THE METABOLISM OF LOW

AND VERY LOW DENSITY LIPOPROTEINS

IN MAN

by

Michael F. Reardon

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

September 1976

This thesis is a report

of my own work

MICHAEL F. REARDON

TABLE OF CONTENTS

ABSTRACT

This thesis is concerned with the metabolism of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) in man. It is presented in the form of seven chapters, the first of which reviews and discusses concepts relevant to this work. The remaining chapters report investigations of the metabolism of these macromolecules in normal and hyperlipoproteinaemic subjects. The significance of these findings to the development of hyperlipoproteinaemia (and the pathogenesis of atherosclerosis) is discussed.

The B apolipoprotein moiety of the above-mentioned lipoprotein species was chosen for study as it alone remains common to these lipoproteins during their entire circulatory life. A method for obtaining the B apolipoprotein free of other VLDL and LDL apolipoproteins was devised. This procedure was based on the relative insolubility of the B apolipoprotein in 5mM NH_4HCO_3, pH 8.0. The isolation procedure was used to obtain B apolipoprotein specific activity data which facilitated many of the analyses and interpretations included in this work.

Using an *in vitro* system employing normal human plasma containing varying concentrations of post-heparin lipolytic activity, it was demonstrated that the sequential transformation of VLDL to LDL was dependent upon lipolytic activity; no conversion of VLDL or intermediate lipoproteins to LDL was observed in its absence. Furthermore, in the presence of lipolytic activity of hepatic origin only, no transformation of the B apolipoprotein into LDL was seen thus suggesting a minor role of hepatic lipases in LDL formation.

A series of *in vivo* investigations on the metabolism of various radioiodinated VLDL and LDL preparations in normal and hyperlipoproteinaemic subjects is also reported. B apolipoprotein specific activity-time curves were analysed for precursor-product relationships between various lipoprotein fractions. From these curves it was deduced that, in man, LDL is ultimately derived from the catabolism of VLDL via an

intermediate lipoprotein species. This intermediate species may be defined (in terms of Svedberg flotation units) as lying in the range s_f 12-60. Likewise, from appropriate kinetic analyses of the rates of disappearance and labelling (*in vivo*) of the B apolipoprotein in various lipoprotein fractions, it was deduced (from turnover data) that, in normotriglyceridaemics, all VLDL is catabolised to LDL. However, in hypertriglyceridaemics, a relatively large proportion of VLDL B apolipoprotein may be removed from the circulation prior to conversion to LDL (probably as the intermediate lipoprotein). The relationship between VLDL B apolipoprotein synthesis and VLDL B plasma pool size described a 'saturable system' suggesting that the rate of VLDL production is a major determinant of VLDL pool size but that in severe hypertriglyceridaemia, VLDL removal mechanisms may also be impaired.

ACKNOWLEDGEMENTS

I wish to thank Dr Noel Fidge and Dr Paul Nestel for their constant advice, assistance and encouragement during the work for this thesis. I am also grateful to Professor Malcolm Whyte and the staff of the Department of Clinical Science who provided help in a variety of ways.

I am indebted to a number of the nursing, resident and clinical staff of Canberra and Woden Valley Hospitals who provided me with skilled and invaluable assistance and accepted responsibility for many of the clinical procedures.

I likewise acknowledge the following persons who assisted in or performed the estimations and procedures listed

Lipid analyses : Mr Eric Pryke

Amino acid analyses : Mr Lewis James (Department of Biochemistry, J.C.S.M.R.)

Dietary regimes : Mrs N. Quinlivan

Lipoprotein sterilisation : Departments of Pharmacy and Bacteriology, Woden Valley Hospital.

I extend thanks to my laboratory colleagues including Gwen Abigail, Carmel Boatwright, Y. Homma, Charles Pirotta, Andrea Poyser, Parissa Poulis and Jenny Schmidt for their assistance, tolerance and amicable exchange.

The final manuscripts were skilfully prepared by my brother Terence, Pauline Wallace and Norma Chin.

The investigations contained within this thesis were carried out from September, 1973 to September, 1976 during the tenure of an Australian National University Post-graduate Scholarship.

- M.F.R.

ABBREVIATIONS

VLDL	very low density lipoproteins
LDL	low density lipoproteins
HDL	high density lipoproteins
FFA	free fatty acid
HLP	hyperlipoproteinaemia
FH	familial hypercholesterolaemia
HEP	heparin dose (in reference to post-heparin plasma)
k	fractional catabolic rate
d.	density
g.	gravitational force

CHAPTER 1

INTRODUCTION

The association between plasma lipids and atherosclerosis has focussed attention on the metabolism of the plasma lipoproteins which transport lipids, not only through plasma, but probably also into the arterial wall. This thesis is concerned with studies into the metabolism of two lipoprotein species, the very low density and low density lipoproteins. Both of these lipoproteins, as well as the intermediate lipoproteins formed during the transformation of very low density into low density lipoproteins, have been implicated in the genesis of the atherosclerotic lesion. The studies to be described have examined the metabolism of these lipoproteins in man, under *in*

vivo and *in vitro* conditions, in an attempt to elucidate some of the mechanisms involved in the catabolism of the very low density lipoproteins both in normal and hyperlipoproteinaemic subjects. The metabolism of the various lipid moieties has been studied by others in great detail and will be discussed later. The present experiments have been concerned with the major common protein of both very low density and low density lipoprotein since the secretion of the lipoprotein macromolecule is dependent on the synthesis of this protein, the B apolipoprotein. The catabolism of very low density lipoprotein is also regulated by other proteins - the C apolipoproteins, which have also been studied in this work. Much less is known of the physiological and clinical significance of the proteins than of the lipids of lipoproteins, though in recent years, this area has come under intense study. This has led to a clearer understanding of the functioning of the entire lipoprotein and of the interactions of the lipid with the proteins.

The interaction between lipids and proteins is fundamental to many biological processes such as the functioning of cell membranes, enzyme reactions, formation of plasma lipoproteins and blood coagulation. Plasma lipoproteins, because of their solubility in aqueous media, are referred to as soluble lipoproteins. This property is not shared by the structural (or membrane) lipoproteins.

The major lipid classes found in plasma are phospholipids, triglyceride, various hydrocarbons including free fatty acids,

cholesterol and fat soluble vitamins (Fredrickson and Lees, 1972). Plasma lipids are largely insoluble in water. They do not occur in a free state in the aqueous plasma environment but in combination with proteins as lipoproteins.

Although lipid-protein complexes were isolated early in the last century, they were at that time considered no more than artifacts. Lipoproteins were first envisaged as metabolic entities following the pioneer work of Macheboeuf (1929) who isolated various water soluble protein-lipid complexes from horse serum using ammonium sulphate - sulphuric acid precipitation.

The ratio of lipid to protein in plasma lipoproteins is highly variable. As the lipid to protein ratio determines the density of the lipoprotein, lipoproteins may be separated by ultracentrifugation into various density ranges. This has led to one system of lipoprotein nomenclature which is based on terms of hydrated lipoprotein density.

1.1 PLASMA LIPOPROTEIN CLASSES

Lipoproteins in human plasma may be divided into five major classes.

1. free fatty acid - albumin complexes
2. high density lipoproteins (HDL)
3. low density lipoproteins (LDL)
4. very low density lipoproteins (VLDL)
5. chylomicrons

1.1.1 Free fatty acid - albumin complexes

Although these complexes are not usually referred to as lipoproteins, they constitute an important group of lipid-protein complexes. Free (or unesterified) fatty acids (FFA) are transported in plasma bound to albumin (Fredrickson and Gordon, 1958). FFA, liberated by lipolysis of adipose tissue triglyceride, serve as an essential energy source (particularly in the post-absorptive state) for skeletal and cardiac muscle as well as liver. These complexes do not float in the ultracentrifuge and may be found in the ultracentrifugal residue (d. 1.21 g/ml fraction).

1.1.2 High density lipoproteins

Plasma HDL may be floated by ultracentrifugation in the salt density range 1.063 - 1.21 g/ml (Havel *et al.*, 1955). These lipoproteins are also referred to as α-lipoproteins since their mobility on paper or starch gel electrophoresis is similar to the α-globulins (Levy and Fredrickson, 1965). Analytical ultracentrifugation studies have demonstrated that lipoproteins in the 1.063 - 1.21 g/ml density range are heterogeneous and may be classified into two populations, HDL_2 (1.063 - 1.12 g/ml) and HDL_3 (1.12 - 1.21 g/ml) (Shore and Shore, 1957).

HDL is characterised by a high protein to lipid ratio (approximately 1). It has a chemical composition (HDL_2 + HDL_3) of approximately 15% cholesteryl ester, 3% free cholesterol, 6% triglyceride, 21% phospholipid with 55% protein (Skipski, 1972). The phospholipid moiety consists of almost 75% lecithin.

The molecular weights of HDL_2 and HDL_3 are 3.6×10^5 daltons and 1.7×10^5 daltons respectively (Scanu and Granada, 1966). HDL_2

molecules range in size from 60 - 140 Å and HDL_3 from 40 - 100 Å (Forte *et al.*, 1968).

