A STUDY OF THE POLYPEPTIDES OF PORCINE SERUM LOW DENSITY LIPOPROTEINS

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry

by

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VLDL - very low density lipoproteins.

LDL - low density lipoproteins

HDL - high density lipoproteins

apoVLDL - protein moiety of VLDL

apoLDL - protein moiety of LDL

apoHDL - protein moiety of HDL

apo-ala, apoLP-ala, R-ala - apoprotein with COOH-terminal alanine

apo-glu, apoLP-glu, R-glu - apoprotein with COOH-terminal glutamic acid

apo-ser, apoLP-ser, R-ser - apoprotein with COOH-terminal serine

Alcohol D.H. - alcohol dehydrogenase (yeast)

CD - circular dichroism

ESR - electron spin resonance

Hb - haemoglobin

IR - infra red

ORD - optical rotary dispersion

NMR - nuclear mass resonance

R_m - relative mobility

SDS - sodium dodecyl sulphate

S_f - flotation coefficient in Svedbergs in salt solution density 1.063 g/cm^3, 26°C.

Chapter 1

INTRODUCTION

1.1 Introduction

The problem of transporting nonpolar lipids in the blood has been overcome in mammals by association of the lipid with specific proteins, to form water soluble lipoproteins. This association occurs in a reproducible fixed ratio of lipid to protein and was first demonstrated by Macheboeuf (1929). More recently the role played by the proteins in lipid transport has been the subject of intensive investigation.

The circulating lipoproteins are divided into classes. Two operational systems of classification are used, one based on density and the other on electrophoretic mobility. The lipoproteins floating in a centrifugal field at a density of less than 1.006 g/cm^3 are classified as VLDL. These have a flotation coefficient, S_f, of between 20 and 400 and move in the pre-β band on paper electrophoresis. Those floating at density 1.006 - 1.063 g/cm^3 (S_f = 0 - 20) are classified as LDL and run as the β band on electrophoresis. HDL float at density 1.063 - 1.21 g/cm^3 and run as the α_1 band on electrophoresis, and the chylomicrons have an S_f of greater than 400 and remain at the origin in electrophoretic separations, Figure 1.

Following the ingestion of a triglyceride rich meal

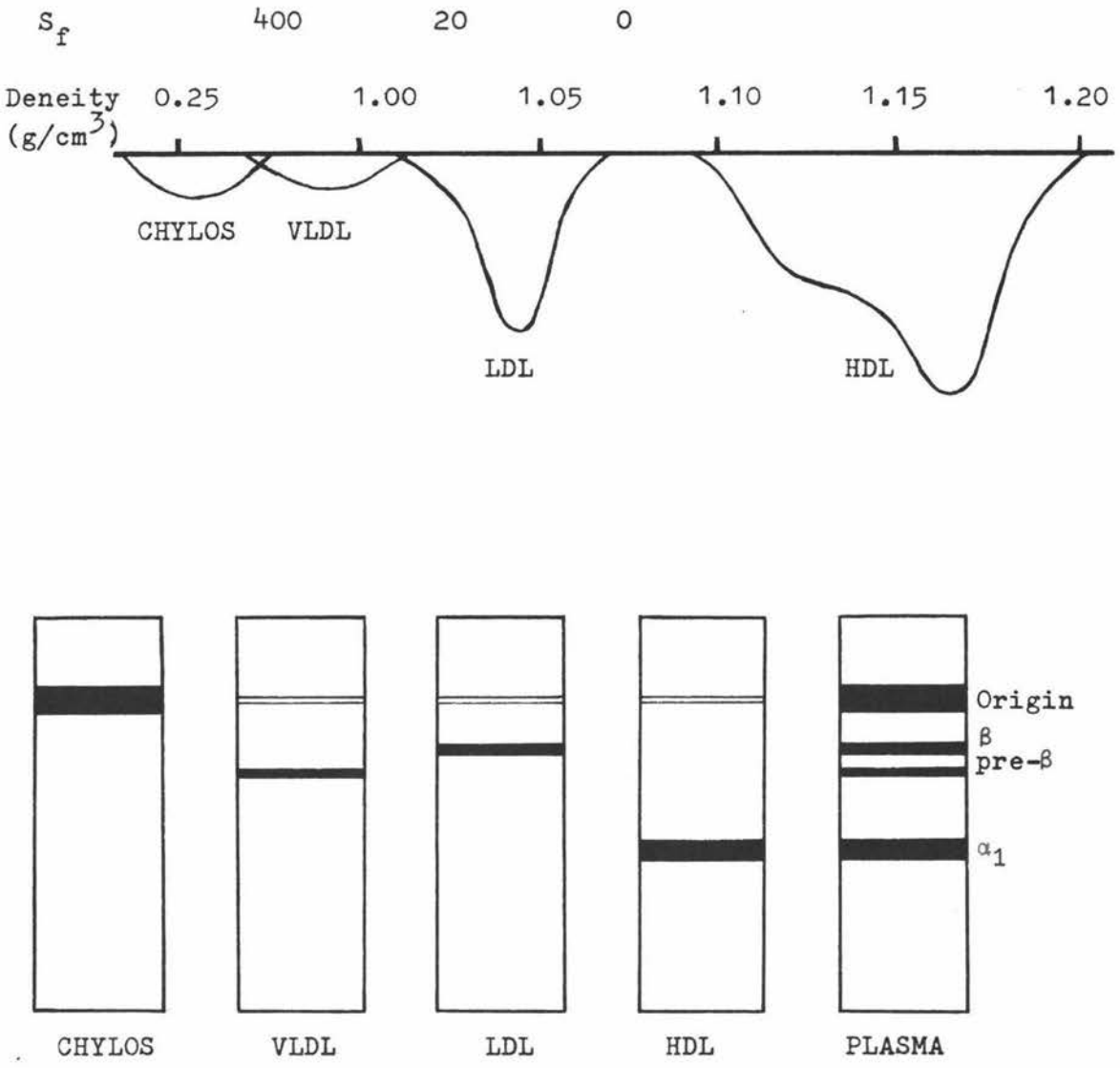


Figure 1. Classification of plasma lipoproteins: (i) by density (Fredrickson, Lux & Herbert, 1973); (ii) by paper electrophoresis (Levy, Lees & Fredrickson, 1966).

the lipid is transported from the intestine mainly as triglyceride in the chylomicrons. Fatty acids synthesized in the liver from carbohydrate and protein are secreted into the plasma as triglycerides bound to VLDL produced in the liver. VLDL is also produced in the intestine (Windmueller, 1968; Ockner et al., 1969), and possibly in other organs as well.

The rate of VLDL release is influenced by the requirement for triglyceride transport from the liver, which is dependent on the rate of fatty acid synthesis in the liver and the level of exogenous medium chain length fatty acids reaching the liver via the hepatic portal vein.

VLDL is thought to be catabolized in the capillary walls of the muscle and adipose tissue by lipoprotein lipase, to release the lipid and form HDL and LDL. Scott & Hurley (1970) have shown, using I^{125} tracer techniques, that LDL is then broken down in the liver, skin and capillary walls.

1.2 Metabolic Relationship Between Lipoprotein Classes

Since it became apparent that the same polypeptides were present in different lipoprotein classes and that VLDL was the major means of transport for esterified lipid, investigation turned to determining the metabolic relationship between the lipoprotein classes.

Evidence now exists for a precursor-product relationship

between VLDL and LDL and at least its partial control by lipoprotein lipase. Fredrickson, Levy & Lees (1967) have shown that in patients whose catabolism of VLDL is slow due to low lipoprotein lipase activity (Type I hyperlipoproteinemia) the plasma LDL concentration is also low. This relationship is also suggested by the results of experiments by Nichols et al. (1968) and Nichols (1969) who showed that heparin, due to its activation of lipoprotein lipase, causes a drop in concentration of plasma VLDL and a concomitant increase in concentration of plasma LDL.

Further support for this hypothesis comes from the results of experiments with radioactive tracers. Fidge & Foxman (1971) working with rats, and Bilheimer et al. (1971) with humans, both isolated VLDL and labelled the apoproteins with I^{125}. The radioactive label was found by Bilheimer et al. (1971) to be distributed in the VLDL apoproteins, before injection, in the proportion: apo-LDL, 45%; apo-ala plus apo-glu, 25%. 10 minutes after intravenous injection of the apoproteins-I^{125}, apo-ala-I^{125} and apo-glu-I^{125} had exchanged between VLDL and HDL, but all the apo-LDL-I^{125} remained in the VLDL. Radioactive LDL appeared subsequently in a fraction of density 1.006 - 1.019 g/cm^3 which reached a maximum after 6 - 12 hours followed by a rapid decline, and then in a fraction of density 1.019 - 1.063 g/cm^3 which reached a maximum after 24 hours and was followed by a slow decline. This process was accelerated by heparin and slowed in patients with Type I hyperlipoproteinemia. Langer (1970) showed in similar experiments with I^{125} labelled VLDL apoproteins, that VLDL is not metabolized to higher density fractions and is not apparently recycled to new VLDL.

Rubenstein & Rubenstein (1972) used radioactive phospholipid and protein to study VLDL metabolism in rats. They found a free two way exchange of tritiated protein and phospholipid-P^{32} between VLDL and HDL. Tritiated protein in VLDL exchanged within 20 minutes with HDL, but not with LDL. Using gel electrophoresis they found exchange between 3 of the 6 bands in apoVLDL and apoHDL.

These results appear to support the hypothesis that LDL is a product of the action of lipoprotein lipase on VLDL and that its formation is to some extent dependent on the rate of lipase activity. Whether all circulating LDL derives from VLDL is not known, nor is it known if LDL has a specific function.

The relationship between HDL and VLDL is less clear. The free exchange of components between them could suggest that some or all HDL apoproteins were precursors to VLDL formation, but further investigation will be required before definite conclusions can be drawn.

1.3 Cofactor Activity of Lipoproteins

Korn (1955) showed that lipoprotein lipase could hydrolyse a triglyceride emulsion only after activation by serum lipoproteins and suggested a possible role of lipoproteins in the control of lipid transport. Although evidence has been presented involving VLDL, LDL and HDL as activators (Bier &

Havel, 1970), it is generally considered that HDL is the activator for human lipoprotein lipase. However, current information does not permit definite conclusions to be drawn on the mechanism, nature and specificity of cofactors in lipoprotein lipase activity. The most serious shortcoming is that most experiments are carried out with crude enzyme preparations and the results may well be influenced by the nature and amount of impurities. Another problem is that the polypeptide preparations being tested for cofactor activity have not always been well characterized and in some cases different amino acid compositions have been reported for peptides with the same COOH-terminal groups.

LaRosa et al. (1970) found that peptides with glutamic acid and alanine COOH-terminal groups (apoLP-glu and apoLP-ala) had cofactor activity with crude lipoprotein lipase preparations. In contrast to this Brown & Baginsky (1972) showed that purified apoLP-ala (of VLDL) inhibits lipoprotein lipase at the level of 2% of the substrate. Bier & Havel (1970) reported activation by HDL and also by VLDL at 0.2% of substrate level. They suggest that either of the polypeptides apoLP-thr and apoLP-glu, being common to both classes, could be the activator.

1.4 Composition of LDL

The composition of the lipoprotein classes is given in Table 1.

Table 1. Composition of Lipoprotein Classes

Composition % by weight	Chylomicrons	VLDL	LDL	HDL	LP-X
Protein	2	5-12	20-22	50	6
Carbohydrate			3-5		
Total Lipid	98	88-95	75-78	50	94
Cholesterol ester	4	10-13	35-41	20	2
Phospholipid	4	13-20	21-23	24	66
Cholesterol	2	3-5	8-9	2	23
Triglyceride	88	50-60	6-8	4	3

LDL also contains traces of lysolecithin, glycolipids, and other unidentified lipids (Skipski et al., 1967). The saturated fatty acids are mainly 16:0 and the unsaturated mainly 18:1 and 18:2. LDL also contains 50% of serum tocopherol (McCormick et al., 1960) and 75% of serum β-carotene and lycopene (Krinsky et al., 1958).

LDL has been found to have a polysaccharide component containing galactose, mannose, glucosamine, fructose and sialic acid. Evidence exists for covalent bonding of protein and carbohydrate and for the terminal position of sialic acid on the polysaccharide chain (Margolis & Langdon, 1966a).

