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Magnetic resonance studies of dynamic organisation of lipids in chloroplast membranes

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Abstract. Spinach chloroplast membranes and aqueous dispersions of their extracted lipids have been studied by spin label (stearic acid) electron spin resonance and carbon-13 nuclear magnetic resonance techniques. Combined with electron microscope studies, first systematic evidence is found for the existence of a dynamic lipid-bilayer structure in the chloroplast membranes.

Keywords. Chloroplast; membranes; lipids; nuclear magnetic resonance; electron spin resonance.

Introduction

It is generally believed that the thylakoid membranes of the plant chloroplasts have a fluid lipid-bilayer structure interacting with membranous proteins (Curatolo, 1987). Curatolo (1987), however has credited this viewpoint to Quinn and Williams (1983), who in turn have stated that it is difficult to reconcile this view due to the fact that the predominant membrane lipid, monogalactosyl diacylglycerol (MGDG) normally takes up hexagonal-II structure when dispersed alone in water and furthermore it forms inverted lipid micellar structure when dispersed together-with the second most abundant lipid, digalactosyl diacylglycerol (DGDG), of the thylakoid membranes.

Unlike most other biological membranes which are rich in phospholipids, the chloroplast thylakoid membranes contain little phospholipid (Kates *et al.,* 1970). Despite the fact that MGDG, the major constituent lipid of chloroplast membranes, does not form an aqueous lipid-bilayer structure (Larsson and Puang-Ngern, 1979), the other lipid constituents such as DGDG, sulphaquinovosyldiacylglycerol and phospholipids may well be responsible for the formation of a lipid-bilayer structure which can serve as a suitable matrix of the thylakoid membrane in which the proteins are embedded (Murphy, 1986; Van Gurp *et al.* 1988; Anderson, 1975). Absence of studies on dynamic state of lipid molecules in membranes led Weier and Benson (1966) to propose a lipid-nonbilayer structure for thylakoid membranes. The present investigation, therefore, was undertaken to systematically study the dynamic organisation of the chloroplast photosynthetic membranes employing spin label electron spin resonance (ESR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy. Together-with earlier studies (YashRoy, 1980, 1990a), convincing evidence has been gathered suggesting the existence of a lipid-bilayer structure in the chloroplast membranes.

Abbreviations used: MGDG, Monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; ESR, electron spin resonance; $_{13}$C-NMR, carbon-13 nuclear magnetic resonance; TMS, tetramethylsilane.

Materials and methods

The chloroplast membranes prepared (Arnon et al., 1956) from fresh spinach leaves were vortexed, under dark conditions, with a dried film of stearic acid spin label for a satisfactory labelling of the membranes in about 20 min. An aliquot of lipids extracted (Nichols, 1963) from the chloroplast membranes (finally dissolved in chloroform) was mixed with the spin label (dissolved in chloroform). The mixture was dried under a stream of nitrogen gas and subsequently vortexed with water for about 20 min under dark conditions for use as model membranes in the ESR studies. The molar ratio of chlorophyll (Vernon, 1960)-to-spin label was kept 20:1 for all ESR preparations. Vortexed lipid-water dispersions made from the lipid extract of the chloroplast membranes were also studied by EM after negative staining (Lucy and Glauert, 1964; YashRoy, 1990a).

Spin labels (figure 1) employed for these studies contained a nitroxide (doxyl)