1.1.3 Low density lipoproteins

Plasma LDL may be floated in the ultracentrifuge in the density range 1.006 - 1.063 g/ml. As with HDL, they are described as β-lipoproteins because of their β-globulin mobility on paper or starch electrophoresis. This density range (1.006 - 1.063 g/ml) also contains small amounts of HDL referred to as HDL_1.

LDL is the major carrier of cholesterol in the circulation. It consists of approximately 25% protein, 38% cholesteryl ester, 10% free cholesterol, 5% triglyceride and 22% phospholipid (Lindgren *et al.*, 1959). Estimates of the molecular weight vary from 2.2 to 2.7×10^6 daltons (Lindgren *et al.*, 1951; Scanu and Wisdom, 1972). LDL is a spherical particle with an average diameter of 220 Å (Forte *et al.*, 1968).

The major LDL fraction of human plasma lies in the d. 1.019 - 1.050 g/ml range. Although lipoprotein particles in the range d. 1.006 - 1.019 g/ml are usually included in the LDL range, recent metabolic studies (Bilheimer *et al.*, 1972; Eisenberg *et al.*, 1973), as well as those in this thesis, indicate that lipoproteins in this class (termed LDL_1) are distinct in their metabolic behaviour from the major LDL class and so should be considered as a separate lipoprotein species. These lipoproteins appear to belong to a remnant lipoprotein class, the characteristics of which are discussed in section 1.5.2.

1.1.4 Very low density lipoproteins

Plasma VLDL float in the ultracentrifuge at salt density 1.006 g/ml. VLDL particles thus have a hydrated density of less than 1.006 g/ml which is the density of plasma (Havel *et al.*, 1955). These lipoproteins are referred to as pre β-lipoproteins because they migrate slightly further on paper or starch electrophoresis than the β-lipoprotein class.

VLDL is a heterogeneous class of lipoprotein particles. It is often further divided into various subfractions based on the flotation rate (in the ultracentrifuge) of various sized particles. The flotation rate (s_f) of a lipoprotein is expressed in Svedberg flotation units (i.e. negative sedimentation units as 10^{-13} cm/sec/dyne/g) in a medium of salt density 1.063 g/ml. Using this form of reference, VLDL particles are in the s_f 20 - 400 range (while LDL_1 and LDL_2 are in the s_f 12 - 20 and 0 - 12 ranges respectively).

The chemical composition of VLDL is variable because of the considerable variation in particle size. Larger VLDL particles contain more triglyceride and phospholipid at the expense of protein and cholesteryl ester. Average values for VLDL composition (s_f 20 - 400) are 55% triglyceride, 20% phospholipid, 11% free cholesterol, 4% cholesteryl ester with 10% protein (Skipski, 1972).

1.1.5 Chylomicrons

In contrast to the other plasma lipoproteins, which are always seen in normal human plasma, chylomicrons are found only after a fatty meal. They are not present in plasma from normal fasting subjects.

Chylomicrons, which are very large triglyceride - rich particles consist of approximately 1% protein, 86% triglyceride, 1% free cholesterol, 6% esterified cholesterol and 6% phospholipid (Skipski, 1972). Chylomicrons do not migrate from the origin on paper or starch electrophoresis. They range in size from 750 - 6,000 Å (Bierman *et al.*, 1966).

The basis of distinguishing between VLDL and chylomicrons (both float in the ultracentrifuge at d. 1.006 g/ml) is that almost all of the triglyceride carried in chylomicrons is considered to be of exogenous origin only, i.e. triglyceride absorbed from the intestine, whereas VLDL carries triglyceride of endogenous origin predominantly derived from the liver. Chylomicrons are usually defined as particles with flotation rates of $s_f > 400$, though, there is some overlap of particles in the VLDL and chylomicron range.

1.1.6 Lp-a

In addition to the major lipoprotein classes described, a further lipoprotein class, Lp-a, may also be isolated in the range d. 1.050 - 1.12 g/ml in the majority of people. This lipoprotein, which exhibits pre-β mobility on agarose electrophoresis, has a molecular weight of approximately 5×10^6 daltons. The functional significance of this lipoprotein, which is similar in lipid but not apolipoprotein composition to LDL is unknown. Its plasma concentration is thought to be determined by polygenic inheritance (Albers *et al.*, 1974).

1.2 APOLIPOPROTEINS

1.2.1 Nomenclature

It is convention to refer to the protein moieties of lipo-

proteins as 'apolipoproteins' or 'apoproteins'. Apolipoproteins may be obtained by the removal of the lipid moieties from the lipoproteins, thus leaving the protein moiety only. This process, usually employing organic solvents, is termed delipidation.

Two systems of apolipoprotein nomenclature are in current use. The system proposed by Alaupovic and colleagues stems from the premise that apolipoproteins are the sole determinants of lipoprotein families which exist as definable physical entities. Thus Alaupovic defines an apolipoprotein as 'a protein which binds through predominantly non-covalent interactions, neutral lipids and phospholipids to form a soluble polydiverse lipoprotein family' (Alaupovic, 1971). Thus, the A apolipoproteins form the LP-A family which are found primarily, but not exclusively in HDL. Similarly, the B apolipoprotein forms the LP-B family constituting almost solely LDL but also present in significant amounts in VLDL. The LP-C family predominates in VLDL and HDL. Further lipoprotein families have been more recently postulated, namely LP-D and LP-E as will be described.

The other system in use for apolipoprotein nomenclature is to classify apolipoproteins according to their carboxy terminal amino acid residue e.g. apolipoprotein-serine (apo-ser) (Fredrickson *et al.*, 1972). Though there are difficulties with both systems, the ABC system is most commonly employed because of the occurrence of different apolipoproteins with the same carboxy terminal amino acid (Kostner and Alaupovic, 1971) and because errors have been made in the initial determination of the apolipoprotein carboxy terminal amino acids (Herbert *et al.*, 1971).

Apolipoproteins may be separated by a variety of techniques that separate proteins according to their respective sizes and/or electrostatic charges. These include gel chromatography on Sephadex G-150 and G-200, affinity chromatography on concanavalin-A sepharose, ion - exchange chromatography on DEAE cellulose, polyacrylamide gel electrophoresis and solubility in weak salt solutions (Brown, Levy and Fredrickson, 1970a, 1970b; Shore and Shore, 1973; Herbert *et al.*, 1973; Kane, 1973; Lee and Alaupovic, 1974).

1.2.2 The B apolipoproteins

After delipidation of VLDL, the resulting apo-VLDL may be fully solubilised only in buffers containing detergents, e.g. sodium dodecyl sulphate. Solubilized apo-VLDL may be resolved into two fractions by chromatography on Sephadex G-150 (Brown, Levy and Fredrickson, 1970a). The protein that elutes in the void volume of the column is identical to the major apolipoprotein of LDL i.e. the B apolipoprotein (Gotto *et al.*, 1972). This protein is therefore common to VLDL and LDL and is also a major apolipoprotein of chylomicrons (Kostner and Holasek, 1972). The B apolipoprotein imposes extreme difficulties in characterisation because of its aqueous insolubility when delipidated with organic solvents. It has been reported to consist of two subunits each having a molecular weight of 250,000 (Smith *et al.*, 1972). Further sub-unit structure has been suggested from studies with maleyated B apolipoprotein (Kane *et al.*, 1970), and with B apolipoprotein of LDL delipidated using sodium deoxycholate and then applied to SDS gels. Molecular weights of 9,500 and 13,000 have been suggested for the fundamental subunits (Chen and Aladjem, 1974).

1.2.3 The C apolipoproteins

The other major protein of VLDL is the C apolipoproteins. These apolipoproteins, which are also found in HDL, may be further fractionated into at least four distinct proteins (C-1, C-11, C-111-1 and C-111-2) by chromatography on DEAE cellulose (Herbert *et al.*, 1973).

Apo C-1 is the first peptide to elute from the ion-exchange column. It is a single polypeptide chain of 57 amino acids of known sequence (Shulman *et al.*, 1975; Jackson *et al.*, 1974) with serine as its carboxy terminal amino acid. Hence, it is also referred to as apo-serine. It was initially named apo-valine due to the incorrect identification of the carboxy terminal amino acid (Brown *et al.*, 1970b). The molecular weight of apo C-1 is approximately 7,000. This peptide has been reported to be an activator of a lipoprotein lipase purified from post-heparin plasma (Ganesan *et al.*, 1971; Ganesan and Bass, 1975), as well as an activator of the plasma enzyme lecithin: cholesteryl acyl transferase (LCAT) (Garner *et al.*, 1972). Apo-C-1 has slight stimulatory activity on lipoprotein lipase activities from a number of sources (except milk lipase) but is inhibitory at higher concentrations of the peptide (Havel *et al.*, 1973a).

The second protein to be eluted from the ion-exchange column is apo C-11, which is a single polypeptide chain of approximately 95 amino acids and a molecular weight of 11,000 (Brown *et al.*, 1970b). It is a potent activator of lipoprotein lipase from all sources (Havel *et al.*, 1973a). The amino acid sequence is, at present,unknown. Glutamic acid is the carboxy terminal amino acid and hence it is also referred to as apo-glutamic acid (apo-glu) (Brown *et al.*,1970b).

