For comparison, the data obtained with the membranes enriched with 15:1 are shown and labeled as control.

(King and Spector, 1978)

Table 9

Phase transition temperature (T_G) of the hydrated pure lipid components from the chloroplast membranes, by differential scanning calorimetry (DSC) and X-ray diffraction techniques. Arranged from Shipley et al., (1973).

LIPID-WATER SYSTEM	T_G ($^{\circ}\text{C}$)
MGDG- H_2O (hexagonal cylinders)	-30 (DSC)
DGDG- H_2O (bilayer)	-50 (DSC)
SQDG I- H_2O (bilayer)	-10 to 20 (X-ray)
SQDG II- H_2O (shows dependence on lipid-to-water ratio):	
$\frac{\text{gm lipid}}{\text{gm lipid} + \text{gm water}}$	
0.35 (bilayer above 25-35 $^{\circ}\text{C}$, but demixed 25-35 (X-ray) two phases - lamellar-gel and crystals - below 25-35 $^{\circ}\text{C}$).	
0.80 (bilayer above 45-55 $^{\circ}\text{C}$, but demixed 45-55 (X-ray) two phases - lamellar-gel and crystals - below 45-55 $^{\circ}\text{C}$).	

* SQDG I is (sulphoquinovosyl diglyceride) containing 48% palmitic acid and **SQDG II contains 71% palmitic acid.

interactions among the different classes of lipids and pigments, may result into a bilayer organization and thus give the much different value of T_c as observed in the present studies. On the other hand, low-temperature studies of Murata and Fork (1977) may give rise to artificial subzero values of T_c due to the possible (partial) phase separation of the component lipids. Additional discrepancies in such measurements may be imagined from the possibility of an altered chlorophyll-to-lipid relationship due to any such phase separation. It is also possible that subzero values of T_c might exist in addition to those around 15 deg. C observed here and elsewhere (Ruuge et al., 1977; Jursinic and Govindjee, 1977). This may occur if there are separate lipid domains such as have been shown in plasma membranes of spleen by Klausner, et al., (1980).

The observation of the temperature-dependent break between 15 and 20 deg. C in the oxidation kinetics in the dark of the cytochrome b-559 in the photosystem-II particles fractionated from the spinach chloroplasts, may well be attributed to the lipids associated with this photosystem-II

particles (see Figure 59) (Yamamoto and Ke, 1979). It is known that the lipids present in these photosystem-II particles retain an overall composition similar to that of unfractionated chloroplasts (Vernon et al., 1971). Hence it suggests that physiologically speaking, the T_c at 15-20 deg. C may well be more important than any subzero values to the functioning of the chloroplast membranes.

2. Rotational Isomerization Model for T_c and the Chloroplast Membranes:

The change in fluidity on phase transition is considered to be due to rotational isomerization around the single C-C bonds of the fatty-acyl chains (Träuble, 1971, Hubbel and McConnell, 1971). Thus, a trans conformer can be converted gauche one by rotating the C-C bond by 120 degree. Above the T_c , these conformations interchange rapidly with the life-time of any one conformation approximately, 10^{-6} second (Seelig and Niederberger 1974). Below the T_c , the hydrocarbon chains are in the all-trans form and therefore, tightly and regularly packed in a

Figure 59

The Arrhenius plot for cyt b_{559} oxidation.

Cytochrome b_{559} -rich photosystem-II subchloroplast fragments fractionated from the spinach chloroplast. The break in the curve occurs at the same temperature (16-18° C) where the T_c of the chloroplast membranes has been found by other methods.

(Yamamoto and Ke, 1979)

crystalline array -- THE ORDERED or THE GEL state. Raising the temperature above the T_c , produces considerable trans-gauche rotational isomerism giving rise to a "loose" or less-ordered arrangement of the fatty-acyl chains -- THE LIQUID-CRYSTALLINE state. This is the trans (t)-gauche (g) isomerization model for T_c phenomenon (Seelig and Niederberger, 1974). According to the "kink" model (Seelig and Niederberger), two gauche rotations in opposite directions (designated g^+ & g^-) separated by an unaltered trans bond forms a kink (designated $g^+ t g^-$ or $g^- t g^+$) and this kink moves up and down rapidly to "melt" or fluidize the fatty-acyl chains.

