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High resolution nuclear magnetic resonance studies of hen's egg yolk plasma lipoproteins

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Abstract. High resolution nuclear magnetic resonance spectra of native or protease-treated hen's egg yolk plasma (very low density lipoproteins) were taken either in water or deuterated water; the protease-treated samples showed a sharpening of choline methyl proton signal of phospholipid, indicating the hindrance of the choline head-group rotation by the phospholipids in the native very low density lipoproteins. With both native and the protease-treated egg yolk plasma, elevated temperatue increased the signal intensity and produced line-sharpening of choline methyl protons and the $-CH_2-\overset{O}{\overset{\|}{C}}-$ protons of the methylene group adjacent to the carboxyl group of esterified fatty acids, indicating prior restriction of mobility of these groups. Total extracted lipids of egg yolk plasma containing traces of chloroform, methanol and water (which keep the sample in one phase) also gave similar temperature dependence. Addition of water to the same sample and sonication resulted in the loss of temperature dependence. Frozen and thawed protease-treated egg yolk plasma also behaved in a similar manner. The absence of temperature dependence in these latter two samples is believed to be due to formation of bilayers of phospholipids following phase separation of triglycerides and phospholipids. The results support a model in which the lipoprotein particles of the egg yolk plasma have a lipid-core structure containing triglycerides in the centre with a monomolecular layer of lecithin at the surface, the polar heads of which are surrounded by proteins.

Keywords. Nuclear magnetic resonance; egg yolk plasma; low density lipoproteins; phospholipids.

Introduction

Hen's egg yolk plasma very low density lipoprotein (VLDL) contains 13%protein, 87% lipids and has a density 0.98 g/ml (Cook and Martin, 1969; Evans *et al.,* 1973; Burley, 1975; Raju and Mahadevan, 1974). A lipid-core structure has been proposed for it (Burley, 1975; Eswaran and Mahadevan, 1972; Kamat *et al.,* 1972).

In an earlier report (Eswaran and Mahadevan, 1972) on the high resolution NMR studies of the lipoprotein of egg yolk plasma, line broadening of the choline methyl proton signal obtained was suggested to be due to a close association of the polar

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Abbreviations used: Low density lipoprotein (VLDL); Nuclcur magnetic resonance (NMR); proton magnetic resonance (PMR).

head of lecithin with polar areas on the apolipoprotein. A similar line broadening of the choline methyl proton signal in hen's egg yolk very low density lipoproteins (VLDL) was observed by Kamat *et al.* (1972) who suggested that this may be due to the interaction of the choline methyl group with the polar head of phosphatidyl ethanolamine, with triglycerides at an interface, or with the protein or polypeptide matrix. In contrast, the NMR signal obtained from the choline methyl protons in cholesterol ester-rich low and high density lipoproteins of human serum (Steim *et al.,* 1968; Chapman *et al.,* 1969 a, b; Leslie *et al.,* 1969) were pronounced and sharp, suggesting a possible difference in the location of the choline group in egg yolk VLDL and in human serum low density lipoprotein.

In this paper we present the proton magnetic resonance (PMR) spectra of the lipoproteins of egg yolk plasma before and after removal of the apolipoproteins by exhaustive treatment with a bacterial protease. The features of the spectra are compared with the PMR spectra of solvent-extracted total egg yolk plasma lipids either in single phase or following sonication in water. With all these samples the temperature dependence of the PMR spectra has also been recorded. The results are discussed in terms of the structural and motional properties of the components of VLDL.

Materials and methods

One-day old, unfertilised White Leghorn eggs were procured from the poultry development section of the University of Agricultural Sciences, Hebbal, Bangalore. Granule-free egg yolk plasma or very low density lipoprotein (VLDL) was obtained by the procedure of Raju and Mahadevan (1974).

Protease treatment

Protein-depleted VLDL were prepared by exhaustive proteolysis of VLDL or egg yolk plasma with a protease (Ramakrishna Rao *et al.,* 1978). About 3 ml of egg yolk plasma or 4 ml of VLDL (180 mg of protein by Folin's method) in 0.05 M Tris-HCl buffer, pH 7.5, was treated with 18 mg of protease (ex. *Streptomyces griseus* Type VI, Sigma Chemicals, St. Louis, MO, USA) dissolved in 100 µl of the same buffer. The solution was taken in a dialysis bag with no air bubble in the tubing so as to keep volume changes to a minimum. The material was dialysed, with magnetic stirring, against three changes of 1 litre of 0.05M Tris-HCl buffer, pH 7.5, at 25-28°C for 48 h in order to remove all dialysable products of proteins digestion as and when formed. A few drops of toluene were added to the dialysing buffer to prevent microbial growth. After 48 h of proteolysis, the material was dialysed D_2O (99.4-99.7%) containing 0.05M NaCl for a further 12 h, with 3 changes. A sample of egg yolk plasma with no protease addition was treated in exactly the same manner to serve as control.