The two final major proteins to be eluted from the column are the apo C-111 peptides. They are polymorphic forms of the same peptide of 79 amino acids of known sequence. To the amino acid in the 74th position of the sequence (threonine) is attached a single carbohydrate chain containing one molecule of galactosamine and galactose plus one or two molecules of sialic acid (Brewer *et al.*, 1974). A third polymorphic form of apo C-111 isolated by iso-electric focussing contains no sialic acid residues (Albers and Scanu, 1971). These three peptides, having a molecular weight of 9,500, are distinguished according to their sialic acid residue content as apo C-111-0, apo C-111-1 and apo C-111-11. A fourth polymorphic form of apo C-111, apo C-111-111 has recently been described, though its sialic acid content has not been confirmed (Kane *et al.*, 1975). It is unstable upon delipidation, losing some of its sialic acid content. The effect of apo C-111 on lipoprotein lipase is similar to that observed for apo C-1 (Havel *et al.*, 1973a).

All the apo C proteins are capable of forming stable complexes with phospholipid (Forte *et al.*, 1974) which may then bind neutral lipids.

The C apolipoproteins have also been referred to as 'D peptides' in the literature (Brown *et al.*, 1969), however, this nomenclature system is no longer employed and should not be confused with the D apolipoprotein (section 1.2.6).

1.2.4 Apolipoprotein E

A further apolipoprotein, enriched in arginine, is found preferentially in cholesteryl ester rich VLDL (Shore and Shore, 1974;

Shelburne and Quarfordt, 1974). The apolipoprotein is also found in HDL from cholesterol fed swine (Mahley *et al.*, 1975) and from HDL of LCAT deficient humans (Utermann *et al.*, 1974). This protein is termed arginine-rich protein or apolipoprotein E (Utermann *et al.*, 1974, 1975). It has a molecular weight of between 3.3 and 3.9×10^4 daltons and has alanine as its carboxy terminal amino acid residue. It elutes off G-150 Sephadex in association with the B apolipoprotein (first fraction).

1.2.5 The A apolipoproteins

The A apolipoproteins, found predominantly in HDL consist of at least two proteins, apoA-1 and apoA-11 (Shore and Shore, 1969; Scanu *et al.*, 1969). Both proteins have carboxy-terminal glutamine (Kostner and Alaupovic, 1971) and so are also termed apo-gln-1 and apo-gln-11.

ApoA-1, the major protein of HDL, is a single polypeptide chain of 245 amino acids of known sequence (Baker *et al.*, 1975). It has a molecular weight of 28,000. It is an activator of lecithin: cholesteryl acyl transferase.

ApoA-11 consists of two identical polypeptides of 77 amino acids of known sequence linked together at the 6th position by a disulphide bond (Brewer *et al.*, 1972). It has a molecular weight of 16,000.

Both apoA-1 and apoA-11 readily bind lipids, A-11 having a greater affinity than A-1. However, when both apolipoproteins are present, A-11 increases the binding capacity of A-1 (Ritter *et al.*, 1974).

1.2.6 D apolipoprotein

A further apolipoprotein, found predominantly in HDL, has been referred to as the 'thin line polypeptide' because of the size of the precipitin line on double diffusion analysis of HDL and anti-HDL (Alaupovic *et al.*, 1972; McConathy and Alaupovic, 1976). This protein is immunologically different to the A, B or C proteins (Kostner, 1974a). It has been designated apolipoprotein D (McConathy and Alaupovic, 1973, 1976) and said to be a completely separate moiety of plasma lipoproteins with a distribution extending from VLDL to HDL. Kostner has claimed that the 'thin line peptide' is always associated with apoA proteins and so has termed it apoA-111. It has a molecular weight of approximately 20,000 with a carboxy terminal amino acid of serine (Kostner, 1974a).

1.3 LIPOPROTEINS AND APOLIPOPROTEINS IN OTHER SPECIES

The plasma lipoproteins described for man occur in many animal species, though the circulating levels of these lipoproteins may vary markedly probably suggesting species differences in lipoprotein metabolism and function. For example, dogs, rats and guinea pigs have a much lower plasma LDL concentration to that observed in man. Guinea pigs have an almost complete absence of circulating HDL while dogs have higher HDL levels to that observed in man (Nichols, 1967; Mahley and Weisgraber, 1974; Schonfeld *et al.*, 1974; Sardet *et al.*, 1972).

Many of the major apolipoproteins isolated from animal species are analogous to those of the human. The B apolipoprotein has been found in many species though no species variation has been

described. A-1 and A-11 apolipoproteins have been described in the rat, A-1 having a molecular weight of 26,000 daltons while A-11 (molecular weight 8,000 daltons) is in the monomeric form rather than the dimer observed in humans (Herbert *et al.*, 1974). A-1 and A-11 have also been isolated from several non-human primates (Blaton *et al.*, 1974; Edelstein *et al.*, 1974; Scanu *et al.*, 1974).

Low molecular weight apolipoproteins analogous to the human C apolipoproteins have also been identified in VLDL and HDL of several animal species. In the rat, four C apolipoproteins have been described, C-1, C-11, C-111-0 and C-111-111. C-11 is an activator of lipoprotein lipase (Henderson *et al.*, 1974). Apolipoprotein E has also been found in several species. The amino acid composition of apo E from cholesterol fed rabbits is similar to that of man (Shore and Shore, 1974; Shore *et al.*, 1974).

Many aspects of human lipoprotein metabolism, particularly those related to lipoprotein biosynthesis, have been deduced from studies in the rat. Some studies cannot be readily carried out in man and it should be borne in mind that similar mechanisms may not necessarily operate in both species.

1.4 BIOSYNTHESIS OF LIPOPROTEINS AND THEIR METABOLIC FUNCTION

1.4.1 Chylomicrons and VLDL of intestinal origin

Chylomicrons are the principal form in which absorbed (or dietary) lipid is transported from the intestine, first, into the lymph channels and then finally via the thoracic duct into the blood-stream. Synthesis of chylomicrons takes place in the small intestine

during active fat absorption.

In the intestinal lumen, dietary triglycerides (emulsified) are hydrolysed by pancreatic lipases at the 1 and 3 positions of the triglyceride molecule, forming free fatty acids and monoglycerides. These products, in combination with bile salts, form micellar structures which are then absorbed into the mucosal cell (Johnston, 1968). The absorption process of triglyceride (and probably cholesterol) appears to be influenced by the nature of the fatty acid in position 2 of the triglyceride molecule (Klauda and Quackenbush, 1971).

The formation of micellar complexes of bile salts, fatty acids and monoglycerides is a prerequisite for the absorption of dietary cholesterol (Simmonds *et al.*, 1967; Sylven and Borgstrom, 1968). Dietary cholesteryl esters are hydrolysed by pancreatic cholesteryl esterase prior to absorption (Shiratori and Goodman, 1965). The intestine is capable of cholesterol (Dietschy and Gamel, 1971), phospholipid (Sinclair, 1929) and triglyceride synthesis (Johnston, 1968). Cholesteryl esters of intestinal origin exhibit a marked specificity for oleic acid and are largely independent of the dietary fatty acid (Karmen *et al.*, 1963; Blomstrand *et al.*, 1964).

The synthesis of the B apolipoprotein by the intestine has been demonstrated in the rat (Windmueller and Levy, 1968; Kessler *et al.*, 1970; Windmueller *et al.*, 1973) and recently in man (Rachmilewitz *et al.*, 1976). The synthesis and assembly of the lipid moieties of chylomicrons occur in the smooth and rough endoplasmic reticulum of the intestinal cell. Final assembly of the lipoprotein takes place in the Golgi apparatus from which it is released into the lymphatics.

VLDL is also synthesised by the intestine even in the fasting state (Jones and Ockner, 1971; Tytgat *et al.*, 1971). It is not known whether chylomicrons and VLDL (of intestinal origin) belong to a single lipoprotein class or whether they represent two functionally distinct lipoprotein species. The B apolipoprotein content of chylomicron and lymph VLDL demonstrate differing relationships with lipoprotein size suggesting that VLDL and chylomicrons are discrete lipoprotein species and VLDL is not transformed into chylomicrons merely by the addition of lipid (Glickman and Kirsch, 1974).

Lymph contains lipoproteins of α and β mobility on paper electrophoresis (Courtice and Morris, 1955). However, examination of the density distribution of the A, B and C apolipoproteins in lymph reveals striking differences to that observed in plasma (Reichl *et al.*, 1973). Plasma LDL and probably HDL appear to be able to pass from the plasma compartment into lymph although probably undergoing some modification (Reichl *et al.*, 1973; Reichl *et al.*, 1975). The ability of plasma VLDL to pass into lymph is not known. The C apolipoproteins of plasma VLDL may be found in lymph (Reichl *et al.*, 1975) but their entry is probably via HDL rather than VLDL, as in these studies, early and rapid loss of VLDL C apolipoproteins to HDL was observed in the plasma.

1.4.2 Very low density lipoproteins

VLDL is the major carrier of triglyceride of endogenous origin in the circulation. The liver appears to be the most important site of VLDL synthesis (Havel *et al.*, 1962; Stein and Shapiro, 1959; Sundler *et al.*, 1973). However, as previously mentioned, the relative

contribution of VLDL of intestinal origin to the plasma VLDL pool in man is not known.