In the lipids of the chloroplast membranes, it will be hard to visualize how such kinks could move (cooperatively) up and down the fatty-acyl chains because of the marked abundance of "stiff" cis double bonds at 9-10, 12-13 and 15-16 carbons; may be such a presumed kink could move more freely up and down between C-1 and C-9 positions than further down the chains. Although Lagaly et al., (1977) showed (see Figure 56) how cis double bond together with a gauche and trans

conformations at adjacent locations (cis-t-g) could form a kink-like structure, yet it is not known what would be the probability of the formation and the movement of such kinks in the poly-cis-unsaturated systems of the chloroplast membrane lipids.

I-C. EFFECT OF LIGHT ON CHLOROPLAST MEMBRANES

Shining light on the chloroplast membranes labelled with stearic acid spin label does not seem to cause any change in the motion or the order of the label as measured by the ESR technique. The line splittings and the line shape of the ESR spectra do not change on exposure to light. (See Figure 34 to 40). It may be argued that because of the high linolenic acid (high poly cis-unsaturation) content of these membranes, there should be considerable disorder inherent in these membranes at all the time under most conditions, including dark. The functional changes caused by light, may therefore, not appreciably alter the mobility of the lipid fatty-acyl chains as seen by the spin-labelling technique. Our studies are in line with the studies by other workers (Ruuge et al., 1977)

using the fatty acid spin label incorporated in the bean chloroplast membranes.

Light induced changes in the fluidity of the spinach chloroplast membranes were observed however, with a different spin probe (2, 2-dimethyl -5, 5-dipentyl-N-oxylozolidine, also designated, 6N11) by measuring the changes in the partitioning of the probe in and out of the membranes under the influence of light (Packer, 1975). These results were interpreted (Torres-Pereira et al., 1974; Packer, 1975) as due to the changes in the lipid organization of these membranes, as these authors could observe the concomittant light induced protein aggregation in the membranes using freeze-etch electron microscopy.

However, it may be argued that the partitioning of the 6N11 spin label may also be affected by the changes in pH and ionic composition that are known to take place across the thylakoid membranes in light (Hind, et al., 1974; Chow et al., 1976). Such changes, if not properly controlled (such controls are not seemingly obvious in the experiments referred to above) are likely to play a greater part than the

suggested membrane lipid organizational changes, in determining the increased partitioning of the spin label into the thylakoid membranes on illumination, as observed in the studies of Packer (1975) and Torres-Pereira et al., (1974).

Tzapin et al., (1971) measured changes in the microviscosity of the Vicia faba chloroplast membranes as a result of illumination. These findings, on the first look, appear to contradict our results, however, it must be realized that the spin probe these workers were using,

the nitroxide radical 2,2,6,6-tetramethyl-4-capriloiloxypiperidine-1-oxyl, depicted the state of the membrane near the headgroups of the lipid organization. Therefore, these studies may not represent the changes in the mobility of the fatty-acyl chains of the membrane lipids, which the present studies reveal not to undergo fluidity changes as a result of illumination.

An effect of light on the ESR signal intensity of the spin-labelled chloroplast membranes was also noted in the present studies (see Figure 34-40). The initial rapid increase of

the ESR signal intensity on illumination is followed by its slow but continuous decrease. Detailed reports on such studies using different spin probes and monochromatic light sources for illumination have discussed the kinetics and the possible mechanisms involved (Tsapin et al., 1974; Subchinski, et al., 1975). The initial increase in the signal has been explained as due to the re-oxidation of the spin probe (which was previously reduced by the plastoquinone pool, shown in Figure 60, of the membrane during the labelling of the membranes) involving the delivery of electrons to the probe, possibly from P700.

As the main interest in the present project was to study the fluidity of the chloroplast membranes, the effect of light was studied for only the two extreme positions, using 5- and 16-doxyl stearic acid spin labels, in the chloroplast membranes in their gel state (measurements at 5 deg. C are well below the T_c observed).

Figures 41 to 43 show that the apparent rate oxidation of the spin label (increase in the ESR signal intensity) is slowed down markedly at 5 deg. C. If the spin label regeneration in light is presumed to be due to its interactions with the

Figure 6a

PIERRE JOLIOT (1978)

Model for the photosynthetic membrane. Chl, photoactive chlorophyll; Z and Y, secondary electron donors of photosystem II; Q, primary electron acceptor of photosystem II; PQ, plastoquinone; Cyt f, cytochrome f; PC, plastocyanin; X, primary electron acceptor of photosystem I.

plastoquinone pool, as discussed above, then it suggests that in the gel state of the chloroplast membranes, the accessibility to the plastoquinone pool (perhaps by diffusion) of the 5 and 16-doxyl positions is slowed down in the gel state of the membrane lipids.

