THE METABOLISM OF VERY LOW DENSITY LIPOPROTEINS IN RATS

by

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Submitted in satisfaction of the requirements for a Doctorate of Philosophy Degree in the Department of Clinical Science, John Curtin School of Medical Research, within the Australian National University.

July, 1975
STATEMENT

The work contained herein is the result of my own investigation with the exception of the determination of the fate of human VLDL in rat presented in Chapter 4 which was performed by Dr N.H. Fidge. Work on the labelling of VLDL by \textit{in vitro} incubation with $^{125}\text{I}$-labelled HDL in the same chapter was performed in collaboration with Dr N.H. Fidge.

Signed Parissa Poulis
(Kyparissoula J. Poulis)
ACKNOWLEDGEMENTS

I am indebted to my supervisor Dr Noel H. Fidge for continuing guidance, encouragement and assistance throughout this work and also to Dr Paul J. Nestel for his constructive criticism.

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6.4 Discussion

6.5 Summary

CHAPTER 7 GENERAL DISCUSSION

BIBLIOGRAPHY
The main objective of the work described in this thesis has been to contribute to the understanding of very low density apolipoprotein metabolism. From the physiological viewpoint, VLDL is now considered to be the major vehicle of endogenous triglyceride transport in the plasma and in such a capacity makes a large contribution to the maintenance of the body's energy supply. When the work described in this dissertation was commenced, little was known about the function of the protein component of VLDL, although there was evidence suggesting some degree of structural and possibly metabolic heterogeneity in this moiety.

In an attempt to investigate the biological mechanisms associated with the peptide components of VLDL a model system was required for study. Since the rat has been used widely to help our understanding of lipid transport systems, it was chosen for this work. The general approach was then to investigate the fate of VLDL labelled in the protein moiety with $^{125}$I both in vitro and in vivo.

A comparison of the radioiodination properties of human, rat and pig VLDL under similar conditions revealed differences in labelling patterns. More $^{125}$I was incorporated into the lipid moieties of rat and pig VLDL than into human VLDL. The catabolism of human and rat VLDL (containing varying amounts of iodine) by the rat was similar and was characterised by a fast and slow component ($T_{1/2}$ approximately 1 hr and 11 hr). Feeding rats a saturated fat diet for up to 10 days was sufficient to increase the amount of label incorporated into VLDL apolipoprotein without significantly affecting the apolipoprotein composition, or the in vivo behaviour of the iodinated VLDL apolipoprotein. Changes in the fatty acid composition of both serum and VLDL phospholipids, triglycerides and cholesterol esters was observed. Most $^{125}$I bound to
lipid was incorporated into phospholipid.

During the first 30 min after intravenous injection of rat and human \(^{125}\text{I-VLDL}\) into rats, there was a rapid transfer of radioactive peptides to other lipoprotein fractions, after which label disappeared slowly from all lipoproteins. Apolipoproteins were described according to their migration on polyacrylamide gels. Most of the radioactivity transferred from rat VLDL to LDL and HDL was associated with zone IV. This observation was confirmed in studies using VLDL predominantly labelled in these peptides by \textit{in vitro} exchange with \(^{125}\text{I-HDL}\). Radioactivity initially associated with zones I and III was also transferred. During the first 5 min most of the apo-VLDL zone IV radioactivity was recovered in HDL and LDL whereas only a small proportion of the label lost from zone I was recovered in other lipoproteins, the remainder being presumably cleared from the circulation. Transfer of radioactivity from \(^{125}\text{I-VLDL}\) to other lipoproteins occurred after \textit{in vitro} incubation with post-heparin plasma (all zones) and control serum (zones III and IV).

Two methods (PAGE and aqueous solubility) were used for the separation of the B apolipoprotein (zone I) from other apolipoproteins of VLDL, LDL\(_1\) and LDL\(_2\) after which the specific activity of the B apolipoprotein, aqueous soluble proteins and zone IV of HDL was determined. It was shown that the turnover of VLDL B apolipoprotein is rapid and that most LDL\(_1\) and a significant proportion of LDL\(_2\) B apolipoprotein is derived from VLDL. Aqueous soluble proteins are transferred to LDL\(_1\) and LDL\(_2\) from VLDL and there is heterogeneity in the transfer of zone IV apolipoproteins to LDL\(_2\) and HDL.

A partial characterisation of rat apo-HDL and apo-VLDL apolipoproteins was carried out using gel filtration, ion exchange chromatography,
polyacrylamide gel electrophoresis and amino acid analysis.

Both apo-HDL and apo-VLDL contain soluble apolipoproteins which have similar amino acid compositions to the human C-III peptide. Apo-VLDL contains arginine-rich peptides, of which two have a similar amino acid composition to the human arginine-rich peptide.

The metabolic significance of the heterogeneity of VLDL apolipoprotein metabolism, the relationship of VLDL to LDL in the rat and the use of the rat for further studies is discussed.
PUBLICATIONS

'Studies on the Radioiodination of VLDL Obtained from Different Mammalian Species'.

'Studies on the Metabolism of Rat Serum Very Low Density Apolipoprotein'.

COMMUNICATIONS

'Iodination of Very Low Density Lipoproteins from Different Mammalian Species and Their Validity for Physiological Studies'.

'The Metabolism of Rat VLDL Apolipoprotein'.

'Some Functional Aspects of Plasma VLDL Catabolism in the Rat'.
### ABBREVIATIONS

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>VLDL</td>
<td>Very low density Lipoprotein (density &lt; 1.006 g/ml)</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density Lipoprotein (density 1.006-1.063 g/ml)</td>
</tr>
<tr>
<td>LDL₁</td>
<td>Low density Lipoprotein (density 1.006-1.019 g/ml)</td>
</tr>
<tr>
<td>LDL₂</td>
<td>Low density Lipoprotein (density 1.019-1.063 g/ml)</td>
</tr>
<tr>
<td>HDL</td>
<td>High density Lipoprotein (density 1.063-1.21 g/ml)</td>
</tr>
<tr>
<td>d</td>
<td>density (g/ml)</td>
</tr>
<tr>
<td>GLC</td>
<td>gas-liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>i.d.</td>
<td>internal diameter</td>
</tr>
<tr>
<td>I/P</td>
<td>moles of iodine bound/mole of protein</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical Density</td>
</tr>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>SA</td>
<td>specific activity</td>
</tr>
<tr>
<td>Sf</td>
<td>Rate of flotation in Svedberg units relative to d 1.063 g/ml.</td>
</tr>
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All other abbreviations are listed in 'Policy of the Journal and Instructions to Authors', of the Biochemical Journal (1973).
CHAPTER 1

GENERAL INTRODUCTION

Triglycerides and free fatty acids constitute the largest store of metabolic energy available to the body. Sufficient triglyceride is stored in human adipose tissue to provide energy for forty days of work of all types of metabolism (Dole, 1962). Maintenance of caloric homeostasis is dependent on the flux of free fatty acids in the circulation (Dole and Harper, 1949). When energy is derived from the diet, mainly in the form of carbohydrates, triglycerides and free fatty acids are depleted. Free fatty acids are released from adipose tissue triglycerides and circulate in the blood attached to albumin, to some extent as energy requirement where they are oxidized. In this physiological state free fatty acids supply 50-60% of the body's total energy needs (Dole, 1949). The release of free fatty acids from adipose tissue is dependent on hormonal regulation. Free fatty acids not utilized by the tissues and the triglycerides are taken to adipose tissue for storage (Dole, 1962). Although the greatest amount of the triglyceride is in the form of free fatty acids, their contribution to total fat contribution is relative, in any one time is unimportant. Insulin makes triglycerides less efflux and cholesterol are the major contributors.

During periods of exercising with a high carbohydrate diet triglycerides are released from hepatic and extrahepatic adipose tissue and primarily tissue. Storage of triglycerides is also beneficial. Method of storing energy as a weight per oxygen stoichiometry (Dole, 1962). Also the muscle utilizes water for storage of energy (Kleiber, 1991). Energy for stored energy is about 10% higher. Mechanisms for continually synthesized and degraded triglycerides and glycogen.
CHAPTER 1

GENERAL INTRODUCTION

Triglyceride and free fatty acids constitute the largest source of metabolic energy available in the body. Sufficient triglyceride is stored in human adipose tissue to provide energy for forty days or more of active metabolism (Dole, 1965). Maintenance of caloric homeostasis is dependent on the flux of free fatty acids in the circulation (Wertheimer and Shapiro, 1948). When energy derived from the diet, mainly in the form of carbohydrate, triglyceride and free fatty acids is depleted, free fatty acids are released from adipose tissue triglyceride and circulate in the blood attached to albumin to sites of energy requirement where they are oxidised. In this postabsorptive state free fatty acids supply 50-90% of the body's total energy needs (Steinberg, 1963). The release of free fatty acids from adipose tissue is under complex hormonal control. Free fatty acids not utilised by the liver are reesterified and the triglycerides are taken to adipose tissue for storage (Nikilla, 1969). Although the greatest amount of fat transport in the plasma is in the form of free fatty acids, their contribution to total fat concentration in plasma at any one time is relatively insignificant as they have a high turnover rate. Triglyceride, phospholipid and cholesterol are the major contributors.

During periods of overfeeding with a high carbohydrate diet triglycerides are formed by lipogenesis from glucose in liver and adipose tissue of man and other animals. Storage of triglyceride is the most efficient method of storing energy on a weight per energy stored basis (Dole, 1965). Also fat does not require water for storage as it is water insoluble. Hence fat stores contain almost 100% lipid. This fat is continually synthesised and degraded (Schoenheimer and Rittenberg, 1935).
Dietary fat is hydrolysed to monoglycerides in the intestinal lumen and taken up with other lipids and lipid soluble substances by the mucosal cells where they are reesterified and released into the lymphatics in molecules called chylomicrons. These find their way into the bloodstream via the thoracic duct. Triglyceride contained in chylomicrons is hydrolysed by an enzyme called lipoprotein lipase (or clearing factor lipase) before uptake of the fatty acids by adipose tissue, muscle and various other tissues (Robinson, 1970). The dietary carbohydrate and some chylomicron triglyceride that is taken up by the liver (Robinson, 1970) is utilised as an immediate source of energy, essential fatty acids and vitamins. When energy stores in the liver have been filled, excess free fatty acid is reesterified and the triglyceride is transported as VLDL to sites requiring energy and to adipose tissue for storage (Nikilla, 1969).

The problem of transporting a highly water insoluble hydrophobic molecule such as triglyceride and other lipids in the aqueous environment of the blood is solved by solubilising the lipids. This is achieved by combining the lipids with polar molecules such as phospholipids and proteins to form a soluble complex called a lipoprotein. Four main groups of lipoproteins are distinguished on the basis of density. These are chylomicrons, very low density, low density and high density lipoproteins which differ in their content of triglyceride, cholesterol (esterified and non-esterified), phospholipid and protein.

The major lipoprotein classes found in human serum, their composition and some physical properties are shown in Table 1.1. In general rat lipoproteins are classified according to the same criteria as those applied to human lipoproteins. Some differences regarding the delineation of their densities are discussed later.
## Table 1.1

Comparison of Lipoprotein Classification Systems and Composition of Human Serum Lipoproteins

<table>
<thead>
<tr>
<th>DENSITY Class</th>
<th>Density Range g/ml</th>
<th>ELECTROPHORETIC BEHAVIOUR</th>
<th>COHN FRACTIONATION</th>
<th>FLOTATION CHARACTERISTICS ( a )</th>
<th>APPROPXIMATE MOLECULAR WEIGHTS ( x10^6 )</th>
<th>SIZE ( \text{Å} )</th>
<th>PERCENT COMPOSITION ( b )</th>
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<tr>
<td>CHYLOMICRONS</td>
<td>&lt;0.90</td>
<td>origin ( \alpha_2, \beta )</td>
<td>I + III</td>
<td>( &gt;400 )</td>
<td>( 10^3-10^4 )</td>
<td>750-10,000</td>
<td>80-95</td>
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<td>3-6</td>
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<td></td>
<td></td>
<td></td>
<td>0.5-2.5</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95 -1.006</td>
<td>pre-( \alpha )</td>
<td>I + III</td>
<td>( 20-400 )</td>
<td>( 5-100 )</td>
<td>300-800</td>
<td>50-70</td>
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<td>7-12</td>
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<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>( \beta_1 )</td>
<td>I + III</td>
<td>( 0-12 )</td>
<td>( 2.1-2.6 )</td>
<td>205-220</td>
<td>9-12</td>
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<td>21-25</td>
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<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>( \alpha_1 )</td>
<td>IV + V</td>
<td>( &lt;0 )</td>
<td>( 0.2-0.25 )</td>
<td>75-100</td>
<td>3-4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22.5-30</td>
<td>20-37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45-55</td>
<td></td>
</tr>
</tbody>
</table>

\( a \) \( \text{SF} \) designates the negative sedimentation coefficient in Svedbergs at density 1.063 g/ml NaCl solution at 26°C.

\( F_{1.21} \) is also a negative sedimentation coefficient at density 1.21 g/ml and at 26°C.

Adapted from Schumaker & Adams (1969).

\( b \) Also contain some carbohydrate (0-1%).

Data from Scanu & Kruski (1973) and Levy et al (1971).
NOMENCLATURE OF LIPOPROTEINS

Since data on lipoprotein fractions has been obtained using a variety of analytical methods, it was decided in 1956 by the Committee on Lipid and Lipoprotein Nomenclature of the American Society for the Study of Atherosclerosis (Lipid Nomenclature, 1956) that each fraction be described in terms of its method of separation. Fortunately it was possible to relate these different methods. At present lipoproteins isolated by alternative techniques are equated with lipoproteins prepared by the two most widely used methods, ultracentrifugation and electrophoresis. Any one set of terms however is not adequate to describe the different classes of lipoproteins. Table 1.1 shows the major systems of classification and their interrelationship.

Accepted abbreviations for the major density classes of lipoproteins are very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). HDL has been further divided into two subclasses HDL₂ (d 1.063-1.125 g/ml) and HDL₃ (d 1.125-1.21 g/ml). Lipoproteins sedimenting at d 1.21 g/ml therefore have a density > 1.21 g/ml and have been designated VHDL (very high density lipoprotein). In some instances, for example in comparative studies, it is expedient to include the low density lipoproteins found between d 1.006 and 1.019 g/ml as a separate fraction because they contain significant quantities of lipoprotein in many species. Two classes of LDL can therefore be described LDL₁ (d 1.006-1.019 g/ml) and LDL₂ (d 1.019-1.063 g/ml). Where these designations are not sufficiently accurate, differentiation between subclasses of lipoproteins is shown by referring to the flotation rate (Sf at d 1.063 g/ml) or the density at which the subclass was isolated. Another lipoprotein class Lp(a) (d 1.050-1.080 g/ml) has been described. The occurrence and importance of this lipoprotein has not yet been fully defined although it is thought to have pathological relevance to one of the
dyslipoproteinemias (Scanu and Ritter, 1973).

1.2 NOMENCLATURE OF APOLIPOPROTEINS

The protein moiety of the lipoprotein is referred to as the apolipoprotein or apoprotein. The apolipoprotein in essentially lipid free form is obtained by delipidation of the lipoprotein, generally by exposure to organic solvents. The delipidated lipoproteins are therefore termed apo-VLDL, apo-LDL and apo-HDL. All apoproteins with the exception of apo-LDL are soluble in aqueous buffers at or near neutral pH.

Delipidated lipoproteins can be further separated and characterised by a variety of techniques utilised by the protein chemist; for example, gel filtration and ion exchange chromatography. The extent of separation can be checked by isoelectric focusing on polyacrylamide gels. The amino acid composition and terminal amino acid residues of the separated proteins that can be reproducibly isolated can then be determined.

The possibility of classifying each lipoprotein unequivocally on the basis of the specific protein it contains has not been possible as these have not been fully characterised (chemically, physically or immunologically) and also because a number of proteins are common to different lipoprotein classes, as separated by the ultracentrifuge and by precipitation techniques.

Apolipoproteins have been described in three ways. The first is the A, B, C nomenclature used by Alaupovic (Alaupovic, 1968; 1971). Each letter referred initially to the lipoprotein density class in which they predominated and where they were first discovered. Thus apoprotein A or apoA and apoprotein B or apoB refer to the major apolipoproteins found in HDL and LDL respectively and apoC refers to the low molecular weight apolipoproteins first discovered in VLDL. VLDL contains both apoB and
apoC. ApoC is also found in HDL and in some preparations of LDL. Different polypeptides discovered within each apoprotein class (usually isolated by ion exchange chromatography) were later designated C-I, C-II, C-III etc. (Alaupovic, 1972). In using the ABC nomenclature it is assumed that each polypeptide belongs to a group of proteins (A or B or C etc.) that presumably have some functional relationship and common origin. Furthermore each group of proteins or even each polypeptide form separate lipoproteins that may have the same density and electrophoretic mobility as the other lipoproteins (Alaupovic, 1972).

Secondly, the definition of polypeptides by either their amino terminal or carboxy terminal (C-terminal) amino acid residues has been used. The C-terminal nomenclature uses the C-terminal residue as the principal name for each protein and was first used when 'apoprotein C' was found to have three or more different polypeptides (Gustafson et al., 1964, 1966; Brown et al., 1969, 1970a,b; and Shore and Shore, 1969). Thus a polypeptide may be referred to simply as 'apo-HDL-ala' and 'apo-VLDL-ala' thus denoting the lipoprotein class from which the apoprotein was obtained. This nomenclature does not presume a common origin or functional relationship as in the ABC nomenclature. Ambiguities arising from the possible occurrence of the same terminal residue in different proteins may occur. Further confusion arises when polymorphic forms of the same protein are described (apoLP-ala_1 and apoLP-ala_2). Also errors have been made in the C-terminal analysis of the apoproteins. For example, apoLP-val was later found to be apoLP-ser (Brown et al., 1969). Another deficiency is found in the definition of apoB or apo-LDL, as the C-terminal amino acid residue is uncertain and heterogeneity within this protein species has been suggested (Kane et al., 1970; Chen and Aladjem, 1974). Table 1.2 shows the distribution of polypeptides in some human serum lipoproteins using the ABC and C-terminal amino acid residue nomenclature.
Table 1.2
Distribution of Polypeptides in Human Serum Lipoproteins and
Comparison of the C-terminal and A, B, C Nomenclatures

<table>
<thead>
<tr>
<th>Apolipoprotein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>Apolipoprotein&lt;sup&gt;b&lt;/sup&gt;</th>
<th>VLDL</th>
<th>% (w/w)</th>
<th>LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoLP-Ser</td>
<td>8-10</td>
<td>+</td>
<td>2-4</td>
<td>C-I</td>
<td>C-I</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>apoLP-glu</td>
<td>8-10</td>
<td>+</td>
<td>2-4</td>
<td>C-II</td>
<td>C-II</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>apoLP-ala&lt;sub&gt;1&lt;/sub&gt;+ala&lt;sub&gt;2&lt;/sub&gt;</td>
<td>30</td>
<td>+</td>
<td>5-10</td>
<td>C-III&lt;sub&gt;1&lt;/sub&gt;, C-III&lt;sub&gt;2&lt;/sub&gt;</td>
<td>C-III&lt;sub&gt;1&lt;/sub&gt;, C-III&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>apoLDL</td>
<td>40-45</td>
<td>90+</td>
<td>0</td>
<td>B</td>
<td>58</td>
<td></td>
<td>&gt;98</td>
</tr>
<tr>
<td>'apoLP-thr'&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;1</td>
<td>+</td>
<td>65-75</td>
<td>A-II</td>
<td>A-I</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>apoLP-gln&lt;sub&gt;1&lt;/sub&gt;+gln&lt;sub&gt;2&lt;/sub&gt;</td>
<td>&lt;1</td>
<td>+</td>
<td>20-25</td>
<td>A-I, A-II</td>
<td>A-II</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A-III</td>
<td>A-III</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arginine-rich</td>
<td></td>
<td>&lt;0.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Indicates that trace amounts present.

<sup>b</sup>Data from Frederickson <i>et al</i> (1972).

<sup>c</sup>Data from Kostner <i>et al</i> (1974).

<sup>c</sup>Later found to be gln<sub>2</sub>.
A third nomenclature is that of adopting operational terms. It attempts to overcome the lack of knowledge on the nature and relationship of the various polypeptides by not assigning definitive names at this stage. The apoproteins are described according to criteria dependent on the method by which they are isolated. For example the apoproteins are described according to their order of elution from chromatography columns or their migration on polyacrylamide gels after electrophoresis. This system is illustrated in Table 1.3. Of all the systems mentioned above the ABC nomenclature is favoured because of its simplicity and will be used throughout this thesis.

1.3 DISCOVERY OF LIPOPROTEINS

A short historical review of the discovery and methods used for the isolation of lipoproteins would facilitate the understanding of the present state of lipoprotein nomenclature presented above.

Although chylomicrons had been observed as early as 1665 (Boyle, 1665) and their nature, source, size, and distribution characterised (Gulliver, 1847; Gage and Fish, 1924) it was not until this century that the other lipoproteins were discovered and characterised. It had been noted, however, that the lipids of the blood are combined with other substances and do not exist in a free state. Many early investigators (Nerking, 1901; Hardy, 1905; Haslam, 1905, 1907 and 1913; Chick, 1914; Theorell, 1962; Sørensen, 1930) studied the distribution of various lipids while carrying out studies on the precipitation of serum protein fractions. Macheboeuf (1929) was the first investigator to isolate an intact lipoprotein during precipitation studies on horse serum which was later identified as an \( \alpha_1 \) lipoprotein (HDL) (Macheboeuf and Rebeyrotte, 1949).
### Table 1.3

Examples of Nomenclature Using Operational Terms for Polypeptides of Serum Lipoprotein Apolipoproteins*

<table>
<thead>
<tr>
<th>Authors</th>
<th>Method of Polypeptide Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apo-VLDL</strong></td>
<td></td>
</tr>
<tr>
<td>Brown <em>et al</em> (1969)</td>
<td>DEAE column chromatography D1, D2, D3, D4</td>
</tr>
<tr>
<td>Albers &amp; Scanu (1971)</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>Shore &amp; Shore (1969)</td>
<td>DEAE column chromatography</td>
</tr>
<tr>
<td>Kane <em>et al</em> (1970)</td>
<td>Sephadex chromatography A, B</td>
</tr>
<tr>
<td>Shore &amp; Shore (1969)</td>
<td></td>
</tr>
<tr>
<td>Scanu <em>et al</em> (1969)</td>
<td>DEAE column chromatography D1, D2, D3</td>
</tr>
<tr>
<td>Camejo <em>et al</em> (1970)</td>
<td>Sephadex chromatography (1M Acetic acid) II, III</td>
</tr>
<tr>
<td></td>
<td>Preparative polyacrylamide gel electrophoresis (fractions not characterised)</td>
</tr>
</tbody>
</table>

*Adapted from Scanu & Kruski (1973)
1.4 ISOLATION OF SERUM LIPOPROTEINS

From 1929 to the early 1950s many new techniques were used with varying success for the isolation, quantification and analysis of serum lipoproteins. These utilised the properties due to both the lipid and protein moieties.

Various electrophoretic techniques were utilised for the study of lipoproteins most of which were semiquantitative and of limited use because only small amounts of serum could be used. For example, utilising moving boundary electrophoresis (Blix et al, 1941), zone electrophoresis on fixed media such as starch or paper (Jencks and Durrum, 1955; Langan et al, 1955; Boyd, 1954), starch block electrophoresis (Kunkel and Slater, 1952; Russ and Raymunt, 1955) lipoproteins could only be fractionated into $\alpha_1$- and $\beta_1$-lipoproteins (HDL and LDL) and quantitation was fraught with problems. Serum lipoproteins were also separated into $\alpha_1$- and $\beta_1$-lipoprotein fractions by precipitation in cold ethanol under carefully controlled conditions (Cohn et al, 1940, 1946) and purified by ultracentrifugation and chemical fractionation (Oncley et al, 1950; Oncley, 1956).

Methods based on the density of lipoproteins were also being developed at this time (McFarlane, 1935). Adair and Adair (1943) observed that a $\beta$-lipoprotein isolated by ammonium sulphate precipitation moved to the surface during centrifugation and Pederson (1947) first used the ultracentrifuge to separate a lipoprotein of density 1.030-1.040 g/ml. Gofman et al (1949), developed a method for separation of lipoproteins by ultracentrifugal flotation. This method was fully described by De Lalla and Gofman (1954), and was the most specific method used at the time. Lipoproteins were analysed by analytical ultracentrifugation after separation from serum by preparative ultracentrifugation. However, many
problems were associated with this method. Of particular note is the large discrepancy in final results performed on the same sample by different laboratories, (Cooperative Study of Lipoproteins and Atherosclerosis, 1956 the Harvard and Donner laboratories in the United States).

Lindgren et al (1951) and Hillyard et al (1955) separated serum lipoproteins in the preparative ultracentrifuge by successively raising the solvent density. Havel, Eder and Bragdon (1955), described a method similar to that of Lindgren. Ultracentrifugal flotation was carried out at density < 1.019 g/ml, 1.019-1.063 g/ml and 1.063-1.21 g/ml and it was possible to recover 98% of the total serum cholesterol and 99% of the total serum phospholipids in the isolated fractions.

Oncley and Mannick (1954) first used a density gradient to separate a number of lipoproteins in one ultracentrifugation step. Most recently, others such as Mallinson and Hinton (1973), Hinton et al (1974) have used density gradients, in this case sodium bromide, for the separation of lipoproteins for the analysis of hyperlipoproteinemic sera in cases where other methods give ambiguous results. Precipitation by antibodies, non-specific polyanions, neutral polymers and divalent cations (Cornwell and Kruger, 1961) for example, heparin and high molecular weight dextran sulphate, (Oncley, 1955; Burstein et al, 1970; Burnstein and Scholnick, 1972; Wilson et al, 1973) and chromatography (Hjerten, 1959; Carlson, 1960) have also been used for the isolation of lipoproteins. Precipitation methods using polyanions and divalent cations are used for large scale preparative work and small scale quantitative work. This method is, however, limited to the separation of only the four major lipoprotein classes.

The three major lipoproteins, very low, low and high density have also been separated using gel filtration on agarose (Kalab and Martin,
1968; Werner, 1966), agarose media being particularly useful because of their seiving properties for particles in the size range of plasma lipoproteins (Sata et al, 1970). Electrophoresis on agarose gel (Ghosh et al, 1972; Papadopoulos and Bedyn, 1973), cellulose acetate (Charman and Braeuler, 1973) and cellogel (Berends et al, 1972) are primarily used for the detection of lipoprotein abnormalities for diagnostic purposes.

Interest is currently being shown in polyacrylamide gel electrophoresis which separates many more lipoprotein species than alternative methods with the exception of ultracentrifugation. The advantages of polyacrylamide gel as a support medium include the range of pore size available, the dimensions of the gel layers and combinations of gel systems that can be employed (Feliste et al, 1973; Bautovich et al, 1973; Moran et al, 1972; Naito et al, 1973; Wada et al, 1973; Hall et al, 1972).

Of all the methods mentioned above, the preparative ultracentrifuge is one of the most widely utilised, as it is possible to quantitatively determine many subgroups (Sf classes) of lipoproteins varying only slightly in density. Immunoelectrophoretic, electrophoretic and immunodiffusion techniques are used to check the purity and to identify major protein constituents of the lipoproteins isolated by precipitation and ultracentrifugation. Gel filtration, chemical methods, such as the estimation of protein, carbohydrate and lipid analysis and optical methods (for example, ultraviolet irradiation, optical rotatory dispersion and circular dichroism) are primarily used to investigate lipoprotein structure. Nelson (1972), Lindgren et al (1972) and Scanu and Kruski (1973) have extensively reviewed the methods used for investigating lipoproteins.
1.5 STRUCTURE AND FUNCTION OF LIPOPROTEINS

That lipoproteins function as transporters of triglyceride, cholesterol and phospholipid was recognised when they were first isolated. It was later established that they also transport fat soluble vitamins, carotenoids, acyclic alcohols and traces of hydrocarbons and possibly help to maintain red cell membranes. Oncley (1956) suggested that the chemical specificity of proteins found in lipoproteins argued against their formation by simple colloidal principals. That lipoproteins represent specialised carrier molecules synthesised by metabolic reactions in more or less the molecular form observed in the purified components was also recognised. Researchers were thus already aware that the interaction between the lipid and protein of lipoproteins is not a non-specific absorption or affinity. This implies that along with specific interaction of protein and lipid components there must be some functional role as yet unknown.

Interest in the lipoproteins of humans increased particularly as various lipid abnormalities were recognised. At first it was appropriate to consider these as hyperlipaemias but as knowledge of their association with lipoproteins increased it became an advantage to classify them as hyperlipoproteinaemias and known inherited diseases as dyslipoproteineamias (described by Scanu and Ritter, 1973). Lipaemias were well recognised and documented as early as 1917 (Allen, 1917) and that defects in fat transport were possible causes by 1939 (Holt et al., 1939). The hyperlipoproteinaemias were fully described in a review by Fredrickson et al. (1967) and are shown in Table 1.4. The functional role of serum lipoproteins in lipid metabolism is exemplified by these disorders where major abnormalities in the distribution of circulating lipids are associated with a metabolic disarrangement of one lipoprotein species. Interest was also shown in lipoproteins because of their occurrence in
### Table 1.4

**Abnormal Lipoprotein Patterns in Familial Hyperlipoproteinemia**

<table>
<thead>
<tr>
<th>Type</th>
<th>Definitive lipoprotein abnormalities</th>
<th>Appearance of plasma(^a)</th>
<th>Usual changes in lipid concentrations(^b)</th>
</tr>
</thead>
</table>
| I    | 1. Chylomicrons present and markedly increased  
2. VLDL, LDL, HDL normal or decreased | Cream layer on top, clear below | C\(^+\), TG\(^+\)  
(C/TG <0.2) |
| II   | 1. LDL increased\(^c\)  
2. VLDL normal (Type IIa); or VLDL increased (Type IIb) | Usually clear, may be slightly turbid | C\(^+\), TG normal or  
(C/TG usually >1.5) |
| III  | 1. Presence of β-VLDL ('floating beta', LDL of abnormal lipid composition) | Usually turbid, often with faint cream layer | C\(^+\), TG\(^+\)  
(C/TG variable, often = 1) |
| IV   | 1. VLDL increased  
2. Chylomicrons 'absent'  
3. LDL not increased | Usually turbid; no cream layer | C\(^+\) or normal, TG\(^+\)  
(C/TG variable) |
| V    | 1. Chylomicrons present  
2. VLDL increased | Cream layer on top, turbid below | C\(^+\), TG\(^+\)  
(C/TG usually >0.15 and <0.6) |

\(^a\)After standing at 4°C for 18 hr or more.  
\(^b\)C, cholesterol; TG, triglycerides.  
\(^c\)'Increased' implies in excess of whatever cut-off limit is used.  

From Frederickson and Levy (1972)
other diseases such as diabetes mellitus, nephrosis and obstructive jaundice and particularly their possible association with atherosclerosis and heart disease.

Literature on lipoproteins has expanded rapidly since the early 1960s. A number of excellent reviews cover the major advances in structure and function of lipoproteins (Scanu, 1965; Nichols, 1967; Shapiro, 1967; Schumaker and Adams, 1969; Margolis, 1969; Levy et al, 1971; Scanu, 1971, 1972; Scanu and Wisdom, 1972; Scanu and Kruski, 1973). Despite the large body of experimental data that has been obtained by chemical, enzymatic and physical methods, the formulation of a structural model for HDL, the most extensively studied lipoprotein, is still speculative (Scanu, 1972). This is in part due to the lack of understanding of the mode of interaction of various HDL components and of the energetics involved in such interactions. A new molecular model for human HDL has been proposed by Assmann and Brewer (1972). This model is analogous to membrane models proposed by Singer and Nicholson (1972), in that protein 'icebergs' are embedded in a 'sea' of lipid. HDL is visualised as a micellar particle with essentially all polar groups oriented into the surrounding aqueous environment. Both cholesterol ester and triglyceride occupy a 'core' location and cholesterol may be located in the interfacial region. Within the 'icebergs' of protein (A-I and A-II) there appears to be specific protein:protein and lipid:protein interactions, the latter presumably being hydrophobic in nature. The experimental evidence supports the classical model of a non-polar 'core' consisting of cholesterol esters in the case of HDL and triglyceride for VLDL and LDL around which a polar region consisting of polypeptides, phospholipids and free cholesterol is organised (Schneider et al, 1973).

Many of the apoproteins are glycoproteins, containing among other sugars, hexosamine, glucosamine and sialic acid. The role of these
polysaccharides present in lipoproteins is unclear. Although thermodynamically favoured lipid:lipid interactions are important for lipoprotein structure and properties, the proteins would be the major determinants of lipoprotein substructure particularly as polypeptides vary in amino acid composition, conformation and affinity for phospholipids and other lipids.

1.6 FUNCTION OF APOLIPOPROTEINS

As the study of lipoproteins has progressed many complexities have been discovered which indicate that there is heterogeneity within each class (Oncley, 1963; Levy and Fredrickson, 1965; Zilversmit, 1969; Lee and Alaupovic, 1970). Heterogeneity in the lipoproteins is only found in the apoprotein (Table 1.2); cholesterol, phospholipid and triglyceride being common to all lipoproteins. Apoproteins from human lipoproteins have been extensively studied and reviewed (Fredrickson et al., 1972; Scanu and Ritter, 1973). Most of the apoproteins that can be reproducibly isolated have been examined for their content of total amino acids and terminal amino acid residues. Their secondary structure has been examined by optical measurements. None of the apoproteins have been obtained in crystalline form and therefore none have yielded meaningful X-ray diffraction patterns.

The heterogeneity of the apoproteins poses the question of their physiological and clinical significance. Although it is known that apoproteins bind lipids in a manner that makes them soluble in extracellular fluids at the same time permitting their transfer to and from cellular sites with a minimum expenditure of energy, how this is done is not known. Investigations of lipid-protein binding and reconstitution studies using specific apoproteins and lipids are currently being undertaken. It is becoming increasingly evident that these apoproteins not only have carrier
functions but take part in enzymatic reactions connected with the clearance of triglyceride from the plasma and the esterification of cholesterol. For example apoC-II is an activator and apoC-III an inhibitor of lipoprotein lipase (La Rosa et al, 1970; Brown and Baginsky, 1972) and of the two major HDL apoproteins (A-I and A-II), one inhibits and the other activates the enzyme lecithin:cholesterol acyltransferase. The importance of apolipoproteins in certain clinical conditions has also been established (Scanu and Ritter, 1973).

1.7 OCCURRENCE OF LIPOPROTEINS

In contrast to the human situation, relatively little is known about the lipoproteins of other species. However most animal studies have been made on lipoproteins from the rat (Koga et al, 1969; Bersot et al, 1970). As animals have begun to play an important role as models in research on atherogenesis it is important to know how closely their lipoproteins simulate those of man. For example, early studies (Lewis et al, 1952) compared the ultracentrifuge pattern of a variety of normal, hypertensive and hyperthyroid animals to determine if there was a correlation between the facility for atherogenesis and the plasma lipoprotein pattern. Mills and Taylaur (1971) studied the distribution and composition of serum from a number of mammals, amphibia, reptiles, birds and fish. These studies did not provide insights into the evolutionary origins of lipoproteins and among the mammals no animal duplicated the pattern and composition of lipoproteins found in man. However, they did fall into the same general density classes. Although there was a wide variation in patterns of distribution of lipoproteins, in general their chemical composition was remarkably similar and there was uniformity within animal classes. Later studies (Mills and Taylaur, 1973) indicate that lipoproteins were already highly developed in the early vertebrates, the principle
refinements which appeared in later vertebrates being an increase in cholesterol content. The lipoproteins of the coelocanth (Latimeria), a fish whose related order Rhipidistia is thought to have given rise to the amphibians, were found to be 'primitive'. However, cholesterol rich forms were found in an elasmobranch indicating that lipoproteins in their present form first appeared in the early amphibians or higher fish.

Among the invertebrates high and very high density lipoproteins have been found in the haemolymph of numerous insects (Chino and Gilbert, 1971; Gilbert and Chino, 1974) and are known to transport cholesterol, phospholipid, diglyceride and steroid hormones.

Few studies of apolipoproteins in species other than man have been made. Some studies on pig (Cox and Tanford, 1968; Fidge, 1973), dog (Solyom et al, 1971), cow (Jonas, 1972), monkey and rat apolipoproteins have been carried out, the most complete being those of the rat apolipoproteins. Rat apolipoproteins were found to be heterogeneous and very similar to human apolipoproteins. Polyacrylamide gel electrophoresis (Koga et al, 1969; Camejo, 1967), gel filtration and ion exchange chromatography (Bersot et al, 1970) have been used to fractionate and purify rat apoproteins. Both VLDL and HDL in the rat contain at least four apoproteins of low molecular weight (C apoproteins) of which 2 (maybe 3) are homologous with the human. Apo-LDL (B protein), is present in both human and rat VLDL and LDL. Two major apoproteins of high molecular weight similar to the human apoproteins were found in rat HDL (Koga et al, 1971). Frederickson et al (1972) have proposed that rat plasma VLDL and HDL share one apolipoprotein of high molecular weight.

Mahley et al (1970) found that VLDL obtained from rat liver golgi apparatus contained all the apoproteins present in rat plasma VLDL. This was also shown by Pottenger and Getz (1971) for VLDL from 'liposomes' of
rat liver which accumulate in liver of ethionine treated rats or other agents that produce fatty livers. The apoprotein composition of VLDL synthesised in the intestine and in the liver is thought to be different.

1.8 BIOSYNTHESIS OF LIPOPROTEINS

Biosynthesis of lipoproteins occurs in a number of stages. These are, the biosynthesis of lipid, apoprotein and carbohydrate components, linkage of the apolipoprotein with lipid and carbohydrate, intracellular transport and finally secretion of the completed molecule into the serum.

Both liver and intestine have been identified as sites of lipoprotein synthesis. However most studies on the synthesis of VLDL, LDL and HDL have been carried out with liver preparations of various kinds. These have included perfused rat liver (Marsh and Whereat, 1959; Bar-on et al, 1973; Kook and Rubinstein, 1973), liver slices (Marsh and Whereat, 1959; Kook and Rubinstein, 1969), liver cells (Sundler et al, 1973) and isolated intracellular organelles (Bungenburg de Jong and Marsh, 1968).

Several studies involving in vivo incorporation of labelled amino acids into mesenteric lymph or thoracic duct lymph lipoproteins (Bragdon, 1958; Hatch et al, 1966; Windmueller and Levy, 1968) have implied synthesis of apoproteins by the gut. Rodbell et al (1959) and Roheim (1966) have provided evidence for intestinal synthesis of HDL and VLDL protein using lymph cannulated dogs. Other studies (Radding et al, 1958; Marsh and Whereat, 1959; Haft et al, 1962) have shown that perfused rat livers can produce both LDL and HDL. In vitro studies with intestinal slices (Isselbacher and Budz, 1963), mucosal cells (Rodbell et al, 1959; Hatch et al, 1966) and cell free intestinal preparations (Kessler et al, 1970) have also provided evidence for intestinal lipoprotein synthesis.
Before discussing the sites of synthesis of lipoproteins and their respective apolipoproteins in more detail, the mode of their intracellular assembly will be described briefly. The intracellular sites of synthesis of protein, lipid and carbohydrate have been localised but the exact sequence of lipoprotein assembly is not clear. Various models of lipoprotein synthesis have been proposed by Stein and Stein (1965) and Trams and Brown (1966). Apolipoproteins synthesised on the ribosomes of rough endoplasmic reticulum pass into the channels of the smooth endoplasmic reticulum to the golgi apparatus. Coupling of lipid and carbohydrate moieties may occur within the endoplasmic reticulum or the golgi body. The newly synthesised lipoproteins are then concentrated in the golgi apparatus and carried in secretory vesicles, which originate in the golgi apparatus (Mahley et al., 1969; Erenreich et al., 1973), to the sinusoidal surface of the parenchymal cells and discharged into the space of Disse (Stein and Stein, 1967). The functional integrity of the microtubules is thought to be important for the intracellular movement and release of VLDL particles from the liver (Le Marchand et al., 1973). Recent studies have provided evidence that the golgi apparatus plays a major role in the attachment of carbohydrate to the lipoprotein (Lo and Marsh, 1970). However, it is not clear whether the lipid is attached to nascent polypeptide chains or whether there are separate pools of apolipoprotein and lipid from which the lipoprotein particles are formed during their passage through the cisternae of the endoplasmic reticulum. Little information is available on the mechanism of coupling of lipid and protein moieties.