In the post-absorptive state, plasma FFA are the sole precursors of VLDL triglycerides released into the circulation by the liver (Havel, 1961). The hepatic uptake of plasma FFA is directly proportional to the plasma FFA concentration (McElroy *et al.*, 1960; Fine *et al.*, 1960). The release of triglyceride by perfused rat livers is related to the FFA concentration of the perfusing medium (Nestel and Steinberg, 1963). In man, the *in vivo* secretion of triglyceride by the liver appears to be highly correlated to the hepatic uptake of plasma FFA (Havel *et al.*, 1970a; Boberg *et al.*, 1972).

Liver and adipose tissue are capable of lipogenesis from carbohydrate (Jansen *et al.*, 1966; Zakim *et al.*, 1969; Ballard *et al.*, 1969). In man, the synthesis of fatty acids is potentially greater in the liver than adipose tissue (Shrago *et al.*, 1971) although adipose tissue is capable of fatty acid synthesis *de novo* (Goldrick and Galton, 1974). Barter *et al.* (1972) have demonstrated significant incorporation of glucose carbon atoms into VLDL triglyceride - fatty acids only in subjects consuming large amounts of carbohydrates. The liver is also capable of phospholipid (Glauman and Dallner, 1968) and cholesterol synthesis (Chesterton, 1968).

The origin of the cholesteryl ester moiety of human VLDL is not clear. In the rat, perfusion of the liver leads to the secretion of newly synthesised cholesteryl ester in VLDL (Roheim *et al.*, 1963). Gidez *et al.* (1967) demonstrated, in rats fed diets of differing fatty acid composition, a similarity in plasma VLDL and hepatic cholesteryl

esters. In man, the cholesteryl ester composition of the various plasma lipoproteins are similar and mostly contain linoleic acid though they can be influenced by the fatty acid composition of the diet (Nestel and Couzens, 1966; Goodman and Shiratori, 1964). Furthermore, the cholesteryl esters of liver and plasma lipoproteins are different in man (Nestel and Monger, 1967). Cholesteryl esters have been shown to transfer *in vitro* from HDL to VLDL in exchange for triglyceride (Rehnborg and Nichols, 1964; Nichols and Smith, 1965). Since the plasma cholesteryl esterifying enzyme, LCAT (lecithin: cholesteryl acyl transferase) is more active, *in vitro*, in the presence of HDL than with VLDL (Akanuma and Glomset, 1968) it is thought that cholesteryl esters are initially formed in HDL and then transferred to VLDL. On the other hand precursor-product relationships between VLDL and HDL cholesteryl ester following the injection of ^{3}H-mevalonic acid into man have not confirmed that VLDL cholesteryl esters were derived from HDL (Barter, 1974). More recently Barter and Lally (1976) have described the existence of two pools of HDL cholesteryl esters one of which may be the source of VLDL cholesteryl esters (the role of LCAT in the formation of cholesteryl esters of VLDL will be discussed further in section 1.6.2).

Synthesis of the B apolipoprotein is necessary for the release of VLDL from the liver and from intestine (Isselbacher *et al.*, 1964; Bar-On *et al.*, 1973). The liver in the rat is capable of synthesising all VLDL apolipoproteins (Windmueller *et al.*, 1973). It is not clear, however, whether VLDL gains its complement of C apolipoproteins prior to release from the liver or, following release from circulatory HDL. The intestine of the rat does not appear to synthesise C apolipoproteins

(Windmueller *et al.*, 1973). Studies on the rates of labelling of VLDL and HDL C apolipoproteins from labelled amino acids during perfusion of rat liver are not consistent; the results appear to be dependent upon the nature of the perfusing medium (Windmueller *et al.*, 1973; Noel and Rubinstein, 1974). However, the relative rates of labelling of VLDL and HDL C apolipoproteins from amino acids may not necessarily indicate the origin of VLDL C apolipoproteins as (1) the hepatic pools from which the C apolipoproteins may be derived are, as yet, not defined (Kook and Rubinstein, 1973) and (2) the rapid and ready exchange of C apolipoproteins observed between VLDL and HDL may possibly be more pronounced with nascent lipoproteins. Examination of VLDL in the Golgi apparatus of rat liver suggests that nascent VLDL obtain some of their C apolipoprotein following release into the circulation (Nestruck and Rubinstein, 1975). The arginine-rich protein of VLDL may also be derived from HDL (Marsh, 1976) though it has been observed in VLDL prior to release from the Golgi apparatus (Nestruck and Rubinstein, 1975).

1.4.3 High density lipoprotein

In the rat, HDL is synthesised by the liver (Windmueller and Levy, 1967) and to some extent by the small intestine (Windmueller and Spaeth, 1972). Hepatic HDL synthesis is independent of VLDL synthesis as demonstrated by studies using orotic acid to inhibit hepatic VLDL production. The liver appears to synthesise all of the apolipoproteins of HDL (Roheim *et al.*, 1966 ; Windmueller *et al.*, 1973; Marsh, 1976) though HDL lipoproteins synthesised by the intestine are devoid of C apolipoproteins (Windmueller *et al.*, 1973).

1.4.4 Low density lipoproteins

Studies examining the synthesis of LDL by perfused rat livers, while not confirming the absence of hepatic LDL synthesis, indicate that it is minor in comparison to VLDL and HDL synthesis (Windmueller *et al.*, 1973). In the above study, it was suggested that the incorporation of radioactivity into the d. 1.006 - 1.063 g/ml range was the result of HDL contamination, though this was not ascertained. Marsh (1974) has suggested the possibility of VLDL being converted to LDL by the hepatic triglyceride lipase, during the secretion of VLDL. This appears unlikely however, as the presence of heparin in liver perfusates does not appear to alter the labelling patterns of the lipoprotein proteins (Windmueller *et al.*, 1973). Thus, in the rat, the origin of LDL is not clear although recent *in vivo* studies of Faergeman *et al.* (1975) suggest that all LDL is derived from VLDL in the circulation. In squirrel monkeys, a small fraction of LDL may be derived from direct secretion but most (at least 80%) of circulating LDL is derived from VLDL catabolism (Illingworth, 1975). LDL, in man, is thought to be the product of VLDL catabolism (Bilheimer *et al.*, 1972; Sigurdsson *et al.*, 1975) and not independently synthesised by the liver (as will be discussed in section 1.5.2).

1.5 LIPOPROTEIN CATABOLISM

1.5.1 Chylomicron catabolism

Upon entering the circulation, chylomicron triglyceride and cholesteryl esters are removed very rapidly. The steps involved in chylomicron catabolism, as proposed by Redgrave (1970) involve the

removal of triglyceride by extra-hepatic tissues followed by removal of the 'remnant'. The remnant lipoproteins are rich in cholesterol, but poor in triglyceride. This proposal was first put forward by Nestel *et al.* (1963) who demonstrated chylomicron triglyceride, but not cholesteryl ester, clearance from the circulation of a hepatectomised dog.

Thus, the first step in chylomicron catabolism involves the hydrolysis of triglycerides by lipases of extra-hepatic origin. Purified lipoprotein lipase is more active against plasma chylomicrons than against chylomicrons isolated from the thoracic duct (Dolphin and Rubinstein, 1974). Havel *et al.* (1973b) have shown that when chylomicrons enter the circulation, there is net transfer of apolipoprotein C-11, the activator of adipose tissue lipoprotein lipase, from HDL to chylomicrons. This stage of lipase mediated chylomicron catabolism is probably similar to that for VLDL utilizing similar mechanisms for the removal of surface components as will be discussed in subsequent sections. Both chylomicrons and VLDL serve as substrates for lipoprotein lipase though chylomicrons are hydrolysed at a greater rate (Fielding and Higgins, 1974).

In animals the remnant particles of chylomicron catabolism are thought to be removed by the liver. Chylomicron cholesteryl ester is removed by the liver (Goodman, 1962; Nestel *et al.*, 1963; Quarfordt and Goodman, 1967; Stein *et al.*, 1969; Redgrave, 1970). The cholesterol taken up by the liver is converted to bile acids or is secreted from the liver as unesterified cholesterol either into the

bile or into the plasma lipoproteins (Nilsson and Zilversmit, 1972). The fate of the chylomicron protein components are not entirely known. The chylomicron C apolipoproteins, which were probably originally derived from HDL, are thought to return to HDL during catabolism.

1.5.2 Catabolism of VLDL

The removal of triglyceride from the circulation is mediated by the enzyme lipoprotein lipase (Korn, 1955). This enzyme (glyceryl ester hydrolase - E.C. 3.1.1.3) acts at the endothelial surface of the capillaries, hydrolysing triglycerides to FFA and glycerol. Some of the resulting FFA are removed from the circulation, mainly into adipose tissue and muscle (Eaton *et al.*, 1969; Bergman *et al.*, 1971). Lipoprotein lipase (which is discussed more fully in section 1.6.1) may be released into the circulation following the intravenous administration of heparin, resulting in an elevation of plasma FFA with concomitant decrease in plasma triglyceride concentration (Levy *et al.*, 1966).