II. DISCUSSION ON LIPID-PROTEIN INTERACTIONS IN CHLOROPLAST MEMBRANES

On a dry-weight basis, the chloroplast membranes contain about 50% protein and 50% lipid (Lichtenthaler and Park, 1963). Although this proportion of the membrane protein sounds high, considering the large average molecular weight of proteins, their actual number with respect to the number of lipid molecules may be much smaller. Therefore a lot of lipid may exist in the membrane which is not directly affected by the presence of the membrane proteins. From the study of lipid-protein interactions in model membrane systems, three states of the membrane lipid have been proposed as a result of the presence of intrinsic

membrane proteins (Curatolo et al., 1977). They are (see Figure 61A) summarized as:

1. Boundary lipid, which surrounds individual intrinsic membrane proteins, and hence may be partially immobilized. This may consist of more than one concentric layers of lipid around the protein. Anderson (1975) has proposed that phospho- and sulpho-lipid may play the role of boundary lipid in the thylakoid membranes (see Figure 2).
2. Trapped or perturbed lipid, which is beyond the boundary-layer of lipid, but is trapped among a number of intrinsic proteins, which physically compress the lipid and thus restricting its motion and increasing its degree of order.
3. Bulk lipid, which occupies a zone largely free of the protein and hence behaves as though it does not interact with proteins in the membrane. The relative proportions of these somewhat arbitrarily-defined categories of membrane lipids tend to vary with temperature (Davoust et al., 1980) (Figure 61B) and with protein-to-lipid ratio as studied in the model systems (Marsh et al., 1978) (Figure 61C).

Studies on model membranes are difficult to

Figure 61

61 A. Three categories of lipids due to lipid-protein interactions in a membrane (Curatolo et al., 1977)

61 B. Proteins (circles) in a lipid bilayer. On lowering the temperature proteins tend to progressively aggregate (lower Figure of 61 B) and (presumably) thereby increasing the amount of "trapped" lipid (Davoust et al., 1980)

61 C. Schematic indication of the molecular packing in cytochrome oxidase-dimyristoyl phosphatidylcholine complexes. (a): at low lipid:protein mole ratio; boundary lipid and perturbed (less fluid) lipid bilayer. (b): at high lipid:protein mole ratio; boundary lipid and unperturbed (more fluid) lipid bilayer. (Marsh et al., 1978)

extrapolate to the biological membranes, wherein a variety of proteins and lipids co-exist. The complexity of the problem is further increased by the presence of pigments, as is the case with the chloroplast membranes. EM studies of Park and Pon (1963) indicate that the spinach chloroplast membranes consist of proteins embedded in the lipid matrix. They reached this conclusion by observing these membranes with EM before and after the lipid-removal (by organic solvents). At present, lipid-protein interactions in biological membranes have been routinely studied by comparing the lipid microdynamical properties (mobility studies) of the biological membrane in question, with the model membranes made from its lipid-extract (Gally et al., 1979; Zumbulyadis and O'Brien, 1979; Kang et al., 1979b).

At 5 deg. C, $2T_{max}$ becomes measurable up to 13 or 14-doxyl positions and it is found (see Figure 46) that at all these positions, the chloroplast membranes are considerably less fluid than the model membranes made from their lipid-extract. The line-shape of the ESR spectra at 5 deg. C (Figures 44 and 45) are indicative of the overall reduction in the motion of the spin label

environment at the reduced temperature. Similar changes in the ESR line-shape on lowering the temperature were noted by Brotherus et al., (1980) in studying the lipid-protein interactions between Na, K-ATPase and a lipid membrane system and by Pauls et al., (1980) in Acanthamoeba microsomal membranes.

Zumbulyadis and O'Brien (1979) studied the rhodopsin-phospholipid interactions by carbon-13 and proton NMR. They found that: (i) the presence of protein (rhodopsin) in egg phosphatidylcholine vesicles results in homogenous broadening of the methylene and methyl resonances; and (ii) this broadening effect increased with the increase in rhodopsin content. This model system, though very different from the chloroplast membranes, supports the observations reported in the present thesis in as far as the increased immobilization of the lipid hydrocarbon chains by the presence of the proteins is concerned.