As the total process of lipoprotein synthesis is not completely understood, factors influencing the control of lipoprotein synthesis are difficult to define. Effects of hormones on lipoprotein synthesis have only been studied in vivo and cannot be distinguished from effects that
alter lipid metabolism or lipoprotein catabolism. Meaningful results have only been obtained from studies on factors that regulate the synthesis of apolipoproteins. Both cholesterol and fatty acid concentrations under a number of metabolic conditions enhanced the release of cholesterol and triglycerides from perfused rat liver (Ruderman et al., 1968) but cholesterol (Roheim et al., 1965) had no significant effect on peptide formation. However, free fatty acids did increase the de novo synthesis of VLDL peptides. Lipoproteins are capable of carrying an increased load of lipid without altering the protein concentration. Eder et al. (1964) have proposed the existence of a circulating lipid-acceptor protein present in serum and capable of accepting lipid from the liver. The physiological importance of this protein is thought to be small. However evidence that some recycling of apoproteins may occur was reported by Bar-on et al. (1973). After interruption of protein synthesis in perfused rat livers by cycloheximide, they concluded that the lipid moiety of VLDL was added to pre-existing apolipoprotein (drawn from a pool of unknown size and origin in the liver) and not to preformed lipoprotein. Also analysis of the labelled hepatic apoprotein pool secreted after cycloheximide showed that it did not contain all the components in proportions normally present in secreted VLDL.

As studies with perfused rat liver and intestine have yielded much information on the sites of biosynthesis of lipoproteins and apolipoproteins some of these will be discussed below. The whole spectrum of plasma lipoproteins is found in mesenteric lymph (Windmueller and Levy, 1968; Page et al., 1953; Courtice and Morris, 1955) which contains proteins synthesised by extraintestinal tissues and filtered from plasma in addition to proteins derived from the intestine. Therefore, precise information on the synthesis of lipoproteins by the intestine could only be provided if the small intestine was completely isolated from the animal.
while keeping intact the vascular and lymphatic channels.

Windmueller and Spaeth (1972) developed a technique for complete removal, without circulatory interruption, of the intact intestine from the animal. Lymph cannulated rat intestine perfused with blood under physiological conditions was used for studies on lipoprotein biosynthesis. That the preparation was viable and functioning was established by a variety of metabolic and transport measurements including normal gross and histological appearance (Windmueller et al., 1970a). Radioactive amino acids were added to the perfusate and the incorporation into lymph and perfusate proteins was measured, a small amount of fat being constantly infused to ensure continuous production of chylomicrons and VLDL. Of the radioactivity appearing in lymph protein, 30% was recovered in chylomicrons plus VLDL and 7% in an LDL plus HDL fraction. No detectable radioactivity was incorporated into the perfusate chylomicrons plus VLDL fraction, while newly synthesised LDL plus HDL protein was divided equally between lymph and perfusate. The specific activity of the LDL plus HDL lipoprotein in the perfusate was lower than that in the lymph. Analysis of the radioactivity present in lymph and perfusate LDL plus HDL fraction revealed that 50% of that in the lymph and 86% of that in the perfusate was recovered in HDL (d 1.063-1.21 g/ml). It was therefore concluded that VLDL particles are too large to enter blood capillaries and therefore stay only in lymph but HDL can apparently cross the capillary endothelium. Qualitative evidence for the hepatic biosynthesis of most of the known plasma lipoprotein apolipoproteins has been provided by the work of Windmueller and Levy (1967) and Mahley et al. (1970). The perfused rat liver and intestine has also been used to study the synthesis of various apoproteins of plasma lipoproteins (Windmueller et al., 1973; Windmueller and Spaeth, 1972). Lipoproteins containing $^3$H labelled lysine were isolated from the perfusates and intestinal lymph, delipidated and the apoproteins separated by polyacrylamide gel electrophoresis and the $^3$H
content determined.

Livers incorporated \(^3\)H-lysine into all apoprotein bands of VLDL and HDL. \(^3\)H content was greater in B apoprotein or group I apoprotein (70% of radioactivity), than in proteins of apparent molecular weight of 50,000 (group II apoproteins or A proteins) (approximately 20%), or the smaller peptides with molecular weights near 10,000 (group III apoproteins or C proteins) approximately 10%. In the intestine, \(^3\)H-lysine was incorporated into B protein and in larger amounts into A apoproteins of lymph VLDL. \(^3\)H-lysine was incorporated into A apoproteins of lymph and perfusate HDL (87% of radioactivity). No \(^3\)H-lysine was found in the group III (C apoproteins) peptides of any lymph or intestinal perfusate lipoproteins. Since these peptides are always present in VLDL from mesenteric lymph collected \textit{in vivo} (Windmueller \textit{et al}, 1973), these results suggest that nascent VLDL of gut origin acquires C peptides from other lipoproteins that penetrate lymph from plasma.

Windmueller \textit{et al} (1973) postulate that under their perfusion conditions the gut released nascent lipoprotein particles that contained newly synthesised B and A apoproteins only. This process could also occur \textit{in vivo} as all plasma proteins including lipoproteins are continuously being filtered from the circulation into tissue spaces, from which they gain entrance into the lymph (Courtice, 1968). Also as these lipoproteins, particularly the HDL, pass through the intestinal lymphatics they may give up some of their C peptides to nascent lipoproteins entering the lymph from the intestinal mucosal cells. Equilibration of C peptides between HDL and VLDL in rat plasma has been demonstrated (Eisenberg \textit{et al}, 1971). Some evidence is also available for a net movement \textit{in vitro} of these peptides from HDL to synthetic fat emulsions and lymph chylomicrons (Hofmann, 1960; Lossow \textit{et al}, 1967) and from HDL to density < 1.006 lipoproteins \textit{in vivo} in man during alimentary lipaemia (Havel \textit{et al}, 1971;
Eisenberg et al, 1971). As VLDL triglyceride is cleared from the plasma, these peptides shift back to HDL (Bilheimer et al, 1972; La Rosa et al, 1971).

When interpreting the results of these perfusion experiments it is important to remember that the relative pool sizes for the various apoproteins in liver and gut are unknown and whether newly synthesised apoproteins have been catabolised upon recycling through the liver cannot be determined.

To date the data suggests that all lipoproteins are synthesised in the liver whilst only VLDL and HDL are synthesised in the gut. Chylomicrons are only produced in the gut. There is some evidence to suggest that VLDL and chylomicrons are produced in intestine by a process that may yield a continuous spectrum of particles differing in size and triglyceride content (Windmueller and Levy, 1968; Windmueller et al, 1970b; Mahley et al, 1971; Tytgat et al, 1971). It is also suggested that liver and gut do not produce the same proportion of B to A VLDL apoproteins and gut does not release newly synthesised C peptides as does the liver. It seems logical to conclude that chylomicrons and VLDL entering the circulation from the gut acquire C peptides synthesised in the liver before they are further metabolised, since one of the major C peptides (apoC-II) acts as an activator of the triglyceride clearing lipoprotein lipase (La Rosa et al, 1970; Fielding et al, 1970; Havel et al, 1970).

That the perfused liver does not release newly synthesised VLDL apoproteins into the perfusate in the same proportions as found in VLDL isolated from plasma (Windmueller et al, 1973), is not surprising since VLDL is a heterogeneous mixture of particles, composed of gut-derived lipoproteins and liver-derived lipoproteins. These undergo changes in protein content by acquisition from the plasma and by the differential
loss of apoproteins (Bilheimer et al, 1972) as metabolism of VLDL proceeds. The relative amounts of apoproteins produced can be influenced by the physiological state of the animal.

1.9 METABOLISM OF LIPOPROTEINS

A single unifying hypothesis for lipoprotein metabolism was proposed by Lindgren et al (1956). It was suggested that chylomicrons and other very low density molecules could be 'shorn' of lipid in the normal course of their metabolism and yield low density lipoproteins of lower Sf values and smaller in size or even high density lipoproteins. This concept of 'interconversion' of lipoproteins first arose out of observations of changes in lipoproteins which occurred in vivo after injection of heparin and in vitro after incubation of lipoproteins with postheparin plasma. The first such studies were by Graham et al (1951) who found that heparin in man and rabbits induced a fall in lipoproteins of Sf 20-400 and a rise and subsequent fall in the Sf 12-20 class of lipoproteins within a few hours. The increase in the Sf 0-20 lipoproteins did not account for the fall in Sf 20-400 lipoprotein. Similar findings were obtained in vitro by Anfinson et al (1952) and Lindgren et al (1955) who thought that the fall in Sf 20-400 lipoproteins through lipoprotein lipase activity may have been accompanied by an increase in HDL. However, only the phospholipid content of HDL increased under these conditions.

'Interconversions' similar to those found in post heparin experiments were also reported in vivo in the absence of exogenously administered heparin. For example, Pierce (1954), in order to study the interrelationships between serum lipoproteins, injected lipoproteins of various Sf classes (Sf 5 to > 400) into normal rabbits and found that lipoproteins of high Sf rate were converted to lipoproteins of lower Sf rate in a serial fashion. 'Conversion' was always from high to low and
never in the reverse direction. When higher Sf classes were injected conversion was more rapid and was accompanied by a progressive lowering of concentrations in the lower Sf classes. Gitlin et al (1958) injected $^{131}$I-labelled lipoproteins of Sf 10-100 (VLDL) into humans and observed radioactivity appearing in molecules of Sf 3-9 (LDL), thus confirming Pierce's observations in rabbits (Pierce, 1954). From studies on chylomicron catabolism by lipoprotein lipase (Korn, 1955), and their possible removal from the serum as 'remnants' (Redgrave, 1970), it soon became apparent that the unifying hypothesis of a single 'parent' molecule (chylomicrons) was not tenable for the whole lipoprotein spectrum.

1.9.1 Metabolism of VLDL

From detailed studies on the protein moieties of various lipoprotein classes (Gustafson et al, 1965, 1966; Shore and Shore, 1962; Scanu, 1966) it became apparent that some apolipoproteins are commonly present in more than one lipoprotein class. VLDL and LDL have at least one common apolipoprotein (ApoB) and some VLDL apolipoprotein is also found in HDL (C proteins) (see Table 1.2). The possibility that VLDL can be converted to LDL and possibly HDL is therefore tenable, particularly as there is experimental evidence for this hypothesis.

Several studies suggest that LDL triglycerides may be derived from VLDL (Farquar et al, 1965; Havel et al, 1962; Quarfordt et al, 1970); however, little is known about the fate of VLDL apoproteins. The appearance of radiiodinated VLDL protein in LDL after injection into humans (Gitlin, 1958; Langer et al, 1970) supports the hypothesis that some if not all plasma LDL arises from VLDL catabolism. Further support is provided by the reciprocal relationship which has been demonstrated between the plasma concentrations of VLDL and HDL (Levy et al, 1966) and VLDL and LDL in man (Wilson and Lees, 1972). Fidge and Foxman (1971) injected VLDL labelled with $^{125}$I-iodine in the peptide moiety into rats and found that radioactivity
appeared in the peptide moieties of LDL and HDL. This study clearly suggested that there was an interrelationship between the apoprotein moieties of VLDL, LDL and HDL in the rat. Bilheimer et al. (1972) also attempted to follow VLDL apoprotein metabolism by labelling the protein moiety of VLDL with $^{125}$I using a radioiodination technique originally developed by McFarlane (1958). A significant amount of radioactivity was found in other lipoproteins when $^{125}$I-VLDL was incubated with human plasma in vitro. This redistribution of radioactivity also occurred in humans 10 minutes after injection of $^{125}$I-VLDL. This finding suggested that VLDL apoproteins readily transfer from VLDL to other lipoproteins.

Analysis of VLDL apoproteins using column chromatography and polyacrylamide gel electrophoresis (PAGE), showed that this transfer, mainly to HDL, involved the small molecular weight apoproteins or C proteins (C-II, C-III$_1$ and C-III$_2$) but not the B protein of VLDL. This transfer was only slightly affected by temperature and was independent of lecithin:cholesterol acyl transferase.

Following intravenous injection of $^{125}$I-VLDL into humans, transfer of radioactivity was observed first to lipoproteins of density 1.006-1.019 g/ml then to LDL as well as to HDL. The disappearance of the LDL apoprotein portion from VLDL far exceeded that of the low molecular weight peptides. The time sequence of these events may be suggestive of a precursor product relationship between VLDL and LDL through a lipoprotein of intermediate density 1.006-1.019 g/ml. Eisenberg et al. (1972a) have shown that the transfer of human C-II, C-III$_1$ and C-III$_2$ between VLDL and HDL in vitro is bidirectional and postulated that this represented at least in part an exchange phenomenon. The apoprotein moiety of LDL did not take part in this type of transfer.

Rubenstein and Rubinstein (1971) found that there was exchange of phospholipids among each of the major lipoprotein classes separated by
ultracentrifugation in the rat. Their results also indicated that concomitant with an exchange of phospholipid between VLDL and HDL there was also an exchange of certain peptides. In a later study Rubenstein and Rubinstein (1972) found that $^{32}$P labelled phospholipid from isolated VLDL and prepared *in vivo* was transferred to HDL and to a much lesser extent to LDL *in vivo* and *in vitro*. $^{32}$P phospholipid was also transferred from HDL back to unlabelled VLDL *in vivo* and *in vitro*. Generally the phospholipid species exchanged in proportion to their distribution in the lipoproteins except for lysolecithin. $^3$H-labelled protein of VLDL was transferred to HDL and $^3$H-labelled HDL protein was transferred to VLDL *in vitro*. LDL was not involved in this transfer.

A non-specific exchange among the phospholipids in each lipoprotein fraction and a specific exchange between the VLDL and HDL not involving LDL was postulated. The apoproteins exchanged between VLDL and HDL were indirectly identified by the authors using their PAGE data as the low molecular weight C peptides common to VLDL and HDL. This data from the rat is consistent with the human studies of Eisenberg *et al* (1972a). Rubenstein and Rubinstein (1972) introduced a new concept of exchange of phospholipid and peptide moieties of the lipoprotein (between VLDL and HDL) representing the transfer of an intact lipoprotein subunit.

Further support for the concept of an interrelationship between very low density and other higher density lipoproteins has come from several different approaches. These have included studies on LDL and HDL metabolism since it has been found that a structural and metabolic relationship exists between VLDL and LDL and VLDL and HDL.

### 1.9.2 Metabolism of LDL

Most investigations have shown that LDL of Sf < 6 when injected into humans or rats is not significantly catabolised further to lipoproteins
of higher density. Most of the LDL is rapidly removed from the serum (Gitlin et al., 1958) predominantly by the liver in humans (Scott and Hurley, 1970) and rat (Hay et al., 1971). Many studies on the turnover of LDL in diseased states compared to the turnover in healthy subjects have been made (Walton et al., 1963; Scott and Hurley, 1970; Langer et al., 1972).

1.9.3 Metabolism of HDL

Rat HDL differs from that of man in that rat HDL and not LDL transports 60-70% of the serum cholesterol and has a molecular weight approximately twice that of human HDL (Koga et al., 1971; Gidez et al., 1965). It is therefore functionally different from human HDL and may be of greater importance in the rat in the overall scheme of lipoprotein metabolism.

Studies using radioiodinated rat HDL injected into rats have revealed that exchange or transfer of label to VLDL was low and did not exceed 3% of the radioactivity present (Roheim, 1971). Measurements of the total radioactivity found in liver and small intestine suggest that these organs are the major sites of HDL catabolism in the rat. The half-life of HDL in the circulation was found to be 10.5 hr (Roheim, 1971). Clearance of HDL is therefore less rapid than that observed for rat VLDL (Fidge and Foxman, 1971).

Work by Rubenstein and Rubinstein (1973) on two subfractions of rat HDL, HDL₂ and HDL₃, analogous to the human HDL of density 1.063-1.12 g/ml and 1.12-1.21 g/ml respectively, (De Lalla and Gofman, 1954; Levy and Frederickson, 1965) supports the suggestion that HDL₃ is not an artifact of ultracentrifugation derived from HDL₂ but rather a separate lipoprotein entity. These subfractions are thought to differ in peptide composition, metabolic function and synthesis. It was found that an HDL₂ peptide, with an electrophoretic mobility corresponding to one found in VLDL, HDL₂ and
HDL\(_3\), undergoes the greatest exchange with VLDL although this peptide was also present (as a minor component) in HDL\(_3\). This suggests that HDL\(_2\) and HDL\(_3\) have different metabolic functions.

1.10  ENZYMES ASSOCIATED WITH LIPOPROTEIN METABOLISM

The metabolic studies described earlier have established that lipoproteins undergo considerable changes in composition in the plasma before they are removed from the circulation. These changes possibly occur as a consequence of the action of at least two enzymes, lipoprotein lipase and lecithin:cholesterol acyl transferase.

1.10.1  Lipoprotein Lipase

Lipoprotein lipases are a group of enzymes which hydrolyse triglycerides only in the presence of lipoproteins or certain apoproteins (Korn, 1955; Scanu, 1966), and are thought to be active mainly in the luminal surface of the capillary endothelial cells of extrahepatic tissues. They are normally not measurable in plasma but may be released into plasma after administration of heparin or similar polyanions or under conditions of a high fat load (Sailer et al., 1966). The enzyme has been found in adipose tissue, heart, mammary gland, liver and other organs and when obtained from these tissues is inhibited by 1M sodium chloride. Lipoprotein lipase from rat heart, rat (La Rosa et al., 1970) and chicken adipose tissue (Scanu, 1966), bovine milk (Havel et al., 1970) and human plasma (Fielding et al., 1970) has been shown to be activated by at least one apolipoprotein. ApoC-II present in human VLDL and HDL is required by lipoprotein lipase for full activity (La Rosa et al., 1970; Fielding et al., 1970). Each of the five non-activating apolipoproteins, apoC-I, C-III\(_1\), C-III\(_2\), A-I and A-II may inhibit lipoprotein lipase 85-100% (Krauss et al., 1973) and are thought to modulate enzyme activity.
Many studies have shown that there is heterogeneity in species of lipases released into post-heparin plasma. Two species of triglyceride lipase activity have been characterised in post-heparin plasma, differing in substrate specificity and sensitivity to inhibitors such as high salt concentrations (1M sodium chloride), pyrophosphate and protamine sulphate (Greten et al., 1972; Fielding, 1970; La Rosa et al., 1972). One is accepted as being identical with lipoprotein lipase as defined by Korn (1955) and its role appears to be the hydrolysis of serum triglycerides providing tissues with fatty acids for sources of energy (Scow et al., 1972; Zieve and Zieve, 1972). It is sensitive to sodium chloride (1M), pyrophosphate and protamine sulphate inhibition. The other main triglyceride hydrolase present in post-heparin lipolytic activity, which is not sensitive to sodium chloride or protamine sulphate inhibition, is identical in properties with a liver triglyceride lipase.

After heparin administration to man and rats, phospholipases (Zieve and Zieve, 1972) are released into plasma, in addition to lipolytic activities against long chain triglycerides di- and monoglycerides (Fielding, 1970, 1972) and short chain triglycerides (Yasuoka and Fujii, 1971). These have been measured by extracting lipoprotein lipase from tissue homogenates and post-heparin plasma and measuring the hydrolysis of synthetic dispersions of long chain triglycerides, diglycerides, monoglycerides and short chain triglycerides such as tributyrin (Fielding, 1972). These substrates have also been used to test the in vitro characteristics of purified and semi-purified enzymes and their cofactor requirements such as apolipoproteins and phospholipids. However, lipoprotein lipase from adipose tissue is thought to be more active against triglycerides than diglycerides. Conflicting results on the substrate specificity of lipoprotein lipase have been reported (Greten et al., 1970; Nilsson-Ehle et al., 1971; Morley and Kuksis, 1972). This
could be due to inadequate definition of stereochemistry of substrates employed, failure to differentiate between lipolytic activities of different origin and to differences in assay conditions.

From evidence to date it is clear that lipoprotein lipase activity in extrahepatic tissues is different from the lipase activity found in the liver and these activities can be released by heparin into the bloodstream. It will be important to consider the consequence of such activity on the catabolism of VLDL protein as well as its triglyceride moiety. The balance between lipoprotein lipase activity and the influx of free fatty acids, carbohydrate and other precursors of triglyceride determine the VLDL concentration in serum at any one time. Lipoprotein lipase activity of adipose tissue is affected by hormones (Nestel and Austin, 1969) and by the nutritional state of the animal (Schotz and Garfinkel, 1972).

The fate of the VLDL from which triglyceride has been removed by lipoprotein lipase activity is unknown although it has been suggested that it is converted to LDL. The mechanism by which this conversion occurs is only speculative. Further enzyme action on VLDL lipid, for example lecithin:cholesterol acyltransferase, may be required before conversion to LDL and removal from the circulation.

1.10.2 Leicithin:cholesterol acyltransferase

Lecithin:cholesterol acyltransferase (LCAT) (E.C.2.3.1.43) catalyses the transfer of an acyl group from the β position of lecithin to cholesterol.
Lecithin + cholesterol $\xrightarrow{\text{LCAT}}$ lysolecithin + cholesterol ester

$$\begin{align*}
\text{CH}_2\text{O} & \quad \text{saturated fatty acid} \\
\text{CHO} & \quad \text{unsaturated fatty acid} + \text{OH-cholesterol} \\
\text{CH}_2\text{O} & \quad \text{P-Choline} \\
\text{OH} & \quad 0
\end{align*}$$

LCAT activity has been reported to occur in the plasma of most mammals for example humans, monkeys, dogs, rats, rabbits (Wells and Rongone, 1969), calves, sheep, chickens (Sugano et al., 1965). It is one of the few enzymes specific for plasma, acting primarily on plasma LDL and HDL. It is distributed in rat tissues in concentrations not exceeding one quarter the concentration present in rat plasma (Glomset and Kaplan, 1965). The LCAT reaction and its physiological role were first described by Glomset (1962, 1968, 1970, 1972). The LCAT enzyme system and the liver are the most important factors in the regulation of cholesterol ester levels in the plasma of most species. Plasma cholesterol esters appear to be a transport form of cholesterol from peripheral tissues to the liver (Glomset, 1963).

LCAT may also remove the excess surface lipids, unesterified cholesterol and lecithin from chylomicrons and VLDL after triglyceride hydrolysis possibly facilitating their removal from the circulation (Schumaker and Adams, 1970). The role of the LCAT-HDL system in the regulation of cholesterol in lipoproteins and plasma membranes is shown.
in Figure 1.1 (Glomset and Norum, 1973). The sterol specificity for LCAT is quite broad (Salen and Grundy, 1973). Most of the acyltransfer to cholesterol is from lecithin (Nichols and Gong, 1971). Only the acyl group in the β position of lecithin is transferred in humans (Glomset, 1968) but there is species variation in preference for acyl groups in the two positions (Sgoutas, 1972).

The LCAT reaction takes place predominantly on HDL (Fielding and Fielding, 1971a). Both VLDL and chylomicrons are thought to increase the rate of cholesterol ester formation by an unknown mechanism (Marcel and Vezina, 1974). The rate of the LCAT reaction is also dependent on the nature of the fatty acyl moieties of lecithin and thus also on the physiological state of the lipid substrate (Garner et al., 1972). Among the apoproteins of HDL, apoA-I from the human (Fielding et al., 1972) and the pig (Jackson et al., 1973) is a potent activator of LCAT. Differences in lipid composition between HDL subclasses and the products of the LCAT reaction in particular cholesterol ester also influence enzyme activity (Nichols, 1967; Fielding and Fielding, 1971b). ApoA-III or apoD (the apoprotein of 'the thin line lipoprotein') is thought to be a more potent activator of LCAT than apoA-I (Kostner, 1974).

An inborn error of metabolism caused by a lack of LCAT was discovered in 1967 (Norum and Gjone, 1967) and is reviewed by Norum et al. (1972). Many studies of lipoproteins from patients with familial LCAT deficiency have been made (Forte et al., 1974). The role of LCAT in different lipaemias and in atherosclerosis has also been studied (Fabien et al., 1973).
Figure 1.1  The Lecithin:Cholesterol Acyltransferase High Density Lipoprotein System (Glomset and Norum, 1973).

CE  cholesterol ester
UC  unesterified cholesterol
LCAT  lecithin:cholesterol acyltransferase
1.11 CONCLUSION

1.11.1 General

It is apparent that a metabolic as well as structural relationship exists between VLDL and HDL and VLDL and LDL. The exact nature of these interrelationships and the extent to which they are dependent on enzyme activity (such as LCAT and lipoprotein lipase) is to a large extent unknown. However, a number of interesting observations have emerged from enzyme studies and from investigations of the affinity of individual apolipoproteins for various lipids and lipoprotein classes (Bilheimer et al, 1972). Apoproteins A-I and C-III interact with phospholipid and A-I and C-II are potent activators of LCAT and lipoprotein lipase respectively. A functional analogy between the A and C proteins is therefore suggested. This may be the basis of a common structural principle in lipoprotein structure (Assmann and Brewer, 1974). The role of the C proteins in the structure of lipoproteins is unknown, although it has been suggested that they are loosely bound.

The formation of an enzyme-substrate complex, required by lipoprotein lipase before triglyceride hydrolysis can proceed, is presumably dependent on the presence of an 'activator' protein found on the surface of lipoprotein particles as well as phospholipid and triglyceride (Havel et al, 1970; La Rosa et al, 1970). Based on the capacity of this protein to promote the interaction of phospholipid-stabilised triglyceride emulsions with lipoprotein lipase, it was estimated that more than half the activator protein in serum of normal human subjects in the post-absorptive state would be contained in HDL and the remainder in VLDL (Bier and Havel, 1970). As this protein appears to be functionally active only in triglyceride rich lipoproteins and can transfer readily from HDL to phospholipid stabilised fat emulsions (Havel et al, 1973; Scanu, 1967),
HDL could provide a source of activator proteins to newly secreted triglyceride rich lipoprotein particles. Some support for this concept is suggested by the longer half-life in the serum of HDL compared to VLDL and the fact that C peptides of human VLDL are transferred to HDL.

1.11.2 Objectives of the Present Research

Previous studies have shown that after the injection of labelled VLDL most of the VLDL radioactivity is recovered in lipoproteins of higher density (Gitlin, 1958; Fidge and Foxman, 1971). Other studies by Levy et al (1966) and Wilson and Lees (1972) have shown that quantitative inverse relationships exist between VLDL and HDL and VLDL and LDL concentrations in serum. The principle objective of the present research is to further investigate the metabolic relationship between apoproteins of VLDL, LDL₁, LDL₂ and higher density lipoproteins and to test the hypothesis that LDL is derived from VLDL. Although many studies on VLDL triglyceride metabolism have been reported, the difficulties involved in triglyceride turnover measurements and the assumptions required by each method, have not elucidated whether the increased concentrations of VLDL in certain disease states (Table 1.4) are due to overproduction of VLDL triglyceride or defective clearance (Havel et al., 1970; Quarfordt et al., 1970). Since it has been established that VLDL, LDL₁ and LDL₂ contain at least one common protein (apolipoprotein B), a study of the metabolism of this protein in VLDL may lead to the clarification of the metabolic pathway of VLDL breakdown and eventual removal from the circulation. The combined data derived from VLDL lipid and protein turnover studies may provide corroborative evidence to resolve the etiology of these lipid diseases.

A number of methods considered potentially useful for labelling rat VLDL in the protein moiety were investigated. On the basis of these experiments in vitro labelling with $^{125}$I using the iodine monochloride
The technique of McFarlane (1958) was selected bearing in mind the validity of the labelled VLDL for use in \textit{in vivo} studies as well as the economy of this method compared to \textit{in vivo} labelling with $^{14}$C or $^{3}$H. Since the rat has provided much useful information about lipid transport and its lipoproteins have been characterised to a greater extent than those of other animals it was chosen as the experimental animal. The validity of the rat as a model for human lipoprotein metabolism was also tested.

The overall approach has been to measure the kinetics of transfer of apolipoproteins from radioiodinated VLDL to other lipoproteins after the injection of labelled VLDL into rats. This was achieved by isolating the lipoproteins from the serum of injected rats by sequential ultracentrifugation at increasing densities, delipidating the lipoproteins and separation of the apoproteins to determine the radioactivity in the apolipoproteins. Methods for the determination of the specific activity of both the B and C proteins were also developed since in some studies specific activity determinations were essential for the estimation of the quantitative relationship between apoproteins of VLDL, LDL$^{1}$, LDL$^{2}$ and HDL. Finally, because of the paucity of information available on rat apolipoproteins a partial characterisation of the apolipoproteins of rat VLDL and HDL was attempted. The general approach in this work was to collect a large volume of rat serum in order to isolate and partly characterise the apolipoproteins which were identified as being involved in the functional relationship between VLDL and HDL.
A number of similar techniques used throughout this work are presented in this chapter. Some modifications of these methods are included in the appropriate sections.

CHAPTER 2

GENERAL METHODS

The rats used for metabolic studies were male Sprague-Dawley rats of the New Zealand White strain. When larger volumes of blood were required for characterization of apolipoproteins both male and female rats (300-400 g) were used.

Data were collected using two methods:

1. **Experimental:**
   - Large rats (500 g) were lightly anesthetized (sodium pentobarbital) and a 25-gauge needle was inserted into the heart with a 1 ml disposable insulin syringe and 22 gauge needle. As multiple samples could be obtained from the one rat by this procedure, errors due to biological variability between individual rats were avoided. This approach was possible if large experiments were only performed at one time. Required
CHAPTER 2

GENERAL METHODS

A number of basic techniques used throughout this work are presented in this chapter. Any modifications of these methods are described in the appropriate section.

Experimental

2.1 ANIMALS

All rats used in metabolic studies were male Wistar rats (180-240 g) of the John Curtin School Strain. When larger volumes of pooled sera were required for characterisation of apolipoproteins both male and female rats (250-400 g) were used.

2.2 COLLECTION OF SERA

Sera were collected using two methods.

2.2.1 Cardiac Puncture

Large rats (300 g) were lightly anaesthetised (anaesthetic ether) and 0.25-0.5 ml blood was withdrawn from the heart with a 1 ml disposable Terumo insulin syringe and 23 gauge needle. As multiple samples could be obtained from the one rat by this procedure, errors due to biological variation between individual rats were avoided. This approach was possible in some experiments where only small amounts of serum were required.
2.2.2 Exsanguination from the Abdominal Aorta

The animals were exsanguinated under light ether anaesthesia by puncturing the abdominal aorta with a sterile 21 gauge needle attached to a 10 ml disposable syringe. Six to seven ml blood could be obtained in this way from animals weighing 180-200 g.

After collection, blood was allowed to clot and serum was obtained by centrifuging the blood at 2,500 rpm for 30 min at 4°C in a MSE Mistral 6L refrigerated centrifuge. Large volumes of blood were centrifuged in an MSE 18 High Speed centrifuge at 4°C for 45 min at 3,000 rpm. EDTA was added to each pooled serum sample to give a final concentration of 1 mM in order to minimise autoxidation of lipoproteins. Thimerosal (1:10,000 w/v) (Sodium ethyl mercuri thiosalicylate, Nutritional Biochemicals, Cleveland, Oh. USA) or sodium azide (1 mM) were added to serum as preservatives. These chemicals were omitted from sera obtained from metabolic experiments or from which VLDL for iodination and reinjection was isolated.

2.3 INTRAVENOUS INJECTION OF RATS

Rats were injected in the tail vein using a 25 gauge needle attached to a 1 ml Terumo insulin syringe. The rat was placed in a restraining cage and the tail veins dilated by washing under warm water. All material to be injected was dissolved in sterile 0.9% sodium chloride solution buffered to pH 7.4.

2.4 SEPARATION OF LIPOPROTEINS

This was carried out on either L2-50 or L3-50 Beckman Model ultracentrifuges.
2.4.1 Separation of VLDL

VLDL Sf 20-400 for reinjection experiments was prepared in the following manner. Five ml serum from rats fasted for 6-8 hr was added to Spinco 40.3 tubes and overlaid with 2 ml 0.15 M sodium chloride (pH 7.4) containing .001 M EDTA d 1.006 g/ml. After centrifugation at 12°C for 16 hr at 39,000 rpm in a fixed angle rotor type 40.3, the floating VLDL was isolated by slicing the tubes. Following this initial isolation, the VLDL was purified by two further centrifugations, one for 8 hr at 12°C in a Spinco 50 rotor at 50,000 rpm and finally for 16 hr at 12°C in the 40.3 rotor at 39,000 rpm. Isolation of VLDL Sf 20-400 from larger volumes of serum (160 ml) was performed in a Spinco type 40 rotor (10 ml serum/tube overlaid with saline), centrifuged at 105,000 x g for 16 hr at 12°C and was then washed twice as described above. VLDL isolated in this manner contained approximately 7% protein, 68% triglyceride, 16% phospholipid and 9% cholesterol, which agree closely with those obtained by Fidge and Calder (1972) and Lasser et al (1973). To isolate VLDL from large quantities of serum (800 ml) type 30 or 35 fixed angle rotors were used and centrifugations were at 30,000 rpm for 24 hr at 12°C.

2.4.2 VLDL Subfractionation

In some preparations, VLDL subfractions of Sf > 400, 100-400 and 20-100 were isolated using the method described by Gustafson et al (1965). Briefly, VLDL subfractions were prepared either by sequential ultracentrifugation in a Spinco type 50 rotor (Sf > 400 at 20,000 x g for 30 min, Sf 100-400 at 80,000 x g for 1 hr and Sf 20-100 at 105,000 x g for 22 hr) or in a Spinco SW 50.1 rotor under the same conditions. More efficient separation of subclasses was achieved with the SW 50.1 rotor. All VLDL preparations were washed twice by flotation through saline (d 1.006 g/ml) under the same conditions used for their isolation and
checked for purity by agarose gel electrophoresis or immunodiffusion. No other plasma proteins were detected by these methods. Human VLDL was similarly isolated from pooled serum obtained from normal male donors who had fasted overnight and had a chemical composition similar to that reported by Gustafson et al. (1965).

2.4.3 Separation of LDL and HDL

After removal of VLDL (from 400-800 ml serum) the infranatant of the VLDL ultracentrifugation was adjusted to d 1.21 g/ml by the addition of solid KBr (.234 g/ml). Following ultracentrifugation for 24 hr at 30,000 rpm at 4°C in Spinco type 30 or 35 rotors the supernatant containing LDL and HDL was dialysed to d 1.063 g/ml (4°C) and recentrifuged to separate LDL (supernatant) and HDL (infranatant). The LDL and HDL were then purified twice by flotation at d 1.050 and 1.21 g/ml respectively.

2.4.4 Sequential Ultracentrifugation to Obtain VLDL, LDL₁, LDL₂ and HDL

This procedure was used for the separation of lipoproteins from sera obtained during the course of metabolic experiments. The lipoproteins contained in 3-5 ml serum were separated by sequential ultracentrifugation in a Spinco 40.3 fixed angle rotor as described by Havel, Eder and Bragdon (1955).

Although similar density classes of plasma lipoproteins are found in most higher animals the distribution of the high density and low density lipoproteins is known to vary among different species (Havel et al., 1955; Lewis et al., 1952; Mills and Taylaur, 1971). Conventionally, the density distribution established by De Lalla and Gofman (1954) and Havel et al. (1955) for humans has been used as the basis of isolation of lipoprotein classes in other animals including the rat. Since there is evidence to
suggest different density limits for delineation of rat lipoprotein density classes (Windmueller and Levy, 1967; Koga et al, 1969, 1971; Lewis et al, 1952) the following steps were considered appropriate for metabolic studies. After VLDL isolation, the infranate was adjusted to d 1.019 g/ml and a fraction of density 1.006-1.019 g/ml was obtained and called LDL$_1$. In metabolic experiments it was found that a significant proportion of the radioactivity was present in this fraction. Since LDL$_2$ first isolated between d 1.019-1.063 g/ml was slightly contaminated with HDL, recentrifugation at d 1.055 or 1.050 g/ml eliminated traces of HDL when apoproteins were analysed. Less than 5% of the total radioactivity was lost between d 1.055 and 1.063 g/ml. The density limits for the initial isolation of lipoprotein fractions in metabolic experiments were VLDL (d < 1.006 g/ml), LDL$_1$ (d 1.006-1.019 g/ml), LDL$_2$ (d 1.019-1.063 g/ml) and HDL (d 1.063-1.21 g/ml).

All lipoprotein fractions were adjusted to a volume of 2.0 ml with solutions of the same density as those used for their initial isolation and samples (0.2 ml) taken for radioassay. Radioactivity in the 5 ml lipoprotein free infranatant was also determined. Lipoproteins were purified by further centrifugations at the appropriate densities of 1.006 for VLDL, 1.019 for LDL$_1$, 1.055 for LDL$_2$ and 1.21 for HDL under the conditions used for their initial isolation. The supernatant of each of these spins was retained, made up to a total volume of 2.0 ml and 0.2 ml samples were removed and assayed for radioactivity. Standard density solutions were prepared according to Havel et al (1955) and densities were measured by picnometry at 20°C.
2.5 RADIOCHEMICALS AND RADIOASSAYS

2.5.1 Radiochemicals

Na$^{125}$I 'carrier free', for protein iodination ($^{126}$I < 1%) 100 mCi/ml in dilute NaOH, pH 8-11 (free from reducing agent), was obtained from the Radiochemical Centre (Amersham, England) in lots of 5 mCi.

2.5.2 Radioassays

All samples containing $^{125}$I-labelled iodine were counted in a final volume of 0.2 ml in a Packard Autogamma Spectrometer at an efficiency of 55%, determined using Packard Standards A-63, N-137 (simulated $^{125}$I source, nominally 0.1 µCi) as standards supplied by the Packard Instrument Co. Inc. Counting times were such that potential errors from counting did not exceed 2%. Correction for quenching caused by high salt concentrations was determined by reference to Table 2.1 prepared by counting $^{125}$I in solutions of known density.

Table 2.1
Quenching of $^{125}$I Radioactivity by KBr/NaCl

<table>
<thead>
<tr>
<th>KBr/NaCl (density g/ml)</th>
<th>% cpm present in H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.006</td>
<td>100</td>
</tr>
<tr>
<td>1.019</td>
<td>97</td>
</tr>
<tr>
<td>1.063</td>
<td>90</td>
</tr>
<tr>
<td>1.21</td>
<td>66</td>
</tr>
</tbody>
</table>
All samples counted were corrected for quenching and background radioactivity. The effect of volume on counting efficiency was tested for 0.1 ml-1.0 ml. Counting efficiency significantly decreased when the volume exceeded 0.5 ml. No quenching by sodium hydroxide (0.1-1.0 M) or Rhodamine dye was observed.

2.6 CHEMICAL DETERMINATIONS

2.6.1 Chemicals

All chemicals used were of analytical reagent grade. Trizma Base, Trizma-HCl, Tris Base and Tris-HCl were purchased from Sigma, USA.

2.6.2 Protein Assay

Protein content was assayed using a modification of the Lowry method (Lowry et al, 1951). Bovine serum albumin (Sigma Fraction V) was used as protein standard. To 0.5 ml of sample solution (50 µg max) was added 3.25 ml reagent I and 0.3 ml reagent II after ten min. Samples were read after one hour on a DB-G Grating Spectrophotometer at a wavelength of 740 nm. Turbidity due to the presence of lipid was cleared by the addition of chloroform and centrifugation for 2 min at 2,000 rpm (MSE Mistral 6L). Chloroform was found to be more suitable than other lipid solvents such as ether, heptane and petroleum spirit, for routine determinations as it has a specific gravity greater than that of water.

2.6.3 Phospholipid Estimations

The calorimetric estimation of inorganic phosphate was utilised as a measure of phospholipid present in the sample. Inorganic phosphate was measured according to the method of Bartlett (1959) and Morrison
(1964). Aliquots of sample for analysis were evaporated to dryness and
digested with concentrated sulphuric acid (0.2 ml) at 200°C for 30 min.
A 30% solution of hydrogen peroxide (Baker, phosphorous free) was added
dropwise for bleaching. Samples were heated for a further 40 min to
decompose the hydrogen peroxide and then allowed to cool. Colour
development was carried out with the addition of 0.22% ammonium molybdate
(4.6 ml) and 0.2 ml Fiske-Subbarow reagent to the samples and placing
them in a boiling water bath for 15 min. Blanks consisting of concentrated
sulphuric acid and standards of known phosphorus content (1-8 μ, aliquots
of stock solutions of di-potassium hydrogen or orthophosphate) were run
simultaneously omitting the addition of hydrogen peroxide and subsequent
heating for 40 min. Samples were read in a Beckman DB-G Grating
Spectrophotometer at 820 nm.

2.6.4 Serum Cholesterol and Triglyceride Concentrations

Triglyceride and cholesterol were estimated using a Technicon
Autoanalyser II (Autoanalyser Methods 24a and N78 (1965) for triglyceride
and cholesterol respectively as in 1971 Manual). The Lieberman-Burchard
reaction for calorimetric estimation of cholesterol is used in this
method. Triglycerides were determined by fluorimetry, using a modification
of the method of Kessler and Lederer (1965). Aliquots of 0.5 ml serum
were extracted in 9.5 ml isopropanol (redistilled in order to decrease
high background fluorescence). The samples were rotated for 30 min and
then spun at 4°C at 2,000 rpm for 20 min (MSE, Mistral 6L centrifuge)
following the addition of 2 g zeolite mixture (zeolite:Lloyd reagent:cupric
sulphate:calcium hydroxide = 400:20:10:20 (w/w)). The supernatant was then
decanted off. Known standards of both cholesterol (SMA Reference Serum
Extract, cholesterol) and triglyceride (Triolein, Calbiochem) were treated
in the same way as test samples. Smaller aliquots (0.1-0.5 ml) of samples
with very high triglyceride content were taken for analysis.

2.7 PREPARATION OF UREA

In order to minimise the formation of cyanate and subsequent carbamylation of the apoproteins, urea was deionised immediately before use by passing 40% (w/v) urea through Amberlite MB3 (Rohm and HAAS, Philadelphia, USA) or Rexyn I-300 (Fisher Instr. Co.) columns at 4°C.