The catabolism of VLDL triglyceride is said to proceed via a step-wise conversion of large (and triglyceride-rich) particles to smaller particles of higher density and lower triglyceride content (Barter and Nestel, 1972). In these studies, precursor-product relationships were demonstrated between triglyceride fatty acids of VLDL subfractions s_f 100-400 and s_f 20-100. Following the injection of heparin into man, there is a rapid reduction in plasma VLDL concentration with concomitant increase in the low density lipoproteins (s_f 5-30) and HDL (Nichols *et al.*, 1968; Levy *et al.*, 1966). Shore and Shore (1962) observed that post-heparin lipolytic activity resulted in the *in vitro* conversion of VLDL s_f 20-400 to smaller lipoprotein particles s_f 11-20.

These remnant lipoproteins, when compared to VLDL, contained more B apolipoprotein and cholesteryl ester but less triglyceride and C apolipoproteins (Eisenberg *et al.*, 1973).

Precursor-product relationships between VLDL and LDL triglyceride fatty acids suggest that the small amount of triglyceride in LDL is derived from VLDL (Havel, 1961; Farquhar *et al.*, 1965; Quarfordt *et al.*, 1970). Administration of nicotinic acid to man, which reduces plasma FFA levels, results in an initial fall in VLDL lipids followed by a later delayed fall in the lipids of LDL, suggesting that the lipids of LDL may be derived from VLDL. The lipids of HDL do not change (Carlson *et al.*, 1968).

Precursor-product relationships between VLDL and LDL protein (total lipoprotein protein) in man using VLDL labelled in its protein moiety with ^{131}I, ^{125}I or ^{65}Se- selenomethionine (Gitlin *et al.*, 1958; Bilheimer *et al.*, 1972; Eisenberg *et al.*, 1973; Eaton *et al.*, 1976) have, as for the triglyceride moiety, suggested that the protein moiety of LDL is derived from VLDL. The interpretation of these relationships however is limited. VLDL and LDL have, in common, the B apolipoprotein (Gotto *et al.*, 1972) as part of their protein moiety. However the apolipoproteins of VLDL are markedly heterogeneous. Although, in some of the other studies, the B apolipoprotein of VLDL and LDL was isolated by polyacrylamide gel electrophoresis and gel chromatography, specific activity data of the B apolipoprotein, which is required for a kinetic demonstration of a precursor-product relationship (Zilversmit, 1960)

between VLDL and LDL was not measured. Isolation of the B apolipoprotein of human VLDL and LDL by immunological techniques during the catabolism of isotopically-labelled human VLDL injected into monkeys demonstrated clearly that the B apolipoprotein of VLDL was transferred into the LDL range (Gulbrandsen *et al.*, 1971).

In two recent studies of the catabolism of isotopically labelled VLDL in man, the specific activities of the B apolipoprotein of VLDL and LDL have been measured. Sigurdsson *et al.*, (1975) isolated the B apolipoprotein of VLDL and LDL (d. 1.006 - 1.063 g/ml) following the injection of VLDL - ^{125}I. The B apolipoprotein was isolated by chromatography on Sephadex G-150. The precursor-product relationships (from seven studies) between VLDL and LDL suggested that most, if not all, of the B apolipoprotein of LDL was derived from VLDL. A further section of these studies involving eight investigations (on normo-triglyceridaemic subjects) demonstrated that, in these subjects at least, all VLDL was converted to LDL. In contrast, the results of a study on a single type 4 hyperlipoproteinaemic subject indicated a further pathway for LDL synthesis (Phair *et al.*, 1975). In this study the subject was injected with ^{14}C-leucine and the appearance of radio-activity in the B apolipoprotein of VLDL and three subfractions of LDL (d. 1.006 - 1.02 g/ml; d. 1.02 - 1.04 g/ml; d. 1.04 - 1.06 g/ml) was observed. A computer-devised model of lipoprotein metabolism was formulated to fit the specific activity data obtained and the results suggested that in this individual, VLDL was catabolised by more than one route. Not all VLDL B apolipoprotein was transferred to LDL.

Similar studies in the rat are also conflicting with respect to

the origin of the plasma LDL. While the nature of the precursor-product relationships of VLDL and LDL B apolipoprotein obtained by Faergeman *et al.* (1975) suggest that LDL is derived solely from VLDL, those of Fidge (personal commun.) indicate a second origin of LDL other than from VLDL. In marked contrast to observations in man, almost 90% of VLDL is removed in the rat predominantly by the liver, without being converted to LDL (Fidge and Poulis, 1975; Faergeman *et al.*, 1975).

In vitro incubations of labelled VLDL in plasma have demonstrated the ready capacity of the VLDL C apolipoproteins to exchange between the various lipoproteins and particularly with HDL (Bilheimer *et al.*, 1972). Similar exchange of phospholipids between VLDL and HDL has been demonstrated (Illingworth and Portman, 1972). These exchange processes are dependent upon the relative lipoprotein concentrations present in the system (Bilheimer *et al.*, 1972; Eisenberg *et al.*, 1972). In contrast, the B apolipoprotein does not exchange between the various lipoprotein fractions and its passage from one lipoprotein species to another is an active conversion process requiring catabolic activity. The transfer of B apolipoprotein, in contrast, to the exchange of C apolipoproteins is solely unidirectional (Bilheimer *et al.*, 1972; Eisenberg *et al.*, 1972). The catabolism of VLDL involves a process of active triglyceride removal from the lipoprotein. During this enzyme-mediated process, the B apolipoprotein is transferred into the LDL class while the loss of VLDL C apolipoproteins to the HDL fraction may still be the result of an exchange process (concentration dependent), the equilibrium of which is determined by the relative concentrations of the accepting lipoproteins (mainly VLDL and HDL) (Schonfeld *et al.*, 1972). From estimates of the

B apolipoprotein content of individual particles, Eisenberg *et al.* (1973) have concluded that the catabolism of one VLDL molecule results in the formation of only one LDL molecule.

In the studies of Bilheimer *et al.* (1972) it was observed that lipoproteins in the s_f 12-20 range (LDL_1) were labelled far more rapidly than LDL_2 (s_f 0-12) following the injection of VLDL-^{125}I into humans. Shore and Shore (1962) had previously described the *in vitro* conversion of VLDL (s_f 20-400) into s_f 12-20 particles during incubation in post-heparin plasma. From these observations, the role of LDL_1 as an intermediate in the conversion of VLDL to LDL_2 was postulated (Bilheimer *et al.*, 1972). Precursor-product relationships between free cholesterol, triglyceride, phospholipid and some cholesteryl ester of VLDL, LDL_1 and LDL_2 have been suggested from studies in monkeys (Illingworth *et al.*, 1974). Analytical ultracentrifugation studies have suggested that the intermediate lipoprotein has an s_f value of 12-60 rather than 12-20 (Eisenberg *et al.*, 1973). These authors also suggested that the conversion of the intermediate lipoprotein to LDL_2 was mediated by a process not clearly influenced by heparin induced lipolytic activity. The catabolism of VLDL to LDL_2 may therefore be a two-step process involving the initial formation of intermediate lipoproteins followed by the formation of LDL.

Few studies have characterised the physical and chemical properties of the remnant or intermediate lipoprotein. Shore and Shore (1962) examined the remnant produced, *in vitro*, by the action of human plasma post-heparin lipolytic activity on VLDL. 65% of the VLDL protein was recovered in the d. 1.006 - 1.022 g/ml range. Lipoproteins in this range contained 15% protein, 7% free cholesterol, 22-26% cholesteryl

ester, 17% phospholipid and 35-39% triglyceride.

Remnants produced *in vitro*, by the action of rat plasma post-heparin lipolytic activity mostly are contained in the $d < 1.006$ g/ml range (s_f 20-100). Diameters of chylomicron remnants range from 400-600 Å (Mjϕs *et al.*, 1975) and 150-300 Å for VLDL remnants (Eisenberg and Rachmilewitz, 1975). When compared to their precursors, these remnants are rich in cholesteryl ester and B protein and depleted of phospholipids, triglyceride and C apolipoproteins.

In the formation of remnants from VLDL (in the rat) not only are C apolipoproteins, triglyceride and phospholipids lost from the lipoprotein but also 40% of esterified cholesterol and 60% of free cholesterol (Eisenberg and Rachmilewitz, 1975). A mechanism by which free cholesterol may be removed, involving LCAT and HDL, has been proposed by Glomset (1968) and will be discussed in section 1.6.2. A process by which esterified cholesterol may be removed other than through uptake by the liver has not been described.

1.5.3 Low density lipoprotein

The catabolism of LDL is less clearly defined than that of VLDL, particularly in man. LDL is not converted to lipoproteins of higher density and so its catabolism presumably involves its removal from the circulation.

Until recently it has been accepted that plasma cholesterol, carried predominantly in LDL in man, is removed primarily by the liver, though a small but significant percentage is excreted through the skin (Bhattacharyya *et al.*, 1972; Nikkari *et al.*, 1974). Cholesterol is

also excreted directly by the gut (Dietschy and Wilson, 1970). Cholesterol, upon being taken up by the liver, is partly converted to bile acids (Bloch *et al.*, 1943) which then enter the gastrointestinal tract where they are largely reabsorbed and returned to the liver via the portal vein, thus completing an 'enterohepatic cycle' of bile salts (Reinke and Wilson, 1967; Wheeler, 1972). The rate of catabolism is dependent upon several enzymes including 7α-hydroxylase which catabolises the first reaction in the pathway of bile acid formation. This enzyme is regulated by the reabsorbed bile acids returning to the liver from the gastrointestinal tract (Shefer *et al.*, 1970). Hepatic cholesterol is also directly secreted into the bile and is partly reabsorbed in the gut.

