HIGH RESOLUTION NMR INVESTIGATIONS ON HEN'S EGG YOLK PLASMA

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1. Introduction

High resolution NMR studies on cholesterol-rich, low and high density human serum lipoproteins [1–4] have clearly indicated much mobility for the lipids in these lipoproteins which supports the lipid-core structure proposed for them [5, 6]. While lipid-proton signals were readily obtained with lower resolution spectrometers, meaningful revelation of the protein-proton signals required the use of higher resolution spectrometer, like the 220 MHz Spectrometer, or higher temperatures of operation or of protein denaturants [4]. The latter two conditions of operation would, however, be expected to modify the native structure.

We report here our observation of the high resolution NMR signals obtained from granule-free egg yolk plasma in its native state which contains triglyceride-rich low density lipoproteins as its chief constituent [6]. These signals are compared with those obtained from the total lipids extracted from plasma. A comparison of the native spectrum is also made with gelled and therefore structurally damaged yolk plasma samples obtained either by freezing and thawing [7] or by treatment with phospholipase C [8]. Since limited proteolysis by trypsin treatment prior to freezing and thawing prevents gelation of yolk plasma [7], the spectrum of such a sample has also been taken for comparison.

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2. Materials and methods

Granule-free egg yolk plasma was obtained by preparative ultracentrifugation of unfertilized egg yolk as described earlier [7]. Yolk plasma, at full strength or following dilution with two parts of distilled water (w/v) was used for NMR analysis.

2.1. *Induction of gelation by frozen storage*

This was achieved by storage of plasma samples at -20° for 6 days prior to thawing. Spectra of the gelled samples were taken either directly or following manual homogenization with two parts of distilled water.

2.2. *Treatment with enzymes*

About 2 g samples of yolk plasma were treated either with 1.5 mg of trypsin (Type III, Sigma Chemical Co., USA) or with 3.7 mg of phospholipase C (ex. *Clostridium welchii*; lot 11 B-1100, Sigma Chemical Co., USA), each enzyme being dissolved in 100 μl of distilled water prior to addition. Following incubation of the samples at 33° for 115 min, 2.7 mg of trypsin inhibitor (Type I-S, Sigma Chemical Co., USA), dissolved in 100 μl water, was added to the trypsin-treated sample and stored frozen for 6 days at -20°. Upon thawing, the still fluid plasma was diluted with 2 parts distilled water before recording the spectrum. The phospholipase C treated sample, which gelled during incubation [8], was diluted as above and manually homogenized prior to NMR measurements.

2.3. *Extraction of total lipids*

Lyophilized yolk plasma was extracted with 15

parts (w/v) of a mixture of $CHCl_3$: MeOH (2:1, v/v) and filtered. The solvents were removed by flash evaporation and a part of the clear deep yellow oil of the total lipids was dispersed in water to give an emulsion with a lipid concentration approximately that found in yolk plasma. A portion of the emulsion was sonicated for a minute and the NMR spectra of both unsonicated and sonicated emulsions were recorded. The total lipids have the following approximate composition: neutral lipids (chiefly triglycerides) (70%), phospholipids (26%) and cholesterol and its esters (4%), lecithin being about 75% of the phospholipids [9].

2.4. *NMR spectrometry*

NMR spectra were recorded in a Varian 100 Mc/s spectrometer at a constant temperature of 31°. The spectrometer was locked in the water signal (~ 5.2τ). All signals were recorded with TMS as internal reference. Line widths were measured at half the signal intensity in c/s.

3. Results and discussion

Figs. 1 to 4 depict the spectra of native yolk plasma, gelled (frozen and thawed) yolk plasma, total lipids, and trypsin or phospholipase C treated yolk plasma, respectively. These figures cover only the portion of the total spectra, i.e. the region from ~ 5.2τ onwards to TMS. Based on earlier reports [10, 11] the signals were assigned as follows: the 6.7 ppm, signal to the protons of choline-methyl group [$N^+(CH_3)_3$]; the 7.2, 7.7, 7.9 and 8.4 ppm signals to the protons of $CH_2(C{=}C)_2$, CH_2CO, $CH_2C{=}C$, and $CH_2C{-}CO$ groups in lipids; the 8.7 ppm signals to the methylene ($-CH_2-$) protons of fatty acid chains and the 9.1 ppm signal to the methyl (CH_3) protons of fatty acids. Table 1 summarizes the treatments and line width of the methylene ($-CH_2-$) signal at 8.7 ppm. A comparison of total integrated area showed that the total number of protons contributing to th signals in both native and gelled samples were the sam dicating a structural rigidity without loss of any group

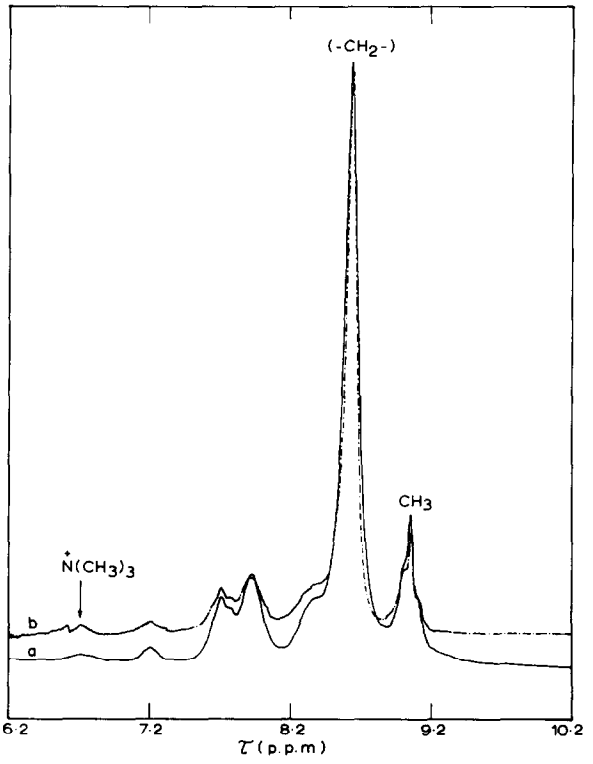

Fig. 1. Spectra of native egg yolk plasma. a) Undiluted; b) diluted (1:2 w/v with water).

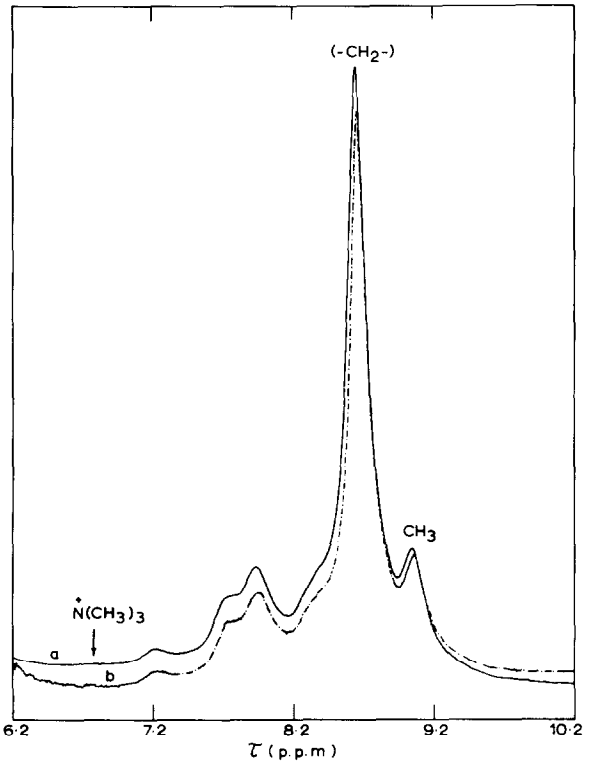

Fig. 2. Spectra of frozen and thawed, gelled yolk plasma. a) Undiluted; b) diluted (1:2, w/v with water).

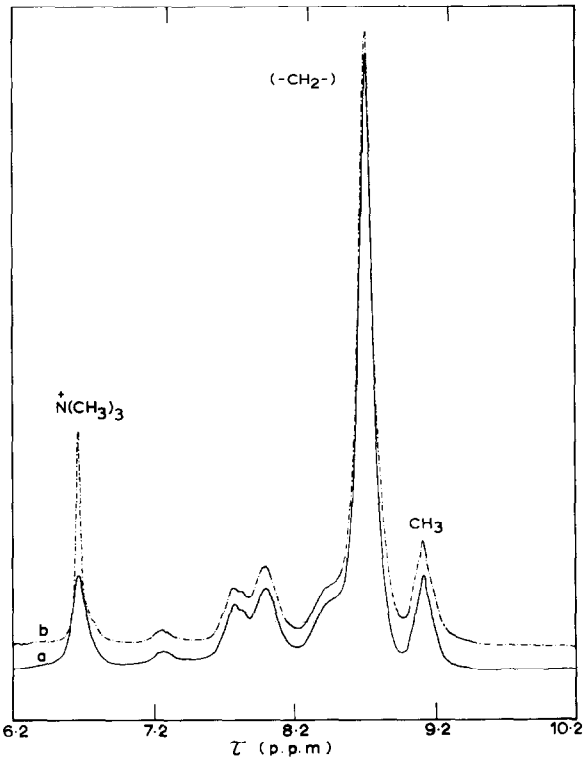

Fig. 3. Spectra of extracted total lipids of yolk plasma in aqueous dispersion. a) Unsonicated; b) sonicated.

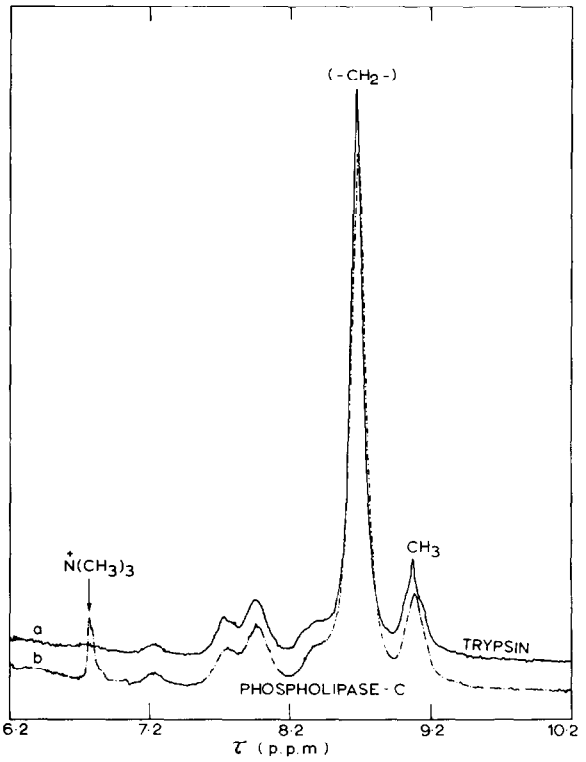

Fig. 4. Spectra of enzyme-treated yolk plasma, diluted 1:2 (w/v) with water. a) Trypsin treated, frozen and thawed; b) phospholipase C treated.

3.1. *Effect of dilution of samples on NMR spectra*

Dilution appeared to have little effect on line broadening of the spectra of both the native (fig. 1a and b, table 1) and the gelled (fig. 2 a and b, table 1) yolk plasma samples, indicating that lowered overall viscosity had no significant effect on the chemical groups contributing to the signals.

3.2. *Comparison of NMR signals from native yolk plasma with those from total lipids*

Spectra of both native yolk plasma (fig. 1a) and total lipids (fig. 3a) appeared similar except for the diminished 6.7 ppm signal in the former. This signal in the total lipids intensified on sonication (fig. 3b) which can be attributed to the greater mobility of the smaller micelles obtained by sonication as reported earlier [12]. The plasma spectrum was essentially the spectrum of the lipids present in the lipoproteins of the plasma (table 1). A similarity in all the signals, including the choline methyl proton signals between the spectra of human serum lipoproteins and the lipids extracted from it has been reported earlier [1]. There were no indications of any protein-proton signals at this resolution and temperature (30°).

3.3. *$N^+(CH_3)_3$ signal at 6.7 ppm*

The virtual reduction of the 6.7 ppm signal from a medium intensity signal in the total lipids (fig. 3a), to a weak and broad signal in the native yolk plasma spectra (fig. 1a and b), and its total absence in the gelle plasma spectra (fig. 2a and b) appears siginificant. It suggests high local viscosity around the polar head group of lecithin in the native plasma sample which is not diminished by dilution (fig. 1b). A close associatio of the polar head of lecithin with polar areas on the apolipoprotein, thereby hindering the mobility of the choline methyl groups, is a possible explanation for the diminished signal. The total absence of the signal

Table 1
Comparison of 8.7 ppm NMR signal from variously treated and from the total lipids of yolk plasma.

Sample treatment	Dilution	Width of NMR Signal at 8.7 ppm in c/s
1. Native	a) undiluted	11
	b) diluted	9
2. Frozen and thawed	a) undiluted	17.4
	b) diluted	15.0
3. Trypsin treated Frozen and thawed	diluted	9.0
4. Phospholipase C treated	diluted	12.0
5. Total yolk lipids		
a) unsonicated	undiluted	11.0
b) sonicated	undiluted	11.0

on gelation following freezing and thawing represents structural alterations around this moiety. Limited proteolysis by trypsin treatment prior to freezing and thawing inhibits gelation and also leads to the retention of this small signal (fig. 4a). Thus gelation rather than freezing and thawing *per se* leads to the disappearance of this signal.

The sharpened 6.7 ppm signal following phospholipase C treatment (fig. 4b) is undoubtedly from the free phosphorylcholine released into the medium from its sequestered location in the lipoprotein when it was a part of the lecithin molecule.

The reduced intensity of the choline-methyl proton signal in the egg-yolk plasma as compared to the total lipid is different from the signal obtained from the choline group in low and high density lipoproteins of human serum [1–4] where pronounced and sharp choline signals almost equivalent to that observed in the total lipids of these lipoproteins [1] were obtained. This suggests a possible difference in the location of the choline group in the human serum and avian egg low density lipoproteins.

3.4. *Signal at 8.7 ppm*

This strong signal in the native yolk plasma arises from methylene protons of the fatty acid chains in the triglycerides and phospholipids. In the frozen and thawed, gelled sample the signal at this position is significantly broadened both in the diluted and undiluted state as seen in table 1. In the case of the sample treated with phospholipase C, where gelation is induced, the signal at this position showed some line broadening whereas the trypsin-treated, frozen and thawed sample where no gelation occurred the line width was identical to that observed in the native yolk plasma. Hence line broadening is primarily due to gelation and not freezing and thawing *per se*.

3.5. *Signal at 9.1 ppm*

This methyl group signal, which is a triplet in the native yolk plasma, is appreciably broadened in the frozen and gelled sample where it collapses into a single line. Whereas in the trypsin-treated frozen and thawed sample, the triplet nature is retained, in the case of phospholipase C treated sample, where gelation is induced, the spectrum showed some line broadening with the collapse of the triplet signal. These suggest that the lipid methyl groups in the native plasma have a high degree of mobility, supporting a lipid-core structure for the lipoprotein in the native state.

Lipid-rich egg yolk low density lipoproteins are believed to have a lipid-core of triglycerides (or cholesterol esters in blood plasma low density lipoproteins) surrounded by a envelope of proteins and the polar groups of amphipatic lipids such as lecithin [5, 6]. Such a structure explains the ready cleavage of the protein envelope by proteolytic enzymes and of lecithin head group by phospholipase C. The information obtained by the NMR studies described here strongly supports this lipid-core structure. The pronounced line broadening of the methylene and methyl signals of lipids indicate restricted motion in these groups during gelation induced by freezing and thawing. Gelation has been attributed mainly to protein–protein aggregation during freeze damage [7]. Such an aggregation could influence the lipid-core structure, individually or collectively, hindering lipid mobility. The prevention of gelation during freezing and thawing by prior trypsin treatment apparently prevents any change in the lipid-core structure.

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References

[1] J.M. Steim, O.J. Edner and F.G. Bargoot, Science 162 (1968) 909.
[2] R.B. Leslie, D. Chapman and A,M. Scanu, Chem. Phys. Lipids 3 (1969) 152.
[3] D. Chapman, R.B. Leslie and R. Hirz, Nature 221 (1969) 260.
[4] D. Chapman, R.B. Leslie, R. Hirz and A.M. Scanu, Biochim. Biophys. Acta 176 (1969) 524.
[5] S. Margolis, in: Structural and Functional Aspects of Lipoproteins in Living Systems, eds. E. Tria and A.M. Scanu (Academic Press, London, N.Y. 1969) p. 369.
[6] W.H. Cook and W.G. Martin, in: Structural and Functional Aspects of Lipoproteins in Living Systems, eds. E. Tria and A,M. Scanu (Academic Press, 1969) p. 579.
[7] S. Mahadevan, T. Satyanarayana and S.A. Kumar, J. Agr. Food Chem. 17 (1969) 767.
[8] S.A. Kumar and S. Mahadevan, J. Agr. Food Chem. 18 (1970) 666.
[9] W.G. Martin, N.H. Tattrie and W.H. Cook, Can. J. Biochem. Physiol. 41 (1963) 657.
[10] D. Chapman and A. Morrison, J. Biol. Chem. 241 (1966) 5044.
[11] D. Chapman, V.B. Kamat, J. deGier and S.A. Penkett, J. Mol. Biol. 31 (1968) 101.
[12] E.G. Finer, A.G. Flook and H. Hauser, Biochim. Biophys. Acta 260 (1972) 49.