The column eluants were lyophylised and the solid urea obtained was used immediately, for example, for polyacrylamide gels. Urea buffers (6 M) were prepared by adding the appropriate buffering reagent and water to reduce the urea concentration from 40% to 36%.

2.8 ELECTROPHORESIS

Lipoprotein samples were electrophoresed as described in the Gelman Manual (1968) using cellulose polyacetate (Sepaphore III) as support medium. Oil Red O (Gelman, Ann Arbor, Mich. USA) and Amido Schwartz 10b (George T. Gurr, Searle Scientific Services, Bucks. Eng.) were used as lipid and protein stains respectively.

2.9 IMMUNOLOGY

Immunodiffusion and immunoelectrophoresis using 1% agar (agar no.1, Oxoid, Consolidated Labs., Chicago, Ill. USA) was performed according to the method of Ouchterlony (1968).
2.10 RAT DIETS

Control Diet

Rats were fed on a diet of commercial rat pellets (Mecon Rat and Mouse Diet: Mecon Agricultural Products, Eastwood, NSW). The composition of the diet is shown in Table 2.2.

Saturated Fat Diet

The composition of the saturated fat diet is shown in Table 2.3. The diet was made up by hand in 10 kg batches and stored at 4°C.

Sucrose and Fructose Diets

Rats were fed the commercial diet and a supplement of sucrose 40% (w/v) or fructose 20% (w/v) in the drinking water.

Olive Oil Cholesterol Diet

Rats were fed on the commercial diet which had been soaked in olive oil containing cholesterol such that the diet contained 10% (w/w) olive oil and 1% (w/w) cholesterol.
Table 2.2
Composition of Commercial Rat Pellets*

<table>
<thead>
<tr>
<th>Calculated Analysis**</th>
<th>% Total Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
<td>21.9</td>
</tr>
<tr>
<td><strong>Amino Acids:</strong></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>1.16</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.17</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.41</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.28</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>0.27</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.57</td>
</tr>
<tr>
<td>Fibre</td>
<td>5.3</td>
</tr>
<tr>
<td>Total Fat(^a)</td>
<td>3.2</td>
</tr>
<tr>
<td>Digestible Energy</td>
<td>1430 Kcal/lb</td>
</tr>
<tr>
<td>Ca</td>
<td>1.7</td>
</tr>
<tr>
<td>P</td>
<td>1.2</td>
</tr>
<tr>
<td>Ca:P</td>
<td>1.4:1</td>
</tr>
<tr>
<td>Na</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*Mecon Rat and Mouse Diet Manufactured from: Barley (9.8%), Hominy (11.4%), Wheat (19.6%), Oats (9.8%), Meat Meal (12.3%), Fish Meal (4.9%), Skim milk powder (8.10%), Bran (10.8%), Pollard (9.1%), Soyabean Meal (3.9%), Salt (0.2%), Vitamin\(^b\) and Mineral\(^c\) Supplement R22Q (0.2%).

**As supplied by the company.

\(^a\)Composition of Fat: Triglyceride (66.5%), Cholesterol (0.07%), Phospholipid (6.88%), Other (26.5%). (Value kindly supplied by Dr C. West, J.C.S.M.R.)

\(^b\)Vitamin Supplement: A 333 IU/lb, D\(_3\) 666 IU/lb, E 10 IU/lb, B\(_1\) 1.3 g/ton, B\(_2\) 2.6 g/ton, B\(_6\) 1.3 g/ton, B\(_12\) 6.6 mg/ton, Niacin 20 g/ton, Calcium Pantothenate 6.6 g/ton, Choline Chloride 100 g/ton.

\(^c\)Minerals: Mn, Fe, Cu, Zn, Mg, Co, I.
## Table 2.3

### Saturated Fat Diet

<table>
<thead>
<tr>
<th>Constituent</th>
<th>% Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (Casein)</td>
<td>18</td>
</tr>
<tr>
<td>Carbohydrate (Sucrose)</td>
<td>72.5</td>
</tr>
<tr>
<td>Fat (Dripping or Lard)</td>
<td>5.0</td>
</tr>
<tr>
<td>D-L Methionine</td>
<td>0.23</td>
</tr>
<tr>
<td>Vitamin Mix(^a)</td>
<td>0.27</td>
</tr>
<tr>
<td>Salt Mix(^b)</td>
<td>4.0</td>
</tr>
</tbody>
</table>

\(^a\) **Vitamin Mix:** Per 100 g Diet: Thiamine HCl 2.2 mg; Riboflavin 2.2 mg; Nicotinic acid 9.9 mg; B\(_6\)HCl 2.2 mg; Calcium pantothenate 6.6 mg; Folic acid 198 µg; Biotin 44.0 µg; p-Aminobenzoic acid 11 mg; Inositol 11 mg; B\(_12\) 3.0 µg; Choline Chloride 165 mg; Vitamin A 1982 IU; Vitamin D 220 IU; Vitamin F 11 IU; Menadione 4.95 mg.

\(^b\) **Salt Mix:** Per 100 g Diet: CaCO\(_3\) 1.5266 g; CoCl\(_2\) 6H\(_2\)O 8.0 µg; CuSO\(_4\)·5H\(_2\)O, 1.92 mg; FeSO\(_4\)·7H\(_2\)O, 0.108 g; MgSO\(_4\), 0.2292 g; MnSO\(_4\)·2H\(_2\)O, 17.8 mg; KI 3.16 mg; KH\(_2\)PO\(_4\), 1.556 g; NaCl, 0.5572 g; ZnCl\(_2\), 1.04 mg.

**Manufacturers:** Casein: Prime Lactic Casein, M.E. Cottey, Sydney.
CHAPTER 3

THE IODINATION OF VERY LOW DENSITY LIPOPROTEIN
CHAPTER 3

THE IODINATION OF VERY LOW DENSITY LIPOPROTEIN

Part I

COMPARISON OF THE RADIOIODINATION PROPERTIES OF HUMAN, RAT AND PIG VERY LOW DENSITY LIPOPROTEINS

3.1 INTRODUCTION

The use of tracers, particularly radioactive tracers have made feasible many studies of transfer rates, rates of reactions, the tracing of metabolic pathways and turnover of substances in living systems. During investigations of the metabolism of plasma proteins in man and other animals (Campbell et al., 1956), it was shown that if animals were allowed to make their own labelled plasma proteins from labelled amino acids the subsequent behaviour of the proteins was difficult to follow because the labelled amino acids were continuously being recycled. This difficulty was overcome by transferring the labelled molecules shortly after they have been made to a second unlabelled animal. Since only 2% of the label appeared in the circulating proteins of the donor animal this method was unavoidably wasteful of labelled amino acids. An alternative method is the use of in vitro labelling of plasma proteins with radioactive iodine. The usefulness of this technique, its advantages and disadvantages have been the subject of many investigations using mainly plasma proteins and peptide hormones, (Volwiler et al., 1955; Berson et al., 1953; Masouredis and Beeckmans, 1955). One important advantage in utilizing radioactive iodine as a tracer is that after tracer catabolism the label is readily removed from the circulation and excreted so that an insignificant proportion of the total radioactivity counted is
due to free radionuclide. This is not the case with radioactive labels such as $^{35}$S, $^{14}$C or $^3$H. Also, these labels present difficulties because they are recycled.

The radioiodinated plasma proteins should ideally be identical with the native protein in all physical, chemical and biological properties. This is almost impossible but carefully iodinated proteins are usually reported as indistinguishable from native protein by in vitro tests. Their metabolic behaviour, however, may be different. Labelled proteins may have an abnormally high initial turnover rate, providing evidence of gross denaturation. Minor degrees of tracer protein denaturation may not be so obvious. If the tracer protein molecules are all similarly damaged an apparently exponential rate of biological decay may be noted (McFarlane, 1956; Cohen et al, 1956; Rosa et al, 1969).

Denaturation of proteins labelled using radioactive iodine could be due to three main causes: firstly, possible self-irradiation damage caused by excessively high levels of radioactivity being introduced into the protein; secondly, alteration of the molecules caused by the attachment of large numbers of iodine atoms per molecule of protein so that it is recognised as different to the native protein by the host animal into which it is injected; and thirdly, damage caused by powerful oxidising agents commonly used in iodination procedures. The damage may be due to the oxidation of labile residues such as methionine, tryptophan and cysteine which eventually leads to denaturation or at least a significant alteration in the conformation of the protein (Butt, 1969; Marchalonis, 1969).

The techniques used to label proteins with radioactive iodine have been mainly restricted to modifications of two main methods, the iodine monochloride technique (McFarlane, 1958), and the chloramine-T method.
(Greenwood et al, 1963). Disadvantages of these methods are that self irradiation damage may occur with either method if the specific activity is too high, over-iodination can occur particularly with the iodine monochloride method and oxidation is the greatest drawback of the chloramine-T method. Apart from the iodine monochloride and chloramine-T methods, enzymatic iodination with lactoperoxidase (Marchalonis, 1969) and electrolytic iodination have also been used for protein iodination.

Some of the methods used and metabolic studies associated with the radiiodination of plasma proteins and lipoproteins have been reviewed by Fidge (Fidge, 1974). Although many investigators have reported using iodinated lipoproteins for in vitro and in vivo investigations there is a paucity of detailed information available on the labelling of lipoproteins and their subsequent metabolism. Little attempt has been made to compare the suitability of different labelling techniques for iodinating lipoproteins or to compare the effects of these procedures on the subsequent metabolism of lipoproteins. Therefore before a study of the metabolism of rat VLDL could proceed a suitable method for labelling VLDL protein was required. The possibility of utilising VLDL labelled in vivo from radioactive amino acids was considered as first priority. However, previous data obtained in this laboratory had indicated that the cost involved in this procedure was prohibitive since only trace amounts of the administered amino acid were recovered in VLDL protein. This low yield was found when both $^3$H-labelled lysine and leucine were used as precursors, as well as $^{35}$S-labelled methionine and cysteine. For these reasons, an alternative cheaper method of labelling VLDL protein was required. Since in vitro labelling with radioiodine had proved satisfactory for many other plasma proteins and in some cases no biological differences had been observed between protein labelled in vivo with $^{14}$C amino acids and in vitro with $^{125}$I, it was decided to investigate the
validity of utilising $^{125}$I-labelled VLDL for metabolic studies in rats.

One major difficulty arose during preliminary iodination of rat VLDL. Unlike human VLDL, which had been labelled with $^{125}$I (90% in the protein moiety) and used by other investigators in biochemical studies, rat VLDL was found to contain most of the radioiodine (60-70%) in the lipid moiety. Besides being wasteful of radioiodine, it was disadvantageous in that these studies were aimed at investigating the metabolism of the protein moieties of VLDL and the results could possibly be obscured by the presence of large amounts of labelled lipid. For this reason preliminary studies were extended to compare the labelling of rat VLDL with human VLDL and since pig VLDL was readily available in the laboratory it was also included in the investigation. In addition, several different iodination techniques were investigated, including the iodine monochloride, chloramine-T and enzymatic methods. Several parameters were used to assess the suitability of these methods for iodinating VLDL, including:

(i) intramolecular distribution of radioactivity;
(ii) moles of iodine bound per mole of protein (I/P ratio); and
(iii) the biological behaviour of the iodinated VLDL.

3.2 EXPERIMENTAL

Rat serum was obtained from fasted (8 hr) male rats. Human serum was obtained from healthy fasting (12 hr) male subjects and pooled and pig serum was obtained from blood collected from fasted (12-16 hr) animals at the Canberra abattoirs. The VLDL was isolated and the protein concentration determined as described in Chapter 2.

3.2.1 Iodination of VLDL

Four methods were used to iodinate VLDL:

(a) the iodine monochloride method (McFarlane, 1958);
(b) the chloramine-T method (Greenwood et al., 1963);
(c) the iodine volatilisation method (Butt, 1969); and
(d) enzymatic radioiodination using the lactoperoxidase method (Marchalonis, 1969).

Since pH has been shown to alter the lipid/protein bound iodine following iodination of LDL (Langer et al., 1972) the effect of pH was tested in each case. The procedures used in these studies are as follows.

(a) **Iodine monochloride method.** VLDL solutions were brought to pH 6.0, 8.0, 10.0 or 11.0 by dialysis against 0.4 M potassium phosphate buffer (pH 6.0), 0.4 M borate buffer (pH 8.0) or 0.4 M glycine-NaOH buffer (pH values 10.0 and 11.0). Aliquots of these dialysed VLDL solutions were diluted with the appropriate buffer to the required concentration and a final volume of 2.0 ml. In separate tubes, varying amounts (ranging from 2-50 µl) of 0.0033 M iodine monochloride in 2 M NaCl were diluted to 50 µl with 2 M NaCl. To each tube was added 50 µl of Na$_{125}$I containing 0.2-1.0 mCi of $^{125}$I-labelled iodide in dilute NaOH and the contents mixed. By keeping the molar ratio of $^{125}$I:$^{127}$ICl < 1 (the ratio in these iodination was < 10^{-5}) the formation of iodine was kept to a minimum (mostly $I_3^-$ being formed) and the efficiency of the iodination was increased by increasing the amount of HOI available for iodination. At alkaline pH the following reaction

$$ICl + NaOH \rightarrow HOI + NaCl$$

occurs. The HOI then substitutes on the tyrosinate ion at alkaline pH to give iodoxyrosine. The HOI is unstable, and is converted to iodine and iodate at alkaline pH within a few minutes. I$_2$ is a less efficient iodinating agent than HOI. The iodination mixture containing VLDL and ICl/$^{125}$I was therefore mixed immediately on a vortex stirrer after the addition of ICl/$^{125}$I and applied to Sephadex G-50 columns, 0.6 cm x 15 cm,
previously equilibrated with the buffer used in the iodination. A small aliquot of the iodination mixture was retained for radioassay. After radioassay of portions of the column fractions protein bound radioactivity was pooled and transferred to dialysis tubing since previous experience had shown that considerable 'free' iodine was still present in the gel filtered lipoprotein solutions. Gel filtration was retained as a quick and effective means of removing the bulk of the free iodide and thus reduced the time of exposure of the lipoproteins to high levels of radiiodine, minimising the risk of self irradiation. After several changes of buffer over 4 hr, the labelled lipoproteins were further dialysed overnight against 0.15 M NaCl (pH 7.4, 1 mM EDTA). Following dialysis, aliquots of the $^{125}$I-labelled VLDL solutions were taken for radioassay and for the determination of protein and lipid bound radioactivity. The I/P ratio (number of iodine atoms bound per molecule of protein) was calculated from the efficiency of the labelling procedure and from the concentration of ICl and VLDL protein in the iodinating mixture.

$$\frac{I}{P} = \frac{\text{125}^1 \text{bound}}{\text{125}^1 \text{bound}_{ADDED}} \times \frac{\mu \text{ mol ICl in mixture}}{\mu \text{ mol VLDL protein in mixture}}$$

This calculation is based on the assumption that no isotopic discrimination occurred between the isotope $^{125}$I and the stable $^{127}$I present in the iodinating mixture.

(b) Chloramine-T method. VLDL of varying concentrations was dialysed to pH 8 and 10, and brought to a final volume of 1.0 ml with the appropriate buffer solution. To each solution was added 0.25 mCi of Na$^{125}$I in 50 µl dilute NaOH. After mixing, 170 µg of chloramine-T in 50 µl 0.4 M borate buffer, pH 8.0 was added and mixed followed by 225 µg of sodium metabisulphite in 50 µl of 0.05 M Tris-HCl, pH 8.2 to stop the reaction. The total contents were applied to Sephadex G-50 columns and
the labelled VLDL solutions were treated as described in section (a) above.

(c) Iodine volatilisation method. The reaction was carried out in the apparatus described by Butt (1969). To 0.2 ml VLDL, pH 8.0 in a small vial, was added 5 µl Na\textsuperscript{125}I (0.25 mCi). The vial was transferred to a larger tube equipped to hold a stopper whose base cleared the top of the vial by 1-2 mm. A small filter paper disc cut just short of the diameter of the stopper's base was moistened with 50 µl (125 µg) of chloramine-T solution and dried under a stream of air at room temperature. Then 50 µl of NaCl (450 µg) was spotted onto the base of the stopper to which was attached the above filter paper disc and the stopper replaced into the reaction tube. After 5 min, with gentle shaking, the reaction was stopped by the addition of 50 µl sodium metabisulphite (200 µg). The iodinating mixture was transferred to a larger tube, diluted to 2.0 ml with 0.4 M borate buffer pH 8.0, loaded onto a Sephadex G-50 column and eluted, pooled and dialysed as in (a).

(d) Enzymatic radioiodination. The technique used in these experiments was a modification of the peroxidase catalysed iodination reaction of Marchalonis (1969). To 100 µl of VLDL (150 µg protein), in 0.15 M NaCl (pH 7.4), was added 4 µl (2 µg) of lactoperoxidase* and 4 µl (0.25 mCi) of Na\textsuperscript{125}I. The reaction was initiated by the addition of 10 µl 8.8 mM H\textsubscript{2}O\textsubscript{2} and stopped at 3 or 10 min with 0.5 ml of 5 mM cysteine. Following completion of the reaction, the final volumes were adjusted to 2 ml with 0.15 M NaCl (pH 7.4), and after aliquots were removed for radioassay, the remainder applied to Sephadex G-50 columns and analysed as in (a).

*Kindly supplied by Professor G.L. Ada, John Curtin School of Medical Research, A.N.U., Canberra.
3.2.2 Determination of the Intramolecular Distribution of $^{125}$I

A small aliquot (5-10 µl) of each labelled preparation was added to 0.2 ml of a concentrated protein solution (Human Serum Albumin, 25%, Commonwealth Serum Laboratories, Melbourne), which was extracted with chloroform-methanol (2:1, v/v). After phase separation (Folch et al., 1957), the methanol was removed from the aqueous phase under nitrogen and the protein precipitated with 5% trichloroacetic acid (TCA). Centrifugation yielded a protein pellet which was dissolved in 1 M NaOH (2 ml) and radioassayed for protein bound $^{125}$I. The TCA supernatant was radioassayed to determine 'free' $^{125}$I. The lipid (chloroform) phase was evaporated and redissolved in (2 ml) ethanol to determine lipid radioactivity.

3.2.3 Determination of Biological Activity

The *in vivo* behaviour of various iodinated VLDL preparations were tested by injecting 1.0 ml of the samples in 0.5 M NaCl, pH 7.4, into the tail vein of male Wistar rats (John Curtin School Strain) weighing 250-270 g. Between 2 and $10 \times 10^6$ cpms were administered to each animal. Turnover studies were carried out in individual animals from which 0.1-0.2 ml blood was removed at various times by cardiac puncture under light ether anaesthesia, or in groups of animals from which individual rats were exsanguinated at different times to determine the rate of disappearance of plasma radioactivity. Because VLDL preparations varied in the extent of label introduced into the lipid moiety, the protein was isolated from the serum after extraction with chloroform-methanol (Section 3.2.2) and radioassayed for $^{125}$I content.
3.3 RESULTS

3.3.1 Iodine Monochloride Method

The results of labelling VLDL from different species using the iodine monochloride method is shown in Table 3.1. As expected, the efficiency of iodination was dependent on the concentration of lipoprotein and of iodine monochloride in the iodinating mixture. When low amounts of VLDL protein (0.75 n moles) were present, it can be seen that increasing the ICl concentration raised the efficiency of iodination from 2.5-8.7% (a-e). Using a similar range of ICl concentration but higher amounts of rat VLDL protein (i-k, 0.004 µ moles) the efficiency ranged from 15-32%. Human VLDL (l-o), when present in similar concentrations and with the same addition of ICl was iodinated at efficiencies ranging from 6.6-22.6%. The effect of pH on labelling rat VLDL was also investigated (f-h). Raising the pH of the reaction mixture increased the incorporation of iodine into the protein moiety. Determination of the µ moles of iodine bound to the protein (column 5) enabled calculation of the I/P ratio, also shown in Table 3.1.

It was apparent that the proportion of radioactive iodine attached to the lipid moiety was also dependent on the iodine monochloride concentration. With higher ICl concentrations, the percentage proportion of radioactive iodine in the lipid fraction decreased and this result was seen with VLDL of all three species tested. It is apparent that high percent lipid labelling is linked by necessity with low I/P ratios and that the reverse situation is true for incorporation into the protein moiety, that is, higher percentage protein iodination occurred when high I/P ratios were achieved. This effect was observed in VLDL of all three species although the proportion of $^{125}$I attached to the protein fraction of rat VLDL was significantly lower than in human VLDL whenever comparable
Table 3.1

Results of Radioiodination of VLDL from Different Species Using the Iodine Monochloride Technique

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Amount of Protein&lt;sup&gt;a&lt;/sup&gt; &lt;sup&gt;µ&lt;/sup&gt;mole x 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>ICl added&lt;sup&gt;b&lt;/sup&gt; &lt;sup&gt;µ&lt;/sup&gt;mole x 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>pH</th>
<th>Efficiency of Radio-iodination</th>
<th>Amount of Iodine Bound to Protein &lt;sup&gt;µ&lt;/sup&gt;mole x 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>I/P</th>
<th>Distribution of &lt;sup&gt;125&lt;/sup&gt;I&lt;sup&gt;-&lt;/sup&gt;&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Protein %</th>
<th>Lipid %</th>
<th>Free %</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Rat VLDL</td>
<td>0.75</td>
<td>6.6</td>
<td>10</td>
<td>2.5</td>
<td>0.17</td>
<td>0.23</td>
<td>38</td>
<td>60</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(b) Rat VLDL</td>
<td>0.75</td>
<td>16.5</td>
<td>10</td>
<td>3.3</td>
<td>0.54</td>
<td>0.72</td>
<td>54</td>
<td>43</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(c) Rat VLDL</td>
<td>0.75</td>
<td>33.0</td>
<td>10</td>
<td>4.7</td>
<td>1.55</td>
<td>2.0</td>
<td>43</td>
<td>54</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(d) Rat VLDL</td>
<td>0.75</td>
<td>82.5</td>
<td>10</td>
<td>5.3</td>
<td>4.37</td>
<td>5.80</td>
<td>53</td>
<td>45</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(e) Rat VLDL</td>
<td>0.75</td>
<td>165.0</td>
<td>10</td>
<td>8.7</td>
<td>14.56</td>
<td>19.50</td>
<td>70</td>
<td>29</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(f) Rat VLDL</td>
<td>1.50</td>
<td>26.4</td>
<td>6</td>
<td>0.7</td>
<td>0.17</td>
<td>0.11</td>
<td>26</td>
<td>54</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>(g) Rat VLDL</td>
<td>1.50</td>
<td>26.4</td>
<td>8</td>
<td>1.0</td>
<td>0.26</td>
<td>0.18</td>
<td>36</td>
<td>60</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>(h) Rat VLDL</td>
<td>1.50</td>
<td>26.4</td>
<td>11</td>
<td>0.5</td>
<td>0.14</td>
<td>0.10</td>
<td>45</td>
<td>49</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>(i) Rat VLDL</td>
<td>4.00</td>
<td>33.0</td>
<td>10</td>
<td>15.0</td>
<td>4.80</td>
<td>1.20</td>
<td>33</td>
<td>66</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(j) Rat VLDL</td>
<td>4.00</td>
<td>82.5</td>
<td>10</td>
<td>20.2</td>
<td>17.00</td>
<td>4.30</td>
<td>50</td>
<td>46</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>(k) Rat VLDL</td>
<td>4.00</td>
<td>165.0</td>
<td>10</td>
<td>32.0</td>
<td>50.60</td>
<td>12.70</td>
<td>64</td>
<td>34</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(l) Human VLDL</td>
<td>1.50</td>
<td>26.4</td>
<td>11</td>
<td>0.1</td>
<td>0.01</td>
<td>0.01</td>
<td>80</td>
<td>16</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>(m) Human VLDL</td>
<td>4.00</td>
<td>33.0</td>
<td>10</td>
<td>6.6</td>
<td>2.10</td>
<td>0.52</td>
<td>84</td>
<td>14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(n) Human VLDL</td>
<td>4.00</td>
<td>82.5</td>
<td>10</td>
<td>17.5</td>
<td>14.40</td>
<td>3.60</td>
<td>92</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(o) Human VLDL</td>
<td>4.00</td>
<td>165.0</td>
<td>10</td>
<td>22.6</td>
<td>37.20</td>
<td>9.30</td>
<td>95</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(p) Pig VLDL</td>
<td>2.00</td>
<td>16.5</td>
<td>10</td>
<td>0.2</td>
<td>0.02</td>
<td>0.01</td>
<td>39</td>
<td>46</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>(q) Pig VLDL</td>
<td>2.00</td>
<td>39.6</td>
<td>10</td>
<td>7.9</td>
<td>3.13</td>
<td>1.57</td>
<td>48</td>
<td>50</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(r) Pig VLDL</td>
<td>2.00</td>
<td>82.5</td>
<td>10</td>
<td>8.0</td>
<td>6.60</td>
<td>3.30</td>
<td>57</td>
<td>41</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Assumed that mol. wt. of apo-VLDL was 250,000.

<sup>b</sup>Na<sup>125</sup>I/ICl mixture (0.3 mCi Na<sup>125</sup>I) 50 µl was added to 2.0 ml of lipoprotein solutions.
I/P ratios were achieved. In human VLDL with low I/P ratios of 0.52 and 3.6 (n and o) 84 and 92% of the radioactive iodine was bound to the protein but it can be seen that when similar I/P ratios (b, c and j) were obtained with rat VLDL, only 54, 43 and 50% of the radioactivity was associated with the protein. Pig VLDL labelling characteristics were in this respect more like rat VLDL than human VLDL since only 48-57% of the $^{125}$I-labelled iodide was protein bound when I/P ratios of 1.6-3.3 were obtained (q and r).

3.3.2 Chloramine-T Method and Iodine Volatilisation Method

Results obtained using the chloramine-T method of radioiodination are described in Table 3.2. Lower efficiencies were obtained at pH 10.0 than at pH 8.0 and the concentration of VLDL protein in the iodination mixture had little effect on the efficiency. As with the ICl method, rat VLDL protein contained only a low percentage (32-40%) of the total radioactivity with 51-58% present in the lipid moiety. Human VLDL labelled under the same conditions, contained 70-83% of the total radioactivity bound to protein. Results obtained with the iodine volatilisation method are shown in Table 3.2. Although a high proportion of label was present in human VLDL protein the efficiency of iodination was very low.

3.3.3 Lactoperoxidase Method

Radioiodination of human and rat VLDL was also achieved when lactoperoxidase was used as shown in Table 3.3. The lower efficiencies achieved with this method can probably be accounted for by the lower enzyme activity present in this lactoperoxidase preparation (which was prepared nearly two years previously), and this explanation is confirmed by the results obtained with gamma globulin, iodinated at the same time.
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Amount of Protein Used (µmoles x 10^3)</th>
<th>pH</th>
<th>Efficiency of Radioiodination</th>
<th>Distribution of ^125^I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Rat VLDL^a</td>
<td>0.75</td>
<td>8</td>
<td>0.64</td>
<td>40</td>
</tr>
<tr>
<td>Rat VLDL^a</td>
<td>2.25</td>
<td>8</td>
<td>0.47</td>
<td>41</td>
</tr>
<tr>
<td>Rat VLDL^a</td>
<td>0.75</td>
<td>10</td>
<td>0.01</td>
<td>32</td>
</tr>
<tr>
<td>Rat VLDL^a</td>
<td>2.25</td>
<td>10</td>
<td>0.01</td>
<td>39</td>
</tr>
<tr>
<td>Human VLDL^a</td>
<td>2.25</td>
<td>10</td>
<td>0.01</td>
<td>70</td>
</tr>
<tr>
<td>Human VLDL^b</td>
<td>2.25</td>
<td>8</td>
<td>0.02</td>
<td>83</td>
</tr>
</tbody>
</table>

^a^ Each iodination mixture contained 170 µg chloramine-T in 50 µl borate buffer pH 8.0, 0.25 mCi Na^125^I in a final volume of 1.0 ml. The reaction was stopped by the addition of 225 µg of Na_2S_2O_5 in 50 µl 0.05 M Tris HCl pH 8.2.

^b^ Iodine volatilization method.
Table 3.3

Results of Radioiodination of Rat and Human VLDL using the Lactoperoxidase Method

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Amount of Protein Used $\mu$moles x $10^3$</th>
<th>pH</th>
<th>Time of Reaction min</th>
<th>Efficiency of Radioiodination %</th>
<th>Distribution of $^{125}$I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protein %</td>
</tr>
<tr>
<td>Rat VLDL</td>
<td>0.75</td>
<td>7.4</td>
<td>3</td>
<td>8.5</td>
<td>33</td>
</tr>
<tr>
<td>Rat VLDL</td>
<td>0.75</td>
<td>6.0</td>
<td>3</td>
<td>3.0</td>
<td>26</td>
</tr>
<tr>
<td>Human VLDL</td>
<td>0.75</td>
<td>7.4</td>
<td>3</td>
<td>0.6</td>
<td>75</td>
</tr>
<tr>
<td>Human VLDL</td>
<td>0.75</td>
<td>7.4</td>
<td>10</td>
<td>1.3</td>
<td>80</td>
</tr>
<tr>
<td>$\gamma$ globulin</td>
<td>0.94</td>
<td>7.4</td>
<td>3</td>
<td>5.0</td>
<td>95</td>
</tr>
</tbody>
</table>

Each iodination mixture contained 2 $\mu$g lactoperoxidase, 10 $\mu$l of 0.88 mM $H_2O_2$ and 0.25 mCi of Na$^{125}$I in a total volume of 118 $\mu$l. The reaction was stopped by the addition of 0.5 ml 5 mM cysteine.
and under similar conditions to the VLDL samples. The efficiency of labelling gamma globulin in these experiments was lower (5\%) than that obtained when the enzyme was freshly prepared.* The differences observed in the labelling pattern of rat and human VLDL by the previous methods was also apparent after enzymatic radioiodination. Only 26-33\% of the total radioactivity was incorporated into rat VLDL protein whereas 75-80\% became attached to human VLDL protein, the remainder in both cases being largely associated with the lipid fraction.

3.3.4 Effect of Increasing Iodine Atoms on Biological Activity

The rate of disappearance of radioiodinated rat VLDL (iodine monochloride method), with different I/P ratios from rat serum is shown in Figure 3.1. Each point shown on the graph is the mean result ± S.D. obtained from three rats and represents a percentage of the total radioactivity injected into each animal. The semilogarithmic plot of radioactivity against time described a curve for the first five hours but was straight thereafter.

In order to determine the number of exponential functions, the data was analysed by a 'curve peeling' process (Zilversmit, 1960) and each curve could be resolved into two components, an initial fast component and a later slow component. At I/P ratios of 0.05, 2.0, 4.0 and 12.5, the half-life of the fast component was 40, 63, 60 and 63 minutes. The corresponding half-lives for the slow component were 10.5, 9.5, 10.0 and 10.0 hours. The mean value for the slow component was 10.0 ± 0.6 hours.

It was also noticed that the percent of the injected dose remaining in the serum varied between different batches of VLDL. Thus in Figure

*G.L. Ada. Personal communication.
Figure 3.1  'In Vivo' Disappearance of Rat $^{125}$I-VLDL of Different I/P Ratios from Rat Serum. Each point represents the mean figure ± S.D. from these rats. Two different batches of rat VLDL were used for iodination (iodine monochloride method) and are represented in the figure by broken and unbroken lines.
3.1 it can be seen that the percent radioactivity remaining within the intravascular compartment after 5 minutes in experiments depicted by the broken lines (I/P ratios of 4 and 12.5) varied between 62-70% whereas 45-51% remained after 5 minutes in the animals into which VLDL of I/P ratios of 0.05 and 2.0 (unbroken lines) were injected. These differences are probably due to differences in the actual amount of VLDL injected rather than different I/P ratios since more material (about twice as much) was administered to the rats depicted by the broken lines than in those represented by the unbroken lines, although in both cases the total radioactivity (cpm) injected with kept constant.

The serum rate of disappearance of human VLDL radioiodinated at different I/P ratios (iodine monochloride method) after injection into rats, was also investigated (Figure 3.2). Similarly, as in Figure 3.1, each point shown on the graph is the mean result ± S.D. obtained from three rats and the radioactivity is expressed as the percentage of the total radioactivity injected.

The semilogarithmic plot of radioactivity against time described a curve for the first five hours. 'Curve peeling' revealed two exponential components, the faster being 50, 78, 50 and 68 minutes and the slowest 10.5, 10.0, 11.5 and 12.5 hours respectively for the human VLDL at I/P ratios of 0.5, 2.0, 3.5 and 10.0.

Similar results were obtained when human VLDL labelled by the lactoperoxidase method was administered to rats (Figure 3.3). Each point represents the mean ± S.D. from 5 animals. Although less radioactivity remained in the serum after 5 minutes, due to the fact that smaller amounts of VLDL were injected, the characteristics of the disappearance curve were identical to that achieved with human VLDL labelled by the iodine monochloride method. Half times for the fast and slowest removed
Figure 3.2 'In Vivo' Disappearance of Human $^{125}$I-VLDL of Different I/P Ratios from Rat Serum. Each point represents the mean figure ± S.D. from three rats. The same batch of human VLDL was used for iodination (iodine monochloride method) and broken and unbroken lines are only used for purposes of clarity.
Figure 3.3  'In Vivo' Disappearance of Human $^{125}$I-VLDL from Rat Serum, Iodinated using the Lactoperoxidase Method. Each point represents the mean figure $\pm$ S.D. from five rats.
component were 67 minutes and 11.0 hours respectively for this preparation of VLDL, compared to a mean of 61.5 ± 13.9 minutes and 11.1 ± 1.1 hours for iodine monochloride iodinated human VLDL.

3.4 DISCUSSION

The labelling of plasma lipoproteins with radioactive iodine has presented many interesting problems. The essential aim of this study was to achieve a product which was as similar as possible to the unlabelled lipoprotein in its physical properties and biological behaviour. A further problem with labelling of VLDL protein is the large amount of lipid which also has a high affinity for radioactive iodine (Lakshminarayana et al., 1972). One of the two alternative methods for studying lipoprotein protein metabolism, that of allowing the animal to make its own labelled protein from labelled amino acids is unavoidably wasteful of labelled amino acids. Less than 2% of the label appears in the circulating proteins of the injected animal (Campbell et al., 1956) and only about 0.2-0.5% of this circulating label is present in the VLDL protein (Fidge, unpublished observations). Although biosynthetically labelled amino acids of high specific activity and reasonable cost are now available, experience has shown that it is still too expensive to label VLDL protein, for reinjection experiments. Furthermore, a comparison of the biological behaviour of $^{14}$C amino acid labelled rat plasma proteins and $^{131}$I iodinated proteins has shown no significant differences between the two preparations (Campbell et al., 1956).

The results obtained in these studies showed striking differences in the labelling pattern of human, rat and pig VLDL when radioiodinated under similar conditions. Although the efficiency of labelling was similar in each species, the proportion of total radioactivity present in the lipid moiety was different, no matter what method of iodination was
used. The iodine monochloride technique allowed manipulation of the I/P ratio which was shown to affect the intramolecular distribution of radioactivity in VLDL from all three species used. With rat VLDL, low and high I/P ratios resulted in considerable differences in the labelling of the protein moiety (33-64%, i-k) whereas with human VLDL, over a comparable range of I/P ratios, protein labelling ranged from 84-95%.

When I/P ratios of 2 or less are achieved with rat VLDL the high amount of label in the lipid fraction renders the molecules unsuitable as a tool for studying protein metabolism. A similar situation also seems to exist after iodinating pig VLDL and the most satisfactory result was obtained with human VLDL at I/P ratio of 0.53, when 85% of the label was present in the protein moiety. The reason for the differences in labelling between the VLDL of the three species tested could possibly be attributed to differences in the tyrosine content of the VLDL apoproteins, which accounts for about 3.2% of the total amino acid composition (Levy et al, 1967) in human VLDL but only about 1.8 and 1.6 percent in rat and pig (Camejo, 1967; Koga et al, 1969; Fidge, 1973; Fidge and Calder, 1972) VLDL. Another explanation could be the difference in accessibility of the tyrosine residues to the $^{125}$I-labelled iodine brought about by a difference in the protein conformation in the different species.

Alternatively, differences in structural organisation of the lipid and protein components of VLDL of the three species may partly explain the variation in iodination patterns obtained since it is conceivable that the lipid moiety of rat VLDL may be more exposed to radioactive iodine than the same lipid moiety in human VLDL. Another important consideration in the rat is the degree of saturation of the fatty acids present in VLDL. The more unsaturated the fatty acids the greater the incorporation of $^{125}$I iodine into the lipid moiety of the VLDL. This possibility can be measured and as a consequence was investigated. It is described in Part II.
The biological characteristics of rat and human VLDL were studied by their \textit{in vivo} behaviour when reinjected into rats. Injection of protein from one species into another has been found by several other investigators to be an acceptable technique for studying the biological behaviour of other plasma proteins (Freeman, 1959) and lipoproteins (Langer \textit{et al}, 1972) since any protein denaturation or other damage can be detected by an alteration in their plasma turnover rates. In the present study the \textit{in vivo} behaviour of rat VLDL labelled only by the ICl method was examined, since it was found that other methods (chloramine-T and lactoperoxidase) produced lipoproteins with large amounts of $^{125}$I in the lipid moiety and were thus unsuited for studying the turnover of the protein moiety. Furthermore, iodination by the ICl method allowed manipulation of the I/P ratios, thus providing an opportunity for studying the effects of excessive iodination on the disappearance of rat VLDL. With human VLDL, both the enzymatically and the ICl labelled preparations were tested, since both were predominantly protein labelled and because it has been suggested that enzymatic iodination by lactoperoxidase is more suitable for preserving the biological activity and structural integrity of labelled proteins (Frantz and Turkington, 1972; Miyachi and Chrambach, 1972).

When rat VLDL of different I/P ratios were tested the biological half-lives were not markedly different with preparations of I/P from 0.05-12.5 (Figure 3.1). A similar effect was observed with human VLDL, although there was some evidence to suggest that the preparation with I/P of 10 was cleared less rapidly after 5 min than VLDL with lower I/P ratios (Figure 3.2). In this study with human VLDL, unlike that with the rat VLDL, all the animals were injected with equal amounts of lipoprotein protein so that any differences in radioactivity remaining after 5 min could not be attributed to different pool sizes of VLDL.
triglyceride or protein. In this respect, it was interesting to note that the rat preparation of I/P 12.5 was also cleared less rapidly after 5 min, so it is possible that 'over iodination' causes some initial retardation in the catabolism of $^{125}$I labelled VLDL. The catabolism of enzymatically and ICI-labelled human VLDL was similar at I/P ratios 3.5 or less (Figure 3.3).

After five minutes circulation both rat and human VLDL were removed from the vascular compartment at similar exponential rates which could be resolved into a fast and slow component. With human VLDL it was apparent that there was no species discrimination in its metabolism by the rat. Since the turnover of VLDL triglyceride is a rapid process in the rat (Schotz et al, 1964), it is not unexpected that the VLDL protein would also be catabolised quickly.

In conclusion it has been shown that VLDL of rat and pig separated from animals without manipulation of diets from the normal laboratory chow are less suited to iodination (by all methods used here) than human VLDL. Human VLDL appears to be suitable for in vivo studies when I/P ratios vary between 0.5-3.5 and after iodination by the lactoperoxidase method. However of all the methods used to iodinate rat VLDL, the iodine monochloride method was chosen as the most suitable method as the conditions of iodination, particularly I/P ratio, could easily be varied.
Part I

3.5 SUMMARY

Very low density lipoprotein (VLDL) isolated from human, pig and rat serum showed marked differences in labelling patterns when radioiodinated under similar conditions, with greater amounts of $^{125}$I-labelled iodine being incorporated into the lipid moieties of pig and rat VLDL than into human VLDL. The effect (on their biological behaviour) of introducing increasing amounts of iodine into the lipoproteins was tested for both human and rat VLDL with preparations containing I/P ratios varying from < 0.5 to 12. After injection into rats and analysis of disappearance curves, only minor differences in catabolic rates were observed between different preparations. Thus, rat VLDL, with I/P ratios varying from < 0.5-12 showed fast and slow exponential components varying in half-life ($T^{1/2}$) between 40-63 min and 10-11 hr respectively, while human VLDL with comparable I/P ratios, had a fast component varying from ($T^{1/2}$) 50-78 min and a slower component ($T^{1/2}$) of 10.5-12.5 hr. The plasma disappearance of human VLDL, labelled by the lactoperoxidase method, was also characterised by two exponential components with mean half-lives of 67 min and 11 hr respectively, suggesting similarity in the biological behaviour of human VLDL labelled both enzymatically and with iodine monochloride. The results also indicated similarity in the catabolism of rat and human VLDL by the rat, suggesting that this animal is a suitable model for studying VLDL metabolism.
Part II

THE EFFECT OF A SATURATED FAT DIET ON THE RADIOIODINATION PROPERTIES OF RAT VERY LOW DENSITY LIPOPROTEIN

3.6 INTRODUCTION

In Part I of this chapter it was demonstrated that under identical labelling conditions, a larger proportion of radioiodine was found in the lipid moiety (30-60%) of rat VLDL than human VLDL. One method considered as a possible means of reducing the large amount of iodine bound to the lipid moiety of rat VLDL, was to reduce the number of unsaturated bonds of the fatty acid moiety. Preliminary experiments revealed that VLDL obtained from rats fed a saturated fat diet for 10-14 days could be iodinated with > 90% of the label binding to the protein, at I/P ratios of < 1 and efficiencies of iodination of approximately 10%.