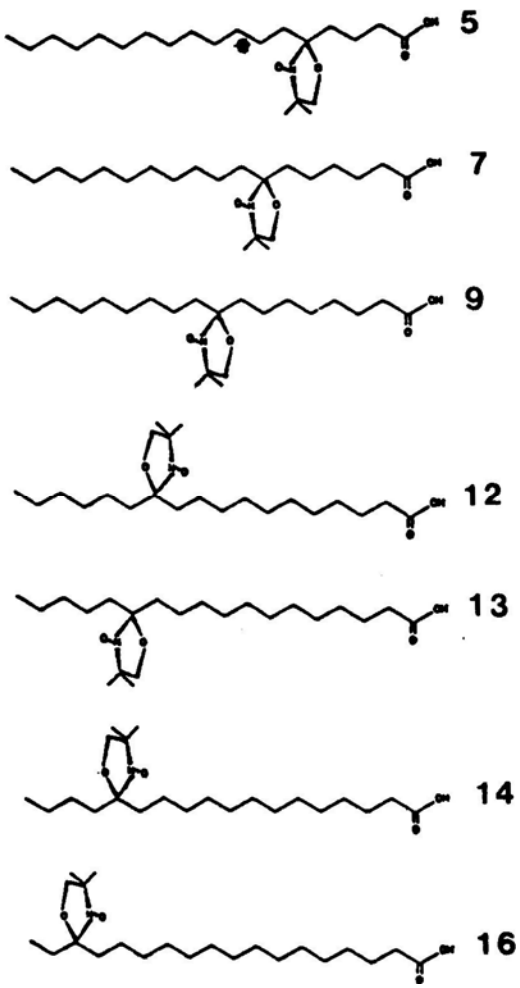

Figure 1. Diagramamatic representation of stearic acid labels showing location of the nitroxyl (doxyl) moiety at 5, 7, 9, 12, 13, 14 and 16 carbons with respect to carboxyl group.

moiety at 5,7,9,12,13,14, or 16th carbon from the carboxyl group of the stearic acid molecule. All ESR spectra were taken under dark conditions at 37°C on the Varian E-9 spectrometer equipped with variable temperature (±0·2°C) control unit. The magnetic field used was 3232 G with scan time of 4 min, microwave power of 5 mW, scan range of 100 G, time constant of 0·3 or 1·0 s and frequency of 9 GHz. In many instances, the maxima and minima of the hyperfine splittings were resolved by increasing the receiver gain about 10-fold. The rotational correlation times and order parameters for ESR studies were also calculated (Cannon *et al.,* 1975).

Natural abundance proton-decoupled ^{13}C-NMR spectra of chloroplast membranes (YashRoy 1990b) (chlorophyll content, 5mg/ml) suspended in deuterated water containing 0·2 M NaCl and 5 mM $MgCl_2$ and sonicated lipid-D_2O dispersions were obtained with Varian XL-100 or Bruker CXP-300 FT NMR spectrometer equipped with a variable temperature (±0·2°C) control unit. The spectra were collected with field frequency lock on deuterium of solvent or external fluorine; the latter was found specially useful for viscous membranes samples. An external tetramethylsilane (TMS) was used as the standard reference. The radiofrequency pulse angle of 45° and acquisition time of 0·4 s were used to obtain the Fourier transform ^{13}C-NMR spectra of chloroplast membranes and sonicated lipid microvesicles. For lipids dissolved in organic solvents such as deutrated chloroform ($CDC1_3$) or methanol (CDOD), the radiofrequency pulse angle used was 22·5° and the acquisition time was 0·8 to 1·0 s.

Results and discussion

Inspection of ESR spectra (figure 2, top-to-bottom) of the spin labelled chloroplast membranes reveals changes in line-shape, line-width and hyperfine splittings, signifying gradual increase in degree of sub-molecular motions of lipid environment from 5-doxyl to the 16-doxyl spin label positions. For example, 5-line spectra of the membrane-incorporated 5,7 and 9-doxyl labels gradually turn into 3-line spectra for 12 to 16-doxyl labels. Also, the line-widths (note the central line) become increasingly narrow in going from 7 to 16-doxyl spin labelled membranes. These variations in the spectra demonstrate existence of a fluidity of flexibility gradient in terms of increasing degree of motions in the segments of the fatty-acyl chains from near lipid headgroups to the terminal (fatty-acyl) methyl groups in the chloroplast membranes.

Model membranes prepared by aqueous dispersion of lipids extracted from the chloroplast membranes, also reveal a similar flexibility gradient. Figure 3 shows the variations in linewidth, W_o (G) of the central spectral line of the ESR spectra of the spin-labelled chloroplast membranes and model membranes made from their lipid-extract. The observation of exceedingly shorter linewidths in the order of 9,12,14 and 16-doxyl positions of the labels incorporated in the two membrane systems signifies the existence of progressively increasing rates of segmental motions towards the terminal methyl groups of the lipid fatty-acyl chains. The similarity of this flexibility gradient between the chloroplast membranes and model membranes made from their lipid-extract indicates a similarity in the organization of the lipids in these two membrane systems. Interestingly, both in chloroplast membranes and their extracted aqueous lipid dispersions there is no significant decrease in linewidth, W_o from 5 to 9-doxyl positions signifying persistence of a high degree of

