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Fluorescence Studies of Protein–Sterol Relationships in Human Plasma Lipoproteins

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Cholesta-5,7,9(11)-trien-3β-ol and its oleate ester were incorporated into human low-density lipoprotein and reconstituted high-density lipoprotein. The unesterified sterol was more efficient than its ester in quenching tryptophan fluorescence, especially in low-density lipoprotein. The results, which indicate that in such lipoproteins unesterified sterols are more closely associated with peptide than are esterified sterols, are used to assess possible structures for the lipoproteins.

The spatial organization of lipid and peptide in lipoprotein structures is still unknown. The most widely accepted view (Gurd, 1960; Schneider *et al.*, 1973) is that lipids such as triglycerides and cholesterol esters are held in a non-polar core and this is stabilized by a surface layer of peptide and polar lipids such as phospholipids and unesterified cholesterol. However, several alternative structures have been put forward (see Margolis & Langdon, 1966; Day & Levy, 1969; Mateu *et al.*, 1972). The distance-dependent transfer of energy between intrinsic and extrinsic peptide and lipid fluorescent probes could provide valuable information on the relationship between the major components, and we have therefore prepared molecules containing conjugated double bonds derived from natural lipids. Here we report the incorporation of the sterol cholesta-5,7,9(11)-trien-3β-ol and its oleate ester into human LD lipoprotein* and reconstituted HD lipoprotein and their ability to quench the fluorescence of the tryptophan molecules present in the peptide moiety.

Experimental

Cholesta-5,7,9(11)-trien-3β-ol was prepared as described by Windaus & Linsert (1928) and the oleate ester obtained by standard methods (Ruzicka & Wettstein, 1936) by using oleic anhydride. Both were assayed spectrophotometrically ($\lambda_{max.}$ 325nm, ϵ12900). Peptide was determined by the method of Lowry *et al.* (1951).

The sterols were incorporated into LD lipoprotein by dispersing them on Celite and incubating with a lipoprotein solution (Ashworth & Green, 1963). However, pure LD lipoprotein solutions cannot be used directly with this method, as adsorption and damage occur. Therefore human plasma was freed of chylomicrons and very-low-density lipoproteins by raising the density to 1.019 with solid KBr and centrifuging at 105000*g* for 24h. The tube was sliced and the upper third discarded. The infranatant solution was dialysed against 0.15 M-phosphate buffer, pH 7.4, and incubated overnight with the sterol dispersed on Celite as described by Ashworth & Green (1963). After removal of the Celite, LD lipoprotein was separated by precipitation with dextran sulphate (Sakagami & Zilversmit, 1962) and dissolved in 0.9% NaCl. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to the solutions incubated with Celite.

Human HD lipoprotein was isolated by ultracentrifugation (Scanu, 1966). The peptide [apo-(HD lipoprotein)] and lipid were separated by solvent extraction at −15°C as described by Scanu & Edelstein (1971). Reconstitution of HD lipoprotein was effected by sonication of apo-(HD lipoprotein) with whole HD-lipoprotein lipid, to which 10–20% by weight of the cholesta-5,7,9(11)-trien-3β-ol or its oleate ester had been added. The conditions used were those used by Forte *et al.* (1971*a*). The solution was passed through a Millipore filter of 100nm pore diameter to remove lipid aggregates. Some samples were passed through a column (20cm × 1.5cm) of Sephadex G-150 to ensure that there were no uncombined peptides present, but this was not found to be a serious problem.

Fluorescence spectra were obtained with a Hitachi Perkin–Elmer MPF 2A fluorescence spectrophotometer operating in the ratio mode and with excitation and emission bandwidths set at 3 and 6nm respectively. Cells of 1 and 10mm path length were used and solutions measured at different dilutions including those for which light transmission was greater than 95% to avoid inner filter effects. Energy transfer between the peptide tryptophan residue and the incorporated sterols was shown by the quenching of the tryptophan fluorescence in the presence of the latter when excitation was carried out at 290nm.

* Abbreviations: HD lipoprotein, high-density lipoprotein; LD lipoprotein, low-density lipoprotein.

Transfer efficiencies were calculated in the usual way (Brocklehurst *et al.*, 1970).

Results and discussion

The absorption and fluorescence spectra of cholesta-5,7,9(11)-trien-3β-ol are shown in Fig. 1. The absorption overlaps the fluorescence emission of tryptophan, and calculation of R_0, the distance at which the transfer efficiency for this pair of chromophores is 50%, gives a value of 2.54nm (25.4Å), based on the values used by Brocklehurst *et al.* (1970) in calculating tryptophan–8-anilinonaphthalene-1-sulphonate energy transfer.

The incorporation of cholesta-5,7,9(11)-trien-3β-ol and its oleate ester into lipoproteins and their efficiency in quenching tryptophan fluorescence are shown in Table 1. Both unesterified and esterified sterols are taken up by LD lipoprotein and incorporated into reconstituted HD lipoprotein. Attempts to incorporate them into native HD lipoprotein from Celite were not very successful, probably because HD lipoprotein contains only 5% as much lipid as LD lipoprotein, and even with the latter uptake represented less than 0.5% of the total lipid. It is unlikely that such amounts would perturb the LD-lipoprotein structure, since *in vivo* exchange and transfer of cholesterol and its esters occur between lipoproteins. Cholesta-5,7,9(11)-trien-3β-ol is incorporated into liposomes as well as cholesterol (R. J. M. Smith & C. Green, unpublished work) and so it should act as a cholesterol analogue in the lipoproteins.

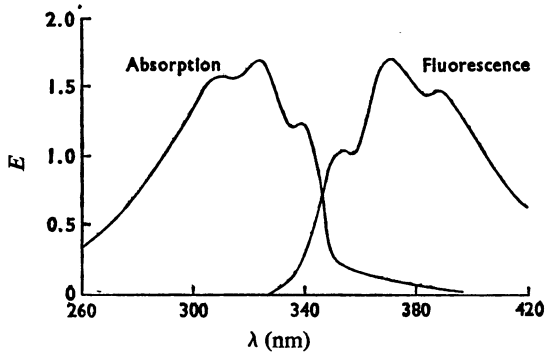

Fig. 1. *Absorption and fluorescence spectra of cholesta-5,7,9(11)-trien-3β-ol*

The fluorescence spectrum is in arbitrary units chosen to equalize the maxima of absorption and emission.

In most experiments quenching of tryptophan fluorescence is only moderately efficient, suggesting that there is no specific association of either sterol with the major tryptophan-containing peptides.

The observed quenching could represent a uniform energy transfer from all tryptophan residues or efficient quenching of some and not of others. However, there is no change in the shape of the fluorescence emission spectrum to indicate differential quenching of tryptophan residues in different environments. In both lipoproteins studied, energy transfer to the unesterified sterol is more efficient than to the sterol ester. This difference, which is very marked for LD lipoprotein but much less so for reconstituted HD lipoprotein, indicates that the peptide is more closely associated with the unesterified sterols than with their esters (cf. Mateu *et al.*, 1972). The association of peptide with a small amount of sterol ester (Pollard & Chen, 1973) is not ruled out, but it seems unlikely in view of the much more efficient quenching by unesterified sterols.

That quenching was caused by energy transfer from tryptophan residues to the probe sterol was con-

Table 1. *Energy transfer between lipoprotein tryptophan and incorporated cholesta-5,7,9(11)-trien-3β-ol or its oleate ester*

Each determination was made in duplicate. The incorporation was calculated from the lipoprotein peptide content, assuming that LD lipoprotein contained on average 500000g of peptide/mol and reconstituted HD lipoprotein 120000g of peptide/mol.

Sterol	Lipoprotein preparation	Sterol incorporation (mol/mol of lipoprotein)	Transfer efficiency
Cholesta-5,7,9(11)-trien-3β-ol	LD lipoprotein	3.4	0.11
		5.5	0.29
		11.7	0.31
		11.9	0.30
	Reconstituted HD lipoprotein	8.3	0.20
		10.9	0.25
Cholesta-5,7,9(11)-trien-3β-yl oleate	LD lipoprotein	2.4	<0.02
		3.6	<0.02
		15.4	0.08
		15.4	0.08
	Reconstituted HD lipoprotein	10.1	0.15
		11.2	0.18

firmed qualitatively by the finding that in all experiments, except those where cholesta-5,7,9(11)-trien-3β-yl oleate was incorporated into LD lipoprotein, there was an increase in fluorescence at 350–420nm (see Fig. 1) on excitation of the tryptophan.

Much is still unknown about LD-lipoprotein peptides (such as the number, if any, of peptide subunits and even the number of tryptophan residues per particle), which makes precise calculations difficult. However, the results do seem consistent with a spherical (or similar) structure of about 20nm (200Å) diameter with peptide and unesterified sterol in a surface layer and sterol esters in the core (Pollard *et al.*, 1969). They are not consistent with the proposals of Mateu *et al.* (1972) for a lipid bilayer structure. Other proposed structures in which the peptide passes through the hydrophobic core containing sterol esters [e.g. structures B, C and D listed by Margolis & Langdon (1966)] are also unlikely, since the hydrophobic amino acids such as tryptophan should be the ones to penetrate the core, and this is not indicated by the fluorescence results. The type of structure suggested by Day & Levy (1969), in which esterified and non-esterified sterols are placed together, is also unlikely in view of the large difference in their quenching efficiencies.

Reconstituted HD lipoprotein contains about 20 tryptophan residues per particle, but as one of the major peptides contains none (Scanu, 1972) no conclusions can be drawn about its location. The degree of quenching by cholesta-5,7,9(11)-trien-3β-yl oleate seems too low for there to be small subunits of the kind postulated for native HD lipoprotein by Forte *et al.* (1971*b*), at least if they are all of similar size and composition. The results will fit a simple spherical structure (Forte *et al.*, 1971*a*) like that for LD lipoprotein but of about half the diameter if the cholesterol esters are assumed not to penetrate the outer monolayer of polar lipids. Other structures in which some of the tryptophan-containing peptide is not closely associated with lipid and projects from the surface are also possible. However, as the inter-chromophore distances calculated for the above model assuming random distribution of cholesta-5,7,9(11)-trien-3β-ol and tryptophan over the surface [2.8–3.4nm (28–34Å)] are close to those calculated according to Brocklehurst *et al.* (1970) from the energy-transfer measurements [3.0–3.2nm (30–32Å)], it seems probable that reconstituted HD lipoprotein has a similar basic structure to that indicated by X-ray-scattering measurements for native high-density lipoprotein fractions (Shipley *et al.*, 1972; Laggner *et al.*, 1973).

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