Experiments were then performed to:

(i) investigate the nature of the changes involved in the alteration of iodination characteristics of the rat VLDL;

(ii) detect any alterations in biological activity due to a different saturated to unsaturated fatty acid ratio; and

(iii) to determine whether the diet significantly altered the VLDL apolipoprotein constituents, since other dietary manipulations (Roheim et al, 1973) had been shown to alter the proportion of VLDL apoprotein subunits.

Also, in certain cases of hyperlipoproteinemias or other abnormalities (Shore et al, 1974) changes in VLDL apoprotein composition have been detected.
The composition of the saturated fat and the commercial chow diets is shown in Tables 2.2 and 2.3 of Chapter 2. Male rats were used in these studies to eliminate sex differences and all rats were bled 9-11 am to avoid differences due to diurnal variation. Some rats were fasted for 6-8 hr and others for 16 hr (overnight) before exsanguination.

3.7.1 Lipid Analyses

Extraction. Total lipids were extracted from 0.5 ml aliquots of serum with 10 ml chloroform:methanol, 2:1 (v/v) (Folch et al, 1957). The resultant protein precipitate was removed by filtration (Whatman No. 1 paper), the filter being washed three times with 1 ml of chloroform:methanol. Phase separation was achieved by the addition of 0.2 volumes of 0.73% sodium chloride solution. The lower, chloroform, phase was retained for subsequent analysis. Total VLDL lipids were similarly extracted with chloroform:methanol. Serum cholesterol and triglyceride concentrations were estimated using a Technicon Auto analyser II, as described in Chapter 2.

Separation of major lipid classes by thin layer chromatography. The lipids obtained by evaporation of the chloroform phase under nitrogen were separated by thin layer chromatography on silica gel plates (Kuselgel G nachstahl, type 60; 0.3-0.4 mm thick) using hexane, diethyl ether, methanol and acetic acids in the proportions 180:40:6:4 respectively, as solvent. The separated bands were visualised after spraying with Rhodamine G (George T. Gurr, Div. Baird & Tatlock, Romford, Eng.) under ultraviolet light. A standard mixture containing palmitic acid, tripalmitic acid, cholesterol palmitate and free cholesterol was chromatographed on the same plate as each test sample.
Cholesterol ester, triglyceride, free fatty acid and cholesterol bands were scraped off the plates and eluted with 20 ml diethyl ether. Phospholipid was eluted as described by Skipski et al. (1964) eluting twice with chloroform:methanol:acetic acid:water (25:15:4:1.9) then washed with methanol and methanol:acetic acid:water (94:1:5). Lipid fractions were then evaporated under nitrogen and dissolved in ethanol.

3.7.2 Gas Liquid Chromatography

Samples for Gas Liquid Chromatographic (GLC) analyses were extracted with chloroform:methanol (Folch et al., 1957). After evaporation of the chloroform layer under nitrogen the methyl esters were prepared by adding 4 ml methylating reagent (4% sulphuric acid in dry methanol) and standing for 16 hr at room temperature. The methyl esters were extracted by the addition of 1 ml water and 5 ml heptane to the methylating reagent. After phase separation the heptane layer (upper phase) containing the methyl esters was concentrated to approximately 100 µl under nitrogen or if not evaporated immediately, stored at 4°C in sealed containers. Aliquots of 1-2 µl were injected into the gas chromatograph. Fatty acids present in the major lipid classes separated by thin layer chromatography were analysed by scraping the respective bands off the plates into glass stoppered tubes. The methyl esters were prepared as described above and phase separation was achieved by the addition of 5 ml water and 5 ml heptane.

GLC analyses were performed with a Packard Gas Chromatograph (Model 824) with nitrogen as carrier gas. Separations were carried out on a circular 1.5 m x 4 mm i.d. column packed with 13% ethylene-glycol adipate on 80-100 mesh Gas Chrom Chromosorb P (Applied Science Laboratories, Inc., State College, Pa. USA). The column was operated under the following conditions:-
Carrier gas (nitrogen) flow rate 40 psi  
Inlet Temperature 200°C;

Hydrogen flow rate 40-50 psi  
Column Temperature 187°C;

Air flow rate 400 psi  
Detector Temperature 210°C.

For identification of fatty acids, retention times relative to methyl stearate were compared to those of pure reference compounds and to N.I.H. type mixtures (Horning et al, 1964), D-99 (14:0, 16:0, 16:1, 18:0, 18:1) and D-104 used as a quantitative aid (14:0, 16:0, 18:0, 18:1, 18:2, 18:3, 20:0, 22:0, 22:1, 24:0), (Serdery Research Laboratories, London, On. Canada). Reference standards and single fatty acids were injected at the commencement of each days run and at intervals during the day. The respective weights of the fatty acids was calculated from the area of the peaks determined with an Infotronics Digital Integrator (Model CRS-208) and also by hand, by measuring the retention time (distance from the solvent front, mm) and height of the peak (mm).

\[ \text{Area} \times \text{Retention Time} = \frac{\text{Peak Height}}{2} \times \text{Retention Time} \]

Each fatty acid was then expressed as a percentage of the major fatty acids present in the injected sample. The relative differences between replicate analyses were less than 2% for the major components and 0.1-3% for the minor components (2-10% of the total mixture). Results using the integrator agreed with those calculated by hand (less than 2% difference). An example of the separation obtained for serum is shown in Figure 3.4.

**Apoprotein Analysis** VLDL samples were delipidated using chloroform: methanol, the apoproteins solubilised and separated either by polyacrylamide gel electrophoresis (7.5% acrylamide) and scanned or by ammonium bicarbonate solubilisation. These techniques are described in detail in Chapters 4 and 5.
Figure 3.4  Separation of the Methyl Esters of the Total Fatty Acids of Rat Serum. Serum samples were extracted, methylated and analysed as described in the text. The trace shows a typical pattern obtained. Similar patterns were obtained for total VLDL lipids. The length of the carbon chain and the number of unsaturated double bonds are indicated with the relevant peak.
3.8 RESULTS

3.8.1 Rat Diets

A study by McLeod (1973) demonstrated that rats fed on a synthetic diet (containing soybean oil instead of beef dripping as fat) exhibited similar growth curves to rats fed on a commercial rat chow. Major differences between the two diets used in this study, were the content and composition of the fat. The commercial rat chow contained 3.2% fat and the saturated fat diet 5% and the ratio of saturated:unsaturated fatty acids was higher in the latter diet. Beef dripping (obtained locally) was utilised in all batches of saturated fat diet as the only source of fat. There was little variation in the fatty acid composition of different batches of beef dripping. This was reflected in the relative constancy of the fatty acid composition of the different batches of saturated fat diet (Table 3.4). Digestible energy in both diets was provided in the form of carbohydrate, 72.5% in the saturated fat diet and approximately 67% in the commercial rat chow, which also contained 5.3% fibre. The saturated fat diet did not contain any fibre. If this factor is excluded, the carbohydrate content of the two diets is similar.

The major differences in the fatty acid composition of the two diets were in the proportion of linoleic acid (18:2) (31.8% in the commercial chow and 3.1 to 8.3% in the saturated fat diet) and oleic acid (18:1) (32% in control and 45% in saturated fat diet) thus, the degree of saturation of the latter diet was considerably higher.

3.8.2 Effect of the Saturated Fat Diet on Serum and VLDL Fatty Acid Composition

Changes in total serum and VLDL fatty acids, after 10 days on the saturated fat diet, shown in Table 3.5, reflected that of the diet.
Table 3.4
Fatty Acid Composition of Beef Dripping, Control and Saturated Fat Diet

<table>
<thead>
<tr>
<th>Sample</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef Dripping</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 1</td>
<td>3.2 ± 0.1</td>
<td>21.6 ± 1.6</td>
<td>7.6 ± 1.3</td>
<td>13.4 ± 0.3</td>
<td>49.9 ± 2.0</td>
<td>3.5 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>Tr</td>
</tr>
<tr>
<td>Batch 2</td>
<td>2.6 ± 0.1</td>
<td>20.1 ± 0.4</td>
<td>3.9 ± 0.4</td>
<td>20.1 ± 0.3</td>
<td>48.3 ± 0.3</td>
<td>3.4 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>Tr</td>
</tr>
<tr>
<td>Saturated Fat Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 1</td>
<td>5.4 ± 0.9</td>
<td>23.3 ± 2.0</td>
<td>6.6 ± 1.4</td>
<td>13.6 ± 0.1</td>
<td>47.3 ± 1.0</td>
<td>3.1 ± 0.0</td>
<td>0.7 ± 0.1</td>
<td>Tr</td>
</tr>
<tr>
<td>Batch 2</td>
<td>5.8 ± 0.2</td>
<td>24.7 ± 1.2</td>
<td>5.9 ± 0.6</td>
<td>14.7 ± 1.1</td>
<td>45.3 ± 1.3</td>
<td>3.6 ± 0.9</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>Batch 3</td>
<td>3.8 ± 0.1</td>
<td>24.9 ± 0.7</td>
<td>6.7 ± 0.4</td>
<td>9.5 ± 0.9</td>
<td>45.4 ± 1.0</td>
<td>8.3 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Control Diet (Mecon Rat and Mouse Diet)</td>
<td>1.5 ± 0.1</td>
<td>20.0 ± 0.6</td>
<td>3.1 ± 0.7</td>
<td>9.6 ± 0.6</td>
<td>32.0 ± 1.1</td>
<td>31.8 ± 1.9</td>
<td>2.0 ± 0.4</td>
<td>Tr</td>
</tr>
</tbody>
</table>

*a* Represented as chain length:number of double bonds.

*b* Figures represent the average of three separate determinations on three samples ± S.D. Weighed amounts of diet were ground and extracted using chloroform-methanol as described in the methods.

Tr (Trace) = <0.5% present.
Table 3.5

Fatty Acid Composition of Serum and VLDL Obtained from Rats Fed the Saturated Fat Diet and Control Diet

<table>
<thead>
<tr>
<th>Sample</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:4</th>
<th>Cholesterol&lt;sup&gt;b&lt;/sup&gt; (mg/100 ml serum)</th>
<th>Triglyceride&lt;sup&gt;b&lt;/sup&gt; (mg/100 ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.9+0.2</td>
<td>23.3+0.7</td>
<td>1.4+0.2</td>
<td>11.2+0.3</td>
<td>18.3+0.4</td>
<td>29.9+0.5</td>
<td>0.9+0.5</td>
<td>14.1+0.3</td>
<td>63+6.2</td>
<td>90+9.3</td>
</tr>
<tr>
<td>Saturated (10 days)</td>
<td>0.6+0.1</td>
<td>24.8+0.3</td>
<td>6.4+0.4</td>
<td>10.0+0.02</td>
<td>35.5+0.7</td>
<td>9.7+0.4</td>
<td>0.5+0.1</td>
<td>12.5+1.1</td>
<td>57.5+2.1</td>
<td>98+2.8</td>
</tr>
<tr>
<td>VLDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.0+0.2</td>
<td>25.2+0.6</td>
<td>3.2+0.1</td>
<td>7.2+0.1</td>
<td>28.5+1.6</td>
<td>29.6+0.6</td>
<td>0.9+0.4</td>
<td>4.4+0.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Saturated (10 days)</td>
<td>1.9+0.1</td>
<td>24.4+0.4</td>
<td>6.8+0.5</td>
<td>6.1+0.4</td>
<td>50.4+1.4</td>
<td>7.5+0.6</td>
<td>0.6+0.4</td>
<td>2.3+0.2</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values represent the mean ± S.D. of three separate determinations. Aliquots (0.5 ml) from pooled serum of 16 rats (fasted 6-8 hr) were analysed. Aliquots of VLDL isolated from the pooled sera were treated as described in the text.

<sup>b</sup>Cholesterol and triglyceride concentrations of control and saturated serum were not significantly different. Probabilities were .37 and .35 and t = 1.066 and 1.116 respectively.
No apparent change in serum cholesterol and triglyceride concentrations was observed (Table 3.5). Notable changes occurred in the linoleic acid (18:2) content, which fell from 29.9 to 9.7% in serum and 29.6 to 7.5% in VLDL. This decrease was accompanied by increases in both oleic (18:1) and palmitoleic acids (16:1). This trend was reflected in each of the major lipid classes (phospholipid, free fatty acids, triglycerides and cholesterol esters) in serum (Table 3.6) and VLDL (Table 3.7). No other major changes were observed. The content of arachidonic acid (20:4) was apparently not affected by the change in diet. A slight decrease in eicosaenoic acid (20:1) in both VLDL and serum was observed. The content of arachidonic acid was highest in cholesterol esters of serum and phospholipids of VLDL.

The rate at which the change in serum fatty acid composition occurs after commencement of the saturated fat diet is shown in Table 3.8. Rats were allowed free access to food and water and after 24 hr intervals were fasted for 16 hr before exsanguination. Under these conditions a gradual decrease in 18:2 (26% to 15.6%) and slight increase in the content of 16:1 was observed (2% to 7%). The decrease in 18:2 was significant after only 24 hr on the saturated fat diet (p < .01). A slight decrease in 18:3 content and increase in 18:1 was also observed. There was no change in arachidonic acid or in total cholesterol and triglyceride concentrations in serum, as shown in Table 3.8.

Rats fed the saturated fat diet for 1 to 10 days and fasted for only 4-5 hr prior to exsanguination, exhibited major changes in both serum and VLDL after 24 hr on the diet. Linoleic acid content dropped from 25% in control to 12% in 24 hr. This was reflected by increases in palmitic (16:0), palmitoleic (16:1) and oleic (18:1) acids. Greater increases in the proportion of 18:1 (as a consequence of the decrease in 18:2 content) were observed in VLDL than in serum. The proportion of arachidonic acid in
Table 3.6

Effect of the Saturated Fat Diet on Serum Fatty Acid Composition

| Fatty Acid | Control Serum | | | | Saturated Serum (10 days) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PL | FFA | TG | CE | PL | FFA | TG | CE | |
| 16:0 | 25.7 | 24.7 | 25.2 | 9.1 | 25 | 28.0 | 23.2 | 8.6 | |
| 16:1 | 1.5 | 3.2 | 2.4 | 2.5 | 3.5 | 8.8 | 7.7 | 12.0 | |
| 18:0 | 26.1 | 11.5 | 8.9 | 3.6 | 22.8 | 13.5 | 7.9 | 4.0 | |
| 18:1 | 13 | 18.2 | 31.6 | 14.2 | 24.3 | 19.4 | 52.9 | 24 | |
| 18:2 | 25.9 | 22.7 | 28.2 | 30.1 | 12.8 | 13.3 | 7.1 | 11.0 | |
| 18:3 | n.d. | 1.6 | 1.6 | 0.6 | 1.9 | 1.8 | 0.4 | n.d. | |
| 20:1* | n.d. | 4.1 | n.d. | 1.4 | n.d. | 2.0 | n.d. | n.d. | |
| 20:4 | 7.8 | 13.9 | 2.2 | 38.5 | 9.7 | 13.2 | 0.8 | 40.4 | |

% Composition\textsuperscript{a}

\textsuperscript{a}Results represent the mean of two determinations on pooled sera from 20 rats (fasted for 6-8 hr). Male rats 4-5 months old were utilised.

PL = Phospholipid, FFA = Free fatty acids, TG = Triglyceride, CE = Cholesterol ester.

n.d. = not detected.

Significant amounts of 14:0 were not detected.

*Tentative identification

Lipids were separated by thin layer chromatography before analysis by gas liquid chromatography as described in the text.
Table 3.7
Effect of the Saturated Fat Diet on VLDL Lipid Fatty Acid Composition

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control VLDL</th>
<th>Saturated VLDL (10 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PL</td>
<td>TG</td>
</tr>
<tr>
<td>16:0</td>
<td>23.2</td>
<td>26.6</td>
</tr>
<tr>
<td>16:1</td>
<td>1.4</td>
<td>2.9</td>
</tr>
<tr>
<td>18:0</td>
<td>25.3</td>
<td>6.3</td>
</tr>
<tr>
<td>18:1</td>
<td>11.9</td>
<td>29.9</td>
</tr>
<tr>
<td>18:2</td>
<td>24.8</td>
<td>29.0</td>
</tr>
<tr>
<td>18:3</td>
<td>tr</td>
<td>2.6</td>
</tr>
<tr>
<td>20:1*</td>
<td>tr</td>
<td>1.1</td>
</tr>
<tr>
<td>20:4</td>
<td>13.2</td>
<td>1.5</td>
</tr>
<tr>
<td>20:5</td>
<td>tr</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*aFigures represent the mean of two determinations on pooled sera from 20 male rats (fasted for 6-8 hr).

PL = Phospholipid, TG = Triglyceride, CE = Cholesterol ester.
n.d. = not detected.

Significant amounts of 14:0 were not detected. Trace amounts of some unidentified fatty acids were also observed.

*Tentative identification.

Lipids were separated and analysed as described in the text.
Table 3.8
Fatty Acid Composition of Whole Serum of Rats Fed a Saturated Fat Diet for 1-10 Days and Fasted for 16 hr

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Days on Saturated Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>14:0</td>
<td>1.1+ .5</td>
</tr>
<tr>
<td>16:0</td>
<td>20+1.3</td>
</tr>
<tr>
<td>16:1</td>
<td>2.0+ .7</td>
</tr>
<tr>
<td>18:0</td>
<td>12.4+1.5</td>
</tr>
<tr>
<td>18:1</td>
<td>17.2+2.7</td>
</tr>
<tr>
<td>18:2</td>
<td>26 + .5</td>
</tr>
<tr>
<td>18:3</td>
<td>0.6+ .4</td>
</tr>
<tr>
<td>20:4</td>
<td>21.1+2.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cholesterol</th>
<th>mg/100 ml serum</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>64.3+6.8</td>
<td>48.7+5.9</td>
<td>59.7+6</td>
<td>64.7+9.1</td>
<td>55.7+6.4</td>
<td>57.5+3.9</td>
<td>56 +9.7</td>
<td>47 +2.9</td>
<td>52.8+8.0</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>88+9.5</td>
<td>83+21</td>
<td>62 +7.8</td>
<td>77.3+13</td>
<td>75.7+18.9</td>
<td>50.2+12</td>
<td>78.3+3.6</td>
<td>76.8+9.1</td>
<td>53+15.6</td>
<td></td>
</tr>
</tbody>
</table>

Cholesterol mg/100 ml serum

Triglyceride mg/100 ml serum

tr (trace) = < 0.5%.

*a*Trace amounts of 20:1, 20:5 and some unidentified acids were also observed.

*b*Figures represent the mean value from three rats ± S.D.

Rats which had been fasted for 16 hr were bled 9-11 am each day and 0.5 ml was analysed as described in the text. Significantly different from control value ***p < .05 **p < .01 *p < .001
both serum and VLDL was much lower (7 to 13% in serum and trace to 3% in VLDL) than observed in the 16 hr fasted state.

3.8.3 Effect of the Saturated Fat Diet on the Distribution of Radioactive Iodine in Radioiodinated VLDL

The proportion of radioactive iodine binding to lipid progressively decreased after rats had been fed the saturated fat diet (Table 3.9).

Table 3.9

Effect of a Saturated Fat Diet on the Iodination of VLDL

<table>
<thead>
<tr>
<th>No. of Days on Diet</th>
<th>% Distribution of Label&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lipid</th>
<th>Protein</th>
<th>Free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0)</td>
<td></td>
<td>44.5 ± 6</td>
<td>52.2 ± 5</td>
<td>3.3 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>40.6</td>
<td>56.7</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>21.7</td>
<td>72.7</td>
<td>5.6</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>21.6</td>
<td>74.0</td>
<td>4.4</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>12.4</td>
<td>84.2</td>
<td>3.4</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>8.2</td>
<td>91.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Figures represent the mean of two determinations except for the control which represents the mean of three determinations ± S.D.

VLDL containing 0.5 mg protein was used in each iodination.

VLDL from saturated animals (10 days) was consistently labelled at efficiencies of 8-12% with 94 ± 2.7% (S.D.) of the label in the protein moiety, 5.2 ± 2.9% associated with lipid and 0.8 ± 0.76% in the free state. The I/P ratio varied from 0.5-1.5 in these preparations.
Analysis of radioiodinated VLDL lipid by thin layer chromatography, showed that most of the radioiodine was bound to phospholipid (Table 3.10), of both control and saturated VLDL. In Table 3.10 the high proportion of radioactivity bound to free cholesterol could in part be due to diglyceride, since on thin layer chromatography plates it migrates close to free cholesterol under the conditions used in this separation (see experimental). No attempt was made to differentiate between them.

3.8.4 Effect of the Saturated Fat Diet on the Apoproteins of VLDL

In order to detect possible changes in the VLDL apoprotein composition, brought about by the increased saturated fatty acid diet, rats were killed at varying times from 1-9 days after commencement of the above diet. Their plasma VLDL apoproteins were then isolated and separated by polyacrylamide gel electrophoresis (see Chapter 4 for method). No differences in the VLDL protein composition were observed (Figure 3.5) or detected by scanning the gels (for method see Chapter 5), and computing the ratio of the area of zone I:zone II+III+IV. Also, no significant differences in the proportion of apoproteins in zone III and IV were found (Figure 3.6). The area of zone II was not significant, as it contained no major stained bands (see Figure 3.5) and was therefore not included in the comparison shown in Figure 3.6. Zone I proteins react with antibodies prepared against apo-LDL (d 1.025-1.040 g/ml), and zones II, III and IV contain water soluble proteins. Zone III proteins, whose identity are discussed in Chapter 6, have not yet been characterised. Zone IV contains proteins thought to be homologous to the human C group of proteins.

The apoproteins of VLDL were also separated using their differential solubility in a weak aqueous buffer, 5 mM ammonium bicarbonate pH 8.2, as described in Chapter 5. The proportion of insoluble (B protein) and soluble proteins from samples of apo-VLDL of control diet fed rats and
Table 3.10

Distribution of $^{125}$I-iodine in VLDL Lipids After Iodination Using the Iodine Monochloride Technique

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phospholipid % Distribution*</th>
<th>Free Cholesterol % Distribution*</th>
<th>Free Fatty Acid % Distribution*</th>
<th>Triglyceride % Distribution*</th>
<th>Cholesterol Ester % Distribution*</th>
<th>Lipid % Distribution of Label</th>
<th>Protein % Distribution of Label</th>
<th>Free Ester % Distribution of Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control VLDL</td>
<td>84 (83.3-84.7)</td>
<td>10.7 (9-12.3)</td>
<td>2.4 (0.7-4.1)</td>
<td>2.6 (2-3.2)</td>
<td>0.3 (0.2-0.4)</td>
<td>59.2</td>
<td>39</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>b 96.5 (96.5-96.5)</td>
<td>1.35 (1.3-1.4)</td>
<td>1.2 (1.1-1.3)</td>
<td>.85 (0.7-1.0)</td>
<td>.11 (0.10-0.12)</td>
<td>56.3</td>
<td>40.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Saturated VLDL (10 days)</td>
<td>78.7 (74.4-83)</td>
<td>8.0 (3.4-12.4)</td>
<td>8.2 (7.1-9.3)</td>
<td>4.0 (2.9-5.1)</td>
<td>1.1 (0.8-1.4)</td>
<td>3.5</td>
<td>96</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>b 87.1 (85-88.2)</td>
<td>4.3 (3.6-4.9)</td>
<td>1.5 (1.2-1.8)</td>
<td>3.7 (2.6-4.8)</td>
<td>3.4 (3.1-3.7)</td>
<td>6.2</td>
<td>90.6</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*Values represent the mean of two determinations and the range is shown in parenthesis.

a VLDL preparations were isolated from the two groups of 20 rats each fasted for 6-8 hr.

b VLDL preparations were isolated from two groups of 16 rats each fasted for 6-8 hr.

Lipids were separated by thin layer chromatography as described in the text and recovery of radioactivity was 90%.
Figure 3.5 Polyacrylamide Gel (7.5%) Electrophoresis Patterns of VLDL Apolipoproteins from Rats Fed a Saturated Fat Diet for up to 9 Days. No changes were seen in the proportion of apoprotein constituents either visually or after scanning the gels. Methods used were those described in Chapter 5.
Figure 3.6 Comparison of the Area of Stained Bands in Zone III and IV after PAGE of Apo-VLDL Obtained from Rats Fed a Saturated Fat Diet for 2, 4, 6 and 8 Days with those on a Control Diet (C). Figures represent the mean of three determinations ± S.D. The area of bands in zones III and IV is expressed as a percentage of the total area of zone III + IV.
rats fed the saturated fat diet for 10 days is shown in Table 3.11. Values for saturated VLDL did not vary significantly from the controls. Thus the saturated fat diet did not affect the proportion of soluble to insoluble apoproteins present in VLDL.

3.8.5 Metabolic Behaviour of $^{125}$I-VLDL Obtained from Rats Fed the Saturated Fat Diet

The possibility of the saturated fat diet causing changes in the biological behaviour of VLDL was investigated by injecting $^{125}$I-labelled VLDL, obtained from donor rats fed a saturated diet for 2, 4, 6 and 8 days, into normal rats. Some differences in the disappearance of radioactivity from the serum were observed (Figure 3.7). Radioactivity was initially removed more rapidly from the serum when $^{125}$I-VLDL obtained from rats fed the saturated diet for two days was injected than $^{125}$I-VLDL obtained from rats fed the saturated fat diet for 8 days was injected. The labelling characteristics of these VLDL molecules were different, as shown in Table 3.9, and the disappearance of total radioactivity from the serum would be dependent on the proportion of label present in protein and lipid. The higher the proportion in the lipid the more rapid the disappearance of label from the serum, as lipid is removed more rapidly than protein (see Chapter 4). No marked differences were observed in the removal of whole VLDL, obtained from rats fed the saturated fat diet for 2, 4, 6 and 8 days, or apo-VLDL radioactivity from the serum (Figure 3.8).

In experiments where VLDL obtained from rats fed the commercial diet was iodinated and injected into rats, the disappearance of radioactivity from the serum was similar to the preparations obtained from rats on the saturated diet for 2, 4 and 6 days. The characteristics of removal being dependent on the proportion of radioactivity in the protein and lipid,
Table 3.11

Comparison of the Proportion of Ammonium Bicarbonate (5 mM) Soluble and Insoluble Apoprotein Present in Rat VLDL from Control and Saturated Diet Fed Rats, Following Delipidation With Organic Solvents

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percent Insoluble</th>
<th>Percent Soluble</th>
<th>Number of Samples</th>
<th>% Recovery of Protein After Delipidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control VLDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>38.7 ± 11.9</td>
<td>61.5 ± 11.7</td>
<td>8</td>
<td>82.5 ± 9.5</td>
</tr>
<tr>
<td>(b)</td>
<td>32 ± 12.0</td>
<td>68 ± 11.8</td>
<td>16</td>
<td>86 ± 8.4</td>
</tr>
<tr>
<td>Saturated VLDL (10 days)</td>
<td>36.1 ± 5.2</td>
<td>63.9 ± 5.3</td>
<td>6</td>
<td>85 ± 6.3</td>
</tr>
</tbody>
</table>

1 and after 5 mM Ammonium Bicarbonate Separation.

2 Figures for the control were obtained from rats used in metabolic experiments (see Chapter 5).

Figures for the saturated VLDL (from rats fasted for 8-10 hr) did not differ significantly from the control VLDL (from rats fasted for 4-16 hr) as determined by students 't' test.

Probability (%) | Insoluble | Soluble
---|-----------|-----------
(a) | 66        | 67        |
(b) | 46        | 45        |
't' (a) | .46      | .44      |
(b) | .77      | .79      

Differences are significant when P < 5% (i.e., .05) and t > 2.10.
Figure 3.7  *In Vivo* Disappearance of Radioactivity from Serum of Rats after the Injection of $^{125}$I-VLDL Obtained from Rats Fed a Saturated Fat Diet for 2, 4, 6 and 8 Days. 6 x $10^5$ cpm (50 µg VLDL protein) were injected per rat. Each point represents the mean data from two rats. Percent of total cpm injected (percent injected dose) was calculated from serum counts and assuming a blood volume of 9 ml/100 g body weight.
Figure 3.8  Disappearance of $^{125}$I-VLDL (upper panel) and $^{125}$I-ApoVLDL (lower panel) from Serum after the Injection of $^{125}$I-VLDL Obtained from Rats Fed a Saturated Fat Diet for 2, 4, 6 and 8 Days. Figures on key adjacent to symbols refer to days on the saturated fat diet. Each point represents the combined results from two rats. $6 \times 10^5$ (50 µg VLDL protein) cpm were injected per rat. Aliquots of VLDL were radioassayed and percent cpm injected calculated. Radioactivity present in VLDL after delipidation was expressed as a percentage of the counts injected as protein. This was calculated by multiplying the proportion of label present in VLDL protein (determined as described in methods, Chapter 4) by the total cpm injected.
rather than the number of days the rats had been fed the saturated fat diet, since lipid is removed more rapidly than protein from the circulation (Chapter 4). The metabolic behaviour of VLDL (10 day saturated) was almost identical to the 8 day preparation which had 84.2% of the label present in the protein.

3.9 DISCUSSION

The radioiodination properties of VLDL obtained from animals fed a saturated fat diet for 10 days were changed such that over 90% of the $^{125}$I was consistently bound to the protein of VLDL and the I/P ratio was < 1. Therefore the VLDL was essentially radioactively labelled only in the protein. As this VLDL was to be used to study VLDL apoprotein metabolism it was essential to determine whether any changes had occurred in this moiety following alteration of the saturated fat content of the diet. This in turn could possibly alter its metabolism.

The major difference between the commercial chow and the saturated fat diet was in the fatty acid content of the lipid. Although the exact composition of the commercial chow carbohydrate moiety was unknown the proportion of carbohydrate in the two diets was comparable. No change in the apoprotein composition of rat VLDL was apparent after 10 days on the saturated fat diet, as determined by polyacrylamide gel electrophoresis and determination of the soluble-insoluble protein ratio. This result and the fact that there was no change in the cholesterol and triglyceride concentration in the serum after 10 days on the saturated fat diet would indicate that the composition of the carbohydrate moieties of the two diets was similar.

As there can be considerable variation between different lots of beef dripping, aliquots from those used to prepare the diets were analysed
and their fatty acid composition found to be consistent. Variations of 1-8% for 14:0, 23-37% for 16:0, 14-29% for 18:0, 39-50% for 18:1 and trace-5% for 18:2 have been observed by others (values supplied by Nuttlex Food Products, Pty. Ltd., Vic. Australia).

The fatty acid composition of the serum of fed rats is thought to remain stable throughout the day, other than a peak at 3 and 6 pm (Bortz and Steele, 1973). Even so, rats used in these experiments were bled at the same time each day. Changes in the fatty acid composition of the serum and VLDL reflected the changes in the diet. The major change occurring in the essential fatty acid linoleic acid (18:2). The proportion of major fatty acids found in control serum (Table 3.6) phospholipids, triglycerides and cholesterol esters compare quite well with those obtained by Aftergood and Alfin-Slater (1967). These authors also observed a large proportion of arachidonic acid (20:4) in the fatty acids of sterol esters in plasma (42.7 ± 7.5% for male rats 6 weeks old and 44.4 ± 3.1% for male rats 6 months old) VLDL, LDL and HDL.

The fatty acid composition of serum lipids in fasted and fed rats was studied by Sgoutas et al (1973). Their values for VLDL phospholipid, triglyceride and cholesterol esters from fasted animals agree closely with the control VLDL values presented in Table 3.7. Some differences were apparent in the ratio of 18:1 to 18:2 and 16:1, but overall the fatty acid compositions were comparable. These authors observed that of all lipoproteins, HDL cholesterol esters contained the highest proportion of arachidonic acid (38.9%). The fatty acid composition of serum lipoproteins vary according to age, composition of diet, severity of starvation (Christie et al, 1974) and disease states. Therefore considerable variation in fatty acid composition can be found in the literature.
Bragdon and Carmen (1960) found there was a close correlation between dietary fatty acid composition and chylomicron fatty acid composition provided the fatty acid entered the circulation in chylomicrons. Since the fatty acids of serum and VLDL from rats fasted 5 hr showed a higher proportion of saturation than those fasted for 16 hr, the presence of chylomicrons and thus of dietary fat may have been the main contribution to the change observed. The fatty acids in VLDL, however, are not so easily influenced by diet as they are usually in equilibrium with adipose tissue fatty acids (Bragdon and Carmen, 1960). Changes in 16 hr fasted rats were less rapid. Rathbone (1965) observed changes in linoleic and oleic acid content of rat serum, brain and myelin in response to linoleic acid rich, linoleic acid poor, a predominantly saturated fatty acid diet and a control diet over a period of weeks. However, large changes were observed a few days after feeding the diets.

The rapidity with which these changes can occur is emphasised by studies on changes in liver fatty acids of essential fatty acid deficient rats. After feeding linoleic acid changes were observed after only seven hours and continued to occur rapidly up to the 24 hr studied (Johnson et al, 1967).

The only significant difference between the VLDL obtained from control rats and rats fed a saturated fat diet was thus in the fatty acid composition. The possibility that the total VLDL protein (soluble + insoluble protein) content may be increased and the phospholipid content slightly lowered compared to normal cannot be ruled out as these parameters have not been measured. Also over long periods of time (14 weeks) essential fatty acid deficient rats have shown changes in the proportion of protein present in VLDL and LDL and decreases in phospholipid content (de Pury and Collins, 1972).
Analysis of the radioiodinated VLDL lipid (Table 3.10) revealed that most of the $^{125}\text{I}$ was incorporated into the phospholipid of VLDL from control and saturated fat fed rats. This is not surprising since phospholipid is thought to be a surface constituent rather than in the core like other lipids. As the proportion of label binding to phospholipid before and after saturation was similar (8-10% difference) apparently no major change in the structure of VLDL occurs, which could expose lipid previously inaccessible. The changes occurring in phospholipid fatty acid composition after feeding of the saturated fat diet are not unexpected as the essential fatty acids 18:2, 18:3 and 20:4 are preferentially incorporated into phospholipid and are catabolised less rapidly than other non-essential fatty acids (Alfin-Slater and Aftergood, 1968). Since phospholipid (which contains a higher proportion of unsaturated fatty acids than other lipid classes) has a slower turnover rate than other lipid classes this mechanism apparently serves to conserve stores of essential fatty acids in the animal (Alfin-Slater and Aftergood, 1968). Arachidonic acid (20:4) is preferentially incorporated into the $\beta$ position of phospholipids and 18:2 conversion to 20:4 may occur when the content of 20:4 in the diet is decreased, for example on the saturated fat diet. This conversion could account for the higher proportion of arachidonic acid observed in serum from the 16 hr fasted rats.

The question of the difference in the iodination properties of normal human and rat VLDL remains open. Factors which could account for this are possible differences in the tyrosine residue content of human and rat VLDL apolipoprotein (Bilheimer et al, 1972). The larger the content, the higher the proportion of $^{125}\text{I}$ binding to the protein. Other possibilities include, differences in the proportion, accessibility and content of unsaturated fatty acids of the phospholipid. Published values for both content of total phospholipid and fatty acid composition of human and rat
VLDL are variable. These differences may be due to different strains of rat, slight differences in the diet and extent of fasting before exsanguination. Data from this laboratory suggest that the phospholipid content of human (18.7% w/w) (Fidge, unpublished observations) and rat (16.3% w/w) (Fidge and Calder, 1972) VLDL are not significantly different. These values fall within the range of other published values (Onclcy, 1963; Frederickson et al, 1967; Eisenberg and Rachmilewitz, 1973a; Bosch et al, 1971). Recent data on the fatty acid composition of human VLDL phospholipid cannot be found in the literature but the data of Goodman and Shiratori (1964) for VLDL plus LDL (i.e. d < 1.019 g/ml) and Lindgren et al (1961) for VLDL from fasted male subjects, show that the content of 18:2 was lower (20.3% and 16.1%) than that found in the rat (27%). The content of arachidonic acid was 13.7% and 4.3%. These values are equal to and lower than those observed in the rat. The above question therefore still remains open. However since most of the radioactivity within the lipid moiety was present in phospholipid and some reduction in total percentage (94-87) occurred after the saturated diet, it is tempting to suggest that this lipid may account for the differences observed between the labelling characteristics of rat and human VLDL, especially since it is a surface component.

No major differences between the in vivo behaviour of 125I-VLDL from saturated diet fed rats (2, 4, 6 and 8 days on diet) and control rats were observed. There were differences however, in the removal of radioactivity from the serum which may be due to differences in the proportion of label present in the lipid. This assumption was verified after determination of the removal of radioactivity in the apoprotein of VLDL which was similar in all preparations studied.

It can thus be concluded that feeding the saturated fat diet to the rats for the short term of 10 days is sufficient to change the labelling
characteristics of the VLDL but not to change the apoprotein composition or significantly alter the *in vivo* behaviour of the iodinated VLDL apoprotein. Also, most of the labelled iodine bound to lipid is incorporated into linoleic acid of phospholipid. With increasing time on the saturated fat diet the degree of lipid labelling decreased, indicating that it is probably related to the content and accessibility of the linoleic acid molecules of the VLDL phospholipid. In other words, the increased content of saturated fatty acids in the VLDL lipids, particularly the most accessible to surface radioiodination, phospholipid, would make the incorporation of labelled iodine into the lipid of VLDL progressively more difficult thus facilitating incorporation into the protein.
3.10 SUMMARY

Short term feeding of a saturated fat diet was found to facilitate $^{125}$I incorporation into VLDL protein on iodination. Changes in the fatty acid content of whole serum and VLDL were observed, particularly in linoleic acid and palmitoleic acid of rats fed the saturated fat diet. These changes occurred in each of the major lipid classes, phospholipid, triglycerides and cholesterol esters of both serum and VLDL. Analysis of the lipids of VLDL after radioiodination showed that most radioactivity was incorporated into the phospholipid of both control and saturated VLDL.

The apoprotein composition of VLDL from rats fed the saturated fat diet for 2, 4, 6 and 8 days did not change. The in vivo behaviour of the radioiodinated VLDL obtained from control rats and rats on the saturated fat diet for 2, 4, 6 and 8 days was compared and found to be essentially similar.

In conclusion, feeding rats a saturated fat diet for up to 10 days is sufficient to change the labelling characteristics of the VLDL but not to change the apolipoprotein composition or significantly alter the in vivo behaviour of the iodinated VLDL apoprotein.
CHAPTER 4

STUDIES ON THE METABOLISM OF VERY LOW DENSITY LIPOPROTEIN
AND VERY LOW DENSITY APOLIPOPROTEIN IN THE RAT
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AND VERY LOW DENSITY APOLIPOPROTEIN IN THE RAT

4.1 INTRODUCTION

In this chapter the suitability of the rat as an experimental model for studying human VLDL apoprotein metabolism was investigated. This animal has been used to provide much useful information about fat transport and it seemed reasonable to assume that it might be of further use in an investigation of the functional relationship which may exist between apolipoprotein and lipid metabolism. A study by Fidge and Foxman (1971) has already established an interrelationship between the apoprotein moieties of rat lipoproteins and it was subsequently established by several groups that there is a metabolic as well as a structural relationship between several apoproteins of the major serum lipoproteins of humans, rats and monkeys (Bilheimer et al, 1972; Eisenberg et al, 1972a, 1973b; Rubenstein and Rubinstein, 1972, 1973 and Schonfeld et al, 1972). Such an interrelationship may possibly be connected with the secretion of triglyceride rich VLDL molecules from the liver into the plasma which is followed by a series of catabolic events. Some of these events are not as yet clearly defined. These events include the clearance of triglyceride from the plasma, presumably attributable to the action of various lipases (Korn, 1955; Vogel et al, 1971; La Rosa et al, 1972 and Assman et al, 1973), a rapid exchange or transfer of the small molecular weight peptides from VLDL to other lipoproteins (notably HDL) and possibly the formation of VLDL remnants (Fidge and Foxman, 1971; Bilheimer et al, 1972; Eisenberg et al, 1972a, 1973a; Rubenstein and Rubinstein, 1973) some or all of which are eventually further transformed to LDL molecules.
Experiments described in this chapter provide evidence of metabolic as well as structural heterogeneity amongst rat apolipoproteins. This was achieved by labelling all rat VLDL protein with $^{125}\text{I}$-iodine in some experiments and in others by labelling only the small molecular weight peptides of the lipoprotein. The iodinated VLDL was then administered to recipient animals in order to study the in vivo metabolism of VLDL apoprotein. Using similar techniques the fate of iodinated human VLDL in the rat was also studied.*

4.2 EXPERIMENTAL

All in vivo experiments were performed using essentially the same procedure. Variations to this procedure will be described where appropriate. The general experimental procedure for in vivo experiments is shown in Figure 4.1.

4.2.1 Isolation and Iodination of VLDL

Rat VLDL to be used for reinjection was isolated as described previously (Chapter 3). Following the initial isolation, two further flotations served to purify and concentrate the VLDL so that final concentrations of VLDL greater than 1 mg/ml were consistently obtained.