The liver has been implicated as the major site of LDL catabolism in the rat (Hay *et al.*, 1971). These authors demonstrated, in normal rats and in male rats treated with oestrogen (which reduces the level of circulating LDL), uptake and degradation of ^{131}I-LDL (d. 1.006 - 1.040 g/ml) in the liver. Enhanced uptake and degradation was observed in the oestrogen treated animals. However, as previously described, VLDL remnants are also rapidly taken up and degraded by rat liver (Faergeman *et al.*, 1974; Faergeman *et al.*, 1975; Fidge and Poulis, 1975; Stein *et al.*, 1974) whereas in normotriglyceridaemic humans at least, VLDL remnants are probably converted to LDL_2 before being removed from the circulation (Sigurdsson *et al.*, 1975). The LDL particle in the rat is far more triglyceride-rich than in the human (Fidge and Calder, 1972) and so its removal may possibly involve different mechanisms.

Recent studies have indicated that peripheral tissues may play an important role in the catabolism of LDL. Sniderman *et al.* (1974)

have compared the catabolism of LDL in normal and hepatectomised pigs and found a more rapid disappearance of LDL in the hepatectomised animals. The bi-exponential disappearance of LDL observed in the intact animal became mono-exponential in the hepatectomised animal. These studies demonstrated that some extra-hepatic tissues had the capacity to remove LDL irreversibly from the plasma compartment and so have indicated that the liver was not the sole site of LDL catabolism. The reason for the increased disappearance of LDL from the hepatectomised animals is not known, though it was suggested that the liver may serve some function which prolongs the life-time of LDL in the plasma-hepatic compartment.

Many recent investigations, mainly employing cultures of human smooth muscle cells (Bierman and Albers, 1975; Albers and Bierman, 1976), swine arterial smooth muscle cells (Weinstein *et al.*, 1976), human fetal aorta cells (Goldstein and Brown, 1975), human skin fibroblasts (Goldstein and Brown, 1974; Stein *et al.*, 1975; Assman *et al.*, 1975) and human lymphocytes (Vijayogopal and Nestel, personal comm.) have demonstrated that cells of many peripheral tissues probably have the capacity to catabolise LDL.

As a result of studies employing cultured skin fibroblasts, Goldstein and Brown have proposed a scheme for LDL catabolism, based on the initial binding of LDL to the fibroblast, in which the rate of cholesterol synthesis and LDL catabolism are linked (Goldstein and Brown, 1975). The binding of LDL to these cultured cells leads to (1) a transfer of the lipoprotein into the cell (2) hydrolysis of cholesteryl ester to free cholesterol within the cell with the subsequent inhibition of the enzyme HMG Co A reductase (a regulatory enzyme in cholesterol synthesis) by free cholesterol and (3) degradation of the LDL protein. Since it appeared that the binding LDL might be

defective or absent in fibroblasts prepared from subjects with homozygous familial hypercholesterolaemia, these investigators have proposed that, in this disorder, defective binding of LDL resulted in (1) a reduced rate of LDL catabolism and (2) a lack of feed-back inhibition of HMG Co A reductase. Both these factors would contribute to elevated plasma cholesterol and LDL concentrations

Goldstein and Brown do not comment on the subsequent fate of the cholesterol from catabolised LDL (other than that it is re-esterified in the fibroblast) although others have suggested that the cholesterol moiety might be transported back to the liver by another protein, possibly in HDL or in the lipoprotein free fraction of plasma (Bailey, 1973; Stein *et al.*, 1975). A possible role for HDL in cholesterol transport is suggested by the marked deposition of cholesteryl esters in peripheral tissues of patients with Tangier disease (an abnormality characterised by a marked reduction in the concentration of plasma HDL).

The quantitative importance of peripheral tissues in LDL degradation is uncertain. Recent studies have shown that membranes of liver cells bind LDL protein with the same affinity as do fibroblasts and other extra-hepatic cells (Bachorik *et al.*, 1976). Calculations have shown that the capacity of extra-hepatic tissues to degrade LDL, based on *in vitro* studies, greatly exceeds the mass of LDL catabolised *in vivo*. The discrepancy probably arises from the artificial conditions of the *in vitro* studies in which cells, deprived temporarily of lipoprotein cholesterol, synthesise surface receptors for LDL resulting in an abnormally high rate of LDL binding and catabolism when the cells are again exposed to LDL. Nevertheless, there is

qualitative agreement between the *in vitro* and *in vivo* findings in homozygous familial hypercholesterolaemia. LDL catabolism is considerably reduced when measured under either condition (Bilheimer *et al.*, 1975).

1.5.4 High density lipoprotein

Rachmilewitz *et al.* (1972) examined the uptake of radioactivity in various tissues of the rat at time intervals following the injection of HDL-^{125}I (previously screened so as to remove most of the labelled C apolipoproteins). Highest percentages of TCA precipitable radioactivity were located in the liver and small intestine. The degree of immunochemical recognition of labelled HDL taken up by intact or perfused rat liver, using an antibody against HDL, decreased progressively with time. These observations ascertained that HDL degradation was occurring in the liver (Rachmilewitz *et al.*, 1972). The removal rates of the different apolipoproteins of HDL have been found to be identical and similar to the removal rate of the slow component of VLDL suggesting a common pool of C apolipoprotein catabolism that involves HDL (Roheim *et al.*, 1972).

1.6 ENZYMES INVOLVED IN LIPOPROTEIN METABOLISM

1.6.1 Lipases

Hahn (1943) first described 'clearing' of chylomicron-rich plasma of dogs following intravenous administration of heparin. The *in vitro* addition of heparin to the plasma caused no reduction in turbidity. Similar findings were reported in humans (Anderson and Fawcett, 1950). These observations led to the concept that intra-

venously injected heparin releases a 'clearing factor' from some tissue site (or sites) in the body and that the 'clearing factor' induces the degradation of chylomicrons in plasma.

Korn (1955) studied the lipase contained in acetone powder extracts of heart muscle and adipose tissue. In the presence of albumin (to accept released FFA) the enzyme in the tissue extracts actively hydrolysed chylomicron triglycerides to FFA and glycerol. Little or no hydrolysis occurred when an artificial emulsion was used as substrate, unless the emulsion was pre-incubated with plasma lipoproteins or whole plasma. The reaction rate could be increased by the addition of small amounts of heparin to the assay system. Korn suggested that the tissue triglyceride lipase was the same enzyme present in post-heparin plasma. This enzyme was referred to as lipoprotein lipase and was inhibited by pre-incubation with protamine sulphate or IM NaCl.

Following intravenous injection of heparin into normal fasting humans, lipoprotein lipase rapidly appears in the plasma. The amount of lipase released is dependent upon the dose of heparin administered (Boberg, 1972). Robinson and Harris (1959) concluded that lipoprotein lipase was located on capillary endothelial surfaces and that heparin was able to displace it into the circulation. A variety of sulphated and non-sulphated polysaccharides produce a similar response when injected intravenously (Robinson *et al.*, 1959). Histochemical studies with perfused adipose tissue and mammary gland also suggest that one site of action of the enzyme is at the luminal surface of the capillary endothelium (Blanchette-Mackie and Scow, 1971; Schoefl and French, 1968). Lipoprotein lipase released into the circulation is rapidly removed by

the liver (Naito and Felts, 1970). The enzyme has been demonstrated in many tissues including heart muscle, skeletal muscle, kidney, spleen, aorta and adipose tissue. The enzyme appears to be absent from the liver (Mayes and Felts, 1968).

Adipocytes, which are capable of very active lipoprotein lipase synthesis, release the enzyme on exposure to heparin, even in the presence of inhibitors of protein synthesis (Stewart and Schotz, 1971, 1974; Stewart *et al.*, 1969; Wing *et al.*, 1967). The inference, from these studies, is that cells may store the enzyme, perhaps in an inactive form. The secretion of the enzyme from the adipocyte into the surrounding tissue has been proposed as an important regulatory mechanism of adipose tissue lipoprotein lipase activity (Garfinkel *et al.*, 1976). This secretory process is influenced by insulin. Adipose tissue lipoprotein lipase may also be influenced by administration of glucose (Schotz and Garfinkel, 1965) and by the nutritional state (Schotz and Garfinkel, 1972; Borensztajn and Robinson, 1970). Interestingly, those hormones that inhibit lipoprotein lipases (i.e. catecholamines, cortisol, ACTH, TSH and glucagon (Nestel and Austin, 1969) stimulate the other major adipose tissue lipases (hormone-sensitive lipases) that hydrolyse stored triglyceride. Conversely, insulin, which potentiates lipoprotein lipase activity, inhibits hormone sensitive lipase. This allows for fine adjustments of fuel flux under different nutritional circumstances. In contrast to the hormone sensitive lipase, lipoprotein lipase activity does not appear to be dependent upon cyclic-AMP (Khoo *et al.*, 1976). In this way, a fine balance of triglyceride uptake, storage and utilisation is achieved during varying nutritional states.