A part of the control and protease-treated samples were analysed for protein content. The protease-treated egg yolk plasma or VLDL contained about 3 % and 2% of the original protein content respectively. The product following protease treatment was therefore essentially the lipid-core or VLDL. Autodigestion and dialysis eventually removed most of the protease protein.

Protein estimation

Protein in control egg yolk plasma or VLDL was determined by the method of Lowry *et al.* (1951). However, in protease-treated samples, the small amounts of residual proteins in the presence of large amounts of lipids was estimated by a slight

modification of Lowry's procedure. Forty five minutes after the addition of the Folin's reagent, 1 ml of $CHCl_3$ was added to 2.2 ml of the estimation mixture and mixed well using a vortex mixer. The emulsion formed was clarified by centrifugation for 10 min and the colour intensity of the top clear aqueous layer was measured colourimetrically..

Induction of gelation by storing frozen samples

This was achieved by placing control or protease-treated egg yolk plasma or VLDL samples directly in NMR tubes and storing at –20°C for 6 days prior to thawing. NMR spectra of these gelled samples were recorded directly.

Total lipids

Extraction of total lipids: The total egg yolk plasma lipids were extracted from lyophilised egg yolk plasma by successive extraction with $CHCl_3$:MeOH (2:1) solvent mixture. The solvents were removed by flash-evaporation at 30°C.

Total lipids in a single clear phase: A clear deep yellow transparent oil was obtained by flash evaporation (when trace amounts of water, methanol and chloroform were present in the mixture). The NMR spectrum of this sample was recorded. Complete drying to remove all traces of solvents led to phase separation since the phospholipids are not soluble in the triglycerides present (Schneider *et al.,* 1968).

Total lipids in water dispersion: A part of the clear yellow single-phase oil of the total lipids was dispersed in water to give an emulsion, with a lipid concentration approximately that found in yolk plasma. The emulsion was sonicated for 20 min in a Branson sonifier, Model S-75 with cooling to prevent excess heating of sample during sonification. The resulting sonified emulsion was opaque cream coloured and viscous in appearance.

PMR spectra

PMR spectra were recorded on a Varian HAA100 NMR spectrometer at a temperature of 31°C. The spectrometer was usually on the water signal (ca. 5.2 τ). All signals were recorded with trimethylsilane as internal reference. The temperature of the sample was varied using Varian temperature accessory and was measured to an accuracy of 1°C. Line widths were measured at half the signal intensity in Hz. The Varian C-1024 time-averaging computer was used to record the spectra of signals whenever necessary.

Results

Figure 1 and 2 show the PMR spectra of choline methyl proton signal of egg yolk plasma and protease-treated plasma in 0.05M NaCl in H_2O and D_2O respectively. Table 1 gives the average line width for the choline methyl proton signal following the treatments. The line width of the average signals of 11.1 Hz in the control egg yolk plasma (or VLDL) sample in water is reduced to 7.7 Hz upon protease treatment. In D_2O, the line width is higher (14.4 Hz) in the native sample and reduces to 9 Hz upon protease treatment. The D_2O effect is reversible and replacement of D_2O by H_2O brings back the egg yolk plasma choline methyl proton signal to 11.5 Hz.

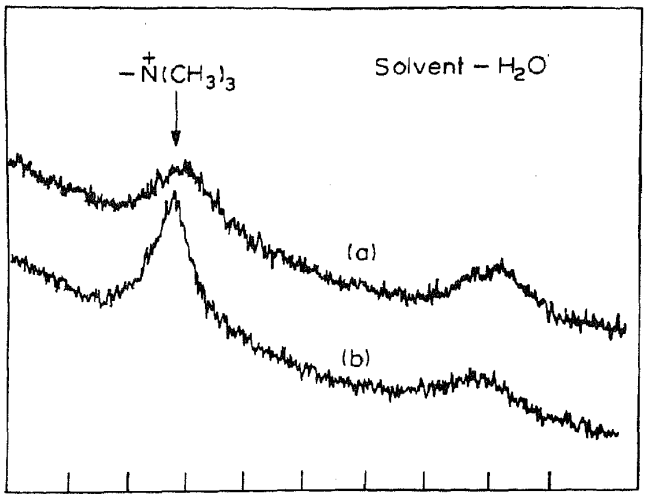


Figure 1. PMR signal of cholinemethyl proton of egg yolk plasma (a) and protease-treated egg yolk plasma (b) in H
Temp. 31 °C; sample in 0.05M NaCl in H_2O.