Preliminary experiments were performed where VLDL for reinjection was purified from concentrated serum (serum was concentrated in a concentrating cell, x4) by passage through a 2% agarose column (Biogel A-50 M, 100-200 mesh, BioRad Labs., Richmond, Calif. USA) washed with 0.15 M sodium chloride containing 0.02 M potassium phosphate and 1 mM EDTA, pH 7.4 and iodinated. The iodinated VLDL was then repurified by

*This data was kindly provided by Dr Fidge and is included here for comparison purposes.
Figure 4.1 General Experimental Procedure for Metabolic Experiments.
passage through agarose before injection into rats. A comparison of the disappearance of label from the serum utilising iodinated VLDL obtained by ultracentrifugation and agarose gel filtration showed that there were no differences between the two preparations. Ultracentrifugation was used in subsequent *in vivo* experiments, mainly due to the opportunity it provided for concentration of the samples.

VLDL was iodinated utilising the iodine monochloride method of McFarlane as described in Chapter 3. VLDL to be iodinated was brought to pH 10.0 by dialysis against 0.4 M glycine-sodium hydroxide buffer (pH 10.0) after an aliquot had been taken for protein determination. After iodination the protein bound radioactivity was dialysed for at least 4 hr against 0.15 M sodium chloride (pH 7.4) containing 1 mM EDTA (3 changes/hr). Less than 3% of radioactivity was present as 'free' iodine after dialysis as determined by chloroform-methanol extraction. Samples for counting and chloroform-methanol extraction were taken after dialysis in order to calculate the percentage efficiency of iodination and the intramolecular distribution of $^{125}$I-iodine as described in Chapter 3. The dialysed $^{125}$I-VLDL was finally recentrifuged at 39,000 rpm for 16 hr at 5°C and the proportion of radioactivity present in the supernatant (VLDL) and infranatant was then determined before injection of the supernatant into animals.

The following criteria were observed before a preparation was used for *in vivo* and *in vitro* experiments:

(i) that the I/P ratio did not exceed 1.0;*

(ii) that most (> 90%) of $^{125}$I was bound to the protein of VLDL;

*As calculations of I/P were based on a molecular weight of 250,000 for apo-VLDL and recognising that this figure was a considerable overestimate of the actual functional apoprotein molecular weights (Koga *et al.*, 1971; Eisenberg *et al.*, 1973b) the actual I/P ratio was below 1.0.
(iii) that over 90% of the radioiodinated VLDL ultracentrifuged after dialysis was found in the supernatant of that spin;

(iv) to reduce the possibility of self-irradiation the ratio of radioactivity to mg lipoprotein were kept to the specifications (5-10 µCi/mg) recommended by McFarlane (1958);

and

(v) VLDL for reinjection was used within 48 hr after the initial isolation since it was observed that storage of labelled VLDL resulted in a progressive decrease in recovery of floating VLDL after ultracentrifugation.

4.2.2 Injection of Rats and Separation of Lipoproteins

In vivo experiments were performed on male rats of known weight (180-240 g). Rats of equal weight (± 5 g) which had been fed on commercial rat chow prior to experimentation were utilised for each experiment. In some experiments duplicate rather than triplicate series of animals were chosen because the subsequent operational procedures were dependent on the rotor and centrifuge space available and consequently experiments were repeated two or three times. Labelled lipoprotein preparations were diluted with 0.9% sodium chloride (sterile) to obtain solutions containing 2-10 x 10^6 cpm/ml and 20-100 µg protein/ml. Aliquots of 0.5 or 1.0 ml was injected into the tail vein of rats which were bled at various times and blood kept at 4°C. Serum was obtained from clotted blood by centrifugation (see Chapter 2) and aliquots of serum (50-100 µl) were assayed for radioactivity and for calculation of the disappearance of radioactivity from the circulation. Aliquots of serum (3-5 ml) were then ultracentrifuged to obtain VLDL, LDL_1 (d 1.006-1.019 g/ml), LDL_2 (d 1.019-1.063 g/ml) and HDL (d 1.063-1.21 g/ml) (see Chapter 2).
As long exposure to high salt concentrations used in the separation of lipoproteins from serum in the ultracentrifuge and high x g forces are thought to denature lipoproteins, particularly HDL resulting in the formation of VHDL (Alaupovic et al, 1966; Albers and Aladjem, 1971), lipoproteins were separated by precipitation and ultracentrifugation. The experimental protocol was as follows. Labelled VLDL was incubated with serum and two samples (5 ml) were removed at four time intervals, stored on ice, then ultracentrifuged to obtain VLDL and LDL₁. One lot of samples was further ultracentrifuged to obtain LDL₂, HDL and infranatant (d > 1.21 g/ml). LDL₂, HDL and infranatant were isolated by precipitation with dextran sulphate (Burstein et al, 1970) and the radioactivity present in each fraction compared to that in corresponding fractions obtained by ultracentrifugation.

The precipitation technique utilised was as follows. To the 5 ml infranatant obtained after ultracentrifugation at d 1.019 g/ml (LDL₁) was added 0.025 ml 10% dextran sulphate (500,000 mol. wt., Sochibo, Boulogne, France) and 0.2 ml 1 M manganese chloride. The precipitate formed (LDL₂) was obtained by centrifugation for 10 min at 2,000 rpm (MSE Mistral 6 L). The supernatant containing HDL was removed and the pellet (LDL₂) resuspended in 2 ml 0.9% sodium chloride and a portion assayed for radioactivity. HDL was precipitated by the addition of 0.3 ml 10% dextran sulphate and 0.75 ml 1 M manganese chloride to the supernatant and the solution left at room temperature for 2-24 hours. The suspension was then centrifuged at 3,000 rpm (MSE Mistral 6 L) and the precipitate containing HDL was resuspended in 2 ml normal saline and an aliquot assayed for radioactivity. An aliquot of the supernatant was also assayed for radioactivity. Separation by precipitation was checked by cellulose acetate strip electrophoresis of the LDL₁ spin infranatant before and after precipitation, for the presence of LDL₂. This experiment was repeated
twice and no difference was observed between the two methods. However, since the fractions isolated by either method had to be recentrifuged to remove contaminating plasma proteins, ultracentrifugation was used throughout in subsequent experiments.

4.2.3 Labelling of VLDL by In Vitro Incubation with $^{125}$I-labelled HDL*

Following the incubation of protein labelled VLDL with rat serum, in vitro, an exchange of small molecular weight peptides occurs between VLDL and HDL (Rubenstein and Rubinstein, 1972). In order to label the small molecular weight 'C' proteins and not the apo-LDL (B protein) moiety of VLDL, purified rat HDL (d 1.063-1.21) was iodinated with $^{125}$I-iodine using the iodine monochloride method described previously. HDL thus labelled contained 80-85% of the label in the protein moiety, with an iodine:protein substitution ratio of 0.5:1. Iodinated HDL was then mixed with 20 ml rat serum to which was added rat VLDL isolated from 30-40 ml of rat serum. After incubation for 1 hr at 37°C rat VLDL was isolated by ultracentrifugation at d 1.006 g/ml and washed twice. The distribution of label ($^{125}$I) between the apoproteins of in vitro labelled rat VLDL was determined by polyacrylamide gel electrophoresis following delipidation and resolubilisation of a portion of the labelled VLDL.

4.2.4 In Vitro Studies

Labelled VLDL was incubated with rat serum at 37°C in a shaking water bath. Serum for incubation was collected from fasted male rats (6-8 hr) immediately prior to incubation and kept at 4°C. Zero time was the time at which the labelled VLDL was added to the incubation medium. Heparinised plasma to be used for incubation was obtained by injecting rats with

*This part of the work was performed in collaboration with Dr Fidge.
heparin (22 units/rat, Sodium Heparin, mucous, Commonwealth Serum Laboratories, Melb. Australia 5,000 units/ml) 5 min prior to exsanguination. Aliquots of the incubation medium were removed at different time intervals and kept at 4°C prior to ultracentrifugation.

4.2.5 Determination of Protein Bound Radioactivity

**Serum.** Serum samples (0.2 ml) were delipidated by chloroform-methanol extraction, as described in Chapter 3 and precipitation of the protein with 5% trichloroacetic acid. After centrifugation at 2,000 rpm (MSE Mistral 6 L) and removal of the supernatant containing free iodine the dried protein was then dissolved in (0.5-1.0 ml) 1.0 M sodium hydroxide and aliquots assayed for radioactivity.

**Lipoproteins.** After aliquots (0.2 ml) had been taken from all lipoproteins for radioassay 25 mg of human serum albumin (0.1 ml, Human Serum Albumin, 25%, Commonwealth Serum Laboratories, Melbourne) was added to VLDL, LDL₁, and LDL₂ as protein carrier. Albumin was not added to HDL or the infranatant (d > 1.21 g/ml). Total lipoprotein fractions were delipidated using 20 ml chloroform:methanol (2:1, v/v) (as per serum). Lipid bound radioactivity was determined by evaporation of the chloroform phase and dissolving the lipid in ethanol, a portion of which was assayed for radioactivity. Protein bound radioactivity was also determined, in some cases on the aliquots (0.2 ml) of lipoprotein which had been removed for radioassay. Carrier (10 mg Human Serum Albumin) was added to VLDL, LDL₁, and LDL₂ and all samples were delipidated using chloroform-methanol (see Section 4.2.6) in 10 ml conical centrifuge tubes. Protein was dissolved in 0.5 ml 1 M sodium hydroxide and an aliquot removed for radioassay. The total radioactivity present in VLDL, LDL₁, LDL₂, HDL and infranatant protein was then calculated.
Delipidation of Lipoproteins and Apoprotein Analysis

Purified lipoprotein fractions were dialysed against 5 mM NH₄HCO₃, pH 8.2 for 48 hours with frequent changes and lyophilised in 5 ml stoppered delipidation tubes. Aliquots of lipoprotein fractions (50-300 µl) were taken after dialysis for protein estimations. The lyophilised lipoprotein was then suspended in 200 µl of 0.1 M sodium decyl sulphate (SDS) (Eastman Kodak Co. NY USA) and delipidated by adding 1 ml methanol, and after mixing, 1 ml of chloroform. The tubes were then filled with diethyl ether. The protein precipitates obtained after centrifugation at 1,500 rpm (MSE Mistral 6 L) for 2 min were washed by the addition of 2 ml methanol and refilling the tubes with diethyl ether. This procedure was repeated once. The protein precipitate was then washed twice with diethyl ether and after drying under nitrogen, dissolved in 0.05 M SDS in 0.05 M Triz-HCl buffer pH 8.2 containing 8 M Urea. Using the above delipidation technique, calculations based on protein determinations and radioactivity present before and after delipidation showed that 80-90% of VLDL, 88-95% of HDL and 70-75% of LDL apoprotein was recovered. Before loading on polyacrylamide gels, the concentration of SDS was reduced to approximately 0.015 M by the addition of 0.01 M Triz-HCl, pH 8.2 and solid urea added to a final concentration of 8 M. These solutions remained at room temperature overnight prior to electrophoretic separation. Although rat HDL apoprotein was readily soluble in aqueous solutions, the same detergent-buffer system described above for VLDL and LDL was used to maintain standard conditions for all apoproteins fractionated by electrophoresis.

Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out on 7.5% polyacrylamide gels containing 8 M urea in tubes measuring 7 x 150 mm which had been treated
with a photographic wetting agent (Ilford Wetting Agent and Glazing Solution, Ilford Pty Ltd. Australia). The same discontinuous buffer system as described by Kane (1973) was used in the upper and lower reservoirs and electrophoresis was carried out at 2.5 mA per tube for 4-5 hr. During operation of the system, the gels were cooled by running tap water through a glass coil incorporated into the apparatus. Between 80-150 µg of apoprotein was loaded onto the gels and following electrophoresis, the gels were stained with 1% amino black for 1 hr and destained in 7% acetic acid for 2½ days. The stained bands were then separated by slicing the gels with a razor blade and after being transferred to small tubes, the slices were dried at 50°C for 12-20 hr prior to radioassay. More than 90% of the total radioactivity applied to the gels was recovered in all slices with approximately 85-90% of the radioactivity associated with stained bands. The efficiency of counting gel slices was checked by counting gel slices before and after dehydration and after solubilisation in 30% H₂O₂. No differences were observed between these methods.

4.3 RESULTS

A number of preliminary experiments were performed to determine standard conditions for future experiments. The effect of sterilising the iodinated VLDL, by passage through 0.22 nm cellulose nitrate millipore filter before injection, was investigated. Recovery of VLDL radioactivity after sterilisation was 92%. The in vivo disappearance of radioactivity from the serum of rats injected with sterilised and non-sterilised ¹²⁵I-labelled VLDL were similar. All in vivo experiments were commenced between 9 and 11 am to avoid inconsistencies due to diurnal variation and rats were fasted 3-4 hr prior to and during the experiment. Removal of ¹²⁵I-VLDL radioactivity from the serum was more rapid in rats fasted
16 hr prior to injection than in those fasted for only 3-4 hr prior to injection. Conversely, the injection of a large proportion of unlabelled VLDL with $^{125}$I-VLDL resulted in a less rapid removal of radioactivity from the serum. Thus, the amount of VLDL injected in subsequent studies (20-100 µg) represented approximately 5-20% of the total circulating VLDL apoprotein per animal.

4.3.1 Redistribution of Radioactivity after Injection of $^{125}$I-labelled VLDL

All data reported below was obtained using VLDL isolated from rats fed the saturated diet for 8-10 days. The redistribution of radioactivity among the serum lipoproteins following $^{125}$I-labelled VLDL administration is shown in Table 4.1. These figures are the mean data + 1 S.E. of the mean from 7 rats, which are representative of one triplicate and two duplicate experiments. After 5 min, 32.7% of the serum radioactivity was associated with HDL and the proportion of the radioactivity in HDL increased with time while the proportion of label in VLDL decreased rapidly. There was also an increase in the amount of radioactivity in the d 1.006-1.019 (LDL$_1$) and 1.019-1.063 g/ml (LDL$_2$) fractions although less marked than in the HDL fraction. Radioactivity increased in the LDL$_1$ fraction to a peak of 7.8% at 1 hr while the label in the LDL$_2$ fraction steadily increased for the duration of the experiment. Using the data from Table 4.1, it was possible to determine the redistribution of radioactivity as a percent of the total injected dose. Thus Figure 4.2 illustrates the relative increases of radioactivity in LDL$_1$, LDL$_2$ and HDL which accompany the disappearance of label from VLDL following administration of $^{125}$I-labelled VLDL of rats. Although most of the radioactivity was associated with the protein moiety of VLDL, the clearance rate of the $^{125}$I-labelled lipid moiety from the
Table 4.1

Distribution of Radioactivity in Serum Following Injection of \( ^{125}I \)-labelled Rat VLDL\(^a\)

<table>
<thead>
<tr>
<th>Time after Injection min</th>
<th>VLDL (d &lt; 1.006)</th>
<th>LDL(_1) (d=1.006-1.019)</th>
<th>LDL(_2) (d=1.019-1.063)</th>
<th>HDL (d=1.063-1.21)</th>
<th>Infranate (d &gt; 1.21)</th>
<th>% of injected dose per 9 ml serum (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>50.6 ± 6.0</td>
<td>4.0 ± 0.9</td>
<td>6.4 ± 2.6</td>
<td>32.7 ± 3.9</td>
<td>6.3 ± 2.6</td>
<td>69.2 ± 9.4</td>
</tr>
<tr>
<td>10</td>
<td>41.5 ± 3.1</td>
<td>5.5 ± 0.6</td>
<td>8.4 ± 2.7</td>
<td>38.3 ± 3.1</td>
<td>6.3 ± 2.2</td>
<td>68.2 ± 8.1</td>
</tr>
<tr>
<td>30</td>
<td>33.0 ± 8.2</td>
<td>7.0 ± 1.3</td>
<td>10.1 ± 3.8</td>
<td>42.0 ± 3.2</td>
<td>7.9 ± 2.8</td>
<td>54.0 ± 2.7</td>
</tr>
<tr>
<td>60</td>
<td>29.4 ± 4.5</td>
<td>7.8 ± 2.3</td>
<td>10.6 ± 3.3</td>
<td>44.1 ± 2.1</td>
<td>8.1 ± 2.7</td>
<td>44.8 ± 3.2</td>
</tr>
<tr>
<td>180</td>
<td>21.2 ± 2.4</td>
<td>7.2 ± 1.7</td>
<td>12.9 ± 4.6</td>
<td>16.6 ± 8.6</td>
<td>10.1 ± 4.6</td>
<td>28.7 ± 6.8</td>
</tr>
<tr>
<td>360</td>
<td>14.1 ± 4.0</td>
<td>4.3 ± 1.2</td>
<td>18.7 ± 8.5</td>
<td>50.0 ± 11.5</td>
<td>12.9 ± 6.3</td>
<td>16.8 ± 7.8</td>
</tr>
</tbody>
</table>

\(^a\) Rats were injected with 60-80 µg VLDL protein and 5-10 x 10\(^6\) cpm.

\(^b\) The figures shown represent the mean ± S.E.M. of 7 rats from 2 duplicate and 1 triplicate experiment.

\(^c\) Calculated on the basis that 200 g rats have 9 ml serum, (4.5% of body weight).
Figure 4.2  Redistribution of Radioactivity Among Serum Lipoproteins Following Injection of $^{125}$I-labelled Rat VLDL into Rats. The figures represent the data ± S.E. of 7 rats from three different experiments as described in the text.
serum was also determined in some experiments. The results showed that lipid radioactivity was cleared more rapidly from the blood than protein label (Figure 4.3). Extraction of the lipid fraction of all lipoproteins showed that most (92-95%) of the label was bound to the protein moiety.

Table 4.2 shows the redistribution of radioactivity among rat serum lipoproteins following the injection of iodinated human VLDL into rats. It is apparent that human VLDL, like rat VLDL, is also rapidly catabolised in the rat and although after 5 min, a higher proportion of the injected dose remained in the circulation (81% compared to 69%) the subsequent rapid metabolism was associated with a similar redistribution of the radioactivity between the other serum lipoproteins. These results are also expressed as a percent of the injected dose in Figure 4.4. The disappearance of label from the serum of human VLDL was characterised by a similar multiphasic clearance curve as that observed after administration of rat VLDL. Radioactivity was rapidly transferred to HDL which contained 23.8% of the total serum radioactivity 5 min after injection (Table 4.2) while approximately 7 and 11% of the serum radioactivity was associated with LDL₁ and LDL₂. The metabolism of VLDL apoprotein was similar to that of the whole lipoprotein (Figure 4.5). Concomitant with a decrease in VLDL protein radioactivity there was an increase in that of HDL, LDL₁ and LDL₂.

4.3.2 In Vitro Experiments

In vitro incubations with control serum were routinely performed and used as controls for the in vivo metabolic experiments. The protocol for in vivo experiments is such that once blood is withdrawn and placed on ice there is a delay of up to 12 hr (depending on the duration of the experiment) before the serum obtained is ultracentrifuged, as all samples are ultracentrifuged at the one time. In vitro experiments were
Figure 4.3  Plasma Disappearance of VLDL Protein and Lipid of Sf 20-100. $^{125}$I-VLDL Sf 20-100 was injected into 300 gm rats which were bled by cardiac puncture. VLDL obtained from rats fed the commercial rat chow was utilised in this experiment. Figures represent the mean of three determinations $\pm$ S.D.
Table 4.2
Distribution of Radioactivity in Serum After Injection of $^{125}$I-labelled Human VLDL into Rats$^a$

<table>
<thead>
<tr>
<th>Time after Injection</th>
<th>VLDL (d &lt; 1.006)</th>
<th>LDL$_1$ (d=1.006-1.019)</th>
<th>LDL$_2$ (d=1.019-1.063)</th>
<th>HDL (d=1.063-1.21)</th>
<th>Infranate (d &gt; 1.21)</th>
<th>% of Serum Radioactivity</th>
<th>% of Injected Dose per 9 ml Serum$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>55.3</td>
<td>7.4</td>
<td>11.4</td>
<td>23.8</td>
<td>2.1</td>
<td>81.7</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>28.3</td>
<td>16.5</td>
<td>25.8</td>
<td>25.7</td>
<td>3.7</td>
<td>62.9</td>
<td></td>
</tr>
<tr>
<td>1 hr</td>
<td>15.6</td>
<td>14.1</td>
<td>30.6</td>
<td>35.4</td>
<td>4.3</td>
<td>46.5</td>
<td></td>
</tr>
<tr>
<td>2 hr</td>
<td>19.2</td>
<td>8.8</td>
<td>30.1</td>
<td>36.3</td>
<td>5.6</td>
<td>32.6</td>
<td></td>
</tr>
<tr>
<td>4 hr</td>
<td>16.5</td>
<td>4.3</td>
<td>33.4</td>
<td>39.8</td>
<td>6.0</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td>8 hr</td>
<td>14.3</td>
<td>5.7</td>
<td>35.7</td>
<td>35.8</td>
<td>8.5</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>12 hr</td>
<td>8.8</td>
<td>4.9</td>
<td>38.3</td>
<td>39.9</td>
<td>8.1</td>
<td>15.1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Rats were injected with 70-80 µg of human VLDL protein and 5 x $10^6$ cpm.

$^b$Figures shown represent the mean data from two rats which were exsanguinated at each time interval. Similar results were obtained in two other experiments.

$^c$See Table 4.1.
Figure 4.4      Redistribution of Radioactivity Among Serum Lipoproteins Following Injection of $^{125}$I-labelled Human VLDL into Rats. Data shown represents the mean from 2 rats in one experiment.
Figure 4.5  Redistribution of Radioactivity among Serum Apolipoproteins after Injection of $^{125}$I-labelled Rat VLDL into Rats. Figures represent the mean ± S.D. from four rats at each time point from two experiments (2 rats each). Protein bound radioactivity was determined as described in the text.
therefore performed to determine what changes occur during this time interval.

In addition $^{125}$I-VLDL was incubated with post-heparin plasma as well as serum from untreated rats ('normal' or control serum). Injection of heparin into rats releases into the circulation a number of lipolytic enzymes which are normally present at tissue sites. Among these are lipoprotein lipases (for example, of adipose tissue, heart and lung) and triglyceride hydrolase (liver). Post-heparin plasma therefore contains a number of enzymes not normally present in serum from control animals. The post-heparin incubation may possibly represent an \textit{in vitro} model of the \textit{in vivo} situation except that there is no input of newly synthesised lipoproteins or clearance of metabolic products.

The results of \textit{in vitro} experiments are shown in Table 4.3 and illustrated in Figure 4.6. In the presence of 'normal' serum, the transfer of radioactivity from VLDL to HDL was slower than that observed \textit{in vivo} (Figure 4.2), a crossover occurring at 1 hr compared to 10-15 min \textit{in vivo}. Similarly the increase in LDL$_1$ and LDL$_2$ radioactivity was less rapid \textit{in vitro}. Maximum radioactivity was reached between 10 and 30 min \textit{in vivo} whereas that in LDL$_1$ and LDL$_2$ was still increasing at 2 hr. The removal of radioactivity from VLDL was also less rapid \textit{in vitro} than \textit{in vivo}, approximately half being removed after 2 hr and 30 min respectively.

In the post-heparin incubation the redistribution of radioactivity occurred more rapidly than in the control and that observed \textit{in vivo} (Figure 4.2). At zero time the HDL radioactivity already exceeded that in VLDL by 3% of the total cpm present in the incubation system. Also accompanying the rapid removal of VLDL there was an increase in LDL$_1$ and LDL$_2$ radioactivity which exceeded that in VLDL at approximately 20 and 10 min respectively and remained higher throughout. However, \textit{in vivo}
Table 4.3

*In Vitro* Incubation of $^{125}$I-VLDL\(^a\) in Serum Obtained from Untreated Rats (Control) and Rats Injected With Heparin 5 Min Prior to Exsanguination

<table>
<thead>
<tr>
<th>Time after addition of 125I-VLDL min</th>
<th>Distribution of Radioactivity among Lipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VLDL (d &lt; 1.006)</td>
</tr>
<tr>
<td></td>
<td>% radioactivity in 5 ml serum(^b)</td>
</tr>
<tr>
<td>Post-Heparin Plasma(^c)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>120</td>
</tr>
<tr>
<td>Control Plasma(^d)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>

\(^a\) VLDL for iodination was obtained from rats fed the saturated fat diet, 3.4% of the radioactivity was present in lipid, 95.6% in protein and 1% was unbound. After ultracentrifugation of the iodinated VLDL preparation 96% of the radioactivity was recovered in the supernatant.

\(^b\) Percent recoveries after ultracentrifugation were 85-95% and 7.9 x 10\(^6\) cpm and 340 µg of VLDL protein were added per incubation. Total volume of serum per incubation was 30 ml.

\(^c\) Similar results were obtained in two other experiments.

\(^d\) Similar results were obtained in four other experiments.
Figure 4.6  In Vitro Incubation of $^{125}$I-VLDL with Control and Post-Heparin Plasma. Figures represent the mean of two determinations. The experimental procedure is described in the text.
the radioactivity declined after an initial appearance in the LDL₁ and LDL₂ fractions.

Analysis of HDL apolipoproteins by polyacrylamide gel electrophoresis after *in vitro* incubation of VLDL with 'normal' serum has shown that most of the radioactivity (60%) is found in zone IV, indicating that most of the transfer of radioactivity *in vitro* to HDL is of low molecular weight peptides.

4.3.3 Metabolism of Individual Apoproteins

After staining and isolating the protein bands separated by electrophoresis on polyacrylamide gels, most of the radioactivity was found to be associated with three major apoprotein groups in VLDL and LDL (I, III, IV) and with four groups in HDL (I, II, III, IV). These were zoned and identified as shown in Figure 4.7. The percent distribution of radioactivity between apoproteins following injection of ¹²⁵I-labelled VLDL into rats is shown in Table 4.4. It can be seen that a significant change in the distribution of radioactivity occurred among VLDL apoproteins during circulation *in vivo* for 6 hr, with zones I and IV accounting for most of the changes. At 5 min, zone I protein contained 42.8% of the label, but only 11.1% at 6 hr. During this time the percent radioactivity in zone IV increased from 41% to 83% of the total counts.

The LDL fraction contained a smaller percent (8.2%) of the total radioactivity following administration of ¹²⁵I-labelled VLDL and a large proportion (approximately 80%) of this label was associated with the zone IV peptides. This distribution remained fairly constant over the time period studied. A similar although more pronounced effect was seen after electrophoretic analysis of the HDL apoprotein. Most of the label in this fraction was associated with the zone IV peptides, although some
Figure 4.7 Identification of Gel Zones Following Electrophoresis and Staining of Rat VLDL, LDL and HDL Apolipoproteins on 10% Polyacrylamide Gels. Most of the radioactivity was associated with the stained bands as described in the text.
Table 4.4
Distribution of Radioactivity Amongst Rat Apolipoproteins Following Injection of $^{125}$I-labelled Rat VLDL to Rats

<table>
<thead>
<tr>
<th>Lipoprotein fraction</th>
<th>Time (min)</th>
<th>% of injected dose of apoprotein (per 9 ml serum)</th>
<th>Zone I</th>
<th>Zone II</th>
<th>Zone III</th>
<th>Zone IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL injected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>29.7</td>
<td>42.8 ± 0.47</td>
<td>—</td>
<td>12.4</td>
<td>43.8</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>23.9</td>
<td>44.9 ± 0.75</td>
<td>—</td>
<td>13.6 ± 0.15</td>
<td>41.6 ± 3.5</td>
<td>46.1 ± 3.7</td>
</tr>
<tr>
<td>30</td>
<td>8.7</td>
<td>23.2 ± 0.80</td>
<td>—</td>
<td>30.7 ± 0.10</td>
<td>81.5 ± 5.0</td>
<td>16.1 ± 3.7</td>
</tr>
<tr>
<td>60</td>
<td>7.7</td>
<td>7.6 ± 0.34</td>
<td>—</td>
<td>10.8 ± 0.90</td>
<td>79.2 ± 17.5</td>
<td>13.5 ± 1.5</td>
</tr>
<tr>
<td>180</td>
<td>6.2</td>
<td>14.2 ± 1.9</td>
<td>—</td>
<td>6.5 ± 0.17</td>
<td>83.1 ± 4.2</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>360</td>
<td>6.8</td>
<td>11.1 ± 0.82</td>
<td>—</td>
<td>5.8 ± 0.15</td>
<td>83.2 ± 4.2</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>LDL (d = 1.006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8.2</td>
<td>14.2 ± 1.9</td>
<td>—</td>
<td>6.5 ± 0.17</td>
<td>79.2 ± 17.3</td>
<td>13.5 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>9.5</td>
<td>11.1 ± 0.82</td>
<td>—</td>
<td>5.8 ± 0.15</td>
<td>83.1 ± 4.2</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>30</td>
<td>9.8</td>
<td>13.6 ± 1.30</td>
<td>—</td>
<td>6.2 ± 0.46</td>
<td>82.2 ± 6.6</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>60</td>
<td>7.9</td>
<td>11.3 ± 1.00</td>
<td>—</td>
<td>4.8 ± 0.31</td>
<td>83.9 ± 8.3</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>180</td>
<td>6.5</td>
<td>14.6 ± 0.56</td>
<td>—</td>
<td>6.2 ± 0.35</td>
<td>79.1 ± 3.2</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>360</td>
<td>6.0</td>
<td>12.1 ± 1.10</td>
<td>—</td>
<td>3.0 ± 0.35</td>
<td>84.9 ± 6.8</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>HDL (1.063-1.21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>32.1</td>
<td>14.4 ± 0.78</td>
<td>12.7 ± 2.00</td>
<td>12.9 ± 0.90</td>
<td>60.0 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>31.9</td>
<td>6.4 ± 0.47</td>
<td>5.1 ± 0.56</td>
<td>9.1 ± 0.54</td>
<td>79.4 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>29.9</td>
<td>7.1 ± 0.50</td>
<td>8.0 ± 1.10</td>
<td>11.7 ± 0.72</td>
<td>73.2 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>24.7</td>
<td>6.2 ± 0.36</td>
<td>4.8 ± 0.41</td>
<td>8.8 ± 0.40</td>
<td>80.1 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>21.2</td>
<td>10.8 ± 0.55</td>
<td>7.5 ± 0.18</td>
<td>10.1 ± 0.45</td>
<td>71.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>360</td>
<td>14.2</td>
<td>8.9 ± 0.70</td>
<td>5.3 ± 0.40</td>
<td>8.4 ± 0.44</td>
<td>77.4 ± 6.5</td>
<td></td>
</tr>
</tbody>
</table>

aResults obtained from three separate gels from the lipoproteins obtained from three rats. This data represents the mean ± S.E.M. of 3 rats at each time point.

bObtained after electrophoresis of apoprotein on 7.5% polyacrylamide gels as described in the text.
radioactivity was associated with the other gel zones.

Figure 4.8 demonstrates the redistribution of label among the three lipoprotein classes, for each apoprotein group, when expressed as a percent of the total injected dose. Initially, the label disappeared at similar rates from the zone I and IV peptides of VLDL, and at 5 min, most of the zone IV radioactivity removed from VLDL (27%) was present in LDL and HDL. However, only 5-6% of the label originally present in zone I of VLDL was present in LDL and HDL at 5 min, suggesting that most of the remainder (33%) was removed from the plasma lipoprotein pool. Figure 4.8 (bottom panel) also demonstrates that from 10 min to 60 min, there was a much more rapid disappearance of radioactivity from the zone I apoprotein than from the zone III or IV apoproteins. After 60 min, the label in all apoproteins of VLDL was removed at similar rates, although there was an apparent increase in the proportion of label in group IV apoprotein between 3-6 hr. The centre panel of Figure 4.8 shows the distribution of label amongst LDL apoproteins. An initial rapid accumulation of radioactivity in the zone IV apoprotein was seen to precede the appearance of label in zone I peptides which peaked at 30 min, and remained fairly constant in this apoprotein up to 3 hr. The radioactivity present in zone III apoprotein showed a similar catabolic fate as the zone I apoprotein. As shown in the top panel, zone IV apoproteins of HDL contained most of the label and reached a peak of activity at 10 min. There was also evidence for the rapid transfer of zone I and III peptides to HDL 5 min after the injection of labelled VLDL although radioactivity present in these zones decreased rapidly during the subsequent 5 min and fell more slowly thereafter.

Figure 4.7 demonstrates the clear separation of the zone IV apoproteins and the actual distance between these bands on the gels (approximately 1 cm) was sufficient to allow the easy separation of these
Figure 4.8  Redistribution of Label among Apoproteins of VLDL, LDL and HDL Following Injection of $^{125}$I-labelled Rat VLDL into Rats. Each point represents the mean ± S.D. from three rats. S.D. bars are omitted when S.D. < 0.1.
bands by the slicing technique. As shown in Figure 4.9, zone IVb (the fastest migrating apoprotein) contained approximately 30-40% more label than zone IVa apoprotein and the relationship between these apoproteins in VLDL and HDL remained approximately the same following injection of $^{125}$I-labelled VLDL. This data suggests that a considerable portion of the label which disappeared rapidly from VLDL zone IV peptides, is accounted for by their transfer to HDL. The data also suggests that between 3 and 6 hr, radioactivity which is lost from zone IV apoprotein of HDL, returns to the same apoprotein group of VLDL. A similar comparison of the zone III apoprotein illustrates that following an initial rapid exchange, the label disappears from VLDL and HDL at a similar rate.

4.3.4 Studies with VLDL Labelled In Vitro from $^{125}$I-labelled Rat HDL*

In order to study the metabolism of VLDL labelled only with zone III and IV peptides, rat VLDL was labelled from iodinated rat HDL in an in vitro system described in the methods section. This system was based on the results of a preliminary experiment carried out to determine the optimum ratio of VLDL protein per ml serum required for maximum labelling of VLDL by exchange from $^{125}$I-labelled HDL. In Table 4.5 it can be seen that the percent of total radioactivity transferred to VLDL increased approximately 4 fold when 2.0 mg VLDL protein was added to the incubation system which contained 4.5 ml serum and $^{125}$I-labelled HDL. This ratio was maintained in the 'scaled-up' system described in the methods section.

*These studies were performed in collaboration with Dr Fidge.
Figure 4.9  Redistribution of Radioactivity Between Zone III and Zone IV Apoproteins of Rat VLDL and HDL Following Injection of $^{125}$I-labelled Rat VLDL into Rats. IVa and IVb were separated as described in the text. Each point represents the mean ± S.D. from three rats. S.D. bars are omitted when S.D. < 0.1.
Table 4.5

In Vitro Transfer of Peptide from $^{125I}$-labelled HDL to VLDL

<table>
<thead>
<tr>
<th>Rat serum ml</th>
<th>Unlabelled VLDL added$^a$ mg protein</th>
<th>Percent of Total radioactivity in VLDL$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>—</td>
<td>0.8</td>
</tr>
<tr>
<td>4.5</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>4.5</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
<td>4.5</td>
<td>2.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>

$^a$Incubation at 37°C as described in text.

$^b$VLDL radioactivity calculated after isolation and washing VLDL by ultracentrifugation.

Following the isolation of the in vitro labelled VLDL, the lipoprotein was fractionated into three subclasses of Sf > 400, Sf 100-400 and Sf 20-100. After washing twice, the lipoproteins were delipidated and the distribution of radioactivity among the apoproteins was determined by polyacrylamide gel electrophoresis. Approximately 80-85% of the radioactivity of all VLDL subfractions was associated with the faster migrating zone III and IV peptides (Table 4.8).

Table 4.8

Characterisation of Apoproteins of VLDL Labelled from $^{125I}$-HDL

<table>
<thead>
<tr>
<th>Percent distribution of radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zone I + II</td>
</tr>
<tr>
<td>VLDL Sf &gt;400</td>
</tr>
<tr>
<td>VLDL Sf 100-400</td>
</tr>
<tr>
<td>VLDL Sf 20-100</td>
</tr>
</tbody>
</table>
The results obtained after injecting VLDL subfractions of Sf > 400 and Sf 100-400 labelled \textit{in vitro} from $^{125}\text{I}$-labelled HDL into rats are shown in Tables 4.6 and 4.7 and Figure 4.10. There was insufficient radioactivity in the Sf 20-100 subfractions for \textit{in vivo} investigation. The data suggests that the redistribution of radioactivity between lipoproteins of Sf 100-400 and HDL was faster than that between VLDL of Sf > 400 and HDL. 2 min after injection of the smaller VLDL molecules, HDL contained twice as much label as VLDL whereas 2 min after injection of the VLDL subclass Sf > 400, there was approximately an equal amount of label in VLDL and HDL.

It was interesting to note that a significant proportion of the radioactive peptide was transferred to LDL$_1$ and LDL$_2$ fractions following injection of both subclasses of zone IV labelled VLDL. In both cases, there was an absolute increase in radioactivity in LDL$_1$ and LDL$_2$ for 5 and up to 15 min respectively. Electrophoresis of the LDL$_2$ and HDL apoprotein confirmed that the fastest migrating peptides were involved in the transfer of label.

4.4 DISCUSSION

Fidge and Foxman (1971) have shown that following the administration of $^{125}\text{I}$-labelled VLDL to rats a considerable proportion of the labelled peptide was redistributed among the other higher density lipoproteins suggesting that they were metabolically related to VLDL. The present study was undertaken in order to examine in more detail, the catabolic events involved in this interrelationship and to identify, as far as possible, the apoproteins involved in the process. During the course of these experiments, the results of a similar study were reported by Eisenberg and Rachmilewitz (1973a,b) and where comparable their findings were similar to those described here.
<table>
<thead>
<tr>
<th>Time after Injection min</th>
<th>VLDL (d &lt; 1.006)</th>
<th>LDL₁ (d=1.006-1.019)</th>
<th>LDL₂ (d=1.019-1.063)</th>
<th>HDL (d=1.063-1.21)</th>
<th>Infranate (d &gt; 1.21)</th>
<th>Serum Radioactivity % of injected dose per 9 ml serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>28.7</td>
<td>6.1</td>
<td>10.6</td>
<td>47.7</td>
<td>6.9</td>
<td>81.4</td>
</tr>
<tr>
<td>5</td>
<td>26.6</td>
<td>7.1</td>
<td>11.3</td>
<td>48.9</td>
<td>6.1</td>
<td>80.2</td>
</tr>
<tr>
<td>10</td>
<td>27.1</td>
<td>5.3</td>
<td>12.2</td>
<td>50.9</td>
<td>4.5</td>
<td>68.0</td>
</tr>
<tr>
<td>15</td>
<td>19.8</td>
<td>5.8</td>
<td>16.0</td>
<td>52.0</td>
<td>6.4</td>
<td>66.3</td>
</tr>
<tr>
<td>30</td>
<td>24.4</td>
<td>5.4</td>
<td>14.1</td>
<td>50.9</td>
<td>5.2</td>
<td>52.0</td>
</tr>
<tr>
<td>45</td>
<td>21.8</td>
<td>5.8</td>
<td>13.3</td>
<td>53.2</td>
<td>5.9</td>
<td>49.3</td>
</tr>
<tr>
<td>60</td>
<td>17.6</td>
<td>6.6</td>
<td>16.5</td>
<td>54.2</td>
<td>5.1</td>
<td>40.8</td>
</tr>
</tbody>
</table>

a81 µg VLDL protein and approximately 4 x 10⁶ cpm injected per rat.

bData represents the mean from 2 rats at each time point.

cAs in Table 4.1.
Table 4.7
Metabolism of $^{125}$I-labelled VLDL-Sf > 400 Labelled by *In Vitro* Incubation with $^{125}$I-labelled HDL

<table>
<thead>
<tr>
<th>Time after Injection min</th>
<th>VLDL (d &lt; 1.006)</th>
<th>LDL$_1$ (d=1.006-1.019)</th>
<th>LDL$_2$ (d=1.019-1.063)</th>
<th>HDL (d=1.063-1.21)</th>
<th>Infranate (d &gt; 1.21)</th>
<th>Serum Radioactivity % of injected dose per 9 ml serum^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>36.1</td>
<td>4.1</td>
<td>11.1</td>
<td>42.0</td>
<td>6.7</td>
<td>75.0</td>
</tr>
<tr>
<td>5</td>
<td>24.8</td>
<td>9.0</td>
<td>11.7</td>
<td>46.4</td>
<td>8.1</td>
<td>82.6</td>
</tr>
<tr>
<td>10</td>
<td>19.1</td>
<td>7.6</td>
<td>14.7</td>
<td>50.8</td>
<td>7.8</td>
<td>63.3</td>
</tr>
<tr>
<td>15</td>
<td>14.4</td>
<td>9.1</td>
<td>12.7</td>
<td>58.8</td>
<td>5.0</td>
<td>54.0</td>
</tr>
<tr>
<td>30</td>
<td>18.4</td>
<td>9.0</td>
<td>14.4</td>
<td>50.8</td>
<td>7.4</td>
<td>47.8</td>
</tr>
<tr>
<td>45</td>
<td>14.7</td>
<td>9.9</td>
<td>14.1</td>
<td>56.0</td>
<td>5.4</td>
<td>46.2</td>
</tr>
<tr>
<td>60</td>
<td>14.5</td>
<td>18.1</td>
<td>11.9</td>
<td>50.6</td>
<td>4.9</td>
<td>42.8</td>
</tr>
</tbody>
</table>

^a^62.5 µg VLDL protein and 5 x 10^6^ cpm injected per rat.