The predominant lipoprotein in obstructive jaundice, LP-X, has a significantly different composition from LDL.

1.5 Molecular Weight of LDL

Numerous values for the molecular weight of LDL have been reported. Calculations for molecular weight must assume that:

1. The molecule is an anhydrous sphere, or values must be assigned for shape and hydration terms.
2. LDL has insignificant preferential salt binding or hydration in the salt solution used.

Early work by Toro-Goyco (1958) and Bjorklund & Katz (1956) gave molecular weights for human LDL of 2.55×10^6 and $2.8 - 3.0 \times 10^6$ daltons. Scanu *et al.* (1968) using sedimentation equilibrium reported a value of $2.2 - 2.3 \times 10^6$ daltons. Studies by Adams & Schumaker (1969) using the high salt flotation technique gave a value of $2.3 - 2.5 \times 10^6$ daltons. Fisher *et al.* (1971) found constants for the shape, hydration and salt binding of the molecule and calculated a value of 2.73×10^6 daltons.

Not only are differences in molecular weights reported between groups, but workers studying individual serum samples have reported differences between samples. Lindgren *et al.* (1969) using human serum LDL found on the average, a higher molecular weight in female subjects (2.36×10^6 daltons) than in male subjects (2.12×10^6 daltons). From the results of banding studies on individual samples Adams & Schumaker (1970) reported marked heterogeneity between samples.

Molecular weight determinations by Janado & Martin (1968) on pig serum LDL also showed marked heterogeneity. Six

subfractions of LDL were isolated, with molecular weights ranging from 2.2×10^6 to 3.8×10^6 daltons. The lower end of the range is comparable to the molecular weights for human LDL.

Fisher (1970) obtained a significantly higher molecular weight of 4.2×10^6 daltons for LDL in patients with Type IV hyperlipoproteinemia.

Mauldin & Fisher (1970) reported aggregation of LDL under conditions of low pH and at neutral pH with high or low ionic strength. This was enhanced at higher LDL concentrations and at high hydrodynamic pressure. These are conditions which prevail during molecular weight determinations by analytical ultracentrifugation, but this would probably not account for the high result obtained by Fisher.

1.6 Particle Size of LDL

Studies with the electronmicroscope have shown human LDL to be a spherical structure. Variations in diameter have been reported between groups, Forte *et al.* (1968) reporting sizes ranging from 200-290 Å and Gotto *et al.* (1968a) finding the particle size range somewhat smaller (216 to 220 Å). Pollard *et al.* (1969) reported an average particle size of 193 Å.

1.7 The Apoproteins

The protein moiety of LDL is generally prepared by extraction of LDL with organic solvents. The apoprotein has a low solubility in water due to aggregation which is favoured by the removal of the lipids. Aggregation is prevented to varying extents by the addition of detergent (sodium decyl sulphate or sodium dodecyl sulphate), urea (8 M) or guanidine hydrochloride with detergent, titration at pH 11.5 or succinylation of native LDL prior to delipidation.

Recently a water soluble, lipid free apoLDL was prepared by Helenius & Simons (1971) by gel filtration of LDL in the presence of sodium deoxycholate, a neutral detergent (Nonidet 40), an anionic detergent (SDS) and a cationic detergent (cetyltrimethyl-ammonium bromide) at micellar concentrations.

Alaupovic et al. (1972) show evidence for 3 distinct immunological determinants, LP-A, LP-B and LP-C which are all present in varying proportions in all classes of lipoproteins. LP-A is the major component in HDL, LP-B in LDL and LP-C in VLDL.

Using heparinized serum Lee & Alaupovic (1970) prepared 6 density subfractions of LDL by ultracentrifugation and reported the presence of LP-A in all six by immunological techniques. The LDL subfractions of plasma from one group consisting mostly of women had virtually no LP-C in the LDL, and a second group consisting mostly men had both LP-A and LP-C in the LDL as minor components. However the presence of the minor components was not consistent in all the subfractions of each group. The amino acid

compositions of the 6 subfractions were similar although not identical (at least 3 amino acids were different when any 2 subfractions were compared). The compositions also differed from their earlier reported composition of LDL and from other literature values. LP-A and LP-C were not considered to be contaminants as their S_f values were confirmed to be in the range 0 - 20 by analytical ultracentrifugation. Neither was it considered that LP-A or LP-C were complexed to LP-B, as LP-A could be removed by precipitation.

Two antigenic determinants were found in LP-B and two polypeptides, LP-B_1 and LP-B_2, separated by diffusion on agar and agarose gels. LP-B_2 was lipid poor but was considered not to be a centrifugation breakdown product, as both forms were sometimes found in fresh LDL. Differences in amino acid composition were found between different LP-B samples, which were considered to be due to varying ratios of LP-B_1 and LP-B_2.

They concluded that LDL is heterogeneous and differences in hydrated densities are explained by subtle changes in the chemical composition of a continuous population of lipoprotein molecules.

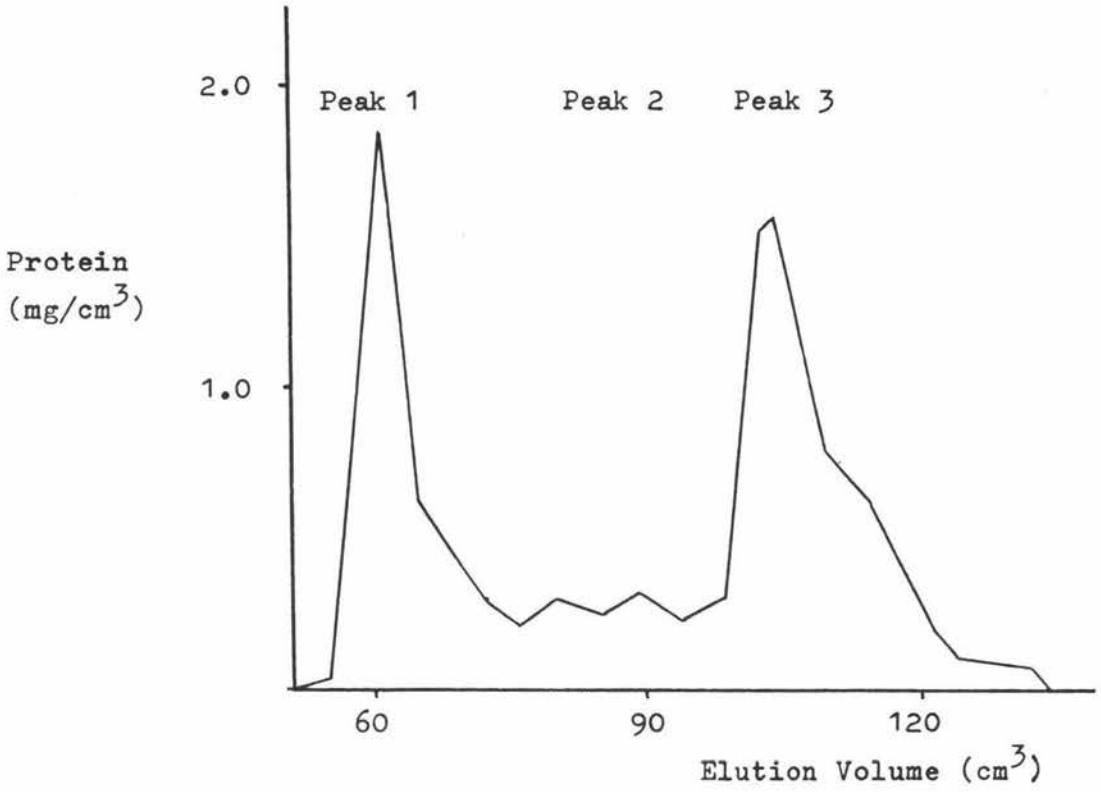
LP-C, the major component of VLDL was recently reported by McConathy, Quiroga & Alaupovic (1972) to be composed of 3 polypeptides: C_I, R-ser; C_{II}, R-glu; and C_{III}, R-ala. All three are required to form LP-C. Kostner & Alaupovic (1971) reported that HDL had two major components LP-A_I and LP-A_{II} both having a glutamine COOH-terminal residue.

Results from the work carried out by Fredrickson's

groups are generally in agreement with that of Alaupovic although a different system of nomenclature is used. Investigation has been centred on VLDL which after delipidation by solvent extraction of lyophilizid VLDL is subjected to gel filtration. Three peaks are eluted from Sephadex G-150, Figure 2, (Brown, Levy & Fredrickson, 1970). 40% of the VLDL apoprotein was found to be immunologically identical to apoLDL. A similar result was obtained by Bersot et al. (1970) in rats. Using polyacrylamide gel electrophoresis, DEAE chromatography, gel filtration and immunological tests they found 25% of the VLDL protein to be identical to LDL protein.

Peak 3 obtained by Brown, Levy & Fredrickson (1970), (Figure 2) contained the peaks D_I, D_{II}, D_{III}, and D_{IV} which are eluted when apoVLDL is run on a DEAE column (Brown, Levy & Fredrickson, 1969) and which have been characterized with respect to terminal groups (Herbert, Levy & Fredrickson, 1971), Table 2. These results confirm the findings of McConathy, Quiroga & Alaupovic (1972); D_I corresponding to C_I (R-ser), D_{II} corresponding to C_{II} (R-glu) and D_{III}, D_{IV} and D_V corresponding to C_{III} (R-ala). D_{II} may be 2 polypeptides as valine was also released to some extent in COOH-terminal determination. D_{III} and D_{IV} differ only in sialic acid content. Fredrickson et al. (1973) has reported LDL to be 90% composed of a single protein of molecular weight 27,000 daltons, with COOH-terminal serine and NH_2-terminal glutamine.

Shore & Shore (1969) using individual samples of human serum, prepared an LDL fraction of density 1.029 - 1.039 g/cm^3. The apoproteins were separated by ion exchange chromatography on



Peak	1	2	3
Immunological identity	LDL	HDL	VLDL
% Total protein applied to column	40	10	50

Figure 2. Gel filtration of apoVLDL using Sephadex G-150, and results of immunological tests (Brown, Levy & Fredrickson, 1970).

Table 2. Properties of the major proteins of VLDL (Herbert, Levy & Fredrickson, 1971).

Peak	NH_2-Terminal	COOH-Terminal	M.W.	% Total apoVLDL
D_I	Threonine	Serine	7,000	10
D_{II}	Threonine	Glutamic acid	10,000	10
D_{III}, $_{IV}$, & $_V$	Serine	Alanine	8,764	30

DEAE cellulose. Two polypeptides were isolated which represented only a 30% recovery of applied protein. The amino acid compositions of the two polypeptides were very similar although not identical and were both different to those of all other known apoproteins. No COOH-terminal groups were found, either by hydrazinolysis or by carboxypeptidase A and B, a result in direct contrast with other workers' findings and with results published earlier by Shore (1957).

VLDL and HDL apoproteins were also run on DEAE, VLDL with a recovery of 50% yielded 6 fractions and HDL yielded 10. None of the VLDL fraction amino acid compositions corresponded to any of the amino acid compositions of R-ser, R-glu or R-ala. Shore & Shore (1968) reported characterization of 2 of the HDL apoproteins; one having COOH-terminal threonine and NH_2-terminal aspartic acid and of molecular weight 16,000 daltons, the other of molecular weight 14,000 daltons having a glutamic acid COOH-terminal and an aspartic acid NH_2-terminal. Two polypeptides were found to be common to VLDL and HDL but there exists a strong possibility that the classes may well have common polypeptides

not eluted from DEAE. The numerous polypeptides with similar amino acid composition are thought by Shore & Shore possibly to be multiple forms of a polypeptide which undergoes minor amino acid composition changes to change lipid binding affinity.

Granda & Scanu (1966) working on pooled human serum found the NH_2-terminal groups for LDL (glutamine) and VLDL (threonine and serine) both results in agreement with Fredrickson. They also published an amino acid composition of LDL which differed considerably from that published by Shore & Shore (1969) but was similar to some of the fractions analysed by Lee & Alaupovic (1970). Koga, Horwitz & Scanu (1969) using pooled rat serum showed the amino acid composition of LDL to be very similar to the composition of human LDL found by Granda & Scanu (1966). They also showed VLDL to have 3 antigenic sites, two were immunologically identical to LDL and HDL and the third was unique. On polyacrylamide gel electrophoresis LDL and apoLDL appeared to move with similar relative mobilities. Both ran as a single band on 3.75% polyacrylamide, a result in conflict with more recent studies showing heterogeneity. However, they observed small amounts of faster moving components in some cases.