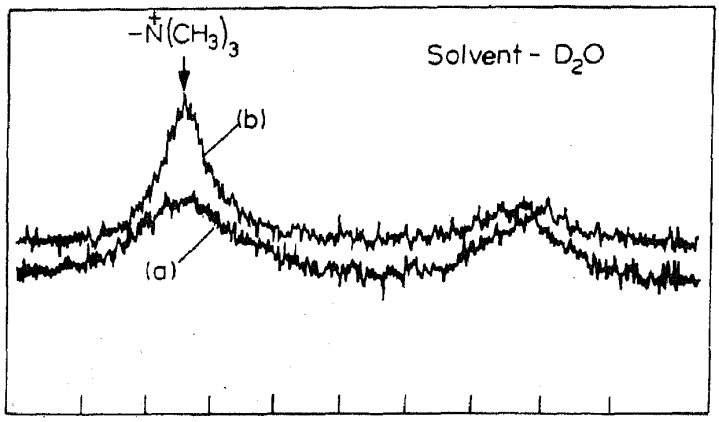


Figure 2. PMR signal of cholinemethyl proton of egg yolk plasma (a) and protease-treated egg yolk plasma (b) in D_2O.
Temp. 31 °C; sample 0.05M NaCl in D_2O

Table 1. Line width of cholinemethyl proton signal of egg yolk plasma and protease-treated egg yolk plasma.

Sample	Average line width (Hz)	
	in H_2O	in D_2O
Egg yolk plasma*	11.1	14.4
Egg yolk plasma (protease-treated*	7.7	9.0
Egg yolk plasma in D_2O and back in H_2O	11.5	—

* Average of 3 experiments; temperature of measurement =31°C.

Figures 3 and 4 show the temperature dependence of the PMR spectra of egg yolk plasma and of plasma treated with protease in 0.05M NaCl in H_2O. At the higher temperatures, the choline methyl proton signals became sharper both in egg yolk plasma and plasma treated with protease. In addition, the triplet signal at 7.7 ppm, and assigned to $-CH_2-\overset{O}{\overset{\|}{C}}-$ protons of methylene group adjacent to the carboxyl group of the esterified fatty acids of triglycerides and phospholipids, becomes very prominent at 60°C with measurable separation, when compared to 30°C where it is a broad single peak. Thus increase in temperature increases the signal intensity and brings about line sharpening of choline methyl protons and gives rise to sharp signals with fine structure of the $-CH2-\overset{O}{\overset{\|}{C}}-$protons. The methylene $(-CH_2-)n$ and methyl ($-CH_3$) groups of the esterified fatty acids are relatively unaffected by increased temperature. The line widths as a function of temperature change for the choline methyl and $-CH_2-\overset{O}{\overset{\|}{C}}-$ protons signals are given in table 2. There is a pronounced and progressive reduction in the line widths of $-CH_2-\overset{O}{\overset{\|}{C}}-$ proton signals