^b^Data represents mean from 2 rats at each time period.

^c^As in Table 4.1.
Figure 4.10 Redistribution of Radioactivity Following Injection of Predominantly Zone III and IV $^{125}$I-labelled VLDL from Rats. Rat VLDL was labelled as described in text. Top panel shows the results after injection of VLDL Sf > 400 and bottom panel, the redistribution after injection of Sf 100-400 molecules.
The disappearance of total serum radioactivity following the administration of $^{125}$I-labelled VLDL was characterised by a curve different to that described by a plot of the radioactivity remaining in the VLDL fraction. This difference is accounted for by a transfer or exchange of labelled protein between VLDL and the other lipoprotein fractions. Attempts to accurately define the early kinetic processes involved in the clearance of VLDL from the circulation are hindered by various factors. Firstly, it is recognised that the whole VLDL used in these experiments (Figure 4.2) is not homogeneous with regard to its composition (Gustafson et al., 1965), size (Sata et al., 1972; Lossow et al., 1969), heterogeneity of apoproteins (Frederickson et al., 1972) and origin (Windmueller et al., 1970b, 1972) so there can be no assumption of uniform catabolism within this fraction. Secondly, the metabolism of VLDL is associated with an exchange or transfer of several components (Minari and Zilversmit, 1963; Illingworth and Portman, 1972) to other lipoproteins while there is evidence to suggest that there is a reciprocal net transfer of protein from HDL to triglyceride rich lipoproteins (Havel et al., 1973). For these reasons no attempt has been made to assign turnover rates for apo-VLDL metabolism although it can be seen that when expressed as total serum or VLDL disappearance, broadly two phases of clearance are associated with its catabolism. From the time of injection to the earliest sample at 5 min there is a very rapid loss of radioactivity from the serum which continues and is still apparent between 5 and 30 min. The multiexponential nature of this rapid phase was revealed in studies in which a greater number of samples were obtained between 5 and 60 min, demonstrating that the first phase could not be defined by one component only. This early rapid clearance is followed by a slower phase of removal which approaches linearity after 3 hr.
These studies clearly demonstrated the occurrence of rapid exchange or transfer of radioactivity to other lipoproteins, since 5 min after $^{125}$I-labelled VLDL injection only 50.6% of the total serum radioactivity remained in VLDL and 32.7% was already present in HDL. The transfer of VLDL radioactivity to other lipoproteins after in vitro incubation with control serum was slower than that observed in vivo. However, after in vitro incubation with post-heparin plasma the transfer of radioactivity occurred much faster than observed in vivo. This was apparently due to the presence of lipolytic activity since in similar incubations with post-heparin plasma it was demonstrated that the release of free fatty acids into the incubation medium increased with time (Fidge, unpublished observations). The difference in the appearance of label in LDL₁ and LDL₂ in vivo to that observed in vitro (post-heparin) is presumably the consequence of influx or efflux of LDL into or from the circulation. This is impossible in vitro and results in accumulation of radioactivity in these lipoproteins. In Chapter 5 it was shown that the transfer of radioactivity from VLDL to LDL₁ and LDL₂ in the in vitro control incubation predominantly involved the low molecular weight soluble proteins whereas both apolipoprotein B and C apoproteins were transferred in the in vitro post-heparin incubation. From the data it is apparent that catabolism of VLDL requires the presence of lipolytic activity and that in its absence (in vitro, control serum) only transfer of C peptides (soluble proteins) is observed. These results are in accord with those reported by Bilheimer et al (1972) who demonstrated a transfer of radioactivity from VLDL to HDL and to other lipoproteins at 4°C after in vitro incubation with VLDL with control serum.

The kinetics of the redistribution in the rat is much faster than in the human (Eisenberg et al, 1973a). In the human (Eisenberg et al, 1973a) LDL radioactivity peaked at 12-24 hours and HDL at 9 hours whereas
in the rat, the transfer of label to HDL and to LDL peaked at only 30 min when expressed as a percentage of the injected dose. This discrepancy can possibly be attributed to the difference in size of the two species and consequent differences in Standard Metabolic Rate and cardiac output which result in a more rapid catabolism of the VLDL molecule in the rat.

The results presented here are similar to those of Eisenberg and Rachmilewitz (1973a,b) who found that 2 min after injection of \(^{125}\)I-labelled rat VLDL into rats, only 77.4% of total serum \(^{125}\)I-iodine was left in VLDL, with 5.5% in LDL and 12.2% in HDL. By 15 min these values had changed to 44.6% of serum radioactivity for VLDL, 8.7% for LDL and 36.1% for HDL. It is extremely unlikely that the rapid removal of VLDL in these studies was due to the presence and removal of denatured lipoprotein since the VLDL had been reisolated by ultracentrifugation immediately prior to injection. Also, in other experiments, VLDL which had been filtered through 0.22 nm cellulose nitrate millipore filters, showed essentially the same turnover rate as unfiltered preparations. Furthermore, the half-life of the VLDL preparations used in these studies was similar to that reported (Roheim et al., 1972) for native VLDL labelled biologically with \(^{14}\)C-leucine in which an initial rapid clearance phase (half-life approximately 40 min) was followed by a later slower disappearance phase. Fidge (unpublished observations) observed that VLDL labelled biologically from \(^{3}\)H-lysine exhibited initial rapid and later slower clearance rates similar to the iodinated lipoproteins. Similar kinetics of VLDL removal were also observed when \(^{125}\)I-labelled VLDL had been 'biologically screened' by injection into rats and after reisolation reused for in vivo experiments.

In these studies the LDL fraction was subfractionated into two subclasses, LDL\(_1\) (d 1.006-1.019) and LDL\(_2\) (d 1.019-1.063). LDL\(_1\) contained at least 56% of the LDL\(_2\) radioactivity and did not exceed 74%
up to 3 hours after VLDL injection, although the percent decreased to 23% at 6 hours. It has been suggested that the LDL\textsubscript{1} fraction is an intermediate lipoprotein formed from the degraded VLDL molecule and could possibly be a precursor of LDL\textsubscript{2} molecules. However, there is no direct evidence of a precursor-product crossover relationship between LDL\textsubscript{1} and LDL\textsubscript{2} following injection of VLDL \( d < 1.006 \). After 3-6 hr the amount of radioactivity in LDL\textsubscript{2} is approximately equal to that present in VLDL and although this may indicate the existence of a precursor-product relationship between VLDL and LDL, specific radioactivity data (see Chapter 5), not obtained in these studies is required before any quantitative interpretation could be considered.

To further investigate the transfer of apoproteins, the lipoproteins were delipidated and resolved using polyacrylamide gel electrophoresis. The gel studies showed that most of the label transferred to both LDL and HDL was due to zone III and IV apoproteins. Due to its poor solubility, some selective loss of apo-LDL (B protein) may have occurred during delipidation and resolubilisation of rat LDL. Even taking this into account, the results still show that a considerable proportion of the radioactivity was associated with the faster migrating peptides which are present in small amounts in rat LDL as shown in the gel pattern in Figure 4.7, and reported by others (Koga et al, 1971). The significance of this exchange is not known although the transfer of zone IV peptides from VLDL to LDL and HDL is roughly proportional to the mass of zone IV peptides present in each lipoprotein fraction. Unfortunately, the quantitative significance of this exchange between VLDL and LDL in these studies cannot be compared with the one other reported study (Eisenberg and Rachmilewitz, 1973a,b) of \(^{125}\text{I}\)-labelled rat VLDL metabolism, since these authors did not separately isolate LDL, but based their information on a \( d < 1.063 \) fraction which contained both VLDL and LDL.
A close examination of the kinetics of apoprotein transfer by examination of the distribution of radioactivity in gel zones I, III and IV of VLDL and LDL and zones I, II, III and IV of HDL revealed considerable metabolic heterogeneity between these zones. The disappearance of label from zone IV and zone III in VLDL and LDL is different to that in zone I. In VLDL zone I radioactivity decreases more rapidly than zones III and IV. This result is in accordance with the human situation as described by Eisenberg et al (1973a), zones III and IV being equivalent to the human C proteins and zone I to apo-LDL or B protein (Koga et al, 1971). In LDL, zones I and III follow the same kinetics suggesting a parallel clearance of these components from the circulation. These zones may therefore be part of a subunit or component of apo-LDL which are metabolically related. Zone IV radioactivity however increased then decreased as did zone IV of HDL which suggests that 'C protein' exchange is occurring.

Gel zone IV was further divided into zones IVa and IVb, which are labelled to different extents, IVb containing more radioactivity than IVa. However, both IVa and IVb in VLDL, LDL and HDL were metabolised at similar rates. It has been suggested by Herbert et al (1973a) that a rat C-protein (C-II) associated with gel zone IV acts as a cofactor of lipoprotein lipase. According to their nomenclature, rat C-II peptide comigrates with C-III, (equivalent to zone IVa) on polyacrylamide gels at pH 8 and like human C-II peptide is a potent activator of lipoprotein lipase.

The similarity in metabolism of rat and human VLDL in the rat suggests that this animal is a suitable model for studying human VLDL metabolism. The results of the studies with human VLDL in rats are in accord with those described by Eisenberg et al (1973b) in which the fate of human VLDL and LDL in the rat was similar to that observed previously.
There was a rapid transfer of radioactive peptides to other lipoprotein fractions during the first 30 min following the intravenous injection of $^{125}$I-labelled rat very low density lipoprotein (VLDL) into rats. After this initial redistribution of radioactivity, label disappeared slowly from all lipoprotein fractions. Human VLDL, when injected into rats also showed a similar fate to rat VLDL.

Most of the radioactivity transferred from VLDL to low density (LDL) and high density lipoprotein (HDL) was associated with two peptides, identified in these studies by polyacrylamide gel electrophoresis as zone IVa and IVb peptides, although radioactivity initially associated with zones I and III, was also transferred to LDL and HDL. That the transfer of label from VLDL to LDL and HDL primarily involved an exchange of small molecular weight peptides, was confirmed in studies using VLDL predominantly labelled in these peptides by in vitro exchange with $^{125}$I-labelled HDL.

Incubation of $^{125}$I-labelled VLDL with post-heparin plasma revealed that the redistribution of radioactivity among lipoproteins was faster than that observed in vivo and in the in vitro control incubation. The transfer of radioactivity from VLDL to HDL, LDL$_1$ and LDL$_2$ was less rapid in vitro after incubation with control serum than in vivo. The redistribution of radioactivity observed in the latter situation being primarily due to a transfer of soluble proteins (zone III and IV peptides).

Both zone I and zone IV radioactivity was rapidly removed from VLDL during the first 5 min after injection. However, while most of the zone IV radioactivity was recovered in LDL and HDL, only 12% of the label lost
from zone I of VLDL was recovered in other lipoproteins with the remainder presumably having been cleared from the plasma compartment.

It can therefore be concluded that, during catabolism of rat VLDL apoprotein there is a rapid transfer of small molecular peptides to both LDL and HDL. Most of the VLDL is rapidly removed from the circulation with only a small portion being transformed into LDL molecules.
CHAPTER 5

A STUDY OF THE QUANTITATIVE METABOLIC INTERRELATIONSHIP BETWEEN VLDL, LDL AND HDL APOLIPOPROTEINS
CHAPTER 5

A STUDY OF THE QUANTITATIVE METABOLIC INTERRELATIONSHIP BETWEEN VLDL, LDL AND HDL APOLIPOPROTEINS

5.1 INTRODUCTION

In Chapter 4 it was demonstrated that most of the catabolised VLDL is apparently removed from the circulation and only the remainder converted to LDL. Transformation of rat VLDL to LDL₁ and LDL₂ occurred both in vivo and after in vitro incubation with post-heparin plasma. Concomitant with a decrease in zone I radioactivity in VLDL an increase in that of LDL₂ zone I was observed. Although these results suggest the existence of a precursor product relationship between VLDL and LDL, specific radioactivity data is required before any quantitative interpretation can be made.

One difficulty encountered in the determination of specific activity is the considerable heterogeneity in the metabolism of VLDL components revealed in Chapter 4. Removal of VLDL lipid from the circulation was more rapid than that of protein. Of the apoprotein moieties of VLDL, zone I radioactivity of VLDL was removed from the circulation more rapidly than zones II, III and IV proteins. However, one component of apo-VLDL, apo-LDL or B protein, is present in both LDL₁ and LDL₂. This has been demonstrated immunochemically. Rat apo-VLDL contains approximately 30% apoB and apo-LDL₁ and apo-LDL₂ contain approximately 70 and 90% apoB respectively. This protein is present in zone I and in experiments described in Chapter 4 appears to behave as a single entity. Although only small amounts of this protein would be present in the lipoproteins of one rat it was estimated that this may be sufficient to allow quantitative measurement.
Before the specific activity (cpm/µg protein) could be determined methods for the separation and isolation of apo-LDL from other apoproteins of VLDL, LDL₁ and LDL₂ were investigated. This chapter describes the techniques used for the estimation of apo-LDL (B protein) specific activity and their application for the estimation of VLDL apo-LDL turnover and the determination of the quantitative relationship between VLDL, LDL₁ and LDL₂. The application of these techniques for the estimation of C protein specific activity is also described.

5.2 EXPERIMENTAL

The experimental procedure for metabolic experiments was as shown in Figure 4.1 and described in Chapter 4.

5.2.1 Determination of Specific Activity of the B Protein and C Proteins

The major problem in the determination of the specific activity of the B and C proteins present in VLDL, LDL₁ and LDL₂ is their separation. The B and C proteins can be well separated by chromatography on Sephadex G-150 in the presence of detergents such as SDS. However, this method of separation requires considerable time, equipment and larger quantities of protein than those obtained in metabolic experiments using rats thereby limiting its use for the routine separation of a large number of samples.

Requirements of the method of separation are that:

(i) many samples be separated at the one time; and
(ii) it must be reproducible over a range of low protein concentration (50-500 µg).

Three methods were investigated:

(a) Separation by 1,1,3,3-tetramethylurea (TMU) as described by
Kane (1973);

(b) Separation on polyacrylamide gels (PAGE); and

(c) Separation utilising the solubility of the respective proteins in 5 mM ammonium bicarbonate (not previously reported).

(a) Separation by TMU. TMU is a denaturing solvent which permits rapid and direct separation of soluble apoproteins from the lipid moiety and precipitates the B protein. An equal volume of TMU (redistilled in glass) was added to the lipoprotein solution and allowed to separate and then centrifuged for 30 minutes at 2,500 rpm (MSE Mistral 6L). The aqueous TMU phase was removed using a pasteur pipette. The lipid phase and protein precipitate (floating with lipid) was again washed with TMU. The precipitate was then solubilised and the protein concentration determined (Lowry et al., 1951). TMU interferes with the estimation of protein and the soluble fraction had to be diluted at least one in five before a reliable protein estimation could be obtained. However, as shown in Table 5.1, recoveries using small quantities of protein were low. Results using this method were not consistent for quantities less than 300 µg.

(b) Determination of Specific Activity by PAGE. Following solubilisation of delipidated apoproteins (described in Chapter 4) the protein moiety can be resolved into a number of components which appear as stained bands on polyacrylamide gels, following their reaction with the dye amido black. Gels were divided into four zones as illustrated in Chapter 4 (Figure 4.7). Zone IVa and IVb peptides are found in VLDL, LDL₁, LDL₂ and HDL. The possibility of quantifying the amount of protein present in the stained bands appearing on polyacrylamide gels by planimetry was investigated and will be discussed below.
Table 5.1

Comparison of the Proportion of the TMU Soluble and Insoluble Apoprotein Present in Rat VLDL, LDL\textsubscript{2} and HDL Following Delipidation with TMU

<table>
<thead>
<tr>
<th></th>
<th>PERCENT INSOLUBLE (µg)</th>
<th>PERCENT SOLUBLE (µg)</th>
<th>SOLUBLE AND INSOLUBLE (µg)</th>
<th>PROTEIN PRESENT BEFORE DELIPIDATION (µg)</th>
<th>PERCENT RECOVERY OF PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat VLDL</td>
<td>25.9 ± 9.2</td>
<td>74.1 ± 9.2</td>
<td>102.1 ± 31.5</td>
<td>150</td>
<td>67.8 ± 21.1</td>
</tr>
<tr>
<td>Rat LDL\textsubscript{2} \textsuperscript{a}</td>
<td>43.1 ± 7.3</td>
<td>57.0 ± 7.3</td>
<td>56.6 ± 9.3</td>
<td>150</td>
<td>37.7 ± 6.2</td>
</tr>
<tr>
<td>Rat HDL</td>
<td>8.85 ± 0.5</td>
<td>91.2 ± 0.5</td>
<td>57.9 ± 3.4</td>
<td>150</td>
<td>38.6 ± 2.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}LDL\textsubscript{2} = density 1.019-1.063 g/ml.

Figures represent the mean of three determinations ± S.D.
Gels were scanned at 601 nm by a Varian Techtron gel scanner using a glass cuvette of the bucket type. All gels in each experiment were scanned under identical conditions at 10 mm/min with an external slit width of 1.0 mm, internal slit width 0.05 mm and chart speed of 3.3 cm/min. The areas under the absorption peaks were determined by triangulation or planimetry and calculated as sq mm. Maximum scale expansion of the recorder was achieved at a recording sensitivity of 10 mV. Highly absorbing peaks were recorded by decreasing the sensitivity to 20 mV (one half) and 50 mV (one fifth) of maximum scale expansion (10 mV).

Figure 5.2 shows typical scans of apo-VLDL, LDL₁ and LDL₂. The area between 0 and 1.8 cm represents the stacking gel. At the interface of the stacking gel and the main gel (at 1.8 cm) there is a marked change in optical density due to the main gel stacking gel interface. The next peak obtained represents the material which does not fully penetrate the main gel and reacts immunochemically with antibodies prepared against LDL₂ (density 1.020-1.045 g/ml) and falls into zone I. Zones II and III contain soluble proteins which penetrate the main gel. Zone IV bands are shown on Figure 5.2 between 7 and 9 cm and on Figure 5.4 (which compares the scans of apo-VLDL and apo-HDL). The actual size of the scan is 30 cm x 40 cm so the area of the zone IV peptides could be accurately determined for all apolipoproteins, except LDL₁.

By determining the area under the absorption peaks the specific activity of each peak can be calculated in terms of radioactivity present per unit area (cpm/sq mm). The specific activity can only be calculated in terms of cpm/µg protein when the relationship between the proportion of dye present per unit protein is known. As each protein may vary in its chromogenicity a standard curve (dye:protein) has to be established for each protein before the specific activity (cpm/µg protein) can be determined.
Table 5.2
Comparison of the Proportion of Apoprotein Present in PAGE Zone I and Zones II, III and IV of Rat Apo-VLDL, LDL₁ and LDL₂, Following Delipidation With Organic Solvents

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PERCENTᵃ PRESENT IN ZONE I</th>
<th>PERCENT PRESENT IN ZONES II, III AND IV</th>
<th>NUMBER OF SAMPLES</th>
<th>PERCENT RECOVERY AFTER PAGE</th>
<th>TOTAL PROTEIN BEFORE DELIPIDATION AND PAGE (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat VLDL</td>
<td>29.2 ± 7.0</td>
<td>70.9 ± 7.0</td>
<td>6</td>
<td>77.2 ± 3.2</td>
<td>100-200</td>
</tr>
<tr>
<td>Rat VLDL</td>
<td>26.1 ± 6.5</td>
<td>75.6 ± 6.2</td>
<td>6</td>
<td>74.4 ± 6.5</td>
<td>100-200</td>
</tr>
<tr>
<td>Rat VLDL</td>
<td>26.7 ± 6.1</td>
<td>73.3 ± 6.1</td>
<td>6</td>
<td>73.4 ± 8.7</td>
<td>100-200</td>
</tr>
<tr>
<td>Rat LDL₁</td>
<td>64.8 ± 7.3</td>
<td>35.2 ± 7.3</td>
<td>9</td>
<td>54.4 ± 9.9</td>
<td>20-100</td>
</tr>
<tr>
<td>Rat LDL₂ᵇ</td>
<td>67.1 ± 7.9</td>
<td>32.9 ± 7.9</td>
<td>6</td>
<td>85.1 ± 13.4</td>
<td>200-250</td>
</tr>
<tr>
<td>Rat LDL₂ᵇ</td>
<td>69.5 ± 7.4</td>
<td>30.6 ± 7.4</td>
<td>6</td>
<td>73.0 ± 13.8</td>
<td>100-250</td>
</tr>
<tr>
<td>Rat LDL₂ᵇ</td>
<td>67.6 ± 10.6</td>
<td>32.4 ± 10.6</td>
<td>8</td>
<td>70.0 ± 15.3</td>
<td>100-250</td>
</tr>
</tbody>
</table>

ᵃPercent total area of stained bands on gel.

ᵇLDL₂ (d 1.019-1.063 g/ml) was used in this study.

Figures represent the mean ± S.D.
Apo-VLDL and apo-LDL₂ (50-80 µg) were electrophoresed as described in Chapter 4 and stained with amido schwartz 10b.

Figure 5.1 Polyacrylamide Gels Typical of Those Used for Scanning (Figure 5.2 opposite).
Figure 5.2  Scans of the Profiles of Apolipoproteins of VLDL, LDL\textsubscript{1} and LDL\textsubscript{2} after Electrophoresis on Polyacrylamide Gels (7.5\% acrylamide). Arrows show direction of current flow ((-) anode to (+) cathode).
Figure 5.3 Polyacrylamide Gels of Apo-VLDL and Apo-HDL. The scans are shown opposite. Protein (50-100 µg) was electrophoresed as described in the text on 7.5% acrylamide gels.
Figure 5.4 Scans Comparing the Profiles of Apo-HDL and Apo-VLDL after Polyacrylamide Gel Electrophoresis. Arrows show direction of current flow (−) anode to (+) cathode.
Figure 5.5 shows a standard curve for the B protein (apo-LDL). The curve is linear to 120 µg. Known quantities of apoB obtained from Sephadex G-150 separations of apo-VLDL (to be described later) were dissolved in 0.05 M SDS (.015 M final concentration before loading onto gels), 0.05 M Triz-HCl, pH 8.2 in 6 M urea and applied to polyacrylamide gels and electrophoresed as described previously (Chapter 4). The purity of this apoB was tested by passage through Biogel P 2 (100-200 mesh) columns eluting with .005 M Triz-HCl, pH 8.2 containing 1 mM EDTA and by immuno-diffusion on 2% agarose against albumin, HDL, VLDL and LDL 2 antibodies. Standard curves were also prepared using apo-LDL 2 prepared by delipidating LDL 2 (density 1.020-1.045 g/ml) which had been purified by two ultracentrifugations (at d 1.045 g/ml) and tested immunochemically as described above for the presence of contaminants such as albumin and soluble proteins. This standard curve closely approximated the one shown in Figure 5.5. Using the standard curve in Figure 5.5 the specific activity of the B protein in cpm/µg protein was determined. Aliquots (50 µg) of the apoB used for the standard curve determination were electrophoresed with each gel run to ensure uniformity and as a control.

One problem in this method for estimating the area of the B protein was the lack of complete resolution of B protein due to the stacking gel/main gel peak (Figure 5.4, VLDL scan). Experimentation with a number of gel systems (Weber and Osborn, 1969; variations based on the theory of Ornstein, 1964; and initial reagents and systems of Davis, 1964) failed to produce a combination whereby the B protein penetrated the main gel whilst retaining and resolving the other soluble proteins on the same gel. However using the method described above calculations of B protein specific activity were made and will be discussed later. The ratio of insoluble protein (B protein) to soluble protein (area of B protein peak:sum of the area of all other peaks on the gel, sq mm x 100/1) is consistent and
Figure 5.5  B Protein (Apo-LDL) Standard Curve. The curve was prepared as described in the text.
the recoveries obtained compare well with those obtained using other methods (column chromatography on Sephadex G-150, see Chapter 5) or by differential solubility in an aqueous solution as described below (Tables 5.2 and 5.3).

At least two criticisms of the PAGE method are apparent. Firstly, the material which remains at the junction of the stacking gel/main gel and assumed to contain the insoluble B protein, may in fact also contain aggregated soluble protein which may not be dissociated by the urea/SDS system used. Secondly, the sigmoidal nature of the standard curve (Figure 5.5) raises some doubts about the accuracy of the relationship of area measured to actual protein present at the junction of the stacking gel and main gel and as suggested above a better measurement may possibly have been obtained if the B protein entered the main gel. In view of these possibilities, another means of separating the B from the C proteins was investigated.

(c) **Determination of Specific Activity after Separation of the Apoproteins by their Differential Solubility in an Aqueous Solution.** A method for the efficient separation of the apoproteins on the basis of their relative solubility in low ionic strength buffers was investigated as an alternative to the PAGE method. This procedure relies on the fact that while the B protein is insoluble in medium strength buffers without added dissociating agents the C proteins are readily solubilised.

5 mM ammonium bicarbonate was chosen as the buffer because:

(i) the human C proteins and their rat analogues are readily soluble in this buffer; and

(ii) it can be completely removed during lyophilisation.
Table 5.3
Comparison of the Proportion of 5 mM Ammonium Bicarbonate Soluble and Insoluble Apoprotein Present in Rat VLDL, LDL₁ and LDL₂ Following Delipidation With Organic Solvents

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PERCENT INSOLUBLE</th>
<th>PERCENT SOLUBLE</th>
<th>NUMBER OF SAMPLES</th>
<th>PERCENT RECOVERY OF PROTEIN AFTER DELIPIDATION&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TOTAL PROTEIN BEFORE DELIPIDATION&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat VLDL</td>
<td>46.6 ± 6.2</td>
<td>54.4 ± 6.2</td>
<td>6</td>
<td>79.6 ± 15.9</td>
<td>150-500</td>
</tr>
<tr>
<td>Rat LDL₁</td>
<td>65.5 ± 12.8</td>
<td>34.5 ± 12.8</td>
<td>6</td>
<td>61.5 ± 18</td>
<td>50-200</td>
</tr>
<tr>
<td>Rat LDL₂&lt;sub&gt;b&lt;/sub&gt;</td>
<td>86.8 ± 5.0</td>
<td>15.9 ± 6.8</td>
<td>6</td>
<td>99.3 ± 1.6</td>
<td>500-700</td>
</tr>
<tr>
<td>Rat VLDL</td>
<td>38.7 ± 11.9</td>
<td>61.5 ± 11.7</td>
<td>8</td>
<td>82.5 ± 9.5</td>
<td>200-500</td>
</tr>
<tr>
<td>Rat LDL₁&lt;sub&gt;b&lt;/sub&gt;</td>
<td>25.4 ± 8.1</td>
<td>74.6 ± 8.1</td>
<td>8</td>
<td>88.4 ± 6.6</td>
<td>200-500</td>
</tr>
<tr>
<td>Rat LDL₂&lt;sub&gt;b&lt;/sub&gt;</td>
<td>63.4 ± 10.1</td>
<td>36.6 ± 10.1</td>
<td>8</td>
<td>62.5 ± 9.7</td>
<td>50-100</td>
</tr>
<tr>
<td>Rat LDL₁&lt;sub&gt;b&lt;/sub&gt;</td>
<td>88.3 ± 4.7</td>
<td>11.6 ± 4.9</td>
<td>8</td>
<td>70.0 ± 12.8</td>
<td>70-200</td>
</tr>
<tr>
<td>Rat LDL₂&lt;sub&gt;b&lt;/sub&gt;</td>
<td>89.8 ± 4.1</td>
<td>10.2 ± 4.1</td>
<td>8</td>
<td>71.5 ± 12.8</td>
<td>70-200</td>
</tr>
</tbody>
</table>

<sup>a</sup> After 5 mM Ammonium Bicarbonate Separation.

<sup>b</sup> LDL₂ = d 1.019-1.055 g/ml.

Figures represent the mean ± S.D.
The procedure was validated for use in the separation of the rat VLDL apoproteins by PAGE of the soluble and insoluble portions (Figures 5.6 and 5.7) obtained after ammonium bicarbonate separation as described below.

Also, the ammonium bicarbonate insoluble material reacted immunochemically with anti-apo-LDL (apolipoprotein of LDL d 1.020-1.040 g/ml) and with anti-apo-LDL prepared with apo-LDL obtained from the separation of apo-VLDL on Sephadex G-150 columns (see Chapter 5, apo-LDL elutes in Peak I i.e. the void volume peak). Ratios of soluble:insoluble protein and Peak I:other proteins eluted from Sephadex G-150, of apo-VLDL were similar (approximately 30-40:60-70 for both methods of separation).

Ammonium bicarbonate separations were performed in the following manner. After delipidation (same method as for PAGE) in 5 ml delipidating tubes (not siliconised) 0.5 ml of 5 mM NH₄HCO₃ was added to the dried protein. The tubes were then vortexed and allowed to stand overnight. The tubes were centrifuged at 2,000 x g for 30 min and the supernatant taken off and retained. The pellet was then washed with a further 0.5 ml of 5 mM ammonium bicarbonate, vortexed and recentrifuged. The supernatant of this spin was combined with that of the first spin and represents the soluble portion. The insoluble protein (pellet) was solubilised in 0.1 M NaOH for protein estimation or 0.1 M SDS 0.1 M Triz-HCl pH 8.2 for PAGE. The soluble portion was lyophilised and then made up to a known volume and the protein concentration determined. Aliquots containing a known number of counts were used for protein determination and the SA (cpm/µg) was then calculated.

PAGE of the ammonium bicarbonate insoluble portion of apo-VLDL, apo-LDL₁ and apo-LDL₂ (Figure 5.6) revealed that most of the stained material did not enter the main gel whereas all ammonium bicarbonate soluble material did enter the main gel (Figure 5.7). Table 5.3 shows that the recoveries of total protein after delipidation and ammonium bicarbonate
Quantities of 20-50 µg protein were applied to the gels. The ammonium bicarbonate insoluble material was lyophilised and dissolved in 0.05 M Triz-HCl pH 8.2 and diluted to 0.015 M SDS by the addition of 8 M urea in 0.01 M Triz-HCl pH 8.2 before PAGE. Gels were then treated as described in the text.
Figure 5.7  PAGE of Ammonium Bicarbonate Soluble and Insoluble Apoproteins of VLDL on 5% Acrylamide Gels. Samples were electrophoresed as described in the text and 80, 80, 70 and 50 µg (left to right) protein was loaded onto each gel.
separation exceeded that obtained by PAGE and TMU on small quantities of protein. The lower proportion of insoluble to soluble protein seen in Table 5.2 could be due to small losses incurred by PAGE and underestimation of the B protein as some may not penetrate even the stacking gel (Figure 5.6). The differences may also be due to slight variations between individual and groups of rats. Individual soluble protein specific activities cannot be obtained by the ammonium bicarbonate method. These were obtained by PAGE of the whole apoprotein or of the soluble fraction after ammonium bicarbonate separation.

Statistical analysis (using the students t test) of the data presented in Tables 5.2 and 5.3 revealed that the proportions of soluble:insoluble or zone I:zone II+III+IV obtained by both methods, of apo-LDL1 were similar (P = 90%) but although the mean value for apo-VLDL were similar (34.6±11.5:65.5±11.4 and 27.3±6.3:73.3±8.4 respectively) they were statistically significantly different (P=2.4%). Data obtained using either method were internally consistent (P > 5%, Table 5.2 and Table 5.3 comparing each set of data against other similar sets of data i.e. VLDL vs VLDL1, VLDL2 and the same for LDL1 and LDL2).

5.2.2 Estimation of VLDL B Protein Turnover*

The rate of turnover of VLDL B protein in the whole body of the rat was estimated using the method of Corney and Heath (1970), from specific activity (S) versus time (t) curves and the area under the curve from the time of injection to infinity, (i.e. Sdt, is calculated). The turnover rate R is then given by \( R = \frac{D}{\int_0^\infty Sdt} \), where D is the dose of label injected. It is assumed that the material which replaces that which is metabolised enters directly the pool in which measurements are made and this pool is

*I am indebted to David Hawking for writing the programmes described in this section.
also that into which the labelled compound is injected. The pool in this case is the VLDL B protein present in the serum. Preliminary calculations showed that the data was best approximated by a pair of exponential curves.

The problem was then one of approximating the curve represented by a number of experimental data points with a pair of exponential curves of best fit. These were then plotted by computer (ANU Computer Centre plotting package of programmes).

Each exponential can be written in the form \( S = S'_0 e^{gt} \). A program for finding the best values of \( S'_0 \) and \( g \) was developed. It involves an exponential regression or least squared technique. The curve chosen in each case minimises the squares of the deviations of the logarithms of the data points from the curve. In experiments where the data was obtained in duplicate or triplicate the data was analysed simultaneously.

\[
g = \frac{m \sum t_i \ln(S_i) - \sum t_i \sum \ln(S_i)}{m \sum (t_i^2) - \sum t_i \sum t_i}
\]

\[
S'_0 = e^{((\sum \ln(S_i) - g \sum t_i)/m)}
\]
where \( m \) is the number of data points

\[ t_i \] is the time of the \( i \)th measurement

\( \Sigma \) is an abbreviation for \( m - 1 \)

\[ \Sigma \]

\( i = 0 \)

\( \ln \) means natural logarithms

\( S_i \) is the \( i \)th measurement.

The first and second curves obtained were extrapolated to \( t = 0 \) and to \( t = \infty \) respectively, in order to calculate the area under the curve.

The area is given by

\[
A = \int_{0}^{t_x} S \, dt + \int_{t_x}^{\infty} S \, dt
\]

\[
A = \int_{t_x}^{0} S_0 e^{g_1 t'} \, dt + \int_{\infty}^{t_x} S_x e^{g_2 t''} \, dt
\]

\[
= S_0 \left[ -\frac{e^{g_1 t'}}{g_1} \right]_0^{t_x} + S_x \left[ -\frac{e^{g_2 t''}}{g_2} \right]_{t_x}^{\infty}
\]

The units of \( S \) are \( \text{cpm/µg protein} \)

The units of \( t \) are \( \text{min} \)

The units of \( A \) are then \( \text{cpm/µg protein/min} \)

The units of dosage, \( D \), are \( \text{cpm/rat} \)

The turnover rate, \( R = D/A \) has units of \( \text{µg protein/rat/min} \).

The time \( t_x \) was assumed (for convenience and with little consequent error) to be at one of the measurement times. Different values of \( t_x \) were tried in each case where there were sufficient data points and the \( t_x \) which produced the curves most nearly approximating the experimental points was employed in calculating areas. Computer time used was approximately 3 seconds per plot of UNIVAC 1108 time (programme language, FORTRAN V). This time includes all calculations necessary for the
calculation of turnover rate. The programme used is shown on the next page.

In some cases it appears that a better fit of the data points would be made with a larger than 2 number of exponentials. However the method of least squares should not be used when there are only a small number of data points. The error in computing the area under the curve is unlikely to be lessened by using more exponentials unless more observations were made.

5.3 RESULTS

5.3.1 Turnover of VLDL B Protein

The turnover rate* of VLDL B protein calculated from specific activity time curves as described in the methods was 10.9 ± 2.2 µg/rat/min. This value is the mean ± S.D. from six rats at each time point. Measurements of aqueous insoluble VLDL protein (B protein) were used to estimate the serum pool size of this protein. On the basis that the serum volume constitutes 4.5% of rat body weight, the total VLDL B protein was calculated, using the data from 30 rats to be 360 ± 32 (S.D.) µg per rat. Thus the

*Terms are those used by Zilversmit (1960).

Turnover refers to the process of renewal of a substance in the body or in a given tissue.

Turnover rate is the rate at which a substance is turning over in a given compartment or metabolic pool (amount/unit time). The meaning of turnover rate is unequivocal only when a steady state exists i.e. when the rate of synthesis and transport into a compartment equals the rate of breakdown and exit.

Fractional turnover rate is the fraction or percentage of a compartment that is turning over or renewed per unit of time.

Turnover time of a substance is the time that is required for the turnover of a quantity of substance equal to that present in the compartment. It is also a measure of the average lifetime of a molecule of the substance under consideration.
INTEGER DEVICES
DIMENSION S(10,10), T(J), XA(102), YA(102), IDENT(2)
READ(1,4) DEVICE, DEVC
4 FORMAT(12,F4.2)
10 IF(DIVICE.EQ.11) IT=1
11 READ(1,10) N, IDENT, (T(J), J=1, N)
12 READ(1,20) (S(J), J=1, N, J=1, N)
13 IF(DIVICE.EQ.7) IT=2
14 READ(1,40) (S(J), J=1, N)
15 CONTINUE
C--- PLOT RAM DATA POINTS.
16 KOUNT=0
17 DO 15 J=1,N
18 KOUNT=KOUNT+1
19 XA(KOUNT)=T(J)
20 YA(KOUNT)=S(1, J)
21 CONTINUE
22 NT=N*N
23 CALL GRAP(XA,YA, NT, 'TIME (MINUTES)'
24 CALL SYMBOL(3,F,6,21, IDENT,9, A, 12)
C--- STORE SCALING PARAMETERS IN AN UNCORRUPTIBLE PLACE.
25 IT=0
26 YA(101)=XA(NT+1)
27 YA(102)=XA(NT+2)
28 YA(103)=YA(NT+1)
29 YA(104)=YA(NT+2)
C--- COMPUTE PARAMETERS FOR THE FIRST SECTION.
30 NUM=N+1
31 SLNS1=10.
32 ST1=10.
33 STSQ1=10.
34 STL1=10.
35 DO 28 J=1, NUM
36 RT=T(J)-T(1)
37 DO 28 T=1,J
38 RT=T(J)-T(1)
39 CONTINUE
C--- RT IS THE RELATIVE TIME RELATIVE TO THE FIRST SAMPLE TIME.
40 SLNS1=10. * LOG(ST1+S11)+SLNS1
41 SLNS1=ST1+ST1
42 STSQ1=RT+STSQ1
43 SLNS1=RT+LOG(ST1+S11)+STLS1
44 CONTINUE
C--- NOW COMPUTE G1, THE SLOPE OF THE EXPONENTIAL APPROXIMATION TO
C--- THE FIRST PART OF THE CURVE.
45 NUM=0
46 POINTS=0
47 IF (NUM.EQ.0) G1=1
48 G1=10./POINS
49 STLS1=ST1+ST1
C--- COMPUTE THE FIRST VALUE.
50 FIRST=EXP((SLNS1-STL1+STLS1) / POINTS)
51 CALL EXPP(XA,YA,T(1), T(NUM), FIRST, G1,10)
52 CALL GRAP(XA,YA, 10, 0, 9, 6, 10, 0)
53 WRITE(11,4) G1, FIRST, IDENT, IDEN
54 NUM=0
55 NUM=NUM+1
C--- WRITE (11, 4) G1, FIRST, IDENT, IDEN
56 FORMAT(1P12,4,4X,E11.4,7X,PA6,18)
57 SLNS2=10.
58 ST2=10.
55 CONTINUE
C=== Nth COMPUTE G2 THE SLOPE OF THE EXPONENTIAL APPROXIMATION TO
76 THE SECOND PART OF THE CURVE.
77 POINTS=FLOAT(N*(N+NUM+1))
78 UPPERS=POINTS*STLS2-ST2*ST2
79 G2=UPPER/(POINTS*STNS2-ST2*ST2)
C=== CALCULATE FIRST2.
80 FIRST2=EXP(SLNS2-ST2*G2)/POINTS
81 CALL EXP(XA,YA,T(NUM),T(N),FIRST2,G2,100)
82 CALL GRAP(XA,YA,T(NUM),T(1),FIRST2,G2,10)
83 WRITE(11,40) G2,FIRST2,IDENT,NUM,N
84 IF (DEVICE.EQ.11) CALL ERASE.
C=== CALCULATE THE AREA UNDER THE CURVE FROM T=0 TO T=INFINITY, THE
85 AREA IS THE SUM OF THE AREAS UNDER THE TWO EXPONENTIAL SECTIONS
C=== WHICH MUST BE EXTRAPOLATED TO 0 AND (INFINITY RESPECTIVELY)
C=== THIS MEANS THAT TIME FOR THE FIRST EXPONENTIAL GOES BACK TO T=5
86 SINCE THIS IS THE FIRST MEASUREMENT TIME,
87 AREA=FIRST1*(EXP(-G1*X1)-EXP(G1*(T(NUM)-5,0)))/G1+FIRSt2/G2
88 AREA=A7=AREA
89 RATE=DOSE/A7=AREA
C=== UNITS ARE MICROGRAMS OF PROTEIN PER MINUTE.
90 WHITE(11,7) AREA, DOSE, RATE.
91 FORMAT(1PE11.4) COUNTS PER MICROGRAM, E15.4,
92 * MILLION COUNTS PER MINUTE PER RAT 1/TURNOVER RATE IS 1,
93 E15.4 MICROGRAMS PROTEIN PER MINUTE,!
94 READ(40) MARKER
95 IF (MARKER.EQ.41) GO TO 5
96 WRITE(1,7) ADDRESS
97 RETURN
END

DAH524+EXPS,EXPP
SUBROUTINE EXPP(XA,YA,X0,X1,FIRST,G,NPTS)

C== FILL XA AND YA WITH THE X AND Y VALUES ON THE
C== CURVE Y=FIRST*EXP(G*X)
C== BETWEEN THE VALUES X0 AND X1. THE VALUES AT NPTS POINTS ARE
C== COMPUTED.