Two major intrinsic proteins are known to be present in the chloroplast membranes. In the freeze fracture observations (see Figure 62), these appear as the protoplasmic face (PF) and the exoplasmic face (EF) proteins which have been

Figure 62. proposed model for the particulate subunits of the chloroplast lamellae. In plastids from plants grown under intermittent light (0-h sample), 75-Å and 80-Å particles are embedded in a lipid phase of the membrane; the more externally localized 75-Å particles cleave with the PF face during freeze fracture, whereas 80-Å particles cleave with the EF face. During greening in continuous light (48-h sample), the light-harvesting pigment protein (shaded material) is inserted into the membrane and primarily associates with the 80-Å "core" unit of the EF face. The total aggregate (80-Å core unit plus various amounts of light-harvesting pigment protein) results in EF particles of discretely variable size classes. Particle spacing in this diagram conforms to average separation distances measured on freeze-fracture micrographs. (Armond et al., 1977)

Figure 63. Diagram showing how the lipids may be "trapped" among the intramembranous proteins in a chloroplast thylakoid membrane (modified from Carter and Staehelin, 1980).

associated with photosystem I and II activities, respectively (Armond et al., 1977). Figure 63 (Carter and Staehelin, 1980) shows that a bulk of the volume of these proteins seemingly lies in the hydrophobic matrix, i.e., the core of the membranes. Such a localization of the proteins agrees with observed lipid-protein interactions in the chloroplast membranes in the present work.

In the present studies, the ordering effect of proteins on the lipid fatty-acyl chains observed may well represent lipids entrapped between the proteins (see Figure 63) as discussed by Curatolo et al., (1977); Chapman et al., (1977) & Marsh et al., (1978).

Some reports in the literature about the lipid-protein interactions in the gel state of the model membranes of lipids containing saturated fatty-acyl chains (Chapman, et al., 1979) seemingly contradict our results. According to these studies (Chapman et al., 1979) in the gel state of the membrane, the intrinsic protein is "squeezed out" of the bilayer (thereby decreasing the lipid-protein interactions) due to the tight packing of the all-trans fatty-acyl chains in the gel state. However, in the

chloroplast membranes, this does not seem to be the case perhaps due to its abundance in unsaturated fatty acids, which might mediate stronger lipid-protein interactions. Evidence in favour of enhanced hydrophobic lipid-protein interactions by increased unsaturation of fatty-acyl chains has been provided by Mateau *et al.*, (1978) on phosphatidic acid-cytochrome c or lysozyme model membrane systems.

Owing to the presence of large amounts of poly-cis-unsaturated fatty acyl chains, such a 'tight' packing of these chains may not be achieved even in the gel state of these membranes. Therefore, the intrinsic protein should not get squeezed out of the lipid-matrix of the chloroplast membranes even in the gel-state. Also, the phase transition above 0 deg. C that was observed in the present studies may or may not represent the only such transition in these membranes (in light of subzero transition temperatures seen by other methods) and thus the chloroplast membranes at 5 deg. C may not be in 'true' gel state.

In a personal communication, Dr. Myer Bloom of the University of British Columbia expressed

the idea that low levels of lipid-protein interactions observed in routine experiments on the biological membranes, could be due to an elastic 'cushion' effect of the proteins on the interacting lipids. Dr. Bloom opined the possible existence of a fluidity gradient increasing from the interior to the exterior of the protein molecules. This idea does not favour a strong immobilization effect of the proteins on the fatty-acyl chains of the lipid molecules interacting with the intrinsic protein. In the chloroplast membranes the rotational correlation times (at least for the 12-doxyl moiety) are only marginally longer when compared with those for the model lipid membranes (see Figure 50). This seems to be in accordance with the above "cushioning effect" of the proteins.

In agreement with the spin label studies of organellar membranes, such as the mitochondrial (Cannon et al., 1975) and chloroplast thylakoid (employing 6, 9 and 12-doxyl fatty-acid spin labels) (Hiller and Raison, 1978) membranes, the present work reveals that the chloroplast membranes are significantly more ordered than the model membranes made from their lipid extract.

The fatty acid spin probe used in the present studies is likely to distribute itself more or less uniformly in the bulk of membrane lipids and it may not preferentially align itself with the "boundary" lipid, if any. Therefore, the lipid-protein interactions observed here should be of a general nature. The ordering and the mild immobilization of the fatty-acyl chains noted here should represent an average over the direct (lipids in direct contact with protein), as well as indirect (lipids not in direct contact with the protein) lipid-protein interactions. Such an ordering and mild immobilization effect in the lipid fatty acyl chains is seemingly contributed more, from the indirect interactions where the mere physical presence of proteins may "trap or perturb" the lipids 'entrapped' among them (see Figure 63). In such a case the fatty acyl chains would have

- (i) a reduced (lateral) space resulting in a reduction in the amplitude of motions, and
- (ii) somewhat decreased rate of motion perhaps due to increased hydrophobic inter- and intra-chain interactions.