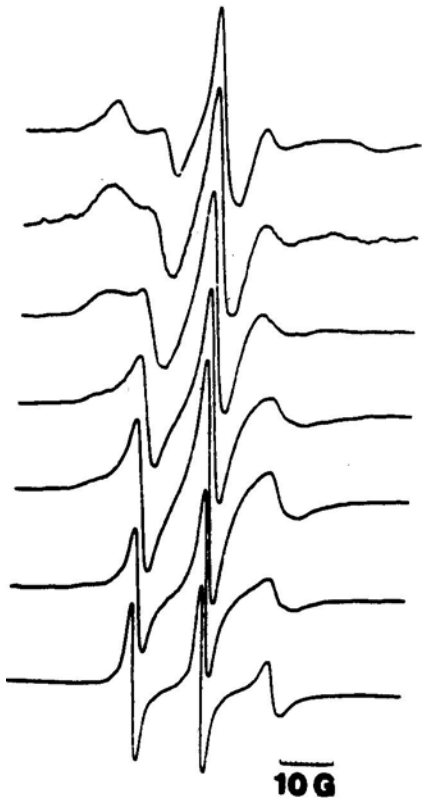

Figure 2. 9-GHz ESR spectra of the chloroplast membranes labelled with (from top to bottom) 5, 7, 9, 12, 13, 14 and 16-doxyl stearic acid spin labels. All the spectra were taken at 37^0C, in dark with the chloroplast membranes suspended in 0·2 M NaCl containing 5mM $MgCl_2$. The spectrometer settings were: magnetic field, 3232 G; time constant, 1 s; scan time, 4 min; microwave power, 10 mw; and modulation amplitude of 1 G.

motional restriction of fatty-acyl chains in this region of the two membranes, This characteristic differentiates the flexibility gradient of a lipid bilayer from that of hexagonal phase, H_{II} as inferable from the observations made by Sternin *et al,* (1988).

A flexibility gradient such as this observed here is known to exist in other biological (excluding chloroplast) membranes *viz.,* mitochondrial (Vignais *et al.,* 1975), sarcoplasmic reticular (Seelig and Hasseilbach, 1971) and plasma (Rottem *et al,* 1970) membranes in addition to many model lipid bilayer membrane systems (McConnell and McFarland, 1972). This has been explained as due to the packing of the lipid molecules in a bilayer, with the lipid headgroups anchored at the lipid-water interface (McConnell and McFarland, 1972). Figure 4 shows multilamellar structures formed by the aqueous lipid-extract of the chloroplast membranes. These structures correspond to the multi-bilayers (liposomes) formed by many aqueous lipid systems that constitute the basis of the lipid-bilayer model for the biological membranes (Bangham, 1968; Mühlethaler *et al,* 1965; YashRoy, 1990a).

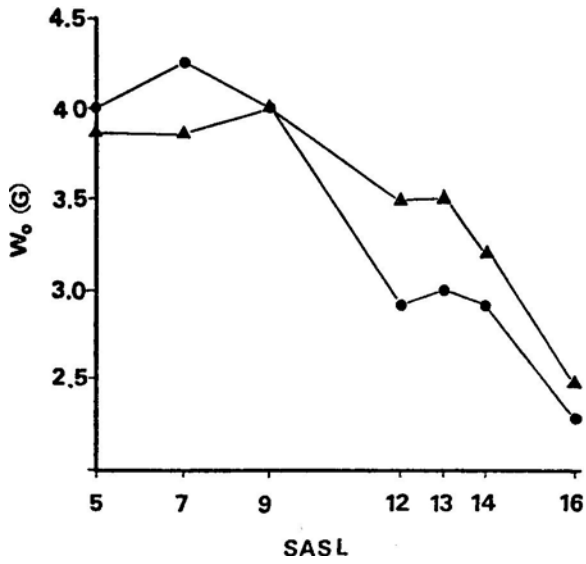

Figure 3. Variation of linewidth, W_o in G of the central line of the ESR spectra recorded in dark from stearic acid spin labelled chloroplast membranes (▲) and their extracted aqueous lipid dispersions (●) with the position of the doxyl moiety (5, 7, 9, 12, 13, 14 and 16) on the stearic acid spin label (SASL).