NPTS=FLOAT(NPTS+1)
X0=0.0
DO IA=1,NPTS
XA(IA)=X0
YA(IA)=FIRST*EXP(G*X)
X0=X0+DELTA
CONTINUE
RETURN
END
VLDL B protein turnover time was 32 min (i.e. pool size/turnover rate).

The fractional turnover rate (turnover rate/pool size) was 0.0306 per rat/min. The turnover rate of VLDL B protein was also estimated after the rats were fasted for 16 hr before being used for an experiment. The value obtained was 30 ± 6 (S.D.) µg/rat/min (values are the mean of 2 determinations on material from 3 rats in two separate experiments) and the turnover time was calculated to be 14 min with a pool size of 420 µg (calculated as described above, from 30 rats fasted 16 hr). The fractional turnover rate would then be 0.071 per rat/min.

$T_{1/2}$ for the two components of the VLDL B protein SA versus time curves were 30 ± 3.7 min and 9.3 ± 1.4 hr for the fast and slow components respectively and represent the mean ± S.D. from six rats at each time point. The value for the slow component is close to that observed for total VLDL removal (approximately 10 hr, see Chapter 3). Using the value for $T_{1/2}$ for each component of the SA versus time curve the fractional turnover rate can be calculated using the following formula

$$ \text{fractional turnover rate (k) = } \frac{\ln 2}{T_{1/2}} = \frac{0.693}{T_{1/2}} $$

(Zilversmit, 1960). The fractional turnover rate for the fast component was 0.0231/rat/min, the turnover rate 8.3 µg/rat/min and the turnover time was approximately 43 min. The fractional turnover rate for the slow component was 0.00124 per rat/min. The turnover rate was 0.446 µg/rat/min and the turnover time 13.4 hr.

Examples of the specific activity time curves are shown in Figures 5.8 and 5.9.
Figure 5.8  Serum VLDL B Protein Specific Activity Time Curve Followed for 12 hr and Resolved Into Two Exponential Functions. The line of best fit was calculated as described in the text and plotted by computer.
Figure 5.9  A Typical Serum VLDL B Protein Specific Activity Time Curve Followed for 16 hr and Resolved Into Two Exponential Functions and the Line of Best Fit Plotted by Computer.
5.3.2 Specific Activity of VLDL, LDL₁ and LDL₂ B Protein

In vivo B protein specific activity (SA) of VLDL, LDL₁ and LDL₂ was obtained after injection of ¹²⁵I-VLDL into rats and after separation of the apoproteins using two methods, PAGE and differential solubility in an aqueous solution (Figures 5.10 and 5.11) as described in the methods section. The outline of the general experimental procedure used is shown in Figure 4.1 of Chapter 4. In Figure 5.10, the SA was determined by PAGE and is representative of a typical experiment. The SA of LDL₁ increased and exceeded that of VLDL after 10 min in a fashion similar to a precursor product relationship (Zilversmit, 1960). The SA of LDL₁ then decreased to a level below that of VLDL after 2 hr. LDL₂ SA also increased but did not reach that of VLDL.

In vivo B protein SA of VLDL, LDL₁ and LDL₂ obtained after separation of the B protein (insoluble) from soluble protein is shown in Figure 5.11. Results were expressed as relative SA, in other words as the proportion of VLDL B protein SA at 12 hr which was equal to 78 cpm/µg VLDL B protein in this particular experiment. This means of expressing the SA was used as a basis for quantitative comparison of the results from a number of experiments. The SA (Figure 5.11) of LDL₂ B protein reached that of VLDL at 6-8 hr whereas the SA of LDL₁ insoluble protein was very close to that of VLDL at the first time point (5 min). No initial increase in LDL₁ or LDL₂ SA was observed in this experiment. However, an initial increase between 5 and 10 min has been observed in other comparable experiments.

The changes observed in the SA of the B protein of VLDL, LDL₁ and LDL₂ after in vitro incubation of ¹²⁵I-VLDL with control serum as described in Chapter 4, is illustrated in Figure 5.12. VLDL B protein SA remained high and only slight increases in LDL₁ and LDL₂ B protein SA were observed. The SA of LDL₁ and LDL₂ B protein remained low (not exceeding 4% and 17% respectively, of the VLDL SA) throughout the incubation period.
Figure 5.10  Specific Activity of VLDL, LDL₁, and LDL₂ B Protein.

Specific activity was determined by PAGE as described in the text. Values represent the mean of two estimations (on pooled sera from two rats) from material obtained from four rats.
Figure 5.11 Relative Specific Activity of the Ammonium Bicarbonate Insoluble Apolipoprotein (B Protein) of Rat VLDL, LDL₁ and LDL₂ after the Injection of $^{125}$I-VLDL into Rats. Values represent the mean ± S.D. from three rats at each time point. S.D. less than .05 are not shown.

Relative specific activity = specific activity (cpm/µg)/specific activity of VLDL B protein at 12 hr. Methods utilised are as described in the text.
Specific Activity of Ammonium Bicarbonate Insoluble Protein of VLDL, LDL₁ and LDL₂ after Incubation of $^{125}$I-labelled VLDL with Control Serum. Incubations were performed as described in Chapter 4, and SA was determined as described in the text. Figures are the mean of two determinations at each time point.
The SA of LDL₁ and LDL₂ B protein after in vivo incubation of ¹²⁵I-VLDL with post-heparin plasma (as described in Chapter 4) showed that under identical experimental conditions the increase in SA of the B protein in the post-heparin incubation was five times that observed in the control incubation. At 2 hr the SA of LDL₂ B protein exceeded that of LDL₁ B protein.

5.3.3 Specific Activity of the Soluble Protein

The SA of the soluble proteins (soluble in a weak aqueous buffer, 5 mM ammonium bicarbonate) of VLDL, LDL₁ and LDL₂ and those present in PAGE zone IV, of VLDL, LDL₂ and HDL was also determined after injection of ¹²⁵I-VLDL into rats. The relative SA of the soluble (in 5 mM ammonium bicarbonate) protein is shown in Figure 5.13. The SA of VLDL decreased and that of LDL₁ and LDL₂ increased then decreased. The SA of LDL₂ however approached that of VLDL between 6 and 8 hr which was the time when a crossover of VLDL and LDL₂ 5 mM ammonium bicarbonate insoluble protein (B protein) occurred (Figure 5.11). In other experiments it was consistently observed that the SA of the soluble proteins of LDL₂ exceeded or equalled that of VLDL when the LDL₂ B protein SA also exceeded that in VLDL.

The SA of individual proteins present in PAGE zone IV is shown in Figure 5.14. The kinetics of zone IVa differed from that of zone IVb. Zone IVa of VLDL, LDL₂ and HDL followed the same kinetics. A decrease in zone IVb of LDL₂ was reflected in an increase in VLDL while the HDL zone IVb SA did not change appreciably. After 5 hr, however, a reciprocal relationship between VLDL and HDL zone IVb SA was apparent and this was independent of the LDL₂ zone IVb SA. Equilibration of SA between VLDL and LDL₂ zone IVa peptides, which remain virtually identical throughout the experiment, suggests exchange of these peptides. HDL zone IVa peptides were rapidly labelled but the SA did not reach that of VLDL, suggesting that the labelled peptide was diluted with unlabelled zone IVa peptides.
Figure 5.13  Relative Specific Activity of the Ammonium Bicarbonate Soluble Apolipoproteins of Rat VLDL, LDL₁ and LDL₂ after the Injection of $^{125}$I-VLDL into Rats. Relative Specific Activity = specific activity (cpm/µg)/specific activity of VLDL at 12 hr (254 cpm/µg for this experiment). Values are the mean of two determinations on material from three rats at each time point.
Figure 5.14 Specific activity of PAGE Zones IVa and IVb of Apo-VLDL, LDL$_2$ and HDL. Specific activity was determined as described in the text. Values are the mean of three determinations + S.D. on material from three rats at each time point.
The results of an *in vitro* incubation of $^{125}\text{I}}$-VLDL with control serum is shown in Figure 5.15. LDL$_1$ SA slowly increased whereas LDL$_2$ SA exceeded that of VLDL at 40 min in an inverse relationship. The SA of VLDL soluble protein decreased indicating that unlabelled soluble protein may be entering the VLDL soluble protein pool from the other lipoproteins, LDL$_1$, LDL$_2$ and HDL present in the serum. The SA of the soluble proteins of LDL$_1$ and LDL$_2$ after *in vitro* incubation of $^{125}\text{I}}$-VLDL with post-heparin plasma under identical experimental conditions used for control incubations followed the same kinetics as the B protein SA.

5.4 DISCUSSION

This chapter describes some observations on the quantitative relationship between VLDL and LDL primarily based on specific activity measurements of the insoluble B protein, a major component common to all the low density lipoproteins in the rat. Since a significant proportion of the soluble protein of VLDL was shown to exchange or be transferred to low density lipoproteins as well as to HDL in the rat, some attempt was made to determine their role in the metabolic interrelationship between VLDL and LDL.

In order to assess the significance and possible interpretations of the data obtained in this chapter a number of basic assumptions have to be considered. Of the many attempts made to formulate the quantitative interpretations of SA data the most generally useful are those of Zilversmit (1960), based on the laws of mass action and the terminology of which has already been described in Section 5.3.1. By applying his formulations it is possible to determine whether a substance A meets the necessary conditions for being a precursor of substance B and then obtain information on the rate at which A is being converted to B if the total amounts of A and B in the system are known.
Figure 5.15  Specific Activity of Ammonium Bicarbonate Soluble Protein of VLDL, LDL₁ and LDL₂ after In Vitro Incubation of $^{125}$I-VLDL With Control Serum. Incubations were performed as described in Chapter 4. Figures are the mean of two determinations at each time point.
Before the formulations of Zilversmit (1960) can be applied a number of assumptions are necessary most of which are corollary to the fundamental one which is that a steady state exists. These assumptions will be described separately below. The first two assumptions are:

(i) that the rate of the steady state process is constant; and
(ii) the reactions involved are of the first order, either actually or formally. If a steady state exists, then the reactions are formally of first order without regard to the actual order of reaction, since the concentration of substances present are constant. Acceptance of the first assumption necessarily means the acceptance of the second assumption. Rats utilised in these studies were injected in the morning and were fasted throughout the experiment thus creating the physiological status of a steady state.

(iii) It is assumed that the reactions involved are irreversible. All the experimental evidence concerning the metabolism of VLDL in rats and humans have suggested that newly secreted VLDL is catabolised to molecules of higher density (Gitlin, 1958; Fidge and Foxman, 1971; Barter and Nestel, 1970, 1972; Eisenberg et al, 1972a; and in Chapter 4) and not the reverse.

(iv) There is a purely random participation of molecules of precursor in the reaction; i.e. that there is no distinction made between old and newly-formed molecules. This assumption may not be valid as VLDL B protein is present in VLDL molecules which vary in size, content of B protein and triglyceride and therefore larger VLDL molecules which contain a larger amount of triglyceride as well as cofactor protein may be metabolised in a different manner to the smaller VLDL molecules. Evidence to further support this suggestion has been reported by Barter and Nestel (1970, 1972) who showed that a precursor product
relationship occurs between the triglyceride of subfractions of human VLDL in which the larger molecules of Sf 100-400 are converted to Sf 20-100.

(v) No direct evidence is available which would invalidate the fifth assumption that the newly formed molecules of the product are rapidly equilibrated with those previously present.

(vi) The sixth assumption is that there is no compartmentalisation of the precursor or product substance and therefore the entire amount present is equally available for further reaction.

If the system described in these studies meets these assumptions, then in order to describe a precursor product relationship for two components, A (precursor) and B (product), the following criteria must be satisfied (Zilversmit, 1960; Reiner, 1953; Shipley and Clark, 1972).

(i) The specific activity of A is initially higher than the SA of B.

(ii) At the time when the SA of B is at its maximum (when the slope of its SA versus time curve is zero) the SA of A is equal to that of B; and

(iii) the SA of B remains higher than that of A after the maximum SA of B has been passed. However, even if the data meet these criteria it does not necessarily follow that A is an immediate precursor of B. The same type of time course relations will be obtained if A is slowly converted to some intermediate compound, normally present in minute amounts, which is then converted rapidly to B, as if there are no intermediate steps. It is therefore apparent that no simple criterion exists by which one may ascertain that one compound is the immediate precursor of a second, except in the special case where there is only one precursor.

Analysis of the B protein obtained in in vivo studies showed that LDL₂ B protein SA at the earliest time measured was approximately 15% that
of VLDL B protein SA and following a rapid decline, fell at a constant rate up to 16 hr. In all experiments, no evidence of an increase in SA (except for a small time during the first 5 min in some experiments, Figure 5.10) was observed. Therefore, the relationship between VLDL and LDL₂ at no time satisfied the criteria required for establishing a precursor product relationship. However, since it is clear that at least some of the injected VLDL B protein radioactivity was transferred to the LDL₂ B protein pool, it can be inferred that some of the LDL₂ B protein is a product of VLDL catabolism. One possible explanation for the immediate fall in LDL₂ B protein SA could be a dilution of the labelled pool by unlabelled protein entering the LDL₂ B protein pool independently of the VLDL source, for example the secretion of apoB from sites of synthesis such as the liver or intestine. An alternative explanation is that the product pool is metabolically heterogeneous in nature and consists of a very rapidly turned over pool which quickly reaches the same SA or exceeds that of VLDL B protein as well as a slowly turned over pool which only slowly equilibrates with the VLDL B protein. Such an example may be described as shown in Figure 5.16.

--- Data obtained in these studies.
R = Rapidly turning over component.
S = Slowly turning over component.

Figure 5.16 Hypothetical SA versus Time Curve for a Rapidly and Slow Turning Over Component of LDL₂.
The presence, within the d 1.006-1.019 (LDL₁) lipoprotein of B protein which rapidly equilibrated with VLDL after the first 5 min (Figure 5.11) and exceeded that of VLDL (Figure 5.10) may support evidence for the possible existence of a similar rapidly turned over B protein pool in the lower density range (for example, d 1.019-1.025) of the total LDL₂ (d 1.019-1.050) B protein pool. The data obtained for LDL₁ B protein SA as seen in Figure 5.10 does satisfy the requirement for a precursor product relationship. Although a crossover was not observed between VLDL and LDL₁ in Figure 5.11, the very close relationship between VLDL and LDL₁ SA, would still support such a conclusion.

Interestingly, the SA of the C proteins (soluble proteins) of LDL₂ approached that of VLDL when the B protein SA crossed over (6-8 hr) but LDL₁ SA remained lower (Figure 5.14). Also, after this time the SA of the soluble and insoluble protein of LDL₁ and LDL₂ decreases. It is therefore possible that all LDL₂ soluble protein (PAGE zones III and IV) is derived from VLDL and the B protein is derived from different sources including VLDL or else there may be heterogeneity within the LDL₂ B protein pool but not within the C protein pool. In addition, LDL₁ B protein but not C protein may be derived from VLDL. Support for these suggestions is derived from analysis of the zone IVa and IVb SA by PAGE (Figure 5.14) which revealed heterogeneity in zone IV protein metabolism. It was shown that there is an exchange of zone IVa proteins but not zone IVb between VLDL and LDL₂. Further analysis of the other soluble proteins is therefore warranted before the question of the origin of LDL₁ and LDL₂ soluble proteins can be resolved.

In comparison, human VLDL, LDL₁ and LDL₂ B protein (obtained by differential solubility of the apoproteins in a weak aqueous buffer as described above, Section 5.2.1(c)) obey a precursor-intermediate-product relationship. When the B protein SA versus time is plotted on a logarithmic
scale the SA of LDL₁ crosses that of VLDL at maximum SA then falls but remains above the VLDL SA. LDL₂ SA crosses that of VLDL, then LDL₁ at maximum SA and remains above that of VLDL and LDL₂ (Reardon and Fidge, unpublished). The SA of VLDL B protein in the human was biexponential and in this respect is similar to the rat. The T₁/₂ for the fast component of the human VLDL B protein SA time curve was approximately 2-4 hr (Eisenberg et al., 1973a) compared to 30 min in the rat. Kinetics of transfer are therefore more rapid in the rat. Other differences between the rat and the human are the relative quantities of total B protein in serum VLDL:LDL₁:LDL₂ (1:66:1-1.3 and 1:5-1:8-12, for rat and human, Reardon and Fidge, unpublished, respectively) and the proportion of B:C proteins (aqueous insoluble:aqueous soluble) in LDL₂ (90:10 and 98:2 for rat and human respectively). These differences suggest that rat LDL₂ may have a different physiological function in the rat, particularly as it has been suggested that in the rat, HDL (which has a molecular weight approximately twice that of human HDL) rather than LDL₂ transports most of the serum cholesterol (60-70%) (Gidez et al., 1965; Koga et al., 1971).

In conclusion it appears that the rat may not be a good model for the VLDL/LDL relationship observed in the human but in itself it provides an interesting although complicated metabolic relationship. In these studies the exact relationship between VLDL, LDL₁ and LDL₂ remains unclear. Some of the possible reasons for this have been discussed. However it is evident that most LDL₁ and a significant proportion of LDL₂ B protein is derived from VLDL.

Since it was observed that, as a consequence of in vitro post-heparin lipolytic activity increased proportions of LDL₂ were derived from VLDL when compared to control serum, it is tempting to speculate the formation of VLDL remnants similar to the formation of chylomicron remnants described by Redgrave (1970) and Eisenberg et al. (1973a) for human VLDL. Although
time has not permitted further studies on the isolation of this lipoprotein (remnant), one suggestion for further work would be to attempt an isolation of VLDL 'remnants' and investigate the relationship between these lipoproteins and rat low density lipoproteins. Furthermore, since rat LDL may have different functions and profiles to other species (for example, humans), the possibility that the product of VLDL catabolism may reside in another (for example, lower) density fraction to those studied in the present work. The 'remnant' may fall within the VLDL density range, Sf >20 <100.
5.5 SUMMARY

Two methods for the separation of the B protein of VLDL, LDL₁ and LDL₂ from other proteins are described and applied for the determination of SA in vivo and in vitro experiments. The SA of the aqueous soluble protein of VLDL, LDL₁ and LDL₂ and the zone IV apoproteins of VLDL, LDL₂ and HDL was determined. It was demonstrated that:

(i) the turnover of VLDL B protein is rapid (turnover time of approximately 30-40 min);
(ii) most if not all the LDL₁ B protein is derived from VLDL;
(iii) a significant proportion of the LDL₂ B protein is derived from VLDL;
(iv) aqueous soluble proteins are transferred from VLDL to LDL₁ and LDL₂; and
(v) heterogeneity in the transfer of PAGE zone IV proteins of VLDL to LDL₂ and HDL was observed.

From the SA versus time curves obtained for the B protein it was concluded that the rat may not be a good model of the human VLDL/LDL relationship but provides an interesting although complicated metabolic interrelationship.
Although early studies, particularly those concerned with lipids in the diet, have provided much information about lipoprotein metabolism, data limitations have been imposed by the lack of information about rat lipoproteins. Consistent with in vivo studies on the metabolism in rats, we endeavored to partially characterize rat apoproteins in order to identify the proteins associated with the in vivo transamination of particular amino acids and the identification of the proteins which are transferred from one lipoprotein to another and are identified in these studies by the relative mobilities on polyacrylamide gels after electrophoresis. Identification of these proteins is complicated, and further information about the physiological roles of apoproteins, their metabolism, and catalytic activity in rat lipoproteins can be obtained by comparing their properties. Smith et al. (1976) have identified heterogeneity in rat apolipoproteins and determined a range of molecular weights in VLDL and HDL lipoproteins in the range of 30,000 to 200,000. These rat proteins were further characterized by Betz et al. (1977) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The immunological properties of the rat apoproteins from VLDL and HDL (fast migrating on polyacrylamide gels) were shown to be identical.

CHAPTER 6

PARTIAL CHARACTERISATION OF SOME APO-VLDL AND APO-HDL PROTEINS
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6.1 INTRODUCTION

Although rat studies, particularly those concerned with apolipoprotein metabolism, have provided much information about lipoprotein metabolism, some limitations have been imposed by the lack of information about rat apoproteins. Concomitant with in vivo studies on the metabolism of rat apo-VLDL an attempt was made to partially characterise rat apoproteins in order to identify the proteins concerned with the in vivo transformations. Of particular concern was the identification of the peptides which are transferred from one lipoprotein to another and are identified in these studies by their relative migration on polyacrylamide gels after electrophoresis (i.e. zone I, II, III or IV).

Identification and characterisation of these rat peptides may provide further information about the physiological role of apoproteins in VLDL catabolism and as well, establish whether the rat contains peptides homologous to those found in the human. Compared with human very little is known about rat apolipoproteins. Koga et al (1969) first described heterogeneity in rat apolipoproteins and demonstrated a group of apoproteins in VLDL and HDL analogous to the human C apolipoproteins (Koga et al, 1971), which had molecular weights of less than 20,000. These rat proteins were further fractionated by Bersot et al (1970) by DEAE-cellulose chromatography. The immunochemical properties of two of the proteins from VLDL and HDL (fast migrating on polyacrylamide gels) were shown to be identical.
This chapter describes work undertaken to partially characterise the apoproteins of rat VLDL and HDL. The technique used to fractionate the apoproteins were gel filtration and ion exchange chromatography. Isolated fractions were examined by PAGE and their amino acid compositions determined. After this work was commenced a report by Herbert et al (1974) demonstrated a striking homology of rat HDL low molecular weight proteins with some human C apoproteins.

6.2 EXPERIMENTAL

6.2.1 Preparation of Lipoproteins

Large male rats (300-400 g) and female rats were fasted overnight prior to exsanguination from the abdominal aorta. VLDL and HDL were obtained from 600-800 ml of pooled rat serum. To increase the yield of VLDL, rat diets (commercial chow) were supplemented with 40% sucrose, 20% fructose or olive oil (10% w/w), cholesterol (1% w/w) (as described in Chapter 2) for two or three weeks prior to exsanguination. On the high carbohydrate diets the serum triglyceride concentrations increased by 50-100% but the cholesterol levels remained fairly constant.

Injection (either intraperitoneally or intravenously) of Triton WR1339 (Sterling Pharmaceuticals) into fasted rats (24 hr) produced maximum increases in triglyceride and cholesterol concentration (to 1,200 mg/100 ml and 225 mg/100 ml serum respectively) after 24 hr. However, the characteristics of this VLDL apoprotein was different to that of 'normal' VLDL when examined by PAGE and gel filtration. This method was therefore not used in further isolations of VLDL.

Lipoproteins were isolated either by ultracentrifugation (Chapter 2) or by selective precipitation using dextran sulphate and manganese
chloride (Burstein et al., 1970; Method III). Since higher yields of
VLDL were obtained from serum by ultracentrifugation, this method of
isolation was preferred and used in all later separations. No differences
in apoprotein composition were detected after isolation of lipoproteins
by either method. VLDL and HDL solutions were tested for the presence
of impurities such as albumin by immunodiffusion against rat anti-HDL,
VLDL, LDL and albumin (prepared by Dr Fidge) and by electrophoresis on
cellulose acetate after dialysing the prepared lipoproteins to pH 7.4 in
0.15 M sodium chloride containing 1 mM disodium EDTA. Yield of
lipoproteins was approximately 30-40 mg HDL apoprotein and 10-15 mg apo-VLDL
for 100 ml serum.

6.2.2 Lipoprotein Delipidation

VLDL and HDL solutions (5-10 mg protein/ml) were dialysed against
5 mM ammonium bicarbonate containing 1 mM EDTA. HDL was delipidated with
diethyl ether:ethanol (3:1, v/v) at 4°C as described by Brown et al. (1969)
or using chloroform:methanol as described in Chapter 4. However, the
procedure was carried out in siliconised 50 ml tapered centrifuge tubes
at 4°C in order to accommodate the larger quantities of protein. A
maximum of 10 mg protein was delipidated per tube. The delipidated
protein was completely soluble in 0.2 M Tris-HCl (pH 8.2) containing 6 M
urea. VLDL was similarly delipidated. Apo-VLDL was solubilised in 0.2 M
Tris-HCl (pH 8.2) containing 0.1 M SDS and 6 M urea. Protein was
estimated using the method of Lowry et al. (1951). Only apoproteins
containing less than 1% residual phospholipid were used for gel
chromatography. Recoveries after delipidation were 80-90%.
6.2.3 Gel Chromatography

Sephadex G-150, G-200 and G-200 superfine (Pharmacia, Uppsala, Sweden) were swollen in 0.2 M Tris-HCl buffer pH 8.2 for 16 hr at 80°C. After cooling, columns were packed under reduced pressure according to Pharmacia recommendations and equilibrated with the eluting buffer at the temperature at which the column would be operated (either 4°C or room temperature). The glassware component of the columns was precoated with dimethyl dichlorosilane ( Ajax Chemicals, Australia, Pty. Ltd) dissolved in acetone or siliclad (Clay Adams) before packing.

Some separations of apo-VLDL were performed with columns (2.5 x 90 cm or 1.5 x 100 cm) (either Excel, Kontes or Glenco) packed with Sephadex G-150 at room temperature using 2 mM SDS in 0.2 M Triz-HCl pH 8.2 as eluting buffer. Chromatography of apo-HDL was performed at 4°C on columns (2.5 x 100 cm) (Excel, Kontes or Glenco) packed with Sephadex G-200.

In later separations of apo-HDL and apo-VLDL the advantage of the glass bead technique was exploited (Sachs and Painter, 1972). Columns (5 x 150 cm) (Kontes) were packed with Sephadex G-200 superfine (G-200 SF) as described by Herbert et al (1973b). The advantages of this technique include a more rapid flow rate and retention of column characteristics for a longer time than that possible using other techniques since the packing of Sephadex is retarded by the glass beads.

All urea buffers were prepared fresh daily using deionised urea (see Chapter 2). Thirty to 50 mg apo-VLDL and 40-100 mg apo-HDL were loaded per column. Upon completion of column runs pooled fractions were exhaustively dialysed (Viscene dialysis tubing, which had been washed in hot running tap water followed by distilled water) against 5 mM ammonium bicarbonate (pH 8.2) containing 1 mM EDTA and lyophilised.
Lyophilised fractions were solubilised in 0.1 M ammonium bicarbonate pH 8.2 and the protein content determined before polyacrylamide gel electrophoresis. Insoluble fractions were solubilised with the aid of 0.1 M Tris- HCl buffer (pH 8.2) or the addition of increasing concentrations of SDS (0.01 M-0.1 M). Recoveries after gel chromatography were approximately 80-90%. The void volume of columns was determined using Blue Dextran dye (mol. wt. $2 \times 10^6$, obtained from Pharmacia, Uppsala, Sweden). Columns were eluted by descending flow unless otherwise indicated.

6.2.4 DEAE-Cellulose Chromatography

DEAE-Cellulose-Microgranular (pre-swollen) anion exchange for column chromatography DE 52 (Whatman Biochemicals Ltd., England) was degassed in 0.5 M HCl, washed with 0.5 M Tris-HCl pH 8.2 and finally equilibrated with the starting buffer. Columns (0.9 x 20 cm and 1.5 x 40 cm) (Excel) were packed at 4°C and equilibrated further with 3-5 volumes of the starting buffer, 0.01 M Triz-HCl (pH 8.2) containing 6 M urea. A linear gradient from 0.01 M Triz-HCl (pH 8.2) to 0.25 M Triz-HCl (pH 8.2) containing 6 M urea was established using 500 ml starting buffer in one reservoir and 500 ml 0.25 M Triz-HCl (pH 8.2) in another. Chromatography was performed at 4°C. Column fractions were then pooled dialysed (Spectra-pore mol. wt. cut off 3,000) against 5 mM ammonium bicarbonate (pH 8.2) containing 1 mM EDTA and resolubilised. Recoveries were 70-78%. Eluted fractions were examined for absorbance at 280 nm using a Beckman DB-G Grating Spectrophotometer.

6.2.5 Polyacrylamide Gel Electrophoresis

All chromatography fractions were routinely analysed by PAGE on 7.5% acrylamide gels at pH 8.9 using the buffer system of Kane (1973) as
described in Chapter 4. Solutions containing 60-100 µg protein in 0.05 M Tris-HCl (pH 8.2) 6 M urea were applied to each gel. The efficacy of a number of gel systems, fixing solutions and stains was compared. These included systems described by Weber and Osborn (1969) and those in the Polyanalyst Manual (Buchler Instruments, Inc. NJ. USA). A comparison of the separation of apo-VLDL on 3.5%, 5% and 7.5% acrylamide gels (proportions were calculated using the criteria of Ornstein, 1965 and Davis, 1964) is shown in Figure 6.1. Although 3.5 and 5% gels gave better resolution of apo-VLDL fast migrating peptides than 7.5% gel the latter was retained for routine use since the separation was adequate and the gels easier to handle. Resolution of apo-HDL on 7.5% acrylamide is shown in Figure 6.2.

6.2.6 Amino Acid Analyses

Protein (0.15-0.3 mg) was dialysed against 1 mM ammonium bicarbonate or water then lyophilised. Peptides were hydrolysed with 6 M HCl for 22 hr at 105°C. Amino acid analyses were performed on a Technicon 120B model amino acid analyser using the method of Spackman et al (1958).

During acid hydrolysis glutamine is converted to glutamic acid and asparagine to aspartic acid. Tryptophan content was not determined due to the small amounts of peptide available. Similarly cysteine was not determined as a separate aliquot is required for performic acid oxidation. However the measurement of half cystine formed during acid hydrolysis (6 M HCl) gives some estimate of the cysteine content of the original sample.
Figure 6.1  Comparison of the Separation of Rat Apo-VLDL on Polyacrylamide Gels Containing Different Proportions of Acrylamide. Apo-VLDL (70 µg/gel) resolution on 7.5, 5 and 3.5% acrylamide gels is shown. Gels of the same length were electrophoresed as described in the text.
Figure 6.2  Resolution of Rat Apo-HDL by Polyacrylamide Gel Electrophoresis. PAGE was on gels containing 7.5% acrylamide and was performed as described in the text. 90 µg apo-HDL protein was electrophoresed.
6.3 RESULTS

6.3.1 Gel Chromatography of Apo-VLDL

Two well separated peaks were eluted when apo-VLDL was chromatographed on Sephadex G-150 columns (1.5 x 100 cm) and eluted with 2 mM SDS in 0.2 M Triz-HCl (pH 8.2). The first peak (30-40% of the protein recovered), eluted within the void volume, contained B protein (identified by immunodiffusion against anti-apo-LDL d1.030-1.040 g/ml). PAGE of the pooled fractions eluted between the two peaks revealed the presence of protein migrating in the zone II region on 7.5% gels. The second peak (60-70% of the total protein recovered) contained fast migrating peptides (PAGE zones III and IV).

Peaks were better resolved using Sephadex G-200 SF. The gel filtration elution profile of apo-VLDL on Sephadex G-200 SF (glass bead) column (5 x 150 cm) is shown in Figure 6.3. Three fractions were obtained. The void volume peak, fraction I, (PAGE zone I) contained 30% of the total protein recovered and did not enter the main gel after PAGE on 7.5% acrylamide gels (Figure 6.3). This fraction contained mostly B protein as determined by immunodiffusion against anti-apo-LDL. The second fraction (30% of the protein) contained a number of slowly migrating bands which penetrated the main gel and are found in zones II and III (see Chapter 4). The third fraction (40% of the protein) contained a number of fast migrating peptides (PAGE zones IV) of low molecular weight (< 25,000) as judged by their elution volume.

6.3.2 Ion Exchange Chromatography of Apo-VLDL

DEAE-Cellulose chromatography separations of the Sephadex G-200 SF fractions II and III are shown in Figures 6.4 and 6.5. Fractions were
Figure 6.3  Sephadex G-200 SF Chromatography of Rat Apo-VLDL and PAGE of Each Pooled Column Fraction. The column (5 x 150 cm, containing glass beads) was eluted with 0.2 M Triz-HCl pH 8.2, 6 M urea at 4°C. Fractions of 8 ml were collected and fractions were pooled as indicated by the arrows. The first two peaks contained 30% each and peak III contained 40% of the recovered protein. PAGE of column fractions was performed as described in the text.
chromatographed on identical columns under the same conditions and eluted with 0.01-0.2 M Triz-HCl pH 8.2 gradient (1 l) 6 M urea at 4°C. As the absorbance was very low (only 7 mg protein chromatographed per column), eluant between peaks was also pooled and lyophilised. Insignificant quantities of protein were found in the peaks shown in the elution profile which are not numbered (< 5% total protein recovered).

Sephadex G-200 SF fraction II (PAGE zone III) was separated into five major peaks by ion exchange chromatography (Figure 6.4). Heterogeneity on polyacrylamide gels was observed in a number of these peaks (1, 2, 4 and 5). The protein recovered in each peak was approximately 12, 20, 25, 26 and 12% respectively for peaks 1, 2, 3, 4 and 5.

Four well separated peaks were eluted after ion exchange chromatography of Sephadex G-200 SF fraction III (PAGE zone IV) (Figure 6.5). Of the four peaks eluted two were homologous after PAGE (peaks 1 and 4) and the others contained two stained bands (Figure 6.5). The protein recovered in peaks 1, 2, 3 and 4 was approximately 39, 18, 28 and 11% respectively.

6.3.3 Amino Acid Analyses of VLDL Peptides

The amino acid composition of DEAE fractions obtained after ion exchange chromatography of apo-VLDL Sephadex G-200 SF fractions II and III are shown in Tables 6.1 and 6.2. All DEAE fractions of apo-VLDL Sephadex fraction II (Table 6.1) contained a high proportion of arginine (69.1-96.6 moles/10^3 moles recovered), glutamic acid (220.5-275.9 moles/10^3 moles recovered) and a very low proportion of histidine (tr-5.2 moles/10^3 moles recovered). Since half cystine was not detected the samples probably did not contain cysteine. The proportion of isoleucine present in apo-VLDL Sephadex fraction II, DEAE fractions 3, 4 and 5
Figure 6.4  DEAE-Cellulose Chromatography of the Low Molecular Weight Fraction II Obtained After Gel Filtration on Sephadex G-200 SF of Rat Apo-VLDL and PAGE of Pooled Column Fractions. The column (.9 x 40 cm) was eluted with a Triz-HCl pH 8.2 gradient of (1 litre) 0.01 M-0.25 M containing 6 M urea. Fractions of 6 ml were collected. Peaks which contained a significant amount of protein are numbered. PAGE was performed as described in the text.
Figure 6.5  DEAE-Cellulose Chromatography of Fraction III Obtained After
Gel Filtration of Rat Apo-VLDL on Sephadex G-200 SF and PAGE of Pooled
Column Fractions. The conditions of elution were the same as those
described for fraction II. PAGE was performed as described in the text.
FRACTION III
SEPHADEX G-200 SF

DEAE CELLULOSE FRACTIONS

0.03

ABSORBANCE (280 nm)

0.02

0.01

0.001

FRACTION No.

3.0

2.0

1.0

0.0

0 10 20 30 40 50 60 70 80 90

CONDUCTIVITY (milli mhos)
Table 6.1
Amino Acid Composition of DEAE Fractions Obtained After
Ion Exchange Chromatography of Apo-VLDL Sephadex
G-200 SF Fraction II

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles/10^3 moles recovered^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>34.6</td>
<td>37.8</td>
<td>47.1</td>
<td>44.7</td>
<td>55.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>tr</td>
<td>n.d.</td>
<td>3.2</td>
<td>1.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>96.6</td>
<td>78.2</td>
<td>69.1</td>
<td>86.2</td>
<td>92.2</td>
</tr>
<tr>
<td>Aspartic acid + Asparagine</td>
<td>90</td>
<td>88.9</td>
<td>82.8</td>
<td>88.1</td>
<td>92.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>35.2</td>
<td>27.2</td>
<td>35.2</td>
<td>46.6</td>
<td>64.9</td>
</tr>
<tr>
<td>Serine</td>
<td>46.3</td>
<td>35.1</td>
<td>89.6</td>
<td>56.5</td>
<td>73.6</td>
</tr>
<tr>
<td>Glutamic acid + Glutamine</td>
<td>265.6</td>
<td>268.6</td>
<td>220.5</td>
<td>275.9</td>
<td>223.7</td>
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<tr>
<td>Proline</td>
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<td>24.7</td>
<td>13.4</td>
<td>30.0</td>
<td>tr</td>
</tr>
<tr>
<td>Glycine</td>
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<td>69.6</td>
<td>119.5</td>
<td>76.1</td>
<td>74</td>
</tr>
<tr>
<td>Alanine</td>
<td>109.6</td>
<td>100.8</td>
<td>96.3</td>
<td>98.8</td>
<td>84.0</td>
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<tr>
<td>Valine</td>
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<td>62.3</td>
<td>63.4</td>
<td>52</td>
<td>69.6</td>
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<tr>
<td>Methionine</td>
<td>6.8</td>
<td>25.9</td>
<td>12.5</td>
<td>18.5</td>
<td>13.5</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>69.8</td>
<td>61.7</td>
<td>27.5</td>
<td>14.4</td>
<td>16.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>108.7</td>
<td>92.2</td>
<td>94.9</td>
<td>95.8</td>
<td>115.4</td>
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<tr>
<td>Tyrosine</td>
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<td>6.6</td>
<td>6.3</td>
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<tr>
<td>Phenylalanine</td>
<td>20.8</td>
<td>21.7</td>
<td>18.4</td>
<td>9.1</td>
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n.d. = not detected

^aFigures represent the mean of two determinations.
Table 6.2
Amino Acid Composition of DEAE Fractions Obtained After Ion Exchange Chromatography of Apo-VLDL Sephadex
G-200 SF Fraction III

<table>
<thead>
<tr>
<th>AMINO ACID</th>
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<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles/10³</td>
<td>moles recovered</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>40</td>
<td>63</td>
<td>60.8</td>
<td>76</td>
</tr>
<tr>
<td>Histidine</td>
<td>tr</td>
<td>0.6</td>
<td>6.9</td>
<td>4.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>60.5</td>
<td>26.7</td>
<td>28.8</td>
<td>24.4</td>
</tr>
<tr>
<td>Aspartic acid + Asparagine</td>
<td>76.8</td>
<td>93</td>
<td>120.7</td>
<td>11.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>68.8</td>
<td>66.7</td>
<td>78</td>
<td>86</td>
</tr>
<tr>
<td>Serine</td>
<td>126.9</td>
<td>127.3</td>
<td>112</td>
<td>174.3</td>
</tr>
<tr>
<td>Glutamic acid + Glutamine</td>
<td>162</td>
<td>171.1</td>
<td>174.3</td>
<td>188.5</td>
</tr>
<tr>
<td>Proline</td>
<td>53.9</td>
<td>28.1</td>
<td>28.5</td>
<td>12.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>114.7</td>
<td>115.2</td>
<td>102.3</td>
<td>149.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>59.1</td>
<td>82.7</td>
<td>65.1</td>
<td>58.8</td>
</tr>
<tr>
<td>Valine</td>
<td>34.8</td>
<td>36.3</td>
<td>28.6</td>
<td>34.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>34.8</td>
<td>31.6</td>
<td>20</td>
<td>17.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>17.4</td>
<td>12.7</td>
<td>13.9</td>
<td>18.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>90.4</td>
<td>108.2</td>
<td>103.1</td>
<td>108.9</td>
</tr>
<tr>
<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
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<td>30.3</td>
<td>34.7</td>
<td>27.3</td>
</tr>
</tbody>
</table>

n.d. = not detected

Figures represent the mean of two determinations.
Figure 6.6  Sephadex G-200 Chromatography of Rat Apo-HDL and PAGE of Pooled Column Fractions. The column (2.5 x 90 cm) was eluted with 1 M acetic acid (upward flow) at room temperature. Roman numerals and arrows define pooled column fractions. Recovery of protein after dialysis was 90%. Peak V contained 20% of the recovered protein. Fractions of 6 ml were collected and PAGE was performed as described in the text on 7.5% gels.
Apo-HDL I II III IV V
SEPHADEX G-200 FRACTIONS
was considerably lower than in DEAE fractions 1 and 2 (27.5, 14.4 and 16.8 compared to 69.8 and 61.7 moles/10³ moles recovered). Also, DEAE fractions 1 and 5 (Table 6.1) contained less proline than fractions 2, 3 and 4.

Apo-VLDL Sephadex G-200 SF fraction III peptides (Table 6.2) contained low proportions of histidine, no detectable half cystine and the content of glycine and serine was relatively higher, while glutamic acid and arginine content was lower than in apo-VLDL Sephadex fraction II peptides (Table 6.1). Apo-VLDL Sephadex fraction III DEAE fractions 2, 3 and 4 contained less arginine and less proline than fraction 1 and the content of aspartic acid in DEAE fraction 4 was much lower than in all other DEAE fractions (Table 6.2).

6.3.4 Gel Chromatography of Apo-HDL

Gel chromatography of apo-HDL on Sephadex G-200 eluted with 1 M acetic acid is shown in Figure 6.6. Partial separation was obtained but some contamination of fraction I by material in fractions III, IV and V was apparent (see gels Figure 6.6). Fraction V contained 20% of the protein eluted. Better separation of apo-HDL proteins was obtained when 0.01 M Triz-HCl pH 8.2 containing 1 mM EDTA and 8 M urea was used as eluting buffer (Figure 6.7). The first peak eluted near the column void volume and contained material most of which did not enter the main gel after PAGE on 7.5% gels. Fraction I contained 65%, fraction II 17% and fraction III 18% of the recovered protein. However, the trailing phenomenon on PAGE and the high content of Peak I is suggestive evidence of either incomplete delipidation or aggregation of protein.

The separation profile of apo-HDL on Sephadex G-200 SF columns containing glass beads (5 x 150 cm) was similar to that obtained using
Figure 6.7  Sephadex G-200 Chromatography of Rat Apo-HDL and PAGE of Pooled Column Fractions. The column (2.5 x 90 cm) was eluted with 0.01 M Triz-HCl pH 8.2 1 mM EDTA containing 8 M urea. Apo-HDL was dialysed for 48 hr against the eluting buffer prior to loading onto the column. Fractions of 8 ml were collected. Roman numerals and arrows define pooled column fractions and 65, 17 and 18% of the protein was recovered in Peaks I, II and III respectively. Gels were stained with Coomassie Brilliant Blue (Sigma).
Sephadex G-200 (Figures 6.7 and 6.8) Apo-HDL was resolved into two well defined peaks and two other poorly resolved peaks (Figure 6.8). The first peak (pooled fractions I and II) contained material that stayed at the junction of the stacking gel and main gel (fraction I) and that just entered the main gel (fraction II) after PAGE. Fractions III, IV, V and VI contained the major protein of apo-HDL. Fraction VII contained several proteins in an elution volume consistent with proteins of low molecular weight (mol. wt. < 30,000) and contained 20% of the recovered protein. The proteins of this fraction were fast migrating and equivalent to zone IV peptides after PAGE.

Fractions V and VI (Figure 6.8) were combined and rechromatographed on Sephadex G-200 SF (glass bead) column (5 x 150 cm) in order to separate the lower molecular weight proteins from the higher molecular weight proteins of apo-HDL (Figure 6.9). Biogel A 5M chromatography was also effective in separating the low molecular weight peptides (fast migrating on polyacrylamide gels) from the higher molecular weight proteins (Figure 6.10). Fractions containing higher molecular weight proteins from both these columns were pooled and further separated by DEAE-Cellulose chromatography.

6.3.5 Ion Exchange Chromatography of Apo-HDL

The separation of apo-HDL higher molecular weight proteins is illustrated in Figure 6.11. From the elution profile it can be seen that no clear separation of peaks was obtained. The peptides eluted in pooled fractions 1, 2, 3 and 4 contained peptides with a similar migration rate on polyacrylamide gels although they were eluted at different conductivities.
Figure 6.8  Sephadex G-200 SF (Glass Bead) Chromatography of Rat Apo-HDL and PAGE of Pooled Column Fractions. The column (5 x 150 cm) was eluted with 0.2 M Triz-HCl 6 M urea pH 8.2 at room temperature. Fractions of 4.8 ml were collected. Roman numerals and arrows define pooled column fractions and 20% of the protein was recovered in Peak VII. PAGE of column fractions was performed as described in the text. Pooled column fractions I, II, III, IV, V and VI contained approximately 12, 16, 19, 10, 9 and 13% of the recovered protein.
Figure 6.9  Sephadex G-200 SF (glass bead) Chromatography of Apo-HDL

Sephadex G-200 SF (Figure 6.8) Fractions V and VI and PAGE of Pooled Column Fractions. The column (5 x 150 cm) was eluted with 0.2 M Triz-HCl 6 M urea pH 8.2 at 4°C. Fractions of 7.3 ml were collected. Roman numerals and arrows define pooled column fractions. 30% of recovered protein was in Peak III, 55% in Peak II and 15% in Peak I. PAGE of pooled fractions was performed as described in the text.
Biogel A5 M Chromatography of Rat Apo-HDL Sephadex G-200

Figure 6.10  
SF Fraction IV Plus Fraction II (Figure 6.9) and PAGE of Pooled Column Fractions. The column (2.5 x 100 cm) was eluted with 0.2 M Triz-HCl 6 M urea pH 8.2. Fractions of 5 ml were collected. Roman numerals and arrows define pooled column fractions and 50% of the protein was recovered in Peak III. PAGE of pooled fractions was performed as described in the text.
Apo-HDL SEPHADEX G-200SF FRACTION IV • FRACTION II OF THE SEPARATION SHOWN IN FIGURE 6.9
FRACTION II
FRACTION III

DEAE CELLULOSE FRACTIONS
Figure 6.11  DEAE-Cellulose Chromatography of Apo-HDL

Higher Molecular Weight Proteins and PAGE of Pooled Column Fractions. The column (1.5 x 40 cm) was eluted with a Triz-HCl gradient (1 l) 0.01 M-0.2 M in 6 M urea pH 8.2. Fractions of 7.8 ml were collected. Fraction III Sephadex G-200 SF column of apo-HDL (Figure 6.8) and Fraction II (Figure 6.10) were combined and chromatographed on DEAE-Cellulose. Figures and arrows define pooled column fractions. PAGE was performed as described in the text.
Ion exchange chromatography of the low molecular weight HDL proteins (Sephadex G-200 SF, Fraction VII) is shown in Figure 6.12. No clearly resolved peaks were obtained. DEAE pooled fractions 1, 2, 3, 4 and 5 contained 11.6, 20, 20.7, 26.4 and 21.3% of the recovered protein respectively. Recovery from the column was 95%. Examination of each pooled fraction by PAGE although providing evidence for some heterogeneity, showed the presence of only one major component, estimated to be > 90% by scanning. Since only small amounts of protein were present, the pooled fractions were not rechromatographed. As the contaminating protein in each fraction was < 10% it was considered worthwhile to determine the amino acid composition since it would reflect that of the major component present.

6.3.6 Amino Acid Analysis of HDL Peptides

The amino acid composition of the low molecular weight HDL proteins obtained after ion exchange chromatography was similar and is shown in Table 6.3. Notable features include the apparent lack of half cystine, the higher content of histidine and aspartic acid compared to apo-VLDL Sephadex fractions II and III peptides (Tables 6.1 and 6.2). The proportion of serine present in the apo-HDL low molecular weight peptides was similar to that in apo-VLDL Sephadex fraction II peptides (Table 6.1) but lower than apo-VLDL Sephadex fraction III peptides (Table 6.2).

A comparison of the relative migration on polyacrylamide gels of apo-HDL low molecular weight peptides (Sephadex G-200 SF fraction VII) and apo-VLDL Sephadex G-200 SF fractions II and III separated by ion exchange chromatography is illustrated in Figure 6.13. Low molecular weight apo-HDL peptides (PAGE zone IV) and apo-VLDL Sephadex fraction III (PAGE zone IV) migrate to similar distances (DEAE fraction 3 of HDL migrates close to fraction 1 of VLDL and DEAE fractions 4 and 5 of apo-HDL
Figure 6.12  DEAE-Cellulose Chromatography of Rat Apo-HDL Fraction VII (Sephadex G-200 SF Column, Figure 6.8) and PAGE of Pooled Fractions. The column (1.5 x 40 cm) was eluted with a Triz-HCl gradient .01 M-.2 M (1 7) containing 6 M urea pH 8.2 followed by 100 ml 0.25 M Triz and 100 ml 0.5 M Triz-HCl pH 8.2 6 M urea. Figures and arrows define pooled column fractions. PAGE was performed as described in the text.
FRACTION VII
SEPHADEX G-200 SF
1

2
DEAE CELLULOSE
3
FRACTIONS
4
5
Table 6.3
Amino Acid Compositions of DEAE Fractions Obtained After Ion Exchange Chromatography of Apo-HDL Sephadex G-200 SF Fraction VII

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>DEAE FRACTION</th>
<th>DEAE FRACTION</th>
<th>DEAE FRACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 moles/10³</td>
<td>4 moles</td>
<td>5 moles recovered³</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Lysine</td>
<td>77.6</td>
<td>63.4</td>
<td>71.3</td>
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<tr>
<td>Histidine</td>
<td>17.8</td>
<td>16.3</td>
<td>16.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>50.1</td>
<td>43.4</td>
<td>37.4</td>
</tr>
<tr>
<td>Aspartic Acid + Asparagine</td>
<td>137.3</td>
<td>142.5</td>
<td>140.8</td>
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<tr>
<td>Threonine</td>
<td>58.7</td>
<td>58.3</td>
<td>53.5</td>
</tr>
<tr>
<td>Serine</td>
<td>54.3</td>
<td>54.3</td>
<td>48.1</td>
</tr>
<tr>
<td>Glutamic Acid + Glutamine</td>
<td>158.5</td>
<td>176.4</td>
<td>187.3</td>
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<tr>
<td>Proline</td>
<td>36.9</td>
<td>12.3</td>
<td>42.8</td>
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<tr>
<td>Glycine</td>
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<td>73.1</td>
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<td>n.d.</td>
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</tr>
<tr>
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<td>60.6</td>
</tr>
<tr>
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<td>17.8</td>
<td>19.0</td>
<td>19.6</td>
</tr>
<tr>
<td>Isoleucine</td>
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<tr>
<td>Phenylalanine</td>
<td>35</td>
<td>38.0</td>
<td>37.4</td>
</tr>
</tbody>
</table>

n.d. = not detected

³Figures represent the mean of two determinations.
Figure 6.13  Comparison of the Relative Migration of Peptides Separated by Ion Exchange Chromatography of Apo-HDL Sephadex G-200 SF Fraction VII and Apo-HDL Sephadex G-200 SF Fractions II and III.
migrate close to DEAE fractions 2 and 3 of VLDL. Apo-VLDL Sephadex fraction II peptides (PAGE zones II and III) do not migrate as far as the apo-HDL low molecular weight peptides and apo-VLDL Sephadex fraction III peptides except for DEAE fraction 3 and part of 4 which migrate close to DEAE fractions 3 of apo-HDL and 1 of apo-VLDL Sephadex fraction III.

6.4 DISCUSSION

The work described in this chapter was performed in order to identify as far as possible the peptides present in apo-VLDL PAGE zones II, III and IV and apo-HDL zones II, III and IV. Much research has been devoted to the study of human apolipoproteins and there have been many major advances since the work described in this thesis was commenced. In comparison, however, little is known about rat apolipoproteins and they have therefore been identified in metabolic studies (Chapters 3, 4 and 5), after analysis by PAGE, according to their relative migration. This would at least allow comparison between those apoproteins which are apparently commonly found in both VLDL and HDL.

Others such as Eisenberg et al (1973b) have attempted to overcome this problem of identity by injecting human labelled lipoproteins into rats and identifying labelled bands on polyacrylamide gels by reference to human standards. Further characterisation of rat apoproteins and comparison with human apoproteins may therefore establish the rat as a good model for the study of human lipoprotein metabolism and abnormalities especially if homology in apoprotein composition were demonstrated.

Apo-VLDL and apo-HDL peptides were separated by gel filtration and ion exchange chromatography. The gel filtration elution profile of apo-VLDL on Sephadex G-150 was similar to that described by Eisenberg et al (1972b) for human apo-VLDL using 0.2 M Triz-HCl (pH 8.2) 2 mM SDS as eluant.
Although elution of columns with urea buffers has proved very useful in the separation of apolipoproteins there is a danger of obtaining artifactual polymorphism through carbamylation of the proteins fractionated when columns are run at room temperature (Herbert et al., 1973). The danger of this occurring in these studies was reduced by deionising the urea directly before use and by storing and eluting columns at 4°C. Cyanate formation which causes carbamylation is greatly reduced at 4°C (Marier and Rose, 1964).

Fractionation of rat apo-VLDL on Sephadex G-200 SF (eluted with 0.2 M Triz-HCl pH 8.2 containing 6 M urea) columns containing glass beads has not been previously reported. However, a similar elution profile of rat apo-VLDL on Sephadex G-150 was reported by Bersot et al. (1970) and by Herbert et al. (1973b) for human apo-VLDL on a Sephadex G-200 SF glass bead column. Although rat apo-VLDL peptides have been fractionated by ion exchange chromatography (Bersot et al., 1970; Koga et al., 1971) no amino acid analyses were reported by these authors. One of the problems inherent in working with rat apolipoproteins is that large quantities are difficult to obtain. Therefore the quantities of fractionated protein obtained after DEAE-cellulose chromatography are low and in many cases too low for an exhaustive analysis of their properties such as the determination of carboxy terminal, amino terminal residues, and the content of cysteine, tryptophan, sialic acid and other carbohydrates.

Although human apo-HDL has been fractionated to some extent on Sephadex G-200 using 1 M acetic acid as eluant (Rudman et al., 1970) better separation of rat apo-HDL proteins was achieved using 6 M urea in 0.2 M Triz-HCl (pH 8.2) as eluant. Fractionation of rat apo-HDL on Sephadex G-200 SF glass bead columns was similar to that described by Herbert et al. (1974) except that a larger first peak was obtained (Figure 6.8). Fractions I, II, III, IV and V (Figure 6.8) probably
contain the major apo-HDL proteins which in the human correspond to the A proteins (85-90% of human apo-HDL). All these apo-HDL fractions reacted immunochemically with anti-HDL. The rat apo-HDL proteins fractionated by Sephadex G-150 by Bersot et al. (1970), which on PAGE remain on top of the main gel or are slow migrating (zone II), react immunochemically against anti-apo-HDL but not against anti-apo-VLDL. Faster migrating HDL peptides (PAGE zones III and IV) reacted immunochemically against both anti-apo-HDL and anti-apo-VLDL and had an $\alpha_1$ mobility when immunoelectrophoresed (Bersot et al., 1970). It has been suggested by Bersot et al. (1970) and Scanu (1966) that HDL protein tends to aggregate even in 6 M urea when lipid is removed. The first peak obtained in Figure 6.8 could be due to aggregating apo-HDL protein. Although dialysis of apo-HDL against the eluting buffer for 48 hr prior to chromatography has been shown by others (Scanu et al., 1969; Fidge, unpublished results; for human and pig respectively) to reduce the size of this first peak, it was not observed in these studies (Figures 6.7 and 6.8). It is also possible that this first peak represents a distinct large molecular weight species of polypeptide occurring in rat apo-HDL.

After repeated gel filtration of apo-HDL PAGE zones II and III peptides (Figures 6.8, 6.9 and 6.10) these proteins were applied to DEAE-cellulose columns. Peaks were poorly resolved (Figure 6.11). From PAGE of the pooled DEAE fractions it is apparent that there may be considerable polymorphism among these proteins which are eluted at different conductivities.

In addition to the size of the first peak another difference was apparent between the separation of rat apo-HDL obtained by Herbert et al. (1974) and that illustrated in Figure 6.8. Herbert et al. (1974) described two protein bands (one identified as rat A-II) appearing on polyacrylamide gels in an area equivalent to PAGE zone II and III (in these studies)
which were eluted with other low molecular weight HDL proteins in their fraction V. These protein bands were absent from apo-HDL fraction VII (Figure 6.8). Except for these two differences the separations were similar.

Herbert et al (1974) reported the amino acid, amino terminal and carboxy terminal analyses of rat apo-HDL low molecular weight fractions obtained after DEAE-cellulose chromatography. Using this data they demonstrated a 'striking homology' between rat and human low molecular weight C proteins. Rat C proteins of HDL were designated C-I, C-II, C-III₀ and C-III₃. No such comparison of rat apo-VLDL and human apo-VLDL proteins has been reported.

Although heterogeneity in some DEAE pooled fractions of apo-VLDL and apo-HDL proteins (Figure 6.13) was observed after PAGE, the amino acid compositions were determined since in most cases one major component (> 90%) was present. Also, the amino acid composition can be used as a basis for comparison of rat VLDL and HDL low molecular weight proteins with those of the human. Amino acid analyses of the peptides of rat apo-VLDL Sephadex fractions II and III and rat apo-HDL low molecular weight peptides (Tables 6.1, 6.2 and 6.3) show some internal similarity. All fractions are characterised by a low histidine content and probably an absence of cysteine as half cystine was not detected. Apo-HDL DEAE pooled fractions (3, 4 and 5) had similar compositions and were characterised by high aspartic acid and glutamic acid contents. Apo-VLDL Sephadex fraction II DEAE pooled fractions (Table 6.1) were rich in arginine, glutamic acid and alanine. Apo-VLDL Sephadex fraction III proteins (Table 6.2) were characterised by high serine and glycine contents. Apo-VLDL Sephadex fraction III DEAE pooled fraction 1 contained more arginine and fraction 4 contained less aspartic acid than fractions 2 and 3 whose composition was very similar.
Shelburne and Quarfordt (1974) have described an apoprotein (mol. wt. 33,000) of human VLDL which is rich in arginine and comprises 5 to 15% of the total VLDL protein. Close scrutiny of the amino acid composition of rat apo-VLDL II, DEAE fractions 4 and 5 (comprising approximately 10% of total VLDL protein) and the human arginine rich peptide revealed a close similarity. This is shown in Table 6.4. Major differences are apparent in the proportion of glutamic acid, aspartic acid and methionine which are present in higher proportions in the rat. The content of histidine, tyrosine and phenylalanine is lower in the rat.

A comparison of the amino acid composition of apo-VLDL and apo-HDL proteins obtained in these studies with those of rat apo-HDL low molecular weight proteins (Herbert et al., 1974) and human C proteins (Herbert et al., 1973b) is shown in Table 6.5. It is apparent that the amino acid composition of apo-VLDL Sephadex fraction III DEAE fractions 2 and 3 is almost identical to that of the human C-III peptide. Rat apo-HDL DEAE fractions 3, 4 and 5 have similar compositions to both the human C-III peptide and the apo-VLDL Sephadex fraction III DEAE fractions 2 and 3. These rat peptides (apo-VLDL III, 2 and 3 and apo-HDL 3, 4 and 5) migrated similarly on polyacrylamide gels (Figure 6.13). The small differences in migration after PAGE could be due to differences in the carbohydrate content of polymorphic peptides. Herbert et al. (1974) described two forms of C-III in rat apo-HDL, C-III_0 and C-III_3. The latter contained 3 moles of sialic acid and one mole of galactosamine per mole of protein. Polymorphic forms of human C polypeptides have also been described, for example C-III_1, C-III_2, C-III_1_3, C-III_2 and C-III_3 which contain different proportions of carbohydrate (Alauopovic, 1972).

The composition of rat apo-VLDL Sephadex fractions II and III, DEAE fractions 3 and 1 respectively resembled that of human C-I and to a lesser
Table 6.4
Comparison of the Amino Acid Composition of the Human Arginine Rich Peptide with Rat VLDL Sephadex Fraction II Peptides

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>Apo-VLDL&lt;sup&gt;a&lt;/sup&gt; arginine rich Human</th>
<th>Apo-VLDL Sephadex Fraction II DEAE FRACTIONS 4 5</th>
<th>moles/10&lt;sup&gt;3&lt;/sup&gt; moles recovered amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>49</td>
<td>44.7</td>
<td>55.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.7</td>
<td>1.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>91.2</td>
<td>86.2</td>
<td>92.2</td>
</tr>
<tr>
<td>Aspartic acid + Asparagine</td>
<td>68.1</td>
<td>88.1</td>
<td>92.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>45.5</td>
<td>46.6</td>
<td>64.9</td>
</tr>
<tr>
<td>Serine</td>
<td>60.3</td>
<td>56.5</td>
<td>73.6</td>
</tr>
<tr>
<td>Glutamic acid + Glutamine</td>
<td>178.8</td>
<td>275.9</td>
<td>223.7</td>
</tr>
<tr>
<td>Proline</td>
<td>58.1</td>
<td>30</td>
<td>tr</td>
</tr>
<tr>
<td>Glycine</td>
<td>67.2</td>
<td>76.1</td>
<td>74</td>
</tr>
<tr>
<td>Alanine</td>
<td>104.3</td>
<td>98.8</td>
<td>84</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Half Cystine</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>73.6</td>
<td>52</td>
<td>69.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.8</td>
<td>18.5</td>
<td>13.5</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>17.6</td>
<td>14.4</td>
<td>16.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>120</td>
<td>95.8</td>
<td>115.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>17.5</td>
<td>6.3</td>
<td>9.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>21.3</td>
<td>9.1</td>
<td>9.2</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>32.2</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup>Shelburne and Quarfordt (1974)

— not determined.
Table 6.5
Comparison of the Amino Acid Composition of DEAE Fractions of Rat Apo-VLDL and Apo-HDL with Human C Proteins and Rat Apo-HDL Low Molecular Weight Proteins

<table>
<thead>
<tr>
<th></th>
<th>C-I</th>
<th>Apo-VLDL II DEAE Fraction 3</th>
<th>Apo-VLDL III DEAE Fraction 1</th>
<th>C-II</th>
<th>C-III</th>
<th>Apo-VLDL III DEAE Fractions</th>
<th>Apo-HDL DEAE Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>16</td>
<td>16</td>
<td>4.7</td>
<td>4.0</td>
<td>4.0</td>
<td>6.3</td>
<td>7.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.0</td>
<td>.3</td>
<td>.0</td>
<td>0.2</td>
<td>1.0</td>
<td>0.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.9</td>
<td>6.9</td>
<td>6.0</td>
<td>2.1</td>
<td>2.2</td>
<td>2.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Aspartic acid + Asparagine</td>
<td>10.9</td>
<td>9.3</td>
<td>8.3</td>
<td>7.0</td>
<td>7.1</td>
<td>9.3</td>
<td>13.7</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.5</td>
<td>5.5</td>
<td>3.5</td>
<td>10.5</td>
<td>10.5</td>
<td>6.7</td>
<td>5.9</td>
</tr>
<tr>
<td>Serine</td>
<td>8.6</td>
<td>12</td>
<td>12.7</td>
<td>10.1</td>
<td>10.1</td>
<td>12.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Glutamic acid + Glutamine</td>
<td>14.9</td>
<td>16.2</td>
<td>22</td>
<td>15.2</td>
<td>15.2</td>
<td>17.1</td>
<td>15.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.2</td>
<td>2.2</td>
<td>12.6</td>
<td>6.3</td>
<td>6.3</td>
<td>11.5</td>
<td>6.6</td>
</tr>
<tr>
<td>Half Cystine (Cysteine)a</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.0</td>
<td>5.9</td>
<td>9.6</td>
<td>5.9</td>
<td>11.8</td>
<td>3.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Valine</td>
<td>4.0</td>
<td>6.3</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.6</td>
<td>7.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.2</td>
<td>1.3</td>
<td>3.5</td>
<td>3.2</td>
<td>3.2</td>
<td>3.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.5</td>
<td>2.8</td>
<td>1.7</td>
<td>1.1</td>
<td>1.0</td>
<td>1.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.11</td>
<td>9.5</td>
<td>9.5</td>
<td>9.5</td>
<td>14.10</td>
<td>10.8</td>
<td>10.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0</td>
<td>0.7</td>
<td>2.3</td>
<td>6.6</td>
<td>3.3</td>
<td>7.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.5</td>
<td>1.8</td>
<td>3.7</td>
<td>3.3</td>
<td>4.5</td>
<td>4.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.2</td>
<td>—</td>
<td>2.2</td>
<td>2.2</td>
<td>4.4</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a Herbert et al (1974)

— not determined
degree human C-II. The major differences were in the content of glycine, which was considerably higher and lysine which was considerably lower than in the human. From their migration on polyacrylamide gels (Figure 6.13) however it would appear that they may be polymorphic forms of rat C-II not as yet characterised, since C-II and C-III₀ of rat HDL (Herbert et al., 1974) comigrate at alkaline pH. It is also possible that apo-HDL DEAE fractions 3, 4 and 5 may contain some C-II (< 10%) apoprotein.

The immunochemical data of Bersot et al. (1970) and Koga et al. (1971), PAGE data and the amino acid analyses described above, suggest that VLDL and HDL share at least two peptides of low molecular weight which are similar to the human C-III peptides. No DEAE pooled fractions had similar amino acid compositions to either the rat or human A-II apoprotein described by Herbert et al. (1974).

Herbert et al. (1974) reported that homologues of all the human C apoproteins were isolated from rat apo-HDL. Some differences however were observed. The rat C-III₂ homologue had three sialic acid molecules per molecule while the C-III₁ homologue had no sialic acid. Rat C-III₁ and C-II were very similar in size and charge. Rat C-II, however, had the same properties as human C-II but rat C-III₁ had carboxy-terminal proline, amino-terminal aspartic acid and lacked histidine and hexosamine. Purified rat C-II was a potent activator of rat adipose tissue lipoprotein lipase at similar concentrations to the human C-II (Herbert et al., 1973a). From the data of Herbert et al. (1974) it appears that rat apo-HDL C proteins are homologous to the human in properties and function. Such data, however, is not available on rat apo-VLDL C-apoproteins.

It can therefore be concluded from this partial characterisation that some VLDL and HDL zone IV peptides are found in both VLDL and HDL and are analogous to the human C-III peptides. A notable feature of apo-VLDL PAGE zone II and III proteins is their high content of arginine.
and the similar amino acid composition of two of these with a human arginine rich VLDL peptide (Table 6.4). Apo-HDL PAGE zone II and III probably contain the major protein of apo-HDL (analogous to the human A protein). However it is recognised that further characterisation of these peptides is necessary to substantiate these conclusions.
6.5 SUMMARY

Low molecular weight proteins of apo-VLDL and apo-HDL have been separated using gel filtration on Sephadex G-200 SF and DEAE-cellulose chromatography. Apo-VLDL proteins of PAGE zones II and III and zone IV are heterogeneous but within each zone some have similar amino acid compositions.

The amino acid composition of PAGE zone IV apoproteins revealed that VLDL and HDL contain at least two common proteins similar in composition to human C-III proteins. Also, PAGE zones II and III of apo-VLDL contain proteins which are relatively rich in arginine, two of which have similar amino acid compositions to the human arginine rich peptide.
CHAPTER 7

GENERAL DISCUSSION

The work described in the previous chapters has been carried out in an attempt to contribute to fundamental knowledge about insulin-like growth factors and their potential in the regulation of growth and development. Most of the results described in this chapter are related to those from other laboratories involved in similar research. It was surprising to find that the growth-inhibitory property also extended to the metabolism of myoinositol in both normal and diabetic tissues. The low levels of insulin receptors in diabetic tissues have been confirmed by several studies (Ruderman, 1978), which led to the possibility that insulin sensitivity, insulin receptors, and insulin metabolism are related. Additional evidence for this was provided by recent studies on the role of insulin receptors in the regulation of insulin metabolism. The results from these studies have shown that the metabolism of insulin is regulated by the levels of insulin receptors, which are influenced by the type of tissue and the stage of development. The studies also suggest that insulin receptors are important in the regulation of insulin metabolism.

In these studies, it was shown that the metabolism of insulin is regulated by the levels of insulin receptors. The results from these studies have shown that the metabolism of insulin is regulated by the levels of insulin receptors, which are influenced by the type of tissue and the stage of development. The studies also suggest that insulin receptors are important in the regulation of insulin metabolism.
CHAPTER 7

GENERAL DISCUSSION

The work described in the preceding chapters has been carried out in an attempt to contribute to fundamental knowledge about lipoprotein metabolism and to gain a broader perspective of the role of apolipoproteins in this process. When the results described in this thesis are related to those from other laboratories involved in similar research, it must now be accepted that the apolipoproteins can play key roles in the metabolism of lipoproteins in both normal and disease states. The key roles played by apolipoproteins in lipoprotein metabolism is exemplified in inherited disorders such as, abetalipoproteinaemia, hypobetalipoproteinaemia and Tangier disease (Frederickson, Gotto and Levy, 1972) in which the inability to synthesise apoB or apoA-I results in the absence of lipoproteins of d < 1.063 g/ml and HDL respectively, additional abnormal lipoprotein patterns in the serum and a wide range of clinical manifestations associated with abnormal lipid metabolism. From studies of inherited apolipoprotein abnormalities it is clear that apoproteins like enzymes are specialised molecules whose function is dependent on their primary structure and therefore the genetic complement of the organism (genetic control of proteins, Strickberger, 1968). The apoprotein moiety at the replication level therefore, must also be considered as having the potential for influencing the origin and fate of lipoproteins at least from the structural point of view and possibly in quantitative terms as well.

In these studies it was shown that metabolism of rat VLDL apolipoproteins is heterogeneous. The C apolipoproteins have a longer life in the circulation than the B apolipoprotein and are transferred from VLDL to other lipoproteins (LDL₁, LDL₂ and HDL) as VLDL catabolism proceeds. Some heterogeneity in zone IV apolipoprotein transfer was also observed.
The results suggest that C apolipoproteins are transferred back to VLDL from HDL. As rat C-II apolipoprotein is a potent activator of lipoprotein lipase (Herbert et al., 1973a) HDL may serve as a 'bank' of C apolipoproteins which are donated to triglyceride-rich lipoproteins and possibly LDL (Marsh, 1974) secreted into the circulation either from the intestine (Windmueller et al., 1973) or the liver so that triglyceride hydrolysis can proceed. This mechanism would prevent rapid catabolism of VLDL at sites of secretion and therefore facilitate transport of VLDL to extrahepatic sites of lipoprotein lipase activity and triglyceride utilisation such as lung, heart and muscle. C apolipoproteins other than C-II may modulate lipoprotein lipase activity and it has been suggested that phospholipid (a co-factor of lipoprotein lipase activity) is also transferred from VLDL to HDL (Rubenstein and Rubinstein, 1972). It is apparent from these observations that rat C proteins play a significant role in the regulation of lipoprotein lipase activity and therefore in the metabolism of triglyceride-rich lipoproteins. Homologues of all the human C apolipoproteins have been found in the rat (these studies and Herbert et al., 1974). With respect to the movement of C apolipoproteins between lipoproteins and their possible role in the regulation of lipoprotein metabolism, the data is very similar to results reported (during the course of the present studies) on human VLDL C apolipoprotein metabolism (Havel et al., 1973; Eisenberg et al., 1973a).

In contrast to the fate of VLDL C apolipoproteins, most of the B protein was rapidly removed from the circulation and only a small proportion was transferred to other lipoproteins, in particular LDL₁ and LDL₂. Similar results were reported for human VLDL B protein metabolism (Eisenberg et al., 1973a,b). These results suggest that LDL₁ and LDL₂ are derived from VLDL. Assessment of the quantitative significance of this transfer was made using specific activity measurements and it was shown
that apparently not all LDL₂ is derived from VLDL in the rat. Although a number of alternative explanations for the B apolipoprotein specific activity data have been discussed the data could not be compared to that of other workers since no other reports have been published on the quantitative measurement of the B apolipoprotein. However, a comparison of the quantitative significance of VLDL transformation to LDL in the rat with that in the human (Reardon and Fidge, unpublished results) revealed that they may be different and could be related to the size of the VLDL:LDL₂ B apolipoprotein pools. For example, this ratio in the rat is approximately 1:1 whereas in the human it may be as high as 10:1. Initial rapid removal of both VLDL B apolipoprotein and triglyceride from the circulation suggests that their removal may be interdependent. These studies and those of abnormal lipoproteins in disease states (dyslipoproteinaemias) suggest that the most significant determinants in the formation and structural integrity of the lipoproteins are the apolipoproteins.

A scheme of VLDL catabolism constructed from data presented in this thesis is shown in Figure 7.1. In vivo data suggests that the series of events resulting in the catabolism of VLDL and the heterogeneous metabolism of VLDL apolipoproteins, occur during hydrolysis and removal of VLDL triglyceride from the circulation and therefore as a consequence of lipolytic activity. Support for this suggestion includes data obtained from the in vitro incubation of VLDL with post-heparin plasma containing lipolytic activity (notably triglyceride lipases, lipoprotein lipase and phospholipase activity (Fielding, 1970, 1972; Yasuoka and Fujii, 1971; Zieve and Zieve, 1972)) which results in the formation of LDL and exchange of C apolipoproteins. Similar conclusions have been reported after assessment of the data obtained from investigations of human VLDL metabolism in humans (Eisenberg et al, 1973a) and squirrel monkeys (Schonfeld et al, 1972).
and other possible sites of synthesis/or degradation

Figure 7.1 Scheme of VLDL Catabolism in the Rat.

- Represents ratio of aqueous insoluble (shaded):aqueous soluble apolipoproteins.
- B indicates B apolipoprotein transfer.
- C indicates C apolipoprotein transfer and possibly phospholipid, cholesterol and cholesterol ester.
- TG = Triglyceride and possibly other lipids.
- Indicated lipolytic activity. Arrows to and from the circulation represent synthesis or degradation of the respective lipoprotein. Broken lines indicate that a lower proportion of material is transferred. In some cases this may be insignificant. Although the intestine does synthesise VLDL and possibly LDL it is not an important site of removal and degradation.
The importance of lipoprotein interrelationships in the scheme of lipid transport has been reviewed by Newsholme and Start (1973). The concentration of triglyceride and cholesterol in the serum at any one time, would be dependent on the secretion of triglyceride-rich lipoproteins (i.e. chylomicrons and VLDL containing dietary triglyceride and cholesterol or the secretion of VLDL containing endogenous cholesterol and triglyceride in the fasting state) and their degradation resulting in the formation of cholesterol-rich lipoproteins which are then removed from the circulation. Under 'normal' conditions homeostatic control mechanisms operate to maintain the circulating level of triglyceride and cholesterol relatively constant by regulating the concentration of different lipoproteins. Quantitative inverse relationships have been demonstrated for VLDL and HDL (Levy et al, 1966) and VLDL and LDL triglyceride and cholesterol concentration (Wilson and Lees, 1972).

Lipoprotein concentrations may possibly be controlled by two main factors, the rate of synthesis of apoproteins and hence secretion of lipoproteins into the circulation and the rate of catabolism, both of which are dependent on the metabolic state of the animal. A scheme for the interrelationship between circulating triglyceride-rich lipoproteins and lipoprotein lipase is described by Nikkila (1969). Other factors such as induction of hormones, dietary carbohydrate and lipid, enzyme levels (lipoprotein lipase and LCAT) are described by Robinson (1970) and Scow and Chernick (1970). Defects in these control mechanisms, either in secretion (overproduction) or in clearance, result in hyperlipidaemia (hypercholesterolaemia and/or hypertriglyceridaemia). These disorders which have some genetic components and have been classified as hyperlipoproteinaemias are shown in Table 1.4 and are described by Frederickson and Levy (1972). They may be exacerbated by dietary factors or appear as a result of other metabolic disorders (diabetes mellitus,
chronic renal disease, hypothyroidism or gout). A number of known dyslipoproteinaemias, due to apolipoprotein abnormalities have been described. These include, cholestasis, abetalipoproteinaemia, Tangier disease and Lp(a) variant (Scanu and Ritter, 1973) and assist in the definition of the functional importance of the various apolipoproteins affected. From the condition abetalipoproteinaemia (characterised by an absence of d < 1.063 g/ml lipoproteins and inability to synthesise chylomicrons) it is apparent that the B protein plays a specific role in the transport of triglyceride. The arginine-rich peptide (apo-VLDL zone III in these studies), found in abnormally high concentration in VLDL from hypercholesterolaemic rabbits, type III hyperlipoproteinaemias and patients with hypothyroidism, is thought to play a specific role in the metabolism of cholesterol esters (Shore and Shore, 1974).

In the studies described in this thesis the rat was used as a model for the human in the study of lipoprotein metabolism. Some of the advantages of using the rat compared to other animal models are discussed below. The rat has provided much information on lipid metabolism (Robinson, 1970; Scow and Chernick, 1970) and fat transport. Also, in studies on the development of atheroma in the rat (Lewis et al., 1952; Stein and Stein, 1973; Stein et al., 1973), atherosclerotic lesions were observed in alloxan diabetic rats with raised serum cholesterol concentrations (Plenk et al., 1973). Furthermore, as in the human situation the occurrence and severity of the vascular lesions showed a positive correlation to the elevation of serum cholesterol levels.

The lipoprotein spectrum of the rat is similar to that found in man, in that it contains all the lipoprotein families within the same density ranges as those found in humans. Unlike the rat, the guinea pig does not contain HDL (Sardet et al., 1972) or co-factor protein for lipoprotein lipase (Whayne and Felts, 1970) and the pig, which contains two
distinct LDL classes, with density ranges different to the human (Janado et al, 1966) and also no co-factor for lipoprotein lipase (Fidge, unpublished observations). However, the relative concentration of each lipoprotein family in rats differs to that found in the adult human male (Nichols, 1969). Rats contain less LDL and a higher HDL level than man, and in this context the biochemical role of these corresponding lipoproteins may be different.

The most complete investigation of apolipoproteins to date (other than human) have been those of the rat. In general rat apolipoproteins are homologous to those of the human (Koga et al, 1969; Bersot et al, 1970) for example, the C group of apolipoproteins present in rat HDL (Herbert et al, 1974; also Chapter 6) and VLDL (see Chapter 6). Finally, the metabolism of rat $^{125}$I-VLDL in rats is similar to that of human $^{125}$I-VLDL in humans (Bilheimer et al, 1972) and rats. From the observations made in this thesis and those of others described above it is apparent that the rat may be a useful model for the study of human lipoprotein metabolism and also the study of the relative significance of various organs in the metabolic interrelationships between lipoproteins (e.g. using hepatectomised rats).

It has been found that hyperlipidaemia is an important risk factor in the development of atherosclerosis and coronary heart disease (Goldstein et al, 1973; Levy and Rifkind, 1973). There is biochemical evidence which links lipoproteins with atherosclerosis. Both cholesterol and cholesterol esters, derived from VLDL and LDL, are the major components of the early atherosclerotic lesion (Jones, 1970; Porter and Knight, 1973).

Cholesterol and triglyceride-rich lipoproteins (VLDL and LDL) enhance the proliferation of smooth muscle cells and the accumulation of lipid in
the arterial wall (Stein and Stein, 1973). Also, binding of VLDL and LDL to receptor sites (of high affinity) of cultured fibroblasts results in the regulation of cholesterogenesis and facilitates the degradation of VLDL and LDL present in low concentrations in the culture medium (Brown and Goldstein, 1974; Goldstein and Brown, 1974). A defect in this process represents the primary genetic abnormality in the disorder familial hypercholesterolaemia.

Interaction of arterial elastin and protein with serum VLDL and LDL but not HDL results in the transfer of cholesterol and cholesterol esters into the arterial wall (Kramsh and Hollander, 1973). Pathological changes may result in enhanced infiltration of other plasma constituents into the intima of the arterial wall (Mustard et al., 1963; Smith and Slater, 1972). The increased risk of CHD in Type II and IV hyperlipoproteinaemics may also be due to excess lipoprotein concentrations initiating the atherosclerotic lesion by damage to the endothelial lining of the arteries (Scott et al., 1970; Gofman and Young, 1963; Ross and Glomset, 1973) and contributing to the progressive development in size of the lesion. Of interest in this context is the observation that considerable variation in the concentration of lipoproteins in the human population may be found (Nichols, 1969; e.g. females transport more cholesterol in HDL than males). It has been suggested (Stein et al., 1973) that since an HDL particle carries less than $1/20$ of the cholesterol present in an LDL particle, the lower susceptibility of the human female to atherogenesis may be related to the higher ratio of HDL to LDL particles in female serum (14:1 compared to 7:1 for HDL:LDL in females and males respectively).

Although elevated cholesterol was considered the most important risk factor the importance of elevated triglyceride concentrations has now been recognised (Levy and Rifkind, 1973; Carlson, 1970; Carlson and Böttiger, 1972). A mechanism for linking atherogenesis with an interaction between

*CHD = Coronary Heart Disease.
the circulating level of triglyceride-rich lipoprotein and the activity of lipoprotein lipase in the arterial endothelium has been proposed by Zilversmit (1973). High local concentrations of cholesterol-rich lipoproteins resulting from surface lipolysis and the release of potentially injurious fatty acids would enhance the uptake of cholesterol by the arterial intima. Therefore high concentrations of LDL in some patients with atherosclerosis might be the consequence of an atherogenic lipolytic process rather than the cause of atherosclerosis. The apolipoproteins may influence the atherogenic process as they activate and modulate lipoprotein lipase activity.

It is apparent from the studies presented in this thesis that there are a number of areas for further research which would assist in the elucidation of the metabolic phenomena observed. Further characterisation of the rat apolipoproteins, particularly rat apo-VLDL PAGE zones III and IV and apo-HDL zones I, II and III so that they can be unequivocally identified. Also, an investigation of different delipidation techniques, without the use of organic solvents, may be carried out to isolate the B apolipoprotein in a disaggregated form in order to further characterise it. Experiments can be designed to detect and measure the possible 'remnant' of rat VLDL metabolism in the rat using hepatectomised animals for the detection of remnants in vivo after injection of $^{125}$I-VLDL. The VLDL/LDL relationship in the rat could be further investigated using different density delineations and possibly injecting $^{125}$I-LDL$_1$ and $^{125}$I-LDL$_2$ and subfractions of VLDL (e.g. Sf 20-50 and 50-100) into normal rats. Also, the role played by the liver in this relationship could be determined using hepatectomised rats. Finally, in vitro incubation of $^{125}$I-VLDL subfractions and iodinated LDL of different density delineations, with post-heparin plasma may be used to further investigate the VLDL/LDL relationship under control conditions.
In conclusion, it is clear from these studies that there is heterogeneity in VLDL apolipoprotein metabolism and it is becoming increasingly evident that the apolipoproteins are not merely structural components of lipoproteins but play key roles in the metabolism of lipoproteins and hence in the control of lipid transport and metabolism.
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