Margolis & Langdon (1966a, 1966b) using pooled human serum prepared LDL by dextran sulphate precipitation and report its homogeneity as tested by analytical ultracentrifuge, polyacrylamide gel electrophoresis and gel filtration. This is in agreement with Granda & Scanu (1966) but at variance with other reports. The amino acid composition was found to be different from all other reports. In an effort to determine the positions of some groups, derivatives of native LDL and apoLDL were formed. The assumption

was made that those groups that reacted fully after delipidation and not in native LDL were exposed only on delipidation. The bromoacetate reaction for histidine and reduction alkylation for disulphide groups showed a 50% and 100% increase in reactivity on delipidation of native LDL. Most lysine residues in native LDL are readily reactive and thought to be on the surface.

Hillyard et al. (1972) working on chick serum and egg yolk apoLDL determined that both sources had the same COOH-terminal (tyrosine) and NH_2-terminal (lysine). The amino acid compositions were very similar, most values being within 10%.

1.8 Structure of LDL

Gotto et al. (1968a) reported a granular surface to LDL particles when negatively stained and observed by electronmicroscope. Pollard et al. (1969) has also reported a fine surface structure which they consider are globular subunits, 50 Å in diameter, and arranged in a dodecahedral pattern with overall isohedral symmetry. This postulate was supported with a 3-D isodensity map constructed from the electronmicrograph.

Small angle X-ray scattering studies on aqueous LDL carried out by Mateu et al. (1971) have indicated a particle size within the range found from electronmicrographs. The electron density distribution was interpreted to support a spherical model with lipids organized as a bilayer covered each side by protein.

The results of optical methods have in some cases been in conflict, and at this stage only generalizations on protein conformation can be made. Scanu & Hirz (1968) obtained a CD spectrum for LDL at room temperature and physiological pH, characterized by one positive (196 nm) and 2 negative Cotton effects (220 and 208 nm), indicating a mixture of α-helix and random structure, although not excluding the possibility of β structure. The CD spectrum was influenced by pH, SDS, urea and guanidine hydrochloride but not by succinylation. Scanu et al. (1969) found the CD spectrum was also influenced by protein concentration and temperature. Low temperature spectra indicated mainly α-helix and at high temperature β structure appeared to dominate (characterized by a single negative Cotton effect near 218 nm). These temperature induced conformation changes were reversible. Native LDL appeared to be less sensitive to temperature induced change, suggesting that the lipid portion stabilizes the protein conformation. IR studies by the same group on LDL at room temperature indicated that only a minor portion of the protein was β structure. This conformational flexibility was also observed by Dearborn & Wetlaufer (1969) using ORD and CD. Their results were similar to those of Scanu & Hirz (1968) confirming the dependence of conformation on temperature and the presence of lipid.

In contrast to this Gotto et al. (1968b) found a major trough at 216 - 218 nm in the CD spectrum of LDL, indicative of β structure, and a slight shoulder indicating the possibility of a small amount of α-helix and/or disordered structure. The CD spectra were supported by IR data interpreted as supporting the

antiparallel nature of β conformation. The reasons for the disagreement between these results lie probably in the conditions of experimentation, which has been shown to influence the spectral behaviour of LDL. Despite some inconsistency it can be concluded that all three conformations probably exist in LDL and that the proportion of each varies with source and preparation of material, temperature, and concentration of protein.

NMR studies have been carried out by Leslie et al. (1969) and Stein et al. (1968). Signals from LDL, coming almost entirely from the lipid, were found to be indistinguishable from aqueous dispersions of lipid extracted from LDL, suggesting that the protein moiety has little influence on the organisation of the lipid. This suggestion is possibly supported by ESR studies by Gotto (1969). The LDL was spin labelled in the protein moiety with N-(1-oxyl-2,2,6,6-tetramethylpiperinyl)-maleimide and the spectra exhibited 2 types of signal; a narrow one indicating a high degree of freedom of tumbling of the free radical, and a broad one indicating some constraint; the lipid posing constraint on the protein.

Degradation experiments have been carried out with lipolytic and proteolytic enzymes. Proteolytic enzymes produce only a partial digestion of native LDL. Structural changes occur on hydrolysis and include an increased tendency to aggregate and increased extractability of lipid (supporting the hypothesis that the protein forms a layer over the lipid).

Conflicting reports have been published on the nature of the peptide released on tryptic digestion. Experiments by

Bernfield & Kelly (1964) showed that the amino acid composition of the digested portion was the same as native LDL, while Margolis & Langdon (1966c) demonstrated the release of a specific peptide with an amino acid composition differing from native LDL. Despite differences in results of hydrolysis products, it can be postulated that the portion of protein not open to proteolytic attack is situated in the interior of the LDL particle.

Phospholipase A is found to hydrolyse up to 97% of phosphatidyl choline, ethanolamine and serine (Nishida, 1968), suggesting that the phospholipids are situated near the surface of the molecule. This is supported by the results of experiments with phospholipase C and D (Bruckdorfer & Green, 1967; Nishida & Cogan, 1969) and also by lipid exchange experiments which have shown phospholipids and cholesterol to exchange much faster than triglycerides.

1.9 Structural Models of LDL

Several problems at present prevent definite answers to the question of a model. Electronmicrographs have reported both smooth and structured surface to the LDL particles. The limited knowledge of the polypeptides and their apparent conformational metastability make it difficult to define a physiological structure. The structural role of water is yet to be investigated fully, and the exact nature of the lipid-protein interaction has yet to be determined. If a precursor-product relationship

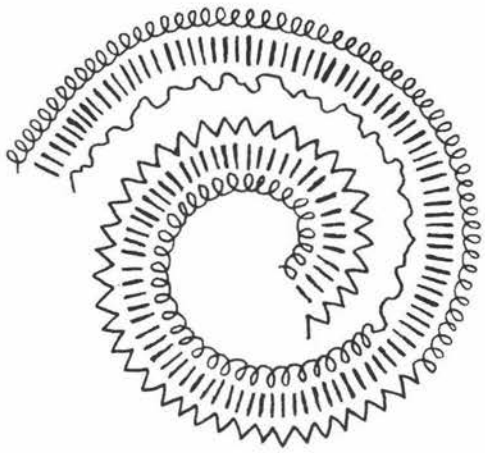
exists between VLDL and LDL, the common polypeptides may occupy similar positions in both classes, or if not a mechanism for their rearrangement has yet to be found.

The solubility properties of lipoproteins led to the concept that the protein and polar lipids (phospholipids) occupy all or most of the lipoprotein surface and the less polar lipids, triglycerides and cholesterol esters form a core. Evidence from experiments by Gustafson (1966) and Oncley (1963) on the surface area occupied by polar components, and from X-ray diffraction data from Mateu et al. (1971) support this hypothesis. The position of cholesterol esters in the core is also shown by analysis of pathological lipoproteins present in obstructive jaundice.

Margolis & Langdon (1966c) claim the most satisfactory model is a bilayer of protein surrounding the lipid and coiled in on itself (Figure 3 (a)). This would account for the limited proteolysis observed with native LDL and the increased proteolysis with delipidated LDL. Synthesis could be by pinching off of the endoplasmic reticulum. An alternative possibility proposed by Margolis & Langdon (1966c) is a structure composed of subunits with peptides extended to cover 70% of the lipid core (Figure 3 (b)). The protein on the exterior would give the antigenic properties and that in the centre would be resistant to hydrolysis.

None of the hypothetical model structures have been demonstrated and although the concepts of lipid transport are clear, the mechanisms and controls are far from understood. A key to both these questions lies in a fuller understanding of the nature and characteristics of the protein moiety.

(a)



(b)

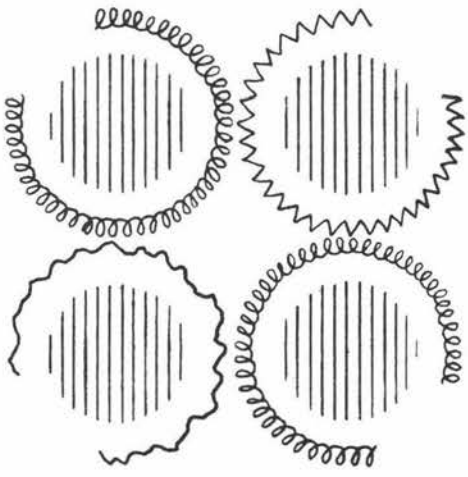


Figure 3. Schematic models of LDL (Margolis & Langdon, 1966c).

α-helix

ß-structure

random structure

lipid

Chapter 2

EXPERIMENTAL METHODS

2.1 Solvents and Reagents

Solvents were purified by distillation, according to Vogel (1957).

Urea was purified by preparing a 10 M stock solution in deionised water and passing it through separate columns (2 x 20 cm) of Amberlite IRA 400 (OH^- form) and IRA 120 (H^+ form). The conductivity of the deionised urea was less than 10 micromho.

2.2 Isolation and Purification of LDL

Blood was collected from freshly killed Large White pigs which had been fasted 24 hours. The clot was allowed to set for 3 hours and the serum was separated by straining through cheese-cloth. Cellular debris was removed by centrifugation in a Sorvall RC-2B centrifuge using a GS-3 rotor at 10,000 g for 15 minutes.

Bacterial growth and enzymatic activity were inhibited by the addition of thiomersal at the rate of 1 cm^3 to 250 cm^3 serum. Thiomersal stock solution was prepared by dissolving

0.7 g thiomersal ($C_2H_5HgSC_6H_4COONa$) in 30 cm^3 of water. The solution was adjusted to pH 8 with 6% sodium borate and made up to 50 cm^3 with water (Hatch & Lees, 1968).

Fractionation of the serum to the lipoprotein classes was carried out by ultracentrifugation according to Lindgren et al. (1961) as modified by Hatch and Lees (1968). Centrifugation was carried out in a Beckman L-2-65B ultracentrifuge at 16°C. At the end of each run the rotor was allowed to come to rest without braking, to avoid disturbance of the layers.

Solutions required:

1. Density 1.006 g/cm^3

   11.40 g NaCl, 0.1 g EDTA.Na_2 and 1 cm^3 1 N NaOH were made up to 1000 cm^3 in a volumetric flask and 3.0 cm^3 additional water added.

2. Density 1.063 g/cm^3

   24.98 g NaBr was added to 100.0 cm^3 of solution 1 to make a solution of density 1.182 g/cm^3. One volume of this solution was mixed with two volumes of solution 1.

Initial preparative runs were done in a 35 rotor (94 cm^3 tubes). 20 cm^3 solution 1 was placed in the tubes which were then capped. The serum was then layered under the solution 1 using a 20 cm^3 syringe with a 15 cm 18 gauge needle. The rotor was run at 95,000 g for 16 hours and the top 20 cm^3 layer containing chylomicrons and VLDL removed with a Pastuer pipette.

The bottom layers were adjusted to density 1.063 g/cm^3 by the addition of solid KBr according to the procedure of Radding and Steinberg (1960). The amount of KBr was determined from the

following equation:

$$X = \frac{V_i(d_f - d_i)}{1 - \bar{V} d_f}$$

where X is the amount of KBr (g) to be added to adjust a volume, V_i, from its initial density, d_i, to the desired final density, d_f. $\bar{V}$ is the partial specific volume of KBr.

To raise the density from 1.006 to 1.063 g/cm^3, 8.34 g potassium bromide was added per 100 cm^3 of serum, the value of $\bar{V}$ used was 0.298 (Baxter and Wallace, 1916).

The serum of density 1.063 g/cm^3 was spun in the same rotor for 22 hours, after which the upper layer of LDL was removed with a Pasteur pipette.

The isolated LDL was washed, to remove adsorbed contaminating proteins, by recentrifugation. This was carried out in a 60 Ti rotor (30 cm^3 capacity tubes). 10 cm^3 of solution 2 was placed in the tubes which were then capped. The unwashed LDL was then introduced as a lower layer as before. The rotor was run at 214,000 g for 12 hours and the washed LDL removed with a Pasteur pipette.

2.3 Preparation of ApoLDL

Three methods were employed to extract the lipid from LDL. Method I was used most frequently. In some cases the LDL was succinylated before delipidation.

2.3.1 Succinylation of LDL

This was carried out according to the method of Scanu et al. (1968) based on that used by Hass (1964) for aldolase. The LDL was constantly stirred and succinic anhydride added in small increments to a final molar ratio of succinic anhydride: lysine of 100:1. The pH was monitored on a Radiometer pH Meter 26 and maintained at pH 8.9 by addition of 1 N NaOH from a Radiometer Titrator 11. The reaction was considered complete when the pH had stabilised after the addition of all the succinic anhydride. The succinylated LDL was dialysed against water, to remove succinic acid, in Visking 24/32 tubing.

2.3.2 Method I (Shore & Shore, 1969)

Washed LDL was made 1 N in NaCl. Cholesterol, cholesterol esters and triglycerides were removed by repeated extractions with diethyl ether at 5°C in a nitrogen atmosphere for 5 hours. The NaCl was removed by dialysis against water and the remaining lipid (mainly phospholipid) was removed by six extractions with mixtures of ether and ethanol in which the ethanol was increased from 20% to 40%. Aqueous and organic phases were kept in the ratio 1:1. Between each extraction the aqueous phase was briefly extracted with diethyl ether. Remaining traces of organic solvent were removed under vacuum. The apoLDL was dialysed against 0.2 M Tris-HCl in 8 M urea at pH 8.4 and concentrated by negative pressure dialysis using Selby 8/32 dialysis tubing.

2.3.3 Method II (Brown, Levy & Fredrickson, 1969)

Washed LDL was dialysed against 0.01% EDTA.Na_2, to remove salts, and then lyophilized. Lipid was extracted by stirring for 12 hours with ethanol:ether, 3:1 (v:v). The protein was centrifuged and extracted twice more for 3 hours with the same solvent. The apoLDL was recovered by centrifugation, rinsed in ether, dried under a stream of nitrogen and dissolved in 0.2 M Tris-HCl in 8 M urea at pH 8.4.

2.3.4 Method III

Washed LDL was freeze dried and extracted with ether at 0°C for 6 hours. The protein was recovered by centrifugation and further extracted for 8 hours with ether:ethanol, 1:1 (v:v). ApoLDL was collected by centrifugation, washed with ether, dried under nitrogen and dissolved in 0.2 M Tris-HCl in 8 M urea at pH 8.4.

2.4 Agarose Gel Electrophoresis

This procedure was carried out on washed LDL by the method of Noble (1968).

Stock solutions:

1. Barbital Buffer 0.05 M pH 8.6

   2.76 g diethyl barbituric acid

15.40 g sodium diethyl barbiturate.

1.5 l water.

2. Agarose (Koch Light)

0.25 g in 50 cm^3 barbital buffer, gently boiled.

3. Agar (Difco Lab.)

0.3 g in 50 cm^3 barbital buffer, gently boiled with constant stirring.

0.5% agarose:agar 4:1 (w:w) was prepared by mixing 40 cm^3 agarose solution with 10 cm^3 agar solution. 2.5 cm^3 of this was spread on each microscope slide (2.5 x 7.0 cm) to form a layer 1.5 mm thick. When the gels had set, wells 1.5 mm wide were cut 1.5 cm from one end and strips of filter paper (Whatman No. 1) were placed in the wells.

The lipoprotein samples (20 - 100 µl) were applied to the filter paper in the wells with 5 µl 0.001% bromophenol blue in water to act as front marker.

The gels were placed in an electrophoretic cell with barbital buffer in the electrolyte compartments. Miracloth wicks, presoaked in barbital buffer were placed on the ends of the slides to make electrical contact. The slides were run at a constant current of 3 mA/slide for 3 hours and then fixed by immersion in 5% glacial acetic acid in 75% ethanol for 30 minutes. The slides were then dried at 50°C and stained.

Stains:

1. Amido Schwarz (Protein Stain)

0.1% Amido Schwarz in 7% acetic acid.

2. Oil Red O Colour Index No. 26125 (Lipid Stain)
630 cm^3 95% ethanol and 0.4 g Oil Red O were warmed with vigorous stirring and 370 cm^3 water added. The mixture was refluxed for one minute and allowed to cool to 40°C at which temperature it was maintained.

3. Sudan Black B Colour Index No. 26150 (Lipid Stain)
0.2 g Sudan Black B was stirred for 1 hour in 100 cm^3 60% ethanol and allowed to stand overnight at room temperature. This solution was diluted 1:1 (v:v) with 60% ethanol prior to use.

2.5 Polyacrylamide Gel Electrophoresis

2.5.1 3.75% Acrylamide Gels

3.75% acrylamide gels were used for running washed LDL (Raymond et al., 1966).

Stock Solutions:

1. Acrylamide.
30.0 g acrylamide and 1.0 g N,N'-methylenebisacrylamide were made up to 100 cm^3 with water and stored in the dark at 4°C.

2. 1.6 g dimethylaminopropionitrile in 100 cm^3 Tris-glycine buffer diluted 2.5 times.

3. 0.48% ammonium persulphate in water.

4. Tris-glycine buffer pH 8.5.

   3.0 g Trishydroxylmethylaminomethane and 14.4 g glycine were made up to 100 cm^3 with water. This solution was diluted 10 times prior to use.

For a typical run of 8 gels (0.7 x 8 cm), 2 cm^3 acrylamide solution, 6 cm^3 water and 4 cm^3 dimethylaminopropionitrile were mixed and deaerated. 4 cm^3 ammonium persulphate solution were added and the gels quickly poured. 0.5 cm^3 distilled water was layered on top of the gels to exclude oxygen and produce a flat top to the gels, which were then left to set for 2 hours.

The gels were placed in an electrophoretic cell with Tris-glycine buffer in the electrolyte compartments. Samples were made more dense than the buffer by addition of sucrose or glycerol and introduced on to the top of the gels with a micro-syringe. 100 μl 0.005% bromophenol blue in water was added to the upper buffer compartment as a front marker. The gels were run at a constant current of 3mA/gel until the front marker approached the end of the gel.

Gels were removed from the tubes with a syringe and fine needle. The front was either measured or marked with a fine piece of wire. Staining was the same as for agarose slides. Destaining was carried out electrophoretically by replacing the gels in the tubes and running at 5 mA/gel with 7% acetic acid in the electrolyte compartments.

2.5.2 7.5% Acrylamide in 8 M Urea Gels

7.5% acrylamide in 8 M urea gels were prepared according to Davis (1964), and used for separation of LDL apoproteins.

The stock solutions were prepared as for Section 2.5.1.

To prepare 8 gels (0.7 x 8 cm), 4 cm^3 acrylamide, 4 cm^3 dimethylaminopropionitrile and 5.67 g urea were mixed and deaerated. 4 cm^3 ammonium persulphate were added and the gels quickly poured. Water was layered on top of the gels as in the previous section. LDL apoproteins were applied to the gels without addition of sucrose. Running conditions, staining and destaining were carried out as in Section 2.5.1.

2.5.3 10% Acrylamide 0.1% SDS Gels

10% acrylamide 0.1% SDS gels were prepared according to Weber & Osborn (1969). The molecular weights of the LDL apoproteins were determined by comparison with proteins of known molecular weight.

Stock Solutions:

1. Acrylamide

   22.2 g acrylamide and 0.6 g N,N'-methylenebisacrylamide were dissolved in water to make 100 cm^3, filtered and stored in the dark at $4^{\circ}C$.

2. Gel Buffer pH 7.0

7.8 g $NaH_2PO_4.H_2O$, 38.6 g Na_2HPO_4 and 2.0 g SDS were made up to 1000 cm^3 with distilled water.

3. Dialysis Buffer pH 7.0.

This buffer was 0.01 M sodium phosphate with 0.1% SDS and 0.1% 2-mercaptoethanol.

For a batch of 8 gels (0.6 x 15 cm), 15 cm^3 gel buffer and 13.5 cm^3 acrylamide solution were deaerated. 1.5 cm^3 ammonium persulphate (15 mg/cm^3, freshly prepared) and 45 µl N,N,N',N'-tetramethylethylenediamine were added and the gels poured and layered with water. The samples and standard proteins of known molecular weight containing about 1 mg protein/cm^3 were dialysed against the dialysis buffer for 3 hours at 37°C. To 50 - 100 µl of each was added:

3 µl 0.005% bromophenol blue in water
1 drop glycerol
5 µl 2-mercaptoethanol
50 µl dialysis buffer.

The gel tubes were placed in the electrophoretic cell and the water removed from the top of the gels. The samples were applied and the gel tubes were filled by layering on gel buffer:water, 1:1 (v:v). The upper electrolyte compartment was filled with the same buffer. Tris-glycine buffer (Section 2.5.1) was used in the lower electrolyte compartment to overcome the accumulation of gas bubbles below the gels.

The gels were run at 8 mA/gel until the bromophenol blue approached the bottom of the gel; about 4 hours.

The gels were removed from the tubes, the fronts marked, and stained for 2 - 3 hours in Coomassie Brilliant Blue made by dissolving 1.25 g Coomassie Brilliant Blue in 454 cm^3 50% methanol and 46 cm^3 glacial acetic acid.

Due to difficulties in replacing these longer gels in the tubes, they were destained by shaking at room temperature in a mixture of 75 cm^3 glacial acetic acid, 50 cm^3 methanol and 875 cm^3 water.

2.6 Determination of Protein

Protein concentrations were determined by the colourimetric method of Lowry *et al.* (1951).

Reagents:

A. 2% Na_2CO_3 in 0.1 N NaOH.

B. 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate.

C. 50 cm^3 A and 1 cm^3 B mixed just prior to use.

D. 1 volume Folin's phenol reagent plus 2 parts of water.

0.5 cm^3 sample containing 10 - 200 μg protein was mixed with 5 cm^3 reagent C and left at room temperature for 10 minutes. 0.5 cm^3 reagent D was added and mixed thoroughly. The colour was read 10 minutes later at 600 nm on a Hitachi 101 Spectrophotometer. Serum albumin (bovine) in water was used as a standard.

2.7 Determination of Lipid

Lipid was determined gravimetrically by the method of Folch *et al.* (1957).

1 cm^3 of solution containing LDL was mixed with 17 cm^3 chloroform:methanol 2:1 (v:v) and filtered through lipid free filter paper. The residue was washed with 3 cm^3 of the same solvent.

4 cm^3 0.37% KCl was shaken with the filtrate and the layers allowed to separate. The top layer was then taken off and the interface washed with the upper phase of the mixture chloroform:methanol:water, 8:4:3 (v:v:v). The lower phase was then placed in a tared vessel, the solvent evaporated off under a stream of nitrogen and the lipid residue weighed.

2.8 Determination of Phosphate

Phosphorus was determined colourimetrically by the method of Bartlett (1959).

2 cm^3 of apoLDL were digested by heating over a small flame with 0.5 cm^3 10 N H_2SO_4 until white fumes ceased to be given off. 4 drops of 30% hydrogen peroxide were added and heating continued for 2 hours. 4.6 cm^3 0.22% ammonium molybdate and 0.2 cm^3 FSR reagent were added and mixed thoroughly.

FSR Reagent was prepared by taking 0.05 g 1-amino-2-

napthol-4-sulphonic acid, 3.0 g sodium bisulphite and 0.1 g sodium sulphite (anhydrous) and making up to 20 cm^3 with water.

The tubes were left to stand 30 minutes and read at 830 nm with a Hitachi 101 Spectrophotometer.

2.9 Determination of the Flotation Coefficient

Freshly prepared LDL in salt solution of density 1.063 g/cm^3 was analysed in a Beckman Model E Analytical ultracentrifuge equipped with a Schlieren optical system. The LDL was placed in a single sector cell and run in an An-D rotor at 52,640 rpm. The temperature was maintained at 20°C. The Schlieren pattern was photographed at speed and subsequently at 16 minute intervals on Kodak plates. The bar angle was set at 80°.

The plates were developed and the distances of peak migration measured by projecting the image through an enlarger. S_f values were calculated according to Schachman (1957).

2.10 Gel Filtration

2.10.1 Separation of Apoproteins

Gel filtration was carried out on a Sephadex G-100 (40 - 120 μ bead size) column (0.9 x 100 cm) equilibrated with

0.2 M Tris-HCl in 8 M urea, pH 8.4 and fitted with a water jacket maintained at 25°C. The soluble apoproteins were applied in 2 cm^3 equilibrating buffer and eluted with the same buffer at 4 cm^3/hour. 2 cm^3 fractions were collected.

Elution volumes were measured volumetrically.

2.10.2 Calibration of Gel Filtration Column

Proteins of known molecular weight were applied to the column and their elution volumes measured. Dextran blue was used to determine the void volume and cupric acetate to determine the totally included volume.

2.11 Determination of Amino Acid Composition

The apoproteins were hydrolysed with 6 N HCl in evacuated sealed tubes at 100°C for 24 hours. The hydrolysates were evaporated to dryness on a Büchi rotary evaporator, redissolved in 2 cm^3 water and evaporated to dryness again. Ammonia was removed by adjustment of the hydrolysate to pH 13 with 1 N NaOH followed by evaporation. The hydrolysate was then taken up in 1 cm^3 water and adjusted to pH 3 with 1 N HCl. The analyses were carried out on a Beckman 120C Amino Acid Analyser which had been calibrated with a known mixture of amino acids. Norleucine was run with the acidic and neutral amino acids and AGP with the basic amino acids.

2.12 Determination of NH_2-Terminal Groups

NH_2-terminal amino acids were determined by the dansylation method of Woods and Wang (1967). The samples containing 0.2 mg protein were dialysed against 0.02 M ammonia for 4 hours to remove traces of Tris and then lyophilized. The protein was then dissolved in 0.5 cm^3 of 0.2 M $NaHCO_3$ in 8 M urea (prepared by dissolving 0.762 g urea in 1 cm^3 0.2 M $NaHCO_3$) and to it was added 0.5 cm^3 dansyl chloride in acetone (25 mg/cm^3). The reaction was heated to 37°C for 4 hours and the products dialysed against water at room temperature for 12 hours.

The dansylated protein samples were transferred to hydrolysis tubes and lyophilized, then hydrolysed with 6 N HCl *in vacuo* at 100°C for 24 hours. The tubes after opening were dried *in vacuo* over NaOH.

The hydrolysate was extracted twice with 25 µl water saturated ethyl acetate, the extracts combined and evaporated to dryness under a stream of nitrogen. The residue was taken up in 5 µl 95% ethanol and 2.5 µl was spotted on each side of a polyamide sheet (7.5 x 7.5 cm). On one side only was spotted 0.5 µl of marker solution N containing dansyl derivatives of arginine, glutamic acid, glycine, isoleucine, phenylalanine, proline and serine. The polyamide sheets were held in a stainless steel rack and developed in each of the solvents shown in Table 3. Elution times and the time allowed for drying the polyamide sheets between runs are also shown.

Table 3. Solvents for development of dansylated amino acids on polyamide sheets

SOLVENT	Time (mins)	
	Development	Drying
1. 1.5% formic acid	10	15
2. Toluene:acetic acid (9:1)	15	5
3. Ethyl acetate:methanol:acetic acid (20:1:1)	10	5
4. (1.6% acetic acid, 0.9% pyridine pH 4.4):ethanol (3:1)	7	5
5. 0.05 M sodium phosphate:ethanol (3:1)	35	5

(All ratios are volume:volume)

The polyamide sheets were washed for reuse in a solution of 1 M ammonia:acetone (1:1) and dried in a stream of warm air.

Chapter 3

RESULTS

3.1 Isolation of LDL and Preparation of Apoproteins

Washed LDL contained 2 - 3 mg protein/cm^3 salt solution. The yield of LDL from serum was 20 - 25 mg LDL protein/100 cm^3 serum.

Washed LDL of 2.5 mg protein/cm^3 contained approximately 10 mg lipid/cm^3.

Following delipidation of unsuccinylated LDL by Method I, 15% of the protein was recovered in buffer solution. Recovery increased to 95% when native LDL was succinylated prior to delipidation by Method I. Delipidation by Methods II and III resulted in a lower recovery of soluble apoproteins from both succinylated and unsuccinylated LDL. Delipidation of unsuccinylated LDL by Methods II and III yielded a similar percentage of soluble apoprotein to Method I. When succinylated LDL was delipidated by Methods II and III, 45% of the protein was recovered in solution.

Phosphorus was determined in apoLDL. Phospholipid was calculated by multiplication of the phosphorus concentration by 25. Residual phospholipid in apoLDL was less than 0.5% by weight.

3.2 Gel Filtration of Apoproteins

The void volume, V_o, determined by the elution of dextran blue, and the totally included volume, V_t, determined by the elution of cuprous acetate were 20 cm^3 and 80 cm^3 respectively. Values varied slightly between runs and to compensate for this the distribution coefficient, $\frac{V_e - V_o}{V_t - V_o}$, was calculated for each protein eluted (where V_e is the elution volume).

When delipidated LDL was subjected to gel filtration on Sephadex G-100 three proteins were eluted; L-1, L-2 and L-3 in order of elution. L-1 was slightly included, L-2 had a distribution coefficient in the range 0.3 - 0.4 and L-3 was nearly totally included. Elution patterns are shown in Figures 4 and 5.

The distribution coefficients of the molecular weight markers are shown in Table 4. The distribution coefficients of the marker proteins were plotted against the logarithm of their molecular weights to give a linear relationship (Figure 6).

The molecular weights of peaks L-1, L-2 and L-3 as determined from the graph are 34,000, 22,000 and 12,500 daltons respectively.

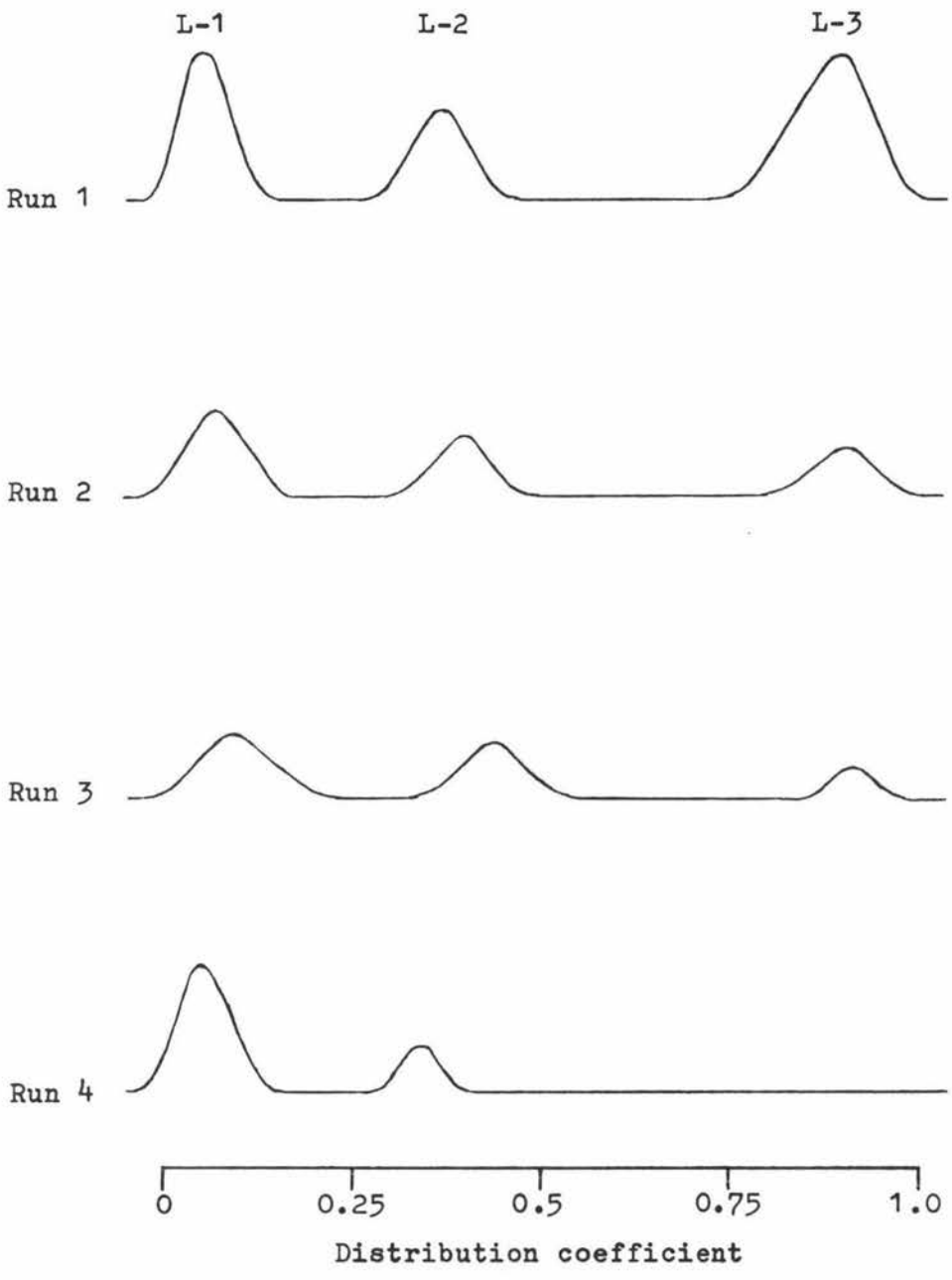


Figure 4: Chromatographic separation of LDL apoproteins prepared by Method I on Sephadex G-100 eluted with 0.2 M Tris-HCl in 8 M urea at pH 8.4.

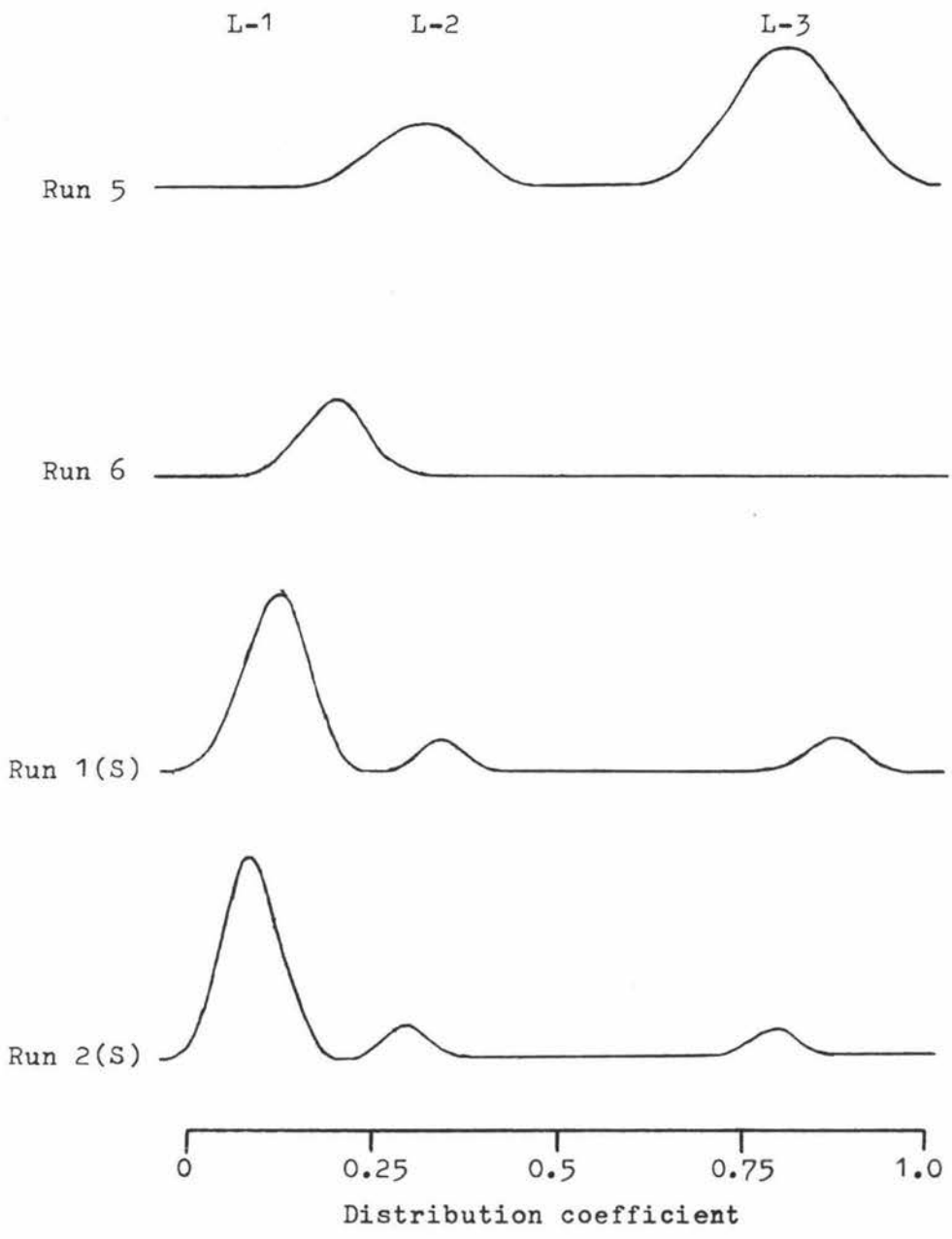


Figure 5: Chromatographic separation of LDL apoproteins prepared by Method II (Run 5), Method III (Run 6) and succinylated apoproteins prepared by Method I (Runs 1(S) and 2(S)) on Sephadex G-100 eluted with 0.2 M Tris-HCl in 8 M urea at pH 8.4.

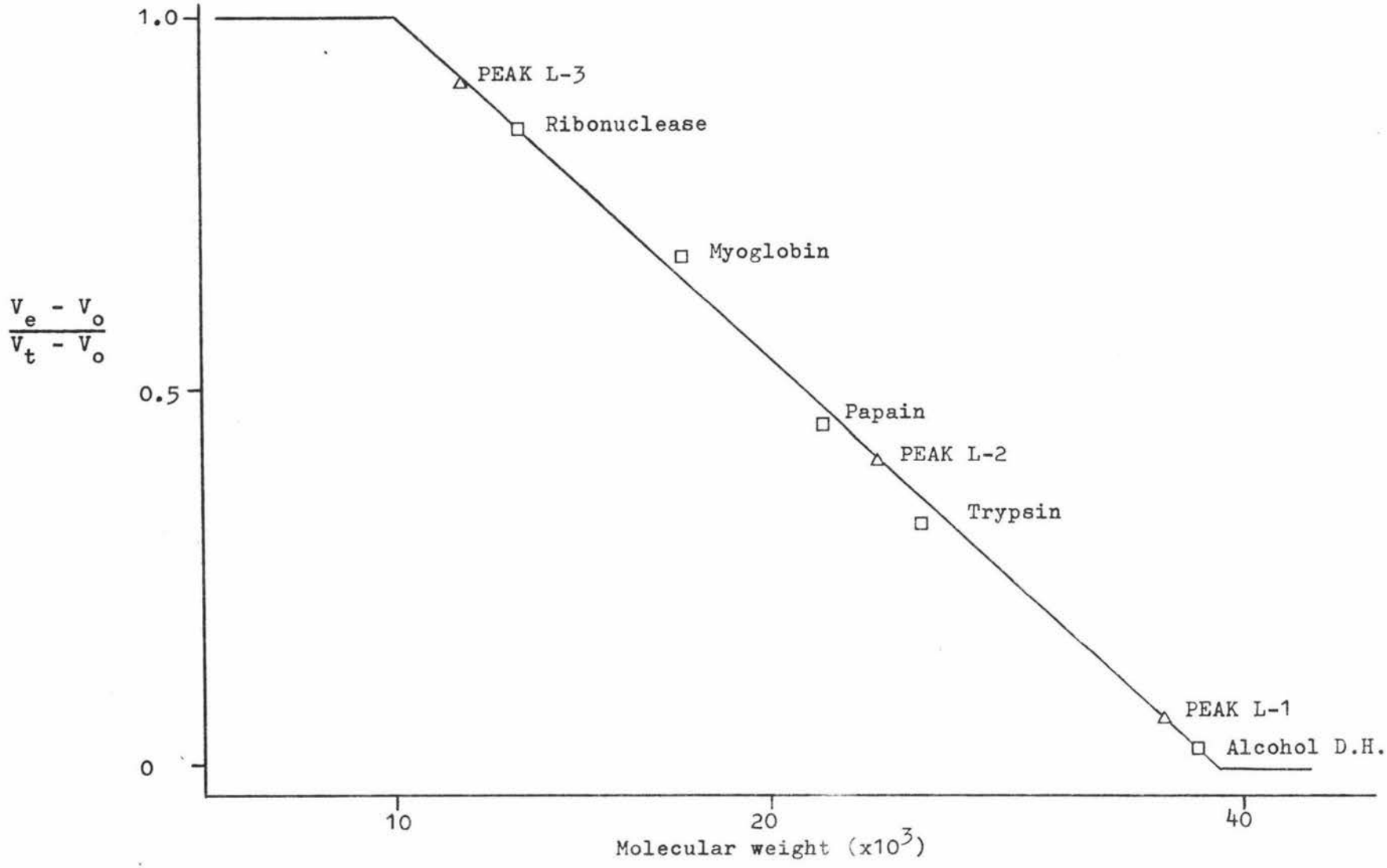


Figure 6: Relationship between distribution coefficient and molecular weight for proteins eluted from a Sephadex G-100 column (0.9 x 100 cm) with 0.2 M Tris-HCl in 8 M urea at pH 8.4.

Table 4: Molecular weights and distribution coefficients of proteins eluted from Sephadex G-100 with 0.2 M Tris-HCl in 8 M urea at pH 8.4

Protein	Molecular Weight	$\frac{V_e - V_o}{V_t - V_o}$
Ribonuclease	13,700	0.85
Myoglobin	16,500	0.68
Papain	21,000	0.45
Trypsin	23,000	0.32
Alcohol D.H. (Yeast)	37,000	0.04

3.3 Gel Electrophoresis

3.3.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis of porcine serum showed 3 bands when stained with Sudan Black, and all the characteristic protein components when stained with Amido Schwarz, Figure 7.

Isolated LDL ran as a single band in the β position as shown by both stains. No protein or lipid impurities were observed.

3.3.2 3.75% Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis patterns for porcine serum are shown in Figure 8. After staining with Sudan Black,

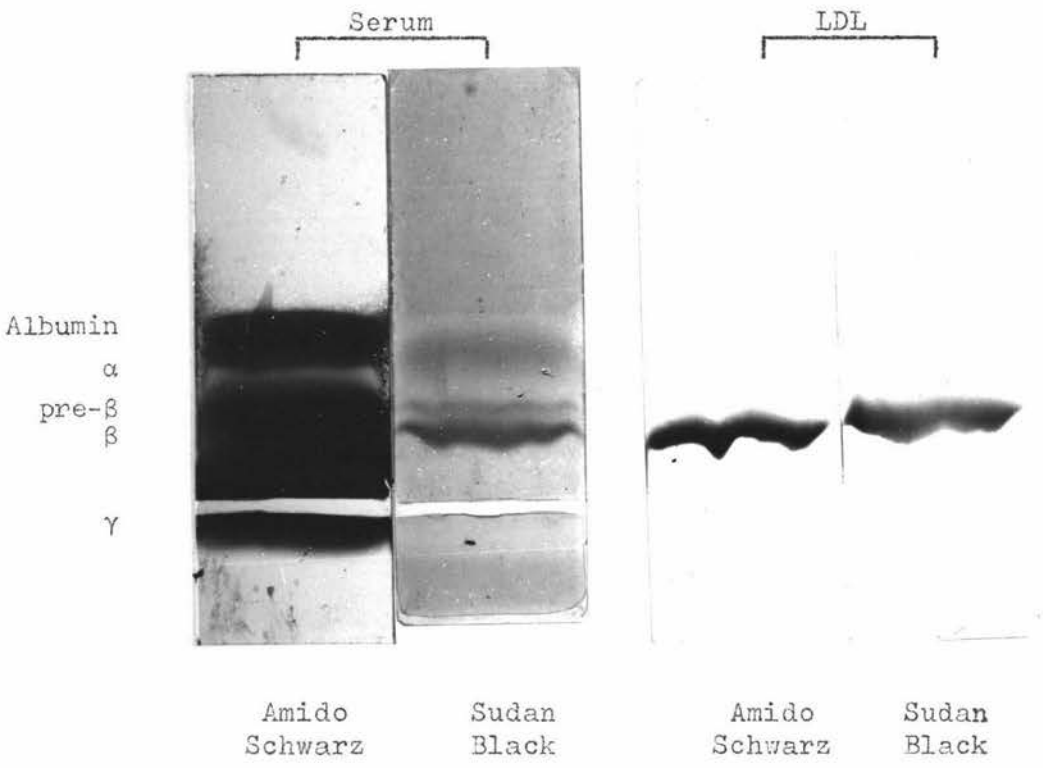


Figure 7: Agarose gel electrophoresis of porcine serum and isolated LDL. Protein was stained with Amido Schwarz and lipid was stained with Sudan Black.

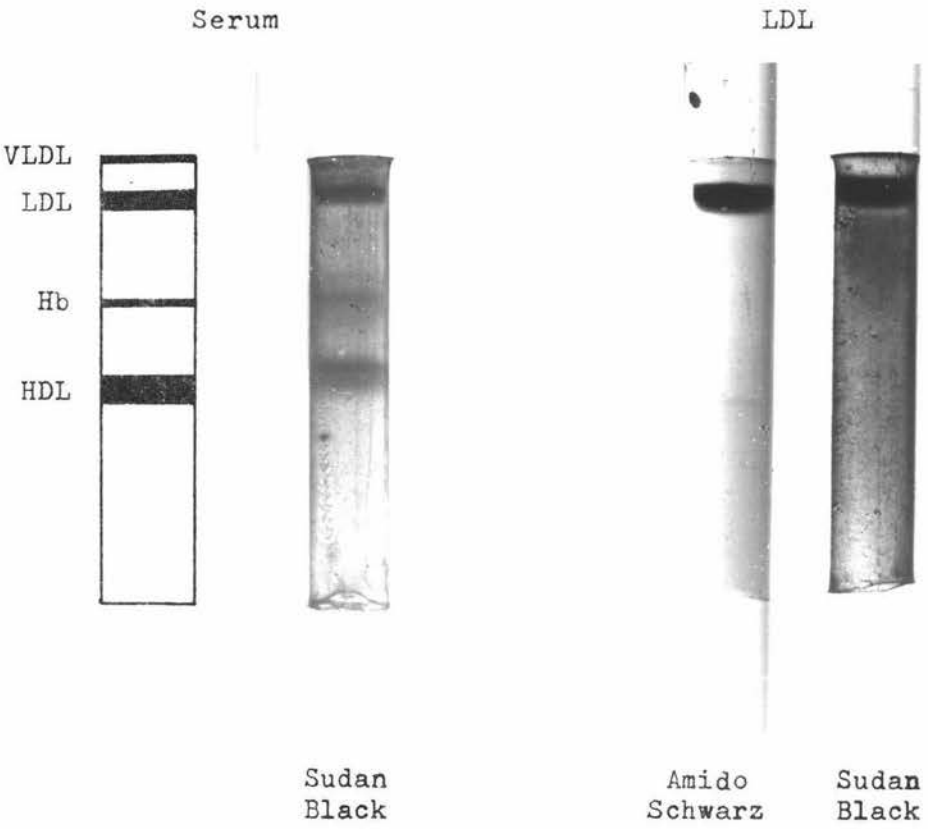


Figure 8: Polyacrylamide gel electrophoresis of porcine serum and isolated LDL in 3.75% acrylamide at pH 8.9.

3 bands of lipid were observed. The relative mobilities were found to be the same when the isolated lipoprotein classes were run separately (Table 5).

Isolated LDL moved as a single band, all the protein being associated with lipid, as shown by Amido Schwarz and Sudan Black stains.

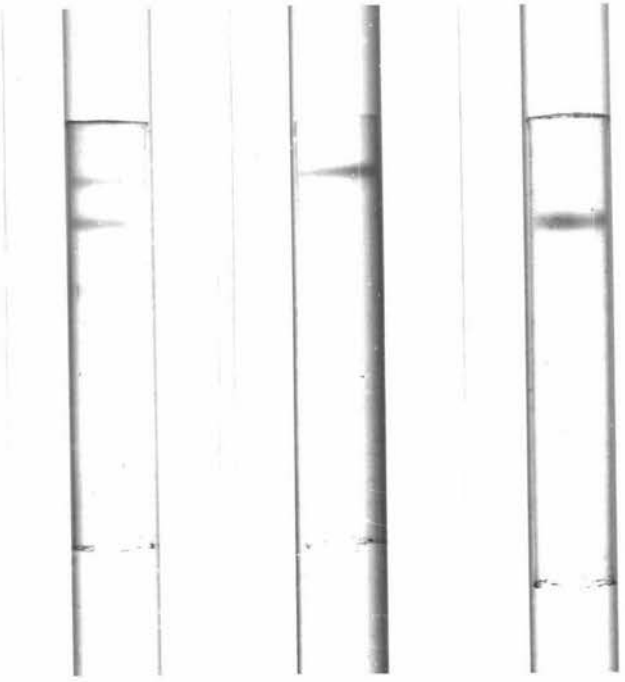
Table 5: Relative mobilities of porcine serum lipoproteins and isolated LDL in 3.75% acrylamide at pH 8.9

Component	R_m
VLDL	0
LDL	0.09
HDL	0.55
Hb	0.37

3.3.3 7.5% Polyacrylamide Gel Electrophoresis in 8 M Urea

LDL delipidated by Method I gave 3 bands on 7.5% polyacrylamide when stained with Amido Schwarz (Figure 9). The 3 bands corresponded in R_m to peaks L-1, L-2 and L-3 obtained by gel filtration of the apoproteins. Peak L-3, after separation from the other apoproteins by gel filtration did not fix on the gel.

Electrophoresis of succinylated LDL delipidated by



apoLDL L-1 L-2

Peak	R_m
L-1	0.16
L-2	0.27
L-3	0.45

Figure 9: Polyacrylamide gel electrophoretic patterns of LDL apoproteins and their relative mobilities. Apoproteins were run in 7.5% acrylamide at pH 8.9. Proteins were stained with Amido Schwarz.

Methods I and II gave variable results. Up to 10 bands with relative mobilities in the range 0 - 0.9 were fixed.

3.3.4 10% Polyacrylamide Gel Electrophoresis in 0.1% SDS

The relative mobilities of the marker proteins are shown in Table 6. The R_m of each protein was plotted against the logarithm of its molecular weight (Figure 10).

The molecular weights of the delipidated apoproteins, as determined from Figure 10 are given in Table 7.

Table 6: Relative mobilities of proteins run in 10% acrylamide with 0.1% SDS.

Protein	M.W.	R_m
Ovalbumin	45,000	0.26
Alcohol D.H.	37,000	0.34
Chymotrypsinogen	25,000	0.49
Chymotrypsin	21,600	0.50
Papain	21,000	0.52
Myoglobin	17,000	0.62
Ribonuclease	13,700	0.67
Lysozyme	13,640	0.70
Cytochrome C	12,400	0.74

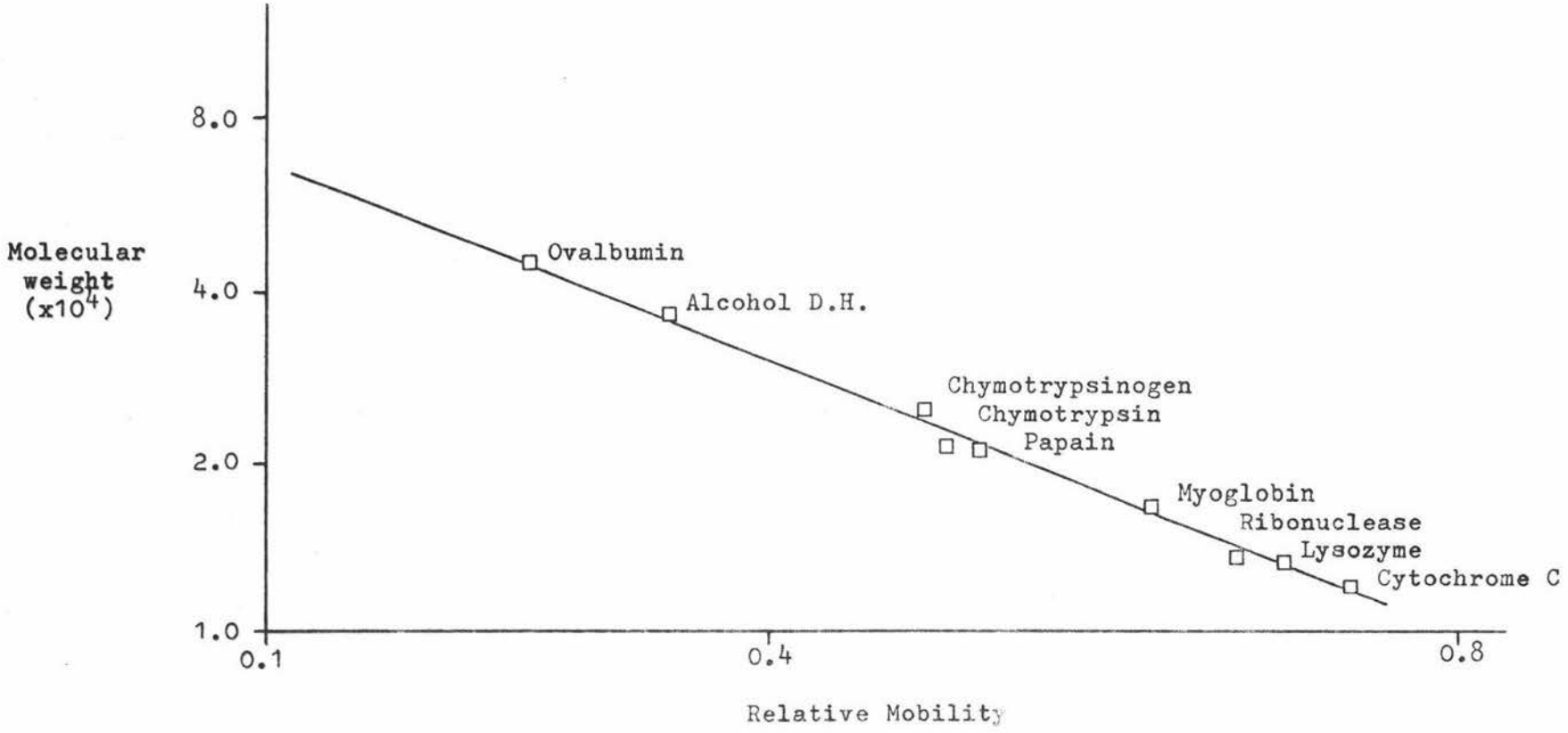


Figure 10: Relationship between relative mobility of proteins and their molecular weights. Proteins were run in 10% acrylamide and 0.1% SDS.

Table 7: Molecular weights of LDL apoproteins determined by comparison of R_m with proteins of known molecular weights run on 10% acrylamide and 0.1% SDS. Apoproteins were prepared by Method I. Runs 1-3, unsuccinylated LDL; Run 4, succinylated LDL.

Run	Molecular Weights			
1	11,500	22,000		
2	11,000	20,000		56,000
3	13,000	25,000		61,000
4	12,000		30,000	

3.4 Determination of Flotation Coefficient

The Schlieren pattern showed the presence of two components; a main peak, and a shoulder on the trailing edge.

The relationship between the logarithm of the distance moved by each peak (log x) and the time taken to move is shown in Figure 11. The flotation coefficients were calculated from the equation:

$$S_f = \frac{1}{\omega^2 x} \cdot \frac{dx}{dt}$$

where x is the distance of the particle from the rotational axis, t is the time in seconds and ω is the angular velocity in radians/second. The flotation coefficient for the peak was found to be 3.4 and for the shoulder 2.3.

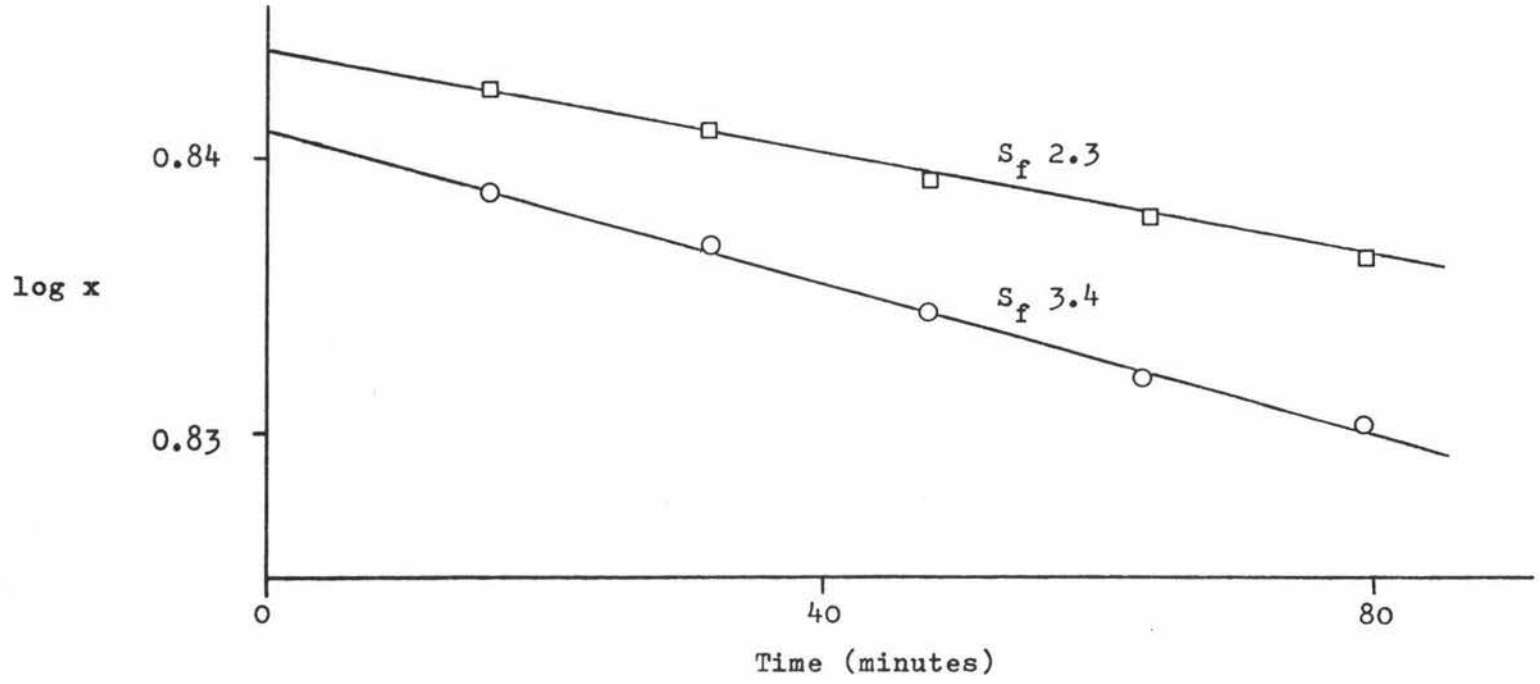


Figure 11: Flotation rate of LDL at 52,640 rpm, 20°C, in a salt solution of density 1.063 g/cm^3.

3.5 Amino Acid Composition

The amino acid composition of apoLDL is shown in Table 8.

Table 8: Amino acid composition (moles/10^3 moles of amino acids) of proteins from porcine serum LDL. Values are for 24 hr hydrolysis. ND, not determined.

	L-1				L-2		L-3	
	Run 1(S)		Run 2(S)		Run 1(S)	Run 2(S)	Run 1(S)	Run 2(S)
Lys	69.3	71.8	70.6	70.2	79.4	72.0	96.8	107.8
His	24.2	25.8	23.6	24.4	6.2	23.2	ND	ND
Arg	35.0	30.5	35.8	34.9	3.1	11.0	ND	ND
Asp	109.2	110.5	106.6	107.1	113.7	96.9	70.8	76.5
Thr	60.9	61.4	62.1	63.2	76.3	62.7	61.4	52.7
Ser	65.2	66.7	71.2	69.4	111.4	122.1	233.8	182.1
Glu	132.2	133.3	127.7	128.8	128.5	123.4	56.7	100.7
Pro	46.8	40.2	41.9	42.6	63.1	35.5	89.7	55.2
Gly	58.6	59.1	53.1	52.9	102.0	134.0	ND	ND
Ala	49.7	50.9	59.5	60.2	83.3	95.6	96.8	86.3
Cys	8.1	3.2	1.6	1.9	15.6	5.0	ND	ND
Val	62.1	62.7	61.4	61.1	50.6	49.5	33.0	45.5
Met	6.2	7.3	13.5	13.9	1.10	5.3	ND	ND
Ile	60.1	81.5	59.2	58.8	24.1	31.2	25.9	28.8
Leu	128.2	128.4	125.9	125.3	64.6	72.3	40.1	69.5
Tyr	36.8	37.9	38.0	37.6	35.5	26.5	ND	ND
Phe	47.3	49.6	48.2	47.7	43.6	38.8	ND	ND

3.6 NH_2 - terminal Amino Acids

A summary of the dansylated amino acids observed is given in Table 9.

Table 9: NH_2-terminal amino acids of LDL apoproteins determined by dansylation. Bracketed amino acids present in trace amounts.

RUN 1(S)	Peak	L-1	ala	gly	(ser)	
		L-2	ala	gly		
		L-3	ala	gly		
RUN 2(S)	Peak	L-1	ala	gly	(ser)	
		L-2	ala	gly		
		L-3	ala	gly		
RUN 3	Peak	L-1	ala	gly		
		L-2	ala	gly	(ser)	(glu)
		L-3	ala	gly	(ser)	(glu)
RUN 4	Peak	L-1	ala	gly		
		L-2	ala	gly		
RUN 5	Peak	L-2	ala	gly		(glu)
		L-3	ala	gly	(ser)	(leu)

Chapter 4

DISCUSSION

4.1 Protein Recovery

The LDL protein obtained from pig serum in the present study was considerably less than the value of 50 mg/100 cm^3 serum found by Janado et al. (1966). However the 20 - 25 mg protein collected from 100 cm^3 serum did not represent a quantitative recovery, as only the upper portions of the floating LDL layers were collected after centrifugation to reduce the possibility of contamination by other serum proteins.

The single determination of lipid content of LDL was in agreement with the value of 80% reported in pigs by Janado et al. (1966) and by Fidge (1973). It is also similar to the proportion of lipid found in human LDL.

The recovery of apoproteins soluble in 0.2 M Tris-HCl in 8 M urea at pH 8.4 was improved significantly by succinylation of native LDL prior to delipidation. Recovery of apoproteins from succinylated LDL varied with delipidation technique and was highest (95%) when Method I was used. The 95% recovery was achieved when the aqueous phase was maintained at its original volume throughout the delipidation process.

ApoLDL precipitated from solution during delipidation could only be redissolved in buffer to a very limited extent.

The delipidation procedures involving lyophilisation (Methods II and III) yielded lower recoveries of soluble apoproteins than Method I. This was due to the difficulty in redissolving the apoproteins solidified by lyophilisation.

Porcine apoLDL appears to be somewhat less soluble than rat apoLDL. Koga et al. (1971) reported that rat serum apoLDL prepared by solvent extraction of LDL at -10°C in the presence of SDS was completely soluble in 0.01 M Tris-HCl in 8 M urea at pH 8.6.

4.2 Homogeneity of LDL

Electrophoresis of LDL on 3.75% acrylamide gels in the absence of urea showed LDL to be a single band which moved only a small way into the gel. This was also found by Fidge (1973).

Analytical ultracentrifugation showed LDL to be composed of two subunits. This was also found by Fidge who carried out sedimentation runs with porcine serum LDL in a solvent of density 1.006 g/cm^3. Although he considered LDL to be essentially homogeneous, a minor, faster sedimenting component was observed.

4.3 Gel Filtration

Gel filtration of both succinylated and unsuccinylated LDL delipidated by Method I gave 3 peaks with similar distribution coefficients. The peaks of unsuccinylated apoLDL were of comparable size but succinylated apoLDL had significantly more protein eluted in peak L-1. This suggests that the protein solubilised on succinylation was all or mostly the peak L-1 protein.

The variation in elution patterns between delipidation techniques suggests that different apoproteins are rendered more or less soluble by the different treatments.

Delipidation by Method II gave an elution pattern lacking peak L-1. The insolubility of this apoprotein is due to a change in its solubility properties, possibly brought about by lyophilisation. Delipidation by Method III produced apoLDL with an elution pattern lacking both peaks L-1 and L-3. Only the protein of peak L-2 remained soluble after this treatment.

Variations in the elution patterns of the apoproteins could be in part due to seasonal variation in LDL. The delipidation experiments were carried out on serum samples collected throughout a 12 month period. Differing demands on the lipid transport system, due to the marked difference in summer and winter fattening diets for pigs, may produce seasonal variations in the apoproteins of LDL. Further investigation would be required to determine if this is a significant factor.

Completeness of delipidation would also have some bearing on the elution patterns. The presence of even small amounts of

lipid could be expected to have an influence on the elution patterns of the apoproteins. Phosphorus determinations showed that less than 0.5% by weight of phospholipid was present after delipidation by Method I, insufficient to effect a measurable change in elution patterns. Phospholipid determinations were not carried out on apoLDL prepared by Methods II and III and so there exists the possibility that the elution patterns of apoproteins prepared by these techniques may have been influenced by the presence of residual phospholipid.

Fidge (1973) found the elution pattern for porcine serum apoLDL on Sephadex G-200 to be a slightly included single peak. It is difficult to relate this result to the elution patterns found in the present study. In both experiments the elution buffers were in 8 M urea and it is unlikely that the differences in elution patterns could be attributed to the slight difference in Tris concentration between the buffers.

Koga et al. (1971), using rat serum, prepared apoLDL by a solvent extraction technique. The elution pattern of the apoproteins from Sephadex G-200 with 0.01 M Tris-HCl in 8 M urea was similar to that in the present study with three peaks being eluted.

The single void volume peak found by Bersot et al. (1970) on gel filtration of rat serum apoLDL, and by Fidge (1973) with pig serum apoLDL could be due to the binding by the apoprotein of abnormally large amounts of the SDS present in the elution buffer used in both studies. SDS binding measurements were not reported by the authors. SDS was not a component of the elution buffer

used in the present study or of that used by Koga et al. (1971), where gel filtration resolved apoLDL into more than one peak.

4.4 Molecular weight determination

The presence of 8 M urea in the elution buffer reduced the fractionation range of Sephadex G-100 from 4,000 - 150,000 daltons to approximately 10,000 - 40,000 daltons. This is a result of the action of urea in reducing the size of the dextran beads and extending the conformation of the proteins. The molecular weights of peaks L-1 and L-3 are close to the limits of resolution of Sephadex G-100 under these conditions and further studies using other grades of Sephadex are required to confirm the molecular weight as determined by gel filtration.

The molecular weight determinations by SDS-polyacrylamide gel electrophoresis were in agreement with the three results from gel filtration. However succinylated LDL gave some bands of higher molecular weight on electrophoresis. These higher values could have been due to aggregates of the lower molecular weight species but are more likely to be due to the binding of larger amounts of SDS.

Molecular weights for the major component of human LDL have been reported by Pollard et al. (1969) as 25,000 - 27,000 daltons and by Scanu et al. (1968) as 36,000 - 38,000 daltons. The higher molecular species found in the SDS-polyacrylamide gel experiments were closer to the molecular weight of 64,000 daltons

reported for human apoLDL by Shore & Shore (1967). Day & Levy (1968) reported an even higher value of 80,000 daltons, determined by gel filtration on Sephadex G-200.

Smith et al. (1972) claim human LDL apoprotein to be homogeneous and in the order of molecular weight 255,000 daltons as determined by SDS-polyacrylamide gel electrophoresis, gel filtration and equilibrium ultracentrifugation. The values obtained from electrophoresis and gel filtration were derived from extrapolation of the behaviour of much lower molecular weight standard marker proteins. From the molecular weight of whole LDL and the percentage composition of protein, Smith et al. calculated that each LDL particle comprises 2 of the 255,000 dalton protein chains.

4.5 The Apoproteins

Amino acid analyses showed significant differences in composition between L-1, L-2 and L-3. The results of NH_2-terminal group determinations suggest that all three consist of two chains with glycine and alanine NH_2-terminal groups.

The amino acid composition of L-1 is in close agreement with that published by Fidge (1973) representing all the apoproteins present in pig serum in the density range 1.019 - 1.063 g/cm^3. L-1 is also very similar in amino acid composition to the apoproteins of human LDL (Granda & Scanu, 1966; Margolis & Langdon, 1966a; Lee & Alaupovic, 1970; Smith et al., 1972), and to the apoproteins of rat LDL (Koga et al., 1971). Rat serum

apoLDL was resolved to three peaks, PI, PII and PIII by gel filtration in studies by Koga et al. (1971). PI, considered to be the counterpart of R-ser or B protein in human LDL, was found to be the predominant component with very close similarity in amino acid composition to L-1 of the present study. Of the other two peaks, PII and PIII, only PII had been analysed, showing an amino acid composition with some similarity to L-2 and L-3, the resemblance not being as close as PI to L-1.

Amino acid analyses of LDL from all species are characterised by higher levels of aspartic acid, glutamic acid, serine and to a lesser extent leucine. These trends are not restricted to LDL but are found to some extent in other lipoprotein classes and are a characteristic of most serum proteins.

Another feature of the amino acid composition is the ratio of hydrophilic to hydrophobic amino acids which is approximately unity. This is not unexpected if equal amounts of polar residues are present on the surface, to interact with the aqueous environment, and non polar residues are present in the core of the particle, to interact with the non polar esterified lipid.

The NH_2-terminal residue determinations were not in close agreement with the results of Fidge (1973) who reports the presence of three major (aspartic acid, glutamic acid and alanine) and two minor NH_2-terminal residues (glycine and leucine). However, four of the five residues are common although discrepancies exist as to their relative proportions. The finding of more than one NH_2-terminal amino acid appears to conflict with other of Fidge's results indicating homogeneity of the LDL apoprotein.

Lee & Alaupovic (1970) divided human LDL into several subfractions according to density and found the amino acid compositions to vary slightly. The variation was considered to be due to the presence of two unequally distributed apoproteins in LDL. If this were the case with porcine LDL the presence of two major NH_2-terminal amino acids would lend support to this hypothesis.

4.6 Conclusion

Studies on the apoproteins of LDL have been severely hampered by the insolubility of the protein moiety. Inconsistencies between workers arising from differing techniques of delipidation and solubilization of the protein have made results difficult to interpret. The development of a standard technique providing complete delipidation resulting in a soluble apoprotein will be a major step toward a complete understanding of the nature of LDL.

Comprehension of the nature of LDL will give a greater insight into its function and its metabolic relationship with other classes of lipoproteins.

SUMMARY

1. LDL (density 1.006 - 1.063 g/cm^3) was isolated from porcine serum by ultracentrifugation.

2. LDL moved as a single band on 3.75% acrylamide gels but analytical ultracentrifugation showed it to have two components, a major one of S_f 3.4 and a minor one of S_f 2.3.

3. Lipid free apoproteins, prepared by solvent extraction of aqueous LDL, were resolved into 3 components (L-1, L-2, L-3) by gel filtration on Sephadex G-100 eluted with 0.2 M Tris-HCl in 8 M urea at pH 8.4.

4. The molecular weights of L-1, L-2 and L-3 as determined by gel filtration and electrophoresis on 10% acrylamide with 0.1% SDS were 34,000, 22,000 and 12,500 respectively.

5. The amino acid compositions of L-1, L-2 and L-3 were not the same. The composition of L-1 was very similar to the composition of human and rat apoLDL.

6. The major NH_2-terminal amino acids of L-1, L-2 and L-3 were alanine and glycine, suggesting that each apoprotein comprised two chains.

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