The small molecular weight apolipoprotein, C-11 (LaRosa *et al.*, 1970; Havel *et al.*, 1970b) is a potent activator of lipoprotein lipase from many sources. This peptide, because of its surface film properties, possibly facilitates access of the enzyme to its substrate (Miller and Smith, 1973). C-11 is transferred from HDL to chylomicrons during alimentary lipemia (Havel *et al.*, 1973b). C-1 and C-111 apolipoproteins have slight stimulatory effects, *in vitro*, on purified lipoprotein lipase from human plasma when present in low concentrations. However, at high concentrations, they markedly inhibit lipolytic activity and may abolish the stimulatory effects of C-11 (Havel *et al.*, 1973a).

At least three different triglyceride lipases may be found in post-heparin plasma. The enzymes differ in their pH optima, inhibition by protamine sulphate or 1M NaCl and activation by the C apolipoproteins. One enzyme, identical to lipoprotein lipase is of extrahepatic origin and is activated by apolipoprotein C-11 but inhibited by NaCl or protamine sulphate (LaRosa *et al.*, 1972; Fielding, 1972; Krauss *et al.*, 1973). A second lipase, also similar in properties to lipoprotein lipase but not found in adipose tissue, is activated by apolipoprotein C-1 and inhibited by 1M NaCl and protamine sulphate (Ganesan and Bass, 1975; Ganesan *et al.*, 1971). Its origin is at present unknown. A third enzyme found in post-heparin plasma differs from the first two lipases in that it is insensitive to inhibition by protamine sulphate or 1M NaCl (LaRosa *et al.*, 1972; Krauss *et al.*, 1973). This enzyme is secreted by the liver (Greten *et al.*, 1974) and has recently been reported to be inhibited by the C apolipoproteins (Kinnunen and Ehnholm, 1976). It is termed hepatic triglyceride lipase

and will hydrolise a triglyceride emulsion in the absence of serum or C apolipoprotein cofactors (Krauss *et al.*, 1973), while for lipoprotein lipase activity the presence of phospholipids and serum cofactors is obligatory.

Post-heparin plasma also contains diglyceride and monoglyceride activities (Greten *et al.*, 1969; Biale and Shafrir, 1969; Nilsson-Ehle and Belfrage, 1972) and phospholipases (Vogel *et al.*, 1971). Zieve and Zieve (1972) have concluded that most of the phospholipase activity of rat plasma originates in the liver. Recent reports have indicated that hepatic triglyceride lipase and possibly lipoprotein lipase also have phospholipase A_1 activity (Ehnholm *et al.*, 1975; Scow and Egelrud, 1976).

1.6.2 Lecithin: cholesteryl acyl transferase

Sperry (1935) demonstrated an enzyme in normal human serum capable of esterifying cholesterol *in vitro*. Glomset (1962, 1968) proposed that the formation of cholesteryl esters in plasma is catalysed by an enzyme which transfers a fatty acyl group from the position 2 of lecithin to the 3 hydroxy group of cholesterol. This plasma enzyme, which is synthesised by the liver (Osuga and Portman, 1971; Simon and Boyer, 1971), is referred to as lecithin: cholesteryl acyl transferase (LCAT) (E.C.2.3.1.43). While no significant fatty acid transfer is observed from phospholipids other than lecithin (Nichols and Gong, 1971), other sterols than cholesterol may be esterified e.g., β-sitosterol, cholestanol, stigmasterol and desmosterol (Salen and Grundy, 1973; Nordby and Norum, 1974). LCAT is stimulated by A-1, C-1 and possibly A-111 apolipoproteins (Fielding *et al.*, 1972; Garner *et al.*, 1972; Kostner, 1974b). The enzyme has been shown to complex

with HDL (Glomset, 1972; Lossow, Shah and Charkoff, 1966).

The importance of LCAT in lipoprotein metabolism has been demonstrated from the lipoprotein patterns observed in patients with familial LCAT deficiency (Glomset and Norum, 1973). All known plasma apolipoproteins may be found in the lipoproteins of these patients though often in abnormal concentrations. All the plasma lipoproteins in LCAT deficiency contain low amounts of cholesteryl ester with elevated levels of free cholesterol and phospholipid. Abnormally large particles, disc shaped, with diameters between 900 and 1200 Å, may be found in the LDL fraction. An abnormal lipoprotein associated with cholestasis, Lp-X, is also found in these subjects. This lipoprotein is characterised by the presence of albumin (65%) and C apolipoprotein (35%) forming its protein moiety (Alaupovic *et al.*, 1969). VLDL in LCAT deficient patients have β rather than pre β migration on paper electrophoresis. The HDL concentration may be reduced by approximately 66%. These abnormalities can be reversed *in vitro* by the addition of LCAT.

The *in vivo* interaction between LCAT and plasma lipoproteins is not clear. LCAT may have several functions. First, in man, it may provide a large proportion of the cholesteryl esters of plasma lipoproteins. The *in vitro* rates of cholesteryl ester formation (Glomset, 1968) are similar to those obtained by *in vivo* measurement of cholesteryl ester turnover (Nestel and Monger, 1967). Secondly, the LCAT reaction may prevent the accumulation of free cholesterol and phospholipids on the surface of lipoproteins, particularly VLDL and chylomicrons, during their catabolism. Finally, LCAT may assist in the

transport of free cholesterol from peripheral tissues to the liver (Glomset, 1968; Glomset and Norum, 1973).

Akanuma and Glomset (1968) have demonstrated, using partially purified enzyme preparations, that HDL is a preferred substrate for LCAT than VLDL. This finding suggested that VLDL cholesteryl esters, in man, are derived from HDL, as implied by the studies of Rehnborg and Nichols (1964) and Nichols and Smith (1965) in which HDL cholesteryl esters transferred to VLDL in exchange for triglyceride. The nature of this exchange is unknown as cholesteryl ester, because of its non-polar nature, would be expected to occupy spaces in the core of the lipoprotein and so not be available for exchange. Glomset *et al.* (1966) have shown that some HDL species react preferentially with LCAT. These "substrate" molecules have higher free to esterified cholesterol and higher lecithin to lysolecithin ratios than other, possibly "older", HDL. These "substrate" molecules are probably species in the HDL_3 class (Fielding and Fielding, 1971).

Recent evidence indicates that free cholesterol in VLDL may also serve as an effective substrate for LCAT in the presence of an undefined plasma component (d. > 1.225 g/ml) (David *et al.*, 1976). The substantial stimulation of LCAT activity by the addition of VLDL and chylomicrons has also been described (Marcel and Vezina, 1973). VLDL is rich in lecithin (Skipski, 1972). The postulated origin of cholesteryl esters in HDL has been disputed by Barter (1974) who observed from kinetic studies that VLDL cholesteryl ester may be derived directly from free cholesterol. It has been proposed that, during the catabolism of VLDL, when the triglyceride moiety is being removed by the action of

lipolytic enzymes, the excess surface lipids (free cholesterol and phospholipid) may be removed by the action of LCAT (Schumaker and Adams, 1970). If LCAT utilises VLDL free cholesterol as a substrate, the LCAT reaction would promote a migration of cholesterol to the core of the lipoprotein as it is converted from the free to the esterified form. Lysolecithin, the phospholipid product of the reaction may be accepted by circulating albumin. If LCAT utilises HDL free cholesterol as a substrate, the removal of surface free cholesterol and phospholipids from HDL (by the action of LCAT similar to that described for VLDL) might result in a transfer of free cholesterol and phospholipid from VLDL to HDL as these lipid moieties transfer between VLDL and HDL. This would result in a net removal of free cholesterol and phospholipid from VLDL. This would be in agreement with the observed net loss of cholesterol (free and esterified) from the VLDL during the formation of remnants (Eisenberg and Rachmilewitz, 1975). In a similar manner to that described for removal of free cholesterol from VLDL, HDL may accept free cholesterol from peripheral tissues and transport them back to the liver in the esterified form for further metabolism in the liver (Glomset, 1968).

1.7 THE HYPERLIPOPROTEINAEMIAS

An abnormally increased concentration of one or more of the plasma lipoprotein families constitutes hyperlipoproteinaemia (HLP). A system for the correct classification of HLP is hampered by the fact that many of the individual steps of lipoprotein metabolism and their controlling factors are incompletely understood.

Primary forms of HLP are generally defined as those which result from a genetic defect and are not the result of a separate endocrine or metabolic disorder. Those HLPs that are the result of other disorders are termed secondary. For example, they may be found in patients with uncontrolled diabetes, hypothyroidism and the nephrotic syndrome.

In classifying hyperlipoproteinaemias, the aim is to be able to define, from inheritable characteristics, a particular abnormal genotype. On the basis of lipoprotein separation obtained by electrophoresis of plasma on paper, Fredrickson *et al*. (1967) have described five abnormal and inheritable phenotypes, classified types 1-5. Although this system is almost universally used for HLP classification, it has become apparent that the original five described phenotypes do not sort out into five distinct genotypes. The classification system has been revised and at present HLPs are categorised into six classes though many more may exist.