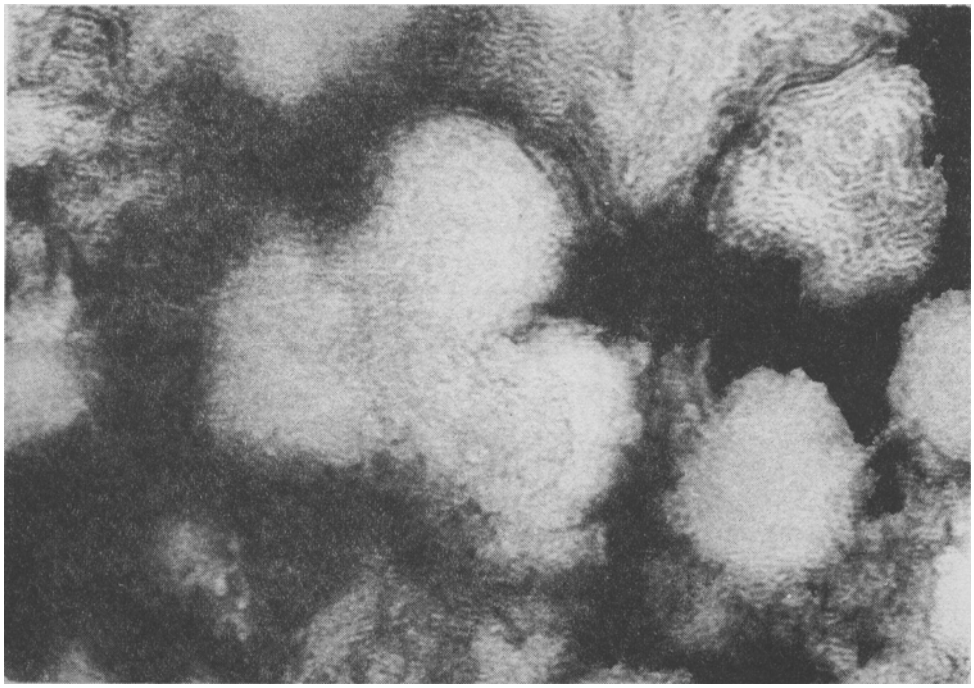

Figure 4. Election micrograph of the aqueous dispersion of the lipids extracted from the chloroplast membranes after staining with phosphotungstic acid (× 190,000).

The above observations of the flexibility gradient are also supported by ^{13}C-NMR studies of the chloroplast membranes and their sonicated aqueous lipid dispersions. Majority of ^{13}C-NMR resonances arising from the chloroplast membranes can be assigned to carbons of linolenic acid (YashRoy, 1987a,b), Figure 5 summarizes the observations on the NMR line-widths of resonances

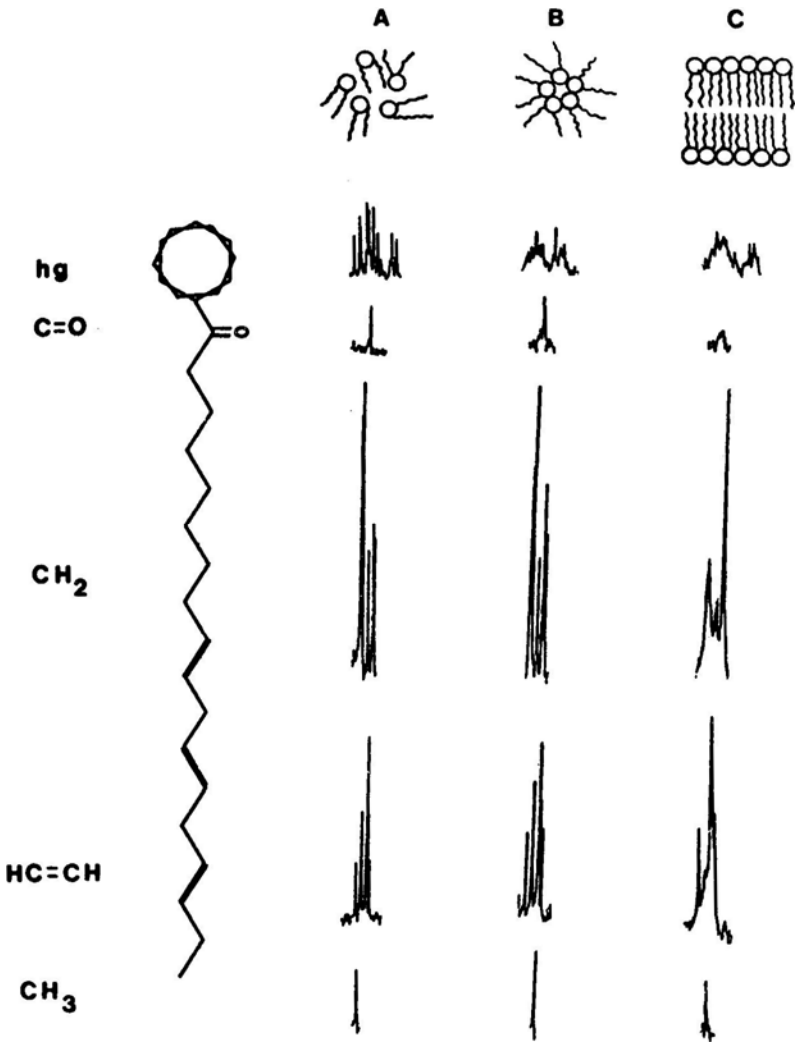

Figure 5. An overall view of the selected resonances of ^{13}C-NMR spectra of lipids extracted from chloroplast membranes. The left hand side shows a sketch of a lipid molecule having a globular headgroup (hg) and single (for clarity) extended fatty acyl chain with one carbonyl group and 3 double bonds. (A) Lipids in deuterated methanol, depicting (top-to-bottom) largely unaggregated (lipid) forms (top) and ^{13}C-NMR resonances corresponding lipid headgroups, fatty acyl carbonyl, $-(CH_2)n$, $-\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-$ and terminal methyl groups. (B) Lipids in deuterated chloroform and (C) lipids dispersed in deuterated water. Note the changes in linewidths from top-to-bottom of each of the lipid preparations (A), (B) and (C).

corresponding to various segments of fatty-acyl chains *e.g*, those located proximal to lipid head-groups, fatty-acyl methyl terminals and intermediate positions. The left-hand-side shows the model diagram of a lipid molecule with a 'globular' headgroup and having a single (for clarity) fatty-acyl chain containing a C=O group and 3 double bonds (representing linolenic fatty-acyl chain found most abundantly in chloroplast membranes). Figure 5A represents some select ^{13}C-NMR resonances arising from the extracted lipids (of chloroplast membranes) dissolved in CD3OD wherein these lipids, by and large, do not form any specific aggregates. This is clearly evident from sharp line-widths of all the ^{13}C-resonances whether head-group, C=O group, fatty-acyl chain (segment) or terminal methyl group. When the same lipids are dissolved in $CDC1_3$, they form aggregates with headgroups packed-in and tails fanning-but 'freely'. This is revealed by the ^{13}C-NMR linewidths (figure 5B) which clearly show broadening of headgroup and C=O group resonances and yet retaining narrowness of fatty-acyl chain resonances. When dispersed in D_2O as unilamellar sonicated microvesicles (YashRoy 1990a) (figure 5C), these lipids show variedly restricted movement consistent with a flexibility gradient, similar to that observed by ^{2}H-NMR and spin label ESR studies of lipid-bilayer systems (Sternin *et al,* 1988). The line-broadening effect is maximum on headgroups and C=O groups and somewhat less on the -(CH_2)n and HC = CH segments and the least on terminal-CH_3 group of the fatty-acyl chains. A similar order of restricted mobility of carbons from headgroups towards the terminal methyl groups of the fatty-acyl chains is noticeable from ^{13}C-NMR spectrum of chloroplast membranes (figure 6). In essence, the fact that the electron

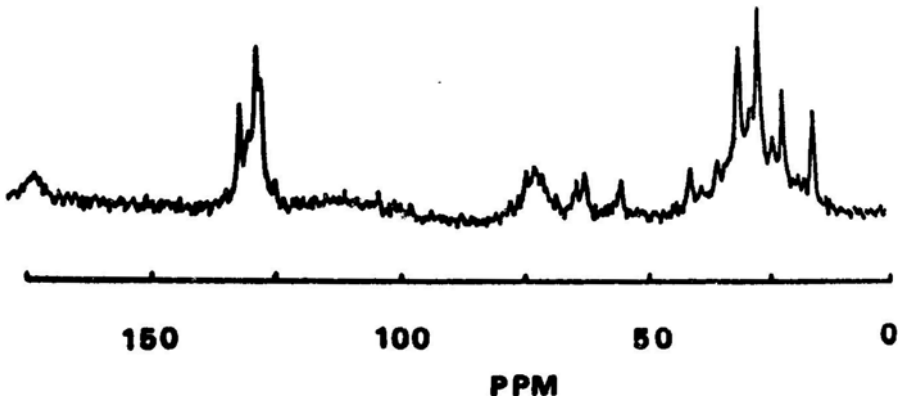

Figure 6. 75-MHz natural abundance ^{13}C-NMR spectrum of spinach chloroplast membranes at 30°C. The membranes were suspended in 0·2 M sodium chloride containing 5 mM magnesium chloride in deutrated water. Chemical shift in parts permillion (ppm) in reference to external TMS (not shown).

microscopically observable liposomal multibilayer structures (figure 4) formed by aqueous dispersion of the lipids extracted from the chloroplast membranes depict a flexibility gradient closely parelleling the flexibility gradient revealed by the native chloroplast membranes (especially notable from ESR spectral line-widths, as in figure 3) strongly suggests that the dynamic organisation of lipids in the chloroplast membranes is largely bilayer.

Acknowledgements

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