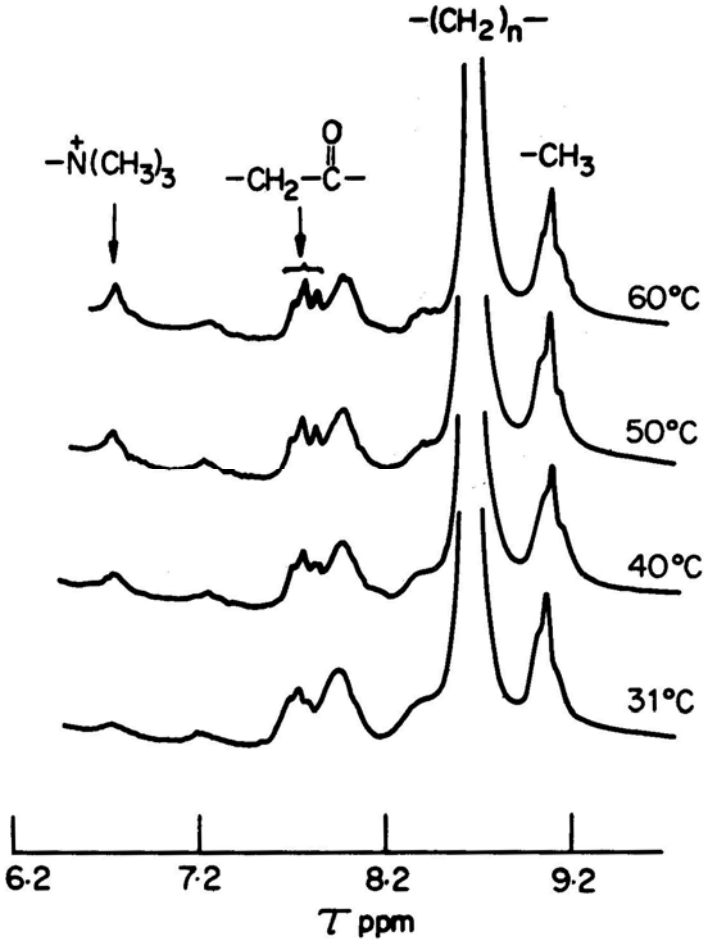
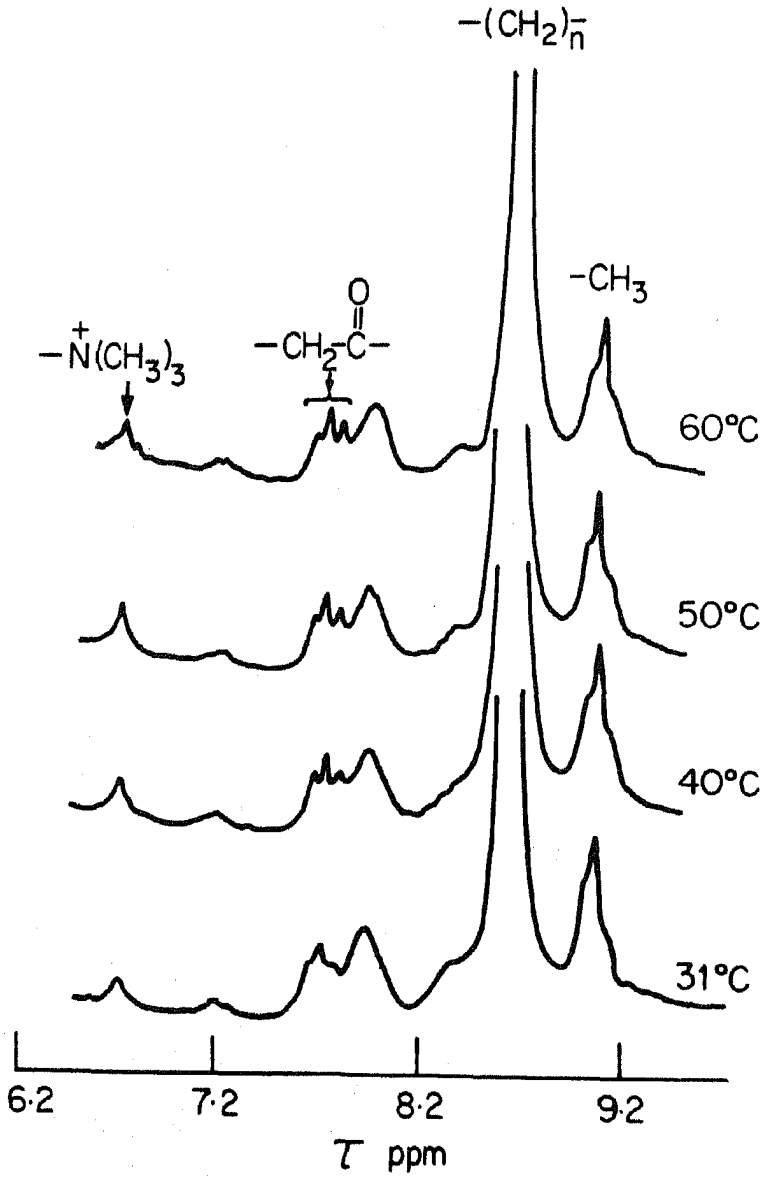


Figure 3. Temperature dependence of PMR spectra of egg yolk plasma in H_2O Samples in 0.05M NaCl in H_2O.

from 11.5-12 Hz at 31°C to 6-7 Hz at 60°C in both egg yolk plasma and protease-treated egg yolk plasma samples. However protease treatment *per se* does not appear to affect the line width of these signals as it affects the line width of the choline methyl proton signals.



Figured 4. Temperature dependence of PMR spectra of protease-treated egg yolk plasma in H_2O. Sample in 0.05M NaCl in H_2O.

The PMR spectra and the temperature dependence of total extracted egg yolk sma lipids is given in figure 5. The clear yellow sample in single phase contained ces of water, methanol and chloroform as already described ref. Experimental). e spectrometer was locked on the large $(-CH_2-)n$ signal which enable the sectionof H_2O, methanol and CHCl; proton signals. It is cearly seen that the bad choline signal is sharpened with an increase in temperature upto 60°C. However, these signals are still somewhat borad compared to the egg yolk plasma or the

Table 2. Temperature dependence of line width of choline methyl and $CH_2-\overset{O}{\overset{\|}{C}}-$ proton signals (in H_2O).

Temp. °C	Egg yolk plasma		Egg yolk plasma protease-treated	
	$N^+(CH_3)_3$ Hz	$CH_2-\overset{O}{\overset{\|}{C}}-$ triplet Hz	$N^+(CH_3)_3$ Hz	$CH_2-\overset{O}{\overset{\|}{C}}-$ triplet Hz
31	11.0	12, 12*	8.5	12, 11.5*
40	10.0	10, 9.5*	5.0	8, 9.5*
50	7.0	6.5, 7, 7.5	6.5	7, 6.5, 6.5
60	—	—	6.1	6.5, 6, 7

* Initial triplet signal present as a shoulder and was not measurable.

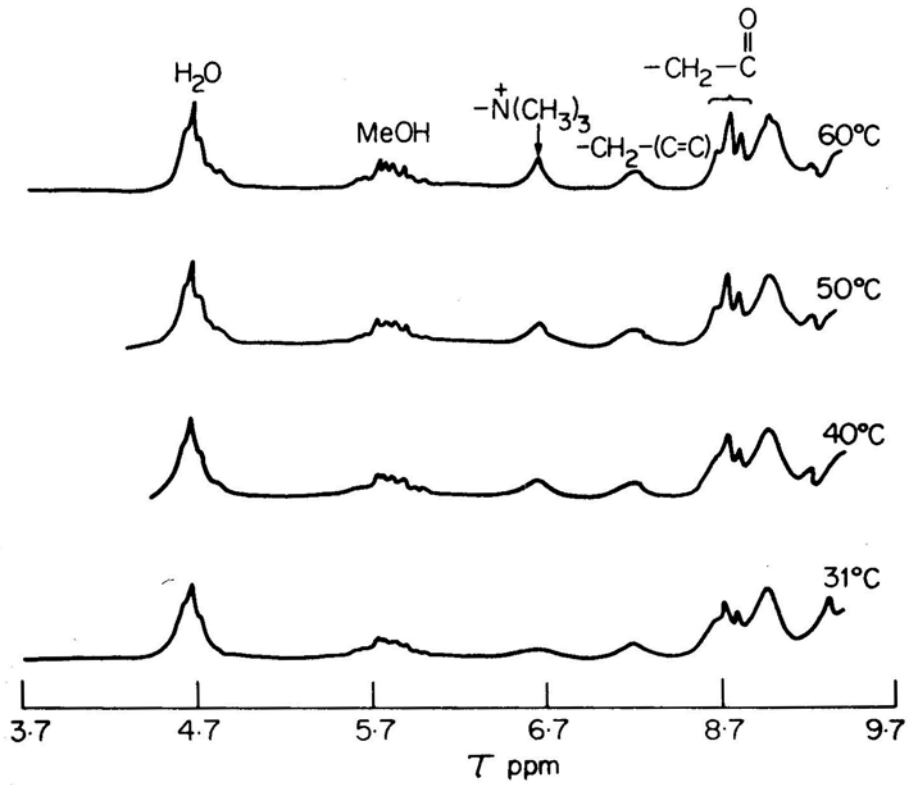


Figure 5. Temperature dependence of PMR spectra of total extracted lipid of egg yolk plasma in single phase.

protease-treated plasma choline methyl proton signals seen in figures 3 and 4. The $-CH_2-\overset{O}{\overset{\|}{C}}-$proton signal is likewise sharpened with temperature.

The PMR spectra at various temperature (31°C to 60°C) of the total lipids of egg yolk plasma were dispersed in water by sonication is reproduced in figure 6. In this the choline methyl proton signals remained broad and unchanged upto 60°C. The $-CH_2-\overset{O}{\overset{\|}{C}}-$ proton signals were likewise unaffected and no triplet signals are seen at the elevated temperatures. However the fatty acid methyl ($-CH_3$) signal appears to sharpen slighcy.

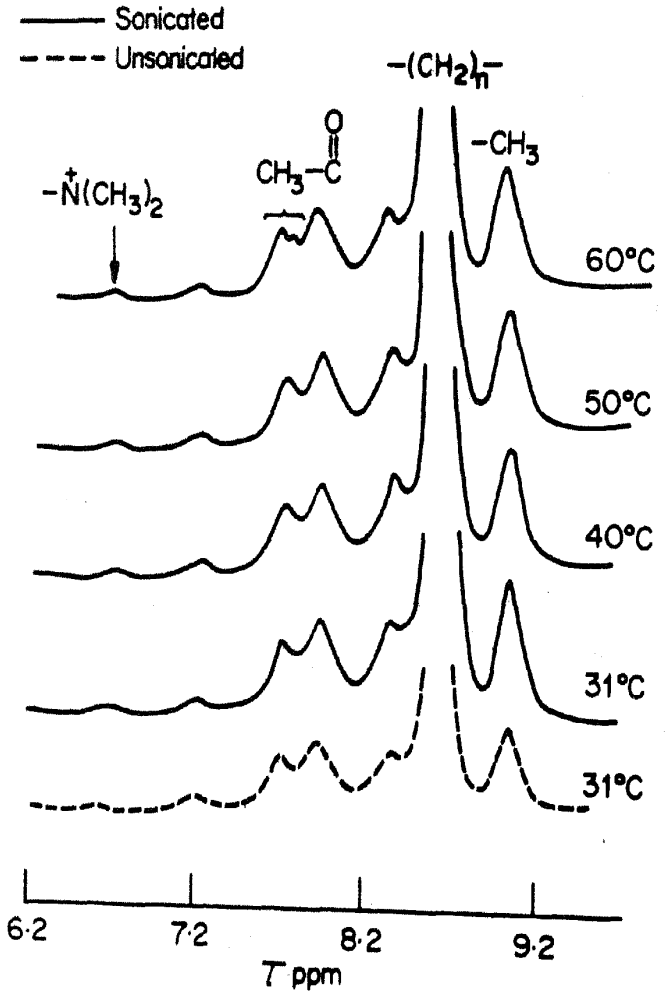


Figure 6. Temperature dependence of PMR spectra of total lipids of egg yolk plasma dispersed in water.

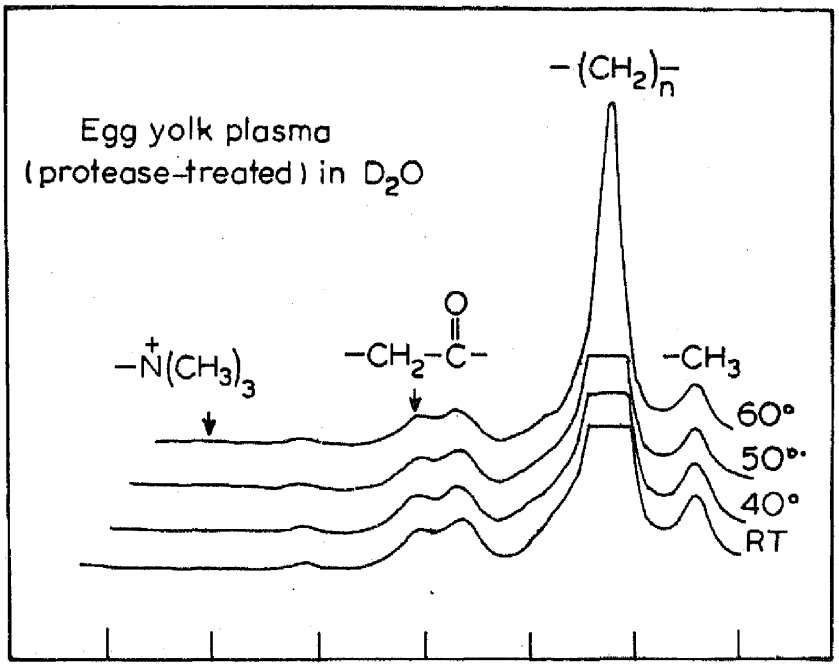


Figure 7. Temperature dependence of PMR spectra of protease-treated egg yolk plasma stored frozen and thawed.

Figure 7 shows the temperature dependence of PMR of protease-treated egg yolk plasma (in D_2O) stored frozen and thawed. Upon storage in the frozen state and thawing, the sample which was initially a clear yellow fluid becomes an opaque yellow gel, obviously caused by phase separation of the triglycerides and phosphorlipids. In the PMR spectra of such a sample, the choline methyl proton signal is almost non-existant and the signals due to $-CH_2-\overset{O}{\overset{\|}{C}}-$, $(-CH_2-)n$ and $-CH_3$

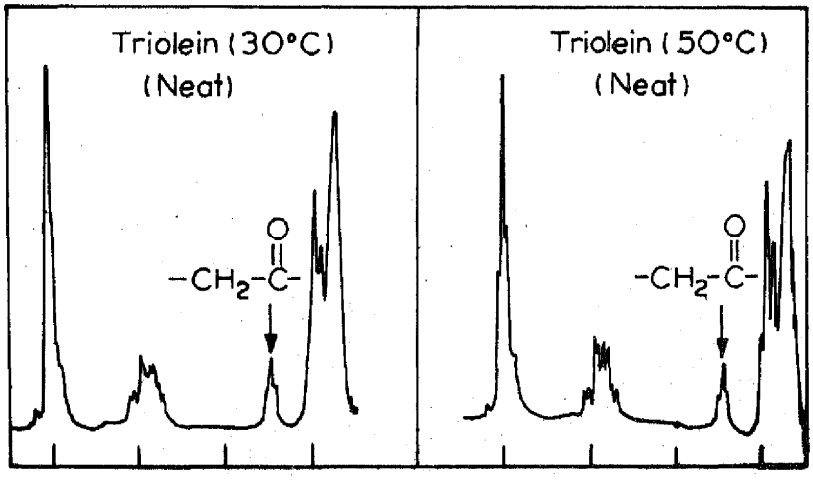


Figure 8. Temperature dependence of PMR spectra of triolein.

groups are quite broad. The spectrum is very similar to that of the total egg yolk plasma lipids dispersed in water (figure 6). Increase in temperature had hardly any effect on any region of the spectrum including choline methyl and $-CH_2-\overset{O}{\overset{\|}{C}}-$ proton regions.

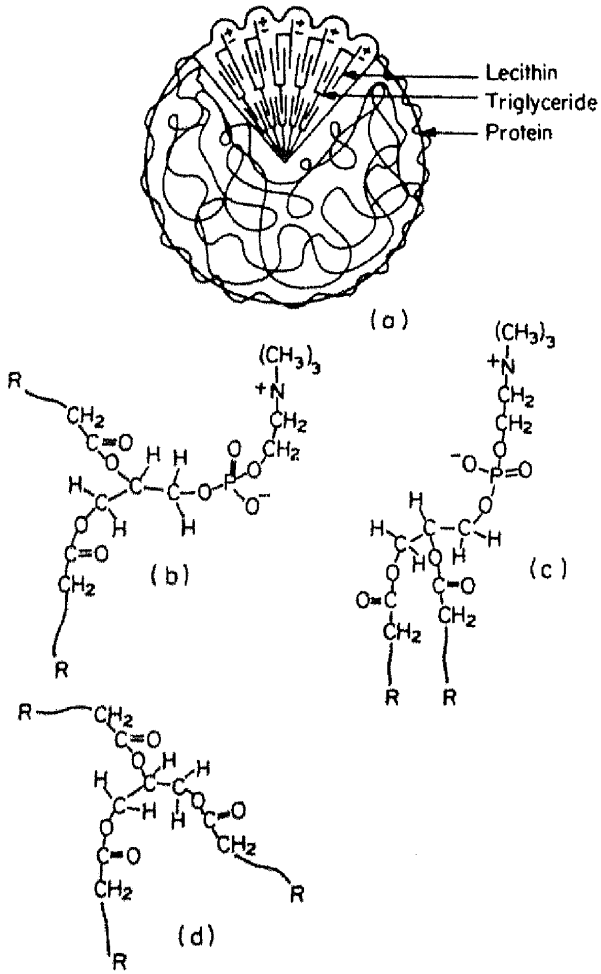
The PMR spectra of triolein, which was taken as a model for an unsaturated yolk triglyceride, was recorded neat at 31 °C and 50°C and is shown in figure 8. The $-CH_2-\overset{O}{\overset{\|}{C}}-$ signals shows a triplet nature at 31°C which is unchanged at 50°C. However all other signals sharpened on going to the elevated temperature.

Discussions

The decrease in the line width of the choline methyl $[-N^+(CH_3)]_3$ protons in the protease-treated samples, in both H_2O and D_2O, clearly support the hypothesis that line broadening is due to the hindrance of choline head group rotation by polypeptide in the native lipoprotein. The temperature-dependent enhancement of signal intensity of both choline methyl and $-CH_2-\overset{O}{\overset{\|}{C}}-$group protons in native as well as protease-teated lipoprotein indicate that temperature preferentially increases the mobility of these groups when compared to the methylene $(-CH_2-)n$ and methyl groups. Similar effects were observed with the total extracted lipids in single phase. However, in the spectra of the extracted lipids dispersed in water the choline methyl and $-CH_2-\overset{O}{\overset{\|}{C}}-$ proton signals are hardly affected by an increase in temperature probably indicating a different state of aggregation of these lipids compared with that in native or protease-treated egg yolk plasma.

A temperature-dependence study on a model triglyceride, triolein, indicated no sharpening of the triplet signal of $-CH_2-\overset{O}{\overset{\|}{C}}-$at the elevated temperature though all other signals sharpened. These protons are apparently quite unhindered for rotation even at the lower temperature and therefore temperature-insensitive. The results therefore suggest that the temperature-dependent$-CH_2-\overset{O}{\overset{\|}{C}}-$ proton triplet signals in egg yolk plasma or protease-treated plasma may arise from the esterified fatty acids of phospholipids.

All these results support a model as shown in figure 9a and figure 10a where the lipoprotein particles of egg yolk plasma is shown as a lipid core structure containing triglycerides in the centre with a monomolecular layer of phospholipids on the surface, the polar head groups of which are surrounded by proteins. Such a structure has been proposed by Kamat *et al.* (1972). In this structure the motion of the choline methyl and $-CH_2-\overset{O}{\overset{\|}{C}}-$ protons are restricted. In the case of choline this may be due to the interaction of the polar groups (phosphate and $N^+(CH_3)_3$) with polar groups of the protein. Protease treatment removes the proteins which, at least partly, releases the choline head group from this interacton. In the case of $-CH_2-\overset{O}{\overset{\|}{C}}-$ group protons, the restriction may be due to the necessary closer packing of both the faty acid chains of phospholipids into the triglyceride core, thereby hindering the freedom of motion. Figure 9b and 9c show lecithin molecules with fatty acids chains 'close' packed with less freedom of movement or 'open' packed with greater



Figured 9. (a) Lipid-core model of VLDL. Partly cut away to show packing of lecithin and triglyceride. Polar head groups of lecithin in close association with protein on the surface of the lipid core. (b) Lecithin molecule with 'open' arrangement of fatty acid chains, (c) Lecithin molecule with 'close'arrangement of fatty acid chains, (d) Triglyceride molecule with 'open' arrangement of fatty acid chains.

rotational freedom. Triglycerides in bulk phase have no restriction of motion of the three fatty chains (figure 9d). On protease treatment, the lipid cores of these lipoprotein particles are released into the medium wherein the triglycerides and phosphorlipids apparency maintain their initial packing arrangement, i.e. triglyceride cores surrounded by a phospholipid monolayer (figure 10b). At this stage, a certain amount of coalition of the lipid cores of these lipoproteins probably occurs, since the volume of these lipid cores is somewhat larger than that of VLDL particles as determined by Sepharose 4B gel filtration (unpublished results). Since the protein restriction has been removed, the signals from choline methyl protons appears sharpened. However, the motion of $-CH_2-\overset{O}{\overset{\|}{C}}-$ grouping of the phospholipid is still restricted, which is reduced at higher temperature as with the whole lipoprotein

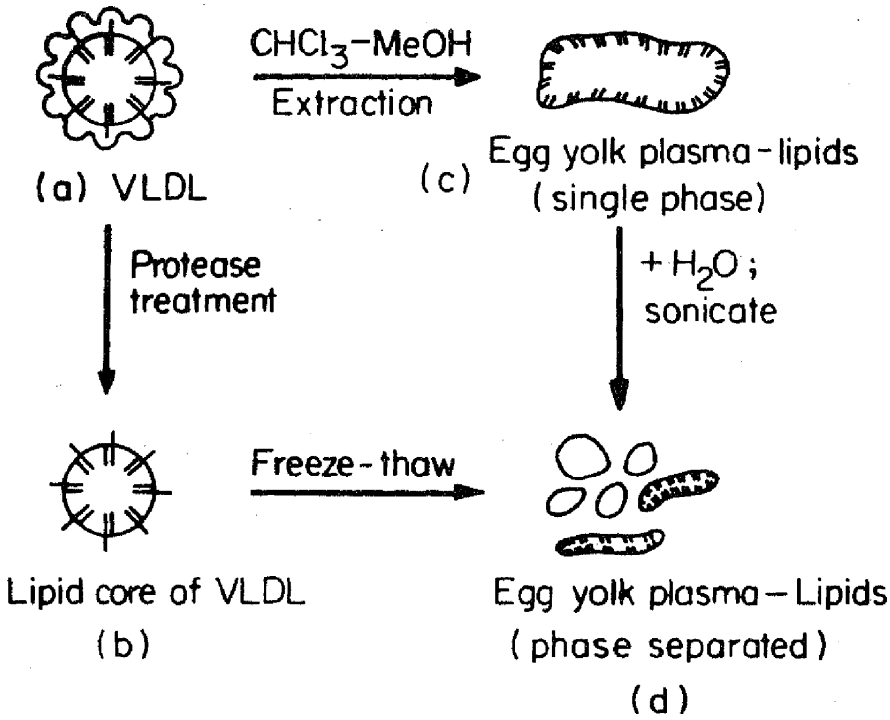


Figure 10. Schematic representation of changes in the packing of lipid components of egg yolk plasma following various treatments. (a) Intact VLDL particle with lipid core and protein coat surface. Phospholipid molecules arranged in monolayer over a bulk triglyceride core. (b) Protease-treated VLDL particle with the protein coat removed. Arrangements of lipids as in (a). (c) Total extracted egg yolk plasma lipids in single phase. Phospholipids shown as monolayer dispersing triglycerides. (d) Frozen and thawed particle showing phase separation of triglycerides (open figure) and phospholipids in bilayer. Same phase separation is obtained by adding water to extracted egg yolk plasma lipids (c).

particle. In the total extracted egg yolk plasma lipid sample containing traces of water, $CHCl_3$ and methanol (figure 10c), where there is no phase separation of triglycerides and phospholipids, the phospholipids probably occur in the monolayer form keeping the triglycerides in some sort of a dispersed state. Such a sample shows a temperature dependence similar to egg yolk plasma protease-treated plasma. Protease-treated egg yolk plasma sample, stored frozen and thawed, becomes opaque probably due to phase separation of the triglycerides and phospholipids (figure 10d). Similar phase separation apparently occurs when water is added to extracted total egg yolk plasma lipid (figure 1 0d). Both these samples showed no temperature dependence of $-N^+(CH_3)_3$ and $-CH_2-\overset{O}{\overset{\|}{C}}-$ proton signals as explained earlier. Phase separation in the frozen and thawed, protease-treated egg yolk plasma sample was also indicated by low angle x-ray scattering which was not obtained with the un-frozen sample (Mahadevan and Chapman, unpublished results).

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