If the boundary lipid, if any, present in the chloroplast membranes, constitutes only a small fraction of the total membrane lipid, which would perhaps incorporate only a small percentage of the spin probe, its contribution to the detectable lipid-protein interactions may not be appreciable. Whatever be their detailed nature, the present studies do indicate the existence of these lipid-protein interactions in the chloroplast membranes.

* * * * *

Figure 64. State of affairs in a bilayer (modified from Horwitz et al., 1973).

CONCLUSIONS

1. Both the chloroplast membranes and the model membranes of their lipid-extract show a comparable flexibility gradient in the fatty-acyl chains as studied by spin label ESR and C-13 NMR techniques. The observed gradient is similar to that known for the lipid-bilayer structures, in general.

2. Together with the electron microscopic evidence of the liposomal structures formed by the model lipid membranes, the above mentioned flexibility gradient suggests that a considerable proportion of lipids in the chloroplast membranes may be organized as bilayers.

3. A technique has been devised for the carbon-13 enrichment of the chloroplast membranes. This procedure practically eliminates the spectral complexity caused by the C13-C13 couplings, normally encountered by the pre-existing enrichment methods.

4. A comparable temperature of liquid crystal-gel phase transition for both the native and the model membranes suggests a similar lipid organization in both the systems.

5. Light illumination does not seem to alter the fluidity of the chloroplast membranes. However, an initial photo-oxidation of the spin label in these membranes seems to be slowed down at 5 deg. C., presumably, as a consequence of a 'gel' state existing at least in parts of these membranes.

6. The lipid fatty-acyl chains in the chloroplast membranes are observed to be more ordered and somewhat less mobile than their counterparts in the model membranes. These differences appear to be due to the "trapping" of lipids amongst the intrinsic proteins of the chloroplast membranes.

SUGGESTIONS FOR FUTURE STUDIES

There is a large variety of spin labels available these days which can be employed for future ESR experiments. Protein spin labels and spin labelled galactolipids should be quite useful.

With the carbon-13 enrichment procedure described here, one could study the C-13 spin relaxation times of these membrane systems.

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*The world is imprisoned in its own
activity except when actions are
performed as a worship of God.*

The Bhagwat Gita

APPENDIX I

During the course of this investigation, it was found that incubating the chloroplast membranes with the spin label from 10 minutes to 10 hours in dark, gave a similar quality of satisfactory labelling of the membranes. At the concentrations of spin label used, almost all the spin label partitions into the membranes; however, when dilute membrane suspensions are used, some label diffuses out of the membranes to appear as sharp liquid-lines (representing loosely-bound label) super-imposed (but resolvable) over the spectra of the label in the membranes. In most cases, when the membranes can be concentrated, the liquid-lines could be eliminated.

At the low concentrations of the stearic acid spin label used, the probability is remote that the label molecules, themselves form some kind of stable aggregates, such as micelles. The phospholipid spin label could form its own lamellar structures. Any such aggregation of the spin label molecules will result in spin-spin interactions, which could not be seen in the present studies of ESR. Therefore, it seems that any such aggregation of the spin label molecules themselves, if at all occurs, may be only a transitory phenomenon, as such structures are likely to fuse with the large excess of the membranes around.

Carleton University
Faculty of Graduate Studies
Ph.D. THESIS EXAMINATION REPORT

Candidate..... **Rakesh Chander Yashroy**
Department..... **Biology**
Date of Examination..... **Tuesday, May 13, 1980 - 2:00 p.m.**
Thesis Title..... **Fluidity and Lipid-Protein Interactions in
Chloroplast Membranes: a Magnetic Resonance Study**

Thesis: ☐ accepted as submitted
☒ acceptable after minor corrections and modifications
☐ acceptable after major modifications as outlined on
attached sheet
☐ rejected

Thesis grade: ☒ Satisfactory ☐ Unsatisfactory

Oral Defence: ☒ Satisfactory ☐ Unsatisfactory

Remarks:

Dean of Faculty, Department of Chemistry and Biology
University of Quebec
Trois Rivières

- External Examiner
Professor J. Sinclair - Thesis Supervisor

Dr. I.C.P. Smith - Co-supervisor

Dr. B. Hellebrege - Chemistry Department

Professor D.R. Gardner - Advisor

Professor K.W. Joy

Professor V.N. Iyer

Dean J.L. Holfeon

Dr. M. Sundaresan - Chairman

Chairman of the Department
H.B. Merriam

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(To be completed in triplicate)

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