The limitations of this classification system include (a) the variability in an individuals phenotype which may depend on changes in nutrition and hormonal status e.g. weight loss in an obese individual may cause the HLP phenotype to change from type 4 to type 2A or 2B; oral contraceptives may cause a change in phenotype from normal to type 4 or 2B; (b) the heterogeneity of phenotypes observed within a family e.g. phenotypes 2A, 2B and 4 frequently aggregate within a family afflicted with hyperlipoproteinaemia.

1.7.1 Type 1 (familial hyperchylomicronaemia)

Type 1 HLP is characterised by a marked presence of plasma

chylomicrons following an overnight fast. In this abnormality, chylomicrons are metabolised very slowly and may persist in the circulation for more than 48 hours after a meal (Quarfordt *et al.*, 1970). VLDL, however, appears to be catabolised at a normal rate (Fredrickson and Levy, 1972). Havel and Gordon (1960) found that following heparin injection, the rate of chylomicron clearance from plasma of type 1 HLPs was 25% of that observed in normals. These patients have a reduced post-heparin lipolytic activity (Fredrickson *et al.*, 1963). Hydrolysis of diglycerides, monoglycerides and phospholipids is normal (Greten, 1972). LaRosa *et al.* (1972) have shown that in type 1 HLP, while the activity of the hepatic triglyceride lipase is normal, lipoprotein lipase activity is markedly reduced. These observations are compatible with low lipoprotein lipase activities measured in adipose tissue biopsies from members of a family with type 1 HLP (Harlan *et al.*, 1967).

1.7.2 Type 2A (familial hypercholesterolaemia)

Familial type 2 HLP is characterised by an increased plasma LDL concentration. Since LDL are cholesterol-rich lipoproteins, it follows that plasma cholesterol levels are also elevated. As this abnormality is often observed in conjunction with an elevation in the plasma VLDL concentration, the type 2 HLP classification is further subdivided into two types (2A and 2B). Type 2A HLP is characterised by an elevation in LDL alone.

The expression of primary type 2 HLP may be related to the inheritance of the 'familial hypercholesterolaemic' gene (Khachadurian, 1964). This autosomal mutant allele has a more pronounced expression

in the homozygous form. The disorder is detectable at birth by analysis of cord blood LDL levels (Kwiterovich *et al.*, 1970).

Abnormal mechanisms resulting in the accumulation of LDL in familial hypercholesterolaemia (FH) may be related to an abnormally high rate of LDL synthesis (over-production) or a reduced rate of LDL clearance. Brown *et al.* (1974), in examining HMG Co A reductase activity and cholesterol synthesis of cultured skin fibroblasts found that the cells cultured from homozygous FH patients had abnormally high HMG Co A reductase activity when compared to cells cultured from normals. Furthermore, this enzyme was not inhibited in cells cultured from type 2A HLP by increasing concentrations of LDL in the culture medium. From these and further observations (reviewed by Goldstein and Brown, 1975) on LDL binding and cholesteryl esterase activities, Brown and Goldstein (1974) have proposed that the primary abnormality in FH involves defective or absent cell surface receptors for LDL. The non-function of this receptor, in type 2A HLP, leads to (1) a decreased rate of catabolism of LDL and (2) lack of product inhibition of HMG Co A reductase activity thereby resulting in an overproduction of cholesterol.

When these observations on skin fibroblasts are extrapolated to intact man, type 2A HLP would result from both mechanisms described, i.e. overproduction and decreased removal. There is little evidence for an overproduction of cholesterol in hypercholesterolaemia in general (Nestel, 1973a). However, in type 2A HLP, homozygous for FH, there is some evidence for an overproduction of LDL. Simons *et al.* (1975) reported an increased production of LDL in four type 2A homozy-

gotes as well as a decreased fractional catabolic rate. These observations are in agreement with later reports of Bilheimer *et al.* (1975) and Thompson and Myant (1976) with single studies on type 2A homozygotes. In the latter study the reduced fractional catabolic rate of LDL was shown to be independent of the plasma LDL concentration. These observations were made on patients before and after plasma exchange (which reduced the size of the LDL pool).

A decreased clearance of plasma cholesterol has been documented in hypercholesterolaemia (Nestel, 1973a). In agreement with these findings are those of the previously described studies (Simons *et al.*, 1975; Thompson and Myant, 1976) where a reduced fractional catabolic rate for LDL was described in patients homozygous for FH. Likewise, in heterozygotes, the fractional catabolic rate of LDL is reduced though an overproduction of LDL has not been observed (Langer *et al.*, 1972). These observations tend to discriminate between homozygotes and heterozygotes for FH. The abnormality in homozygous FH is probably an overproduction of LDL with a concomitant decreased clearance in accordance with the predictions of the Brown and Goldstein model. However, in patients heterozygous for FH, only a reduced clearance of LDL may be demonstrated from *in vivo* turnover studies. Brown and Goldstein have reported that the abnormality of LDL catabolism in fibroblasts from patients heterozygous for FH is a partially reduced binding of LDL leaving values of LDL degradation and cholesterol synthesis that are intermediate between those for normals and homozygotes (Brown and Goldstein, 1974). In heterozygotes, LDL and cholesterol turnover may be normal though at the expense of increased pools.

Turnover studies are carried out in the steady state and reflect long-term equilibrium conditions. It may therefore not be possible to detect the initiating abnormality and consequently the failure of the *in vivo* studies to correlate with the findings in tissue culture do not necessarily rule out the validity of the latter studies in demonstrating the initiating defect. On the other hand, it is possible that the *in vitro* conditions uncover only potential abnormalities that do not contribute significantly *in vivo*.

The abnormality of type 2A HLP does not appear to depend on the structure or chemical composition of the LDL molecule. However LDL isolated from type 2A HLP is not identical, in chemical composition to that of normal individuals (Slack and Mills, 1970; Brown *et al.*, 1973; Bagnall, 1972). However, as suggested by Simons and colleagues, this abnormal lipid composition is a consequence, rather than cause, of type 2A HLP. These authors have compared, simultaneously, the catabolism of autologous LDL and LDL from normal subjects in type 2A homozygotes and found the rate of catabolism to be similar for both types of LDL (Simons *et al.*, 1975).

1.7.3 Type 2B or combined hyperlipoproteinaemia

The phenotype corresponding to 2B HLP in the revised Fredrickson classification has been termed combined hyperlipoproteinaemia by others. It is characterised by an increased plasma concentration of both VLDL and LDL. The expression of this phenotype need not necessarily be related to the type 2A genotype previously described. Rose *et al.* (1974) and Goldstein *et al.* (1973) have described patients with the type 2B phenotype who have no familial

grouping of type 2A HLP and so have concluded that this condition (combined HLP) is distinct from type 2B HLP. Type 2B HLP might best be restricted to the phenotype which describes an environmentally induced elevation of VLDL seen in association with type 2A HLP. Combined HLP appears to be an inheritable phenotype and so must be considered a distinct HLP class.

Combined HLP, which is characterised by moderate elevations of plasma VLDL and LDL only, may be seen in association with obesity, impaired glucose tolerance and hyperuricaemia as in secondary type 4 HLP. A clear distinction between type 4 and combined HLP is possible on the basis of LDL concentration. The biochemical abnormality associated with this disorder has not been elucidated.

1.7.4 Type 3 hyperlipoproteinaemia

Type 3 HLP is characterised by the presence of an unusual class of cholesteryl ester rich lipoproteins in the d. 1006 g/ml range (Fredrickson and Levy, 1972). These lipoproteins have β rather than pre β migration on paper or agarose electrophoresis and may be found throughout the whole VLDL spectrum (Quarfordt *et al.*, 1971). As a result of an elevated concentration of these 'abnormal' lipoproteins, the plasma triglyceride and cholesterol concentrations are both elevated (Lees *et al.*, 1973).

It is uncertain whether this disease involves an accumulation of abnormal lipoproteins or an abnormal concentration of a remnant of VLDL or chylomicron catabolism (Quarfordt *et al.*, 1970). VLDL of type 3 HLP appears similar in core and surface layer composition to normal

VLDL (Sata *et al.*, 1972) as are the apolipoproteins of chylomicrons in this disease (Havel *et al.*, 1973). Newly secreted chylomicrons have a normal cholesterol to triglyceride ratio which increases abnormally only during the clearing of the alimentary lipaemia. These findings suggest that the abnormal particle with its high cholesterol:triglyceride ratio develops as a result of defective lipoprotein catabolism.

VLDL from type 3 HLP contain a disproportionally high amount of apolipoprotein E which is usually associated with cholesteryl ester rich VLDL populations (Shore and Shore, 1973; Havel and Kane, 1973; Shore *et al.*, 1974). Utermann *et al.* (1975) have recently demonstrated a deficiency of a specific component of this apolipoprotein in type 3 HLP and claim that this may be the underlying defect of the abnormality. However, the studies of Bilheimer *et al.* (1971) suggest that the abnormal accumulation of remnants in type 3 HLP is not due to a defective lipoprotein. In comparing VLDL catabolism in type 3 HLP and type 4 HLP subjects, these authors demonstrated that the catabolism of VLDL isolated from type 3 HLP subjects, when injected into type 4 HLