J. Biosci., Vol. 11, Numbers 1–4, March 1987, pp. 299–309. © Printed in India.

Differential scanning calorimetric studies of native and freeze-damaged very low density lipoproteins in hen's egg yolk plasma

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Abstract. Lipid thermal transition patterns of the very low density lipoproteins in native and variously treated egg yolk plasma and extracted total very low density lipoproteins lipids have been recorded by differential scanning calorimetry in the temperature range 220–300 K, after lowering the freeze endotherm of free water in the sample with ethylene glycol.

Three distinguishable patterns of lipid endotherms, designated types 1, 2 and 3 were obtained, respectively, from (i) native very low density lipoproteins in egg yolk plasma, (ii) freeze damaged very low density lipoproteins in gelled egg yolk plasma and (iii) extracted total lipids of very low density lipoproteins dispersed in water. Protein-depleted 'lipid core' particles of very low density lipoproteins obtained by exhaustive proteolysis of egg yolk plasma gave type 2 lipid transition pattern suggesting similarities in its lipid association with that of the freeze damaged very low density lipoproteins. Freezing the 'lipid cores' of very low density lipoproteins led to phase separation and gave type 3 lipid transition pattern of water-dispersed, phase-separated total very low density lipoprotein lipids. Relative heat uptake of native very low density lipoproteins in egg yolk plasma was about 15% lower than the freeze damaged sample or of the extracted total lipids.

Treatments which prevented aggregation and gelation of very low density lipoproteins in egg yolk plasma during frozen storage, namely with additives such as glycerol or NaCl, gave subsequent lipid transition pattern intermediate between type 1 and 2, indicating that while very low density lipoprotein aggregation is prevented, additives do not altogether prevent changes in lipid association in these particles.

Keywords. Differential scanning calorimetry; very low density lipoproteins; egg yolk plasma lipids.

Introduction

Hen's egg yolk plasma (EYP), obtained by high speed centrifugation of egg yolk (Schmidt *et al.,* 1956), consists of very low density lipoproteins (VLDL) and lipid-free proteins, the livetins (Cook and Martin, 1969). Irreversible gelation of EYP occurs when stored frozen below - 6°C and thawed, and this has been shown to be due to freeze damage and aggregation of the VLDL component of EYP (Saari *et al.,* 1964; Raju and Mahadevan, 1974). Aggregation of the protein component of VLDL as a consequence of changes in non-covalent lipid-protein association during frozen storage has been suggested as the reason for gelation (Mahadevan *et al.,* 1969; Raju and Mahadevan, 1976). Additives such as glycerol, sugars or NaCl, or treatment with proteolytic enzymes such as trypsin are known to prevent freeze-induced gelation of yolk (Lopez *et al.,* 1955). Their action is believed to be due to the prevention of VLDL protein aggregation either by partial proteolysis or by preventing drastic changes in protein-lipid interaction during frozen storage (Mahadevan *et al.,* 1969).

Abbreviations used: EYP, Egg yolk plasma; VLDL, very low density lipoproteins; d.s.e., differential scanning calorimetry.

This communication reports the results of a thermal analysis of native, freeze damaged or variously treated VLDL in EYP and of extracted VLDL lipid using a differential scanning calorimeter. The work was undertaken on the premise that altered physical states of VLDL in EYP and their lipid may manifest themselves with altered patterns of thermal transitions of the lipid, and that a comparison of these patterns may throw light on the nature of lipid-lipid and lipid-protein association in the native and gelled states. The results are discussed in relation to the 'lipid core' model structure proposed for VLDL in EYP based on high resolution nuclear magnetic resonance and other evidences (Kamat *et al.,* 1972; Easwaran and Mahadevan 1972; Easwaran *et al.,* 1980).

Materials and methods

EYP was prepared from the yolk of fresh white eggs by preparative ultracentrifugation at 137000 *g* for 4·5 h in the cold. The EYP was decanted free of sedimented granules and stored in ice till use.

Frozen storage of EYP and induction of gelation

Native EYP or EYP containing non-gelling additives such as 5% NaCl (w/w), 10% glycerol (w/w) or following trypsin treatment (see below) were stored at — 25°C for a minimum period of 6 days before thawing. While the native EYP had gelled (owing to freeze damage of VLDL in it) the treated samples were still fluid.

For trypsin treatment, about 2 g of EYP was treated with 6·7 mg of trypsin (type III, Sigma Chemical Co., USA) dissolved in 100 μl water and incubated at 33°C for 4 h. Following digestion, 10 mg of soyabean trypsin inhibitor (type 1–S, Sigma Chemical Co., USA) dissolved in 100 μl H_2O was added. One half of the trypsinized EYP was stored at - 25°C, while the other half was used immediately in calorimetric studies.

Total lipids, phospholipids and neutral lipids

The total lipids were extracted from lyophilised EYP as described earlier (Easwaran *et al.,* 1980).

For calorimetric studies, one portion of the total lipids was dispersed in enough water to give an opaque yellow emulsion with a lipid concentration equivalent to that in EYP.

A second portion of total lipids was dispersed in excess cold acetone and the precipitate of phospholipids collected by centrifugation in the cold. The precipitate was washed twice with cold acetone and the final pale yellow precipitate dried *in vacuo* and dispersed in a water-glycol mixture (2:1) prior to calorimetry.

A third portion of total lipids, dissolved in redistilled $CHCI_3$, was loaded on a column of silicic acid (25 g of Kieselgel 0·05–0·2 mm; Merck, Germany) in $CHCI_3$ in the cold (4°C). The column was eluted with cold $CHC1_3$ and the initial 150 ml of nearly colourless eluate flash evaporated at 30°C. The pale straw coloured oil residue of neutral lipids, chiefly triglycerides, accounted for over 66% of the total lipids

loaded. Carotenoids were not eluted with this fraction under these conditions. The neutral lipid fraction was directly used in calorimetric studies.

Protease treatment of EYP

Protein-depleted EYP was prepared by extensive proteolysis of about 8 g of EYP with 53 mg of protease (ex *Streptomyces griseus)* essentially as described by Easwaran *et al.* (1980). After 48 h of proteolysis and dialysis, the material was dialyzed against 0·05 M NaCl solution in the cold for a further 16 h. A sample of EYP with no added protease was treated in exactly the same manner to serve as control.

A part of the protease-treated EYP and control EYP were analyzed for protein content. Calorimetric studies were conducted on the protease-treated EYP prior to and following frozen storage at – 25°C for 48 h.

Protein estimation

Lipid-rich native and protease-treated EYP samples were analyzed for proteins by a slight modification of the Folin's procedure as described by Easwaran *et al.* (1980). Optical density was measured at 500 nm in a Unicam SP 600 spectrophotometer, using crystalline bovine serum albumin as the standard.

Differential scanning calorimetry (d.s.c.)

All thermal transition heating curves were obtained with a Perkin-Elmer differential scanning calorimeter DSC-2 using helium as the purge gas in the 'head' and nitrogen in the outside chamber. Liquid nitrogen was used as the sample coolant. In order to obtain comparable results, all measurements were made with the following settings: cooling rate: 320°/min; heating rate: 5°/min and 1 m cal/s full scale sensitivity. All measurements were recorded up to 300 K (27°C) since initial experiments indicated no thermal transitions beyond this temperature except that of thermal denaturation of EYP.

Samples (10–15 mg) containing either 23 or 33% ethylene glycol were sealed in aluminium pans. Gelled EYP samples mixed with glycol gave a foamy paste which was clarified by centrifugation at 40000g for 10 min. Samples were cooled to varying temperatures (220–270K) prior to heating and were always maintained at the lower temperature for at least 5 min before heating.

Relative heat uptake was calculated from the area under the d.s.c. curves obtained with a planimeter.

Results and discussion

Initial experiments indicated that fresh EYP gave no thermal transitions when heated from its freezing point (272 K) to temperature of thermal denaturation. However on freezing to 220 K before heating, an endothermic transition peak at 280 K was obtained which immediately followed free water's freeze endotherm.

Separate experiments showed that transitions were due to EYP lipids and not to its protein components.

Lipid transitions occurring at the water melting temperature were thereafter obtained by the addition of ethylene glycol. This additive was useful as it also suppressed freeze induced gelation of EYP. Preliminary trials indicated that most samples could undergo a few cycles of freeze-thaw in the d.s.c. while registering identical thermal transition pattern. Fresh samples were used whenever such cycles started inducing a change in the endotherm pattern.

Thermal transition pattern (type 1) of native VLDL in EYP

Figure 1A shows transition curves obtained from native VLDL in fresh EYP con taining ethylene glycol (23% final concentration) cooled to 220, 250 or 260 K before

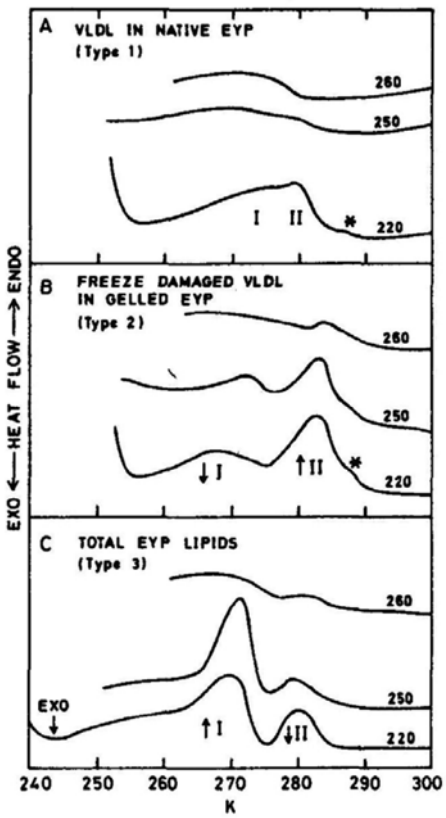

Figure 1. A. D.s.c. heating curves (type 1) of native VLDL in EYP sample containing ethylene glycol (23% final conc). Sample temperature before heating indicated on each curve. I and II represent the two regions of the curve described in text. Abscissa-K (degrees absolute). Ordinate-heat flow; upward endothermic and downward exothermic. **B.** Type 2 transitions of freeze damaged VLDL in gelled EYP sample obtained by frozen storage of EYP at –25°C for 11 days before thawing. Sample contained 23% ethylene glycol. Arrows represent relative shifts of I and II regions compared to A. C. Type 3 transitions of total EYP lipids dispersed in water and containing 33% glycol. Arrows represent relative shift of I and II compared to B. Exo, Exothermic transition.

heating. At 250 K and above broad nondescript transitions were obtained. At 220 K, a transition starting around 254 K and levelling of as a shoulder (I) at 274 K follo wed by a small peak(II) at 280 K was obtained with a small shoulder at 288 K (asterisk). This pattern of thermal transition is designated as type 1. Use of 33% glycol instead of 23 % glycol gave a similar pattern except that the onset of transition at 252-253 K was more clearly seen.

Thermal transition pattern (*type 2*) *of freeze damaged VLDL in gelled EYP*

The thermal transition pattern of freeze damaged VLDL in gelled EYP (figure 1B) was clearly different from that of native VLDL in EYP and is designated as type 2. With the sample frozen to 220 K, a two peaked pattern was obtained, a first small peak (I) at 267 K and a second larger peak (II) at 282 K. A small shoulder (asterisk) at 288 K was also seen. In comparison to type 1, type 2 showed separation of peaks I and II and a relative reduction in height of I compared to II (arrows in figure 1B). Unlike type 1, type 2 gave similar transition, though of reduced magnitude even when cooled to 250 K. However the pattern broadened when cooled to 260 K as in type 1.

Thermal transition pattern (*type 3*) *of total EYP lipids*

The thermal transition patterns for total extracted EYP lipids dispersed in water and glycol (figure 1C) were qualitatively different from types 1 and 2 and is termed as type 3. Cooled to 220 K, type 3 pattern showed a large gradually rising curve peaking at 270 K (I) followed by a smaller peak II at 281 K. The peaks were closer and relative heights reversed compared to type 2 (arrows in figure 1C). An exother mic transition at 243 K was also seen. With sample cooled to 250 K, peak I shifted to 272 K and became sharper and larger while peak II diminished. Cooling to 260 K gave broad transitions as in types 1 and 2.

These patterns were qualitatively similar to transitions obtained with neat, neutral fraction (chiefly triglycerides) of EYP lipids as given in figure 2A. With sample cooled to 220 K, an exothermic transition at 243 K, a slowly rising large endothermic transition peaking at 273 K (I) and a smaller 282 K peak (II) were obtained. Cooling to 250 K showed similar increase of I and lowering of II as in type 3, while cooling to 260 K gave the broadened pattern. The dependence of the pattern of endothermic transitions on the degree of cooling is undoubtedly due to the various polymorphic forms that these mixtures of triglycerides can crystallize into (Ladbrooke and Chapman, 1964).

The phospholipids fraction (figure 2B) in water and glycol, when cooled to 220 K (or 250 K) gave a very broad transition from 253 K ending with a minor peak at 288 K. When starting temperature was increased to 260 K, the peak at 288 K decreased and a new one at 280 K was obtained. This transition at 288 K is apparently visualized as a minor shoulder in native and freeze damaged (types 1 and 2) samples in figure 1 (asterisk).

Thermal transition pattern of protease treated 'lipid core' of VLDL

Lipids of VLDL in a state of aggregation as close as possible to their native state was

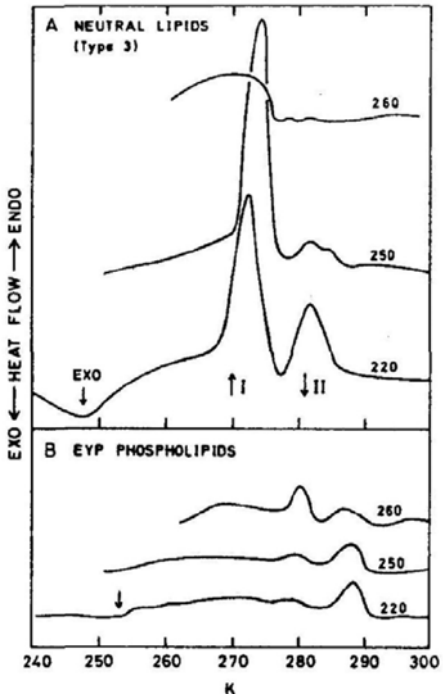

Figure 2. A. D.s.c. heating curves of neutral EYP lipids (triglycerides) in the neat form. **B.** Transitions of phospholipid fraction of EYP dispersed in water and containing 33% glycol. Other details as in figure 1.

obtained by exhaustive proteolysis of EYP with concomittant dialysis. Protein analysis before or after proteolysis indicated that about 2–3% of the original proteins remained (Easwaran *et al.,* 1980). The thermal transition patterns of 'lipid core' of VLDL are given in figure 3A. With samples cooled to 220 K, a small peak I at 268 K and a larger peak II at 272 K resembling type 2 transition of freeze damaged VLDL in EYP (figure 1B) was obtained. Cooling to 250 K retained the pattern while at 260 K the pattern broadened but still discernible when compared to the freeze damaged VLDL sample. Removal of proteins of VLDL therefore yielded 'lipid core' particles where lipids were associated in a manner similar to lipids in freeze damaged VLDL in gelled EYP (type 2) and unlike that in native VLDL (type 1)·

Protein depleted 'lipid core' particles stored frozen for 2 days at — 25°C and thawed changed from a translucent yellow fluid to an opaque yellow; viscous mate rial resembling solvent extracted EYP lipids dispersed in water. The d.s.c. curves of frozen and thawed 'lipid core' cooled to 220 K (figure 3B) resembled type 3 pattern of total EYP lipids with peak I larger than peak II and this difference becoming greater when cooled to 250 K prior to heating. Freeze damage (by phase separation) of 'lipid core' of VLDL occurred even in the presence of 33% of glycol and gave similar transition patterns.

Thermal transition patterns of VLDL in EYP samples treated with non-gelling agents

D.s.c. curves of samples treated with the non-gelling agents glycerol or NaCl prior to frozen storage are given in figure 4 (curves A and B). These patterns were intermedi-

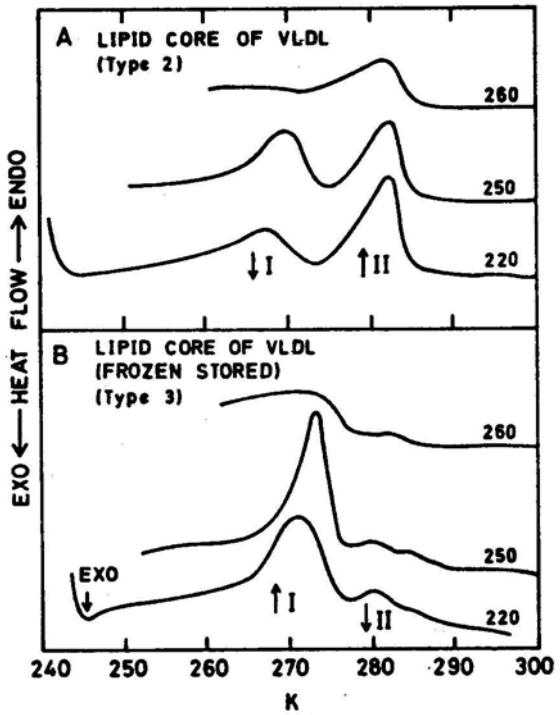

Figure 3. A. D.s.c. heating curves of a protease-treated 'lipid-core' of EYP containing 33% glycol. **B**. Transition curves of protease-treated 'lipid-core' of EYP stored frozen at –25°C for 2 days before addition of glycol (33% final conc). Other details as in figure 1.

ate between type 1 and type 2, with peak II larger than I as in type 2, but I appearing as a shoulder of II as in type 1. Obviously these additives while preventing gelation did not altogether prevent changes in lipid association.

Partial proteolysis of EYP by trypsin treatment prior to frozen storage also prevented gelation. In figure 4, transition curves of trypsinized EYP samples before (curve C) and after frozen storage (curve D) are shown, the former identical to native (type 1) while the latter similar to the freeze damaged VLDL in gelled EYP (type 2). Apparently partial degradation of VLDL proteins prevented gel formation but not the changes in lipid association during frozen storage.

Relative heat uptake by VLDL in variously treated EYP and of lipids extracted from EYP

Table 1 gives the per cent heat uptake by VLDL in and variously treated EYP samples and of extracted lipids relative to native VLDL in fresh EYP. All values were calculated from the area under the curve for samples cooled to 220 K prior to heating. The enthalpy of lipid transition of native VLDL in fresh EYP was about 85% of that observed for VLDL in frozen stored EYP samples, whether gelled, or non-gelled by additives, or for the total extracted lipids. While the freeze damaged ‘lipid core' had similar enthalpy as other frozen stored samples, unfrozen, protein depleted ‘lipid core' of VLDL showed a relative heat uptake intermediate between native VLDL in fresh EYP and the other frozen samples.

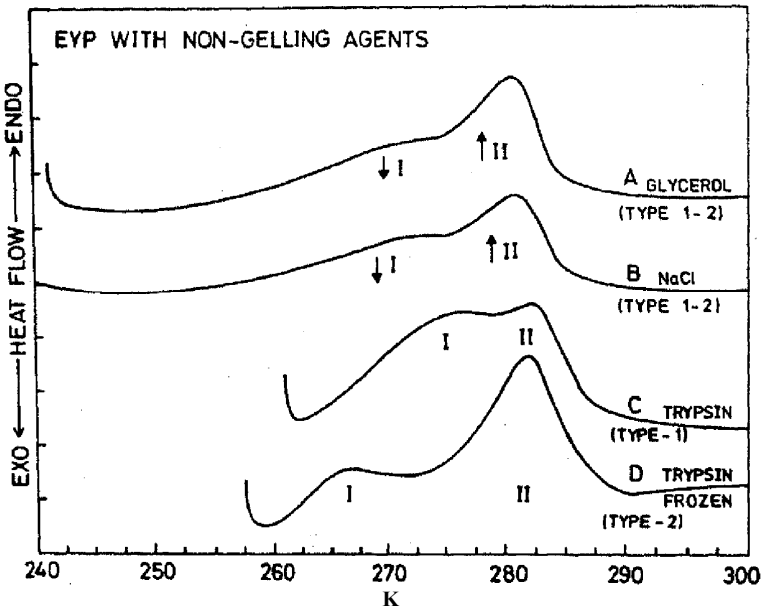

Figure 4. D.s.c. heating curves at EYP samples treated with various non-gelling agents prior to frozen storage. All samples cooled to 220 K before heating. EYP samples containing either 10% glycerol (w/w) (A) or 5% NaCl (w/w) (B) before thawing, and stored for 7 days at – 25° C. Glycol (33% final conc.) added prior to d.s.c. run. Curves C and D-EYP samples treated with trypsin and trypsin inhibitor as described in 'materials and methods'. C-unfrozen sample with 23% glycol; D-sample frozen for 6 days at –25°C before addition of 23 % glycol.

Table 1. Relative heat uptake during lipid thermal transition by native and variously treated EYP and EYP lipids.

Sample	Per cent
Native VLDL in fresh EYP	100
Freeze damaged VLDL in frozen stored (gelled) EYP	118
Total lipids of EYP dispersed in water*	117
Phospholipids of EYP dispersed in water*	23
'Lipid core' of VLDL (protease treated EYP)*	108
'Lipid core' of VLDL (frozen stored-phase separated)*	120
EYP (frozen stored) in 10% glycerol	117
EYP (frozen stored) in 5% NaCl	118

*Calculated on equivalent EYP basis.

A comparison of the thermal transition patterns of native (type 1) or freeze damaged (gelled) VLDL in EYP (type 2) and of total lipids extracted from EYP (type 3) in the temperature range described indicate that the endotherms are primarily due to the lipids, particularly the abundant neutral triglycerides. However, the differences in the two major zones of transitions I and II, clearly indicate that the lipids can exhibit at least 3 different polymorphic forms which are undoubtedly dependent on differences in lipid-lipid and possibly lipid-protein association states.

Of these 3, type 1 lipid association is unique in having a lower enthalpy of transition probably due to protein lipid interactions in the native VLDL particles. Steim *et al.* (1969) have reported a 25% lower enthalpy of transition of the lipids in the membranes of *Achyloplasma laidlawii* as compared to lipids extracted from it, and the difference has been attributed to the interaction of proteins with a faction of the phospholipids of the membrane. High resolution NMR studies have suggested protein interactions with lecithin head group in VLDL particles of EYP (Easwaran *et al.,* 1980). A lesser interaction with surface proteins occurs in type 2 since protein-depleted 'lipid cores' of VLDL gave a pattern similar to the freeze damaged VLDL in EYP. Type 3 lipid association in phase separated, extracted total lipids has no inter actions with proteins at all, and a similar pattern is obtained from frozen stored, phase separated 'lipid cores' of VLDL.

Based on these results, the gross structure for native, freeze damaged (gelled) and protein depleted 'lipid cores' of VLDL in EYP are shown schematically in figure 5. Native VLDL in fresh EYP (figure 5a) is represented (in cross section) as discrete large spheres with a core of triglycerides in the centre and monolayer of phospho lipids at the surface oriented radially. The polar head groups of the phospholipids at the surface of the sphere is in close association with the VLDL proteins around the lipid core as inferred from proton magnetic resonance studies (Easwaran *et al.,* 1980).

The similarity of transition patterns of protein-depleted 'lipid-core' particles of VLDL and freeze damaged (gelled) VLDL *(i. e.* type 2), suggests that lipid association

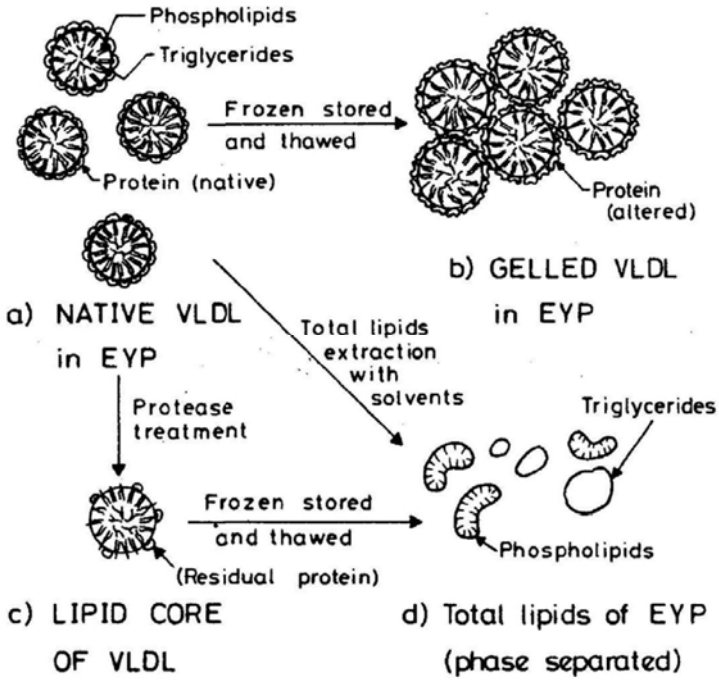

Figure 5. Schematic representation of lipid and protein association. (a), Native VLDL particles; (b), freeze damaged and gelled VLDL particles; (c), 'lipid core' particles obtained by protease treatment; (d), phase separated, total lipids.

Arrows indicate direction of change following various treatments.

in both these samples are similar. As there can only be negligible lipid-protein inter action in the former, it is suggested that negligible protein-lipid interaction occurs in the latter also. Freeze damaged VLDL particles are therefore represented (figure 5c) with a lipid core similar to protein-depleted 'lipid core' particles having surface pro teins with an altered conformation which reduces their interaction with the lipids but increases intra or inter-particle protein interaction leading to aggregation and eventual gelling. Protein fractions with an ability to form gels even in low concentrations have been isolated from VLDL (Raju and Mahadevan, 1975).

Though the protein-lipid interaction in the gelled particles was less than in native VLDL particles, there were still enough influence of the protein to prevent phase separation in the lipids to give type 3 transitions, which both extracted lipids and frozen protease-treated 'lipid cores' of VLDL gave. The proteins therefore prevented large scale aggregation of the discrete lipid core units needed for a phase separation.

Both total EYP lipids in water and frozen stored protease treated 'lipid core' of VLDL are depicted as a conglomeration of various sized particles following phase separation of the neutral and phospholipids (figure 5d), the former existing as globules of fat and the latter as bilayered vesicles or other ordered forms resulting in some crystallinity. A preliminary low angle X-ray scattering experiment suggested some crystallinity in the phase separated sample (unpublished observations). Owing to the large variety of lipid molecules with varying fatty acid composition, the ordered structures would themselves be a mosaic of patterns.

Gelation of VLDL of EYP following freeze damage therefore appeared to be chiefly due to protein aggregation following disruption or alteration in lipid-protein interaction. This leads to alterations in lipid-lipid interaction but these changes are not sufficient to cause drastic differences in viscosity since frozen stored, trypsin treated EYP was still fluid, though the lipid thermal transition pattern was similar to that of gelled EYP. Additives like NaCl and glycerol while preventing gelation of EYP on frozen storage, did not altogether prevent changes in lipid association.

Acknowledgement

Much of this work was done in the laboratory of Proof. D. Chapman, Department of Chemistry, University of Sheffield, Shefield, UK during 1973 when the author was supported by a visitorship of the British Council, UK and a travel grant from the University Grants Commission, New Delhi.

The author thanks Proof. Chapman and Dr. V. B. Kamat for stimulating discuss ions and Dr. Bryan Cater and Mrs. Sue Hawes for initial help with the d.s.c.

Research partly financed by a grant from the US Department of Agriculture, under PL-480 Project No. FG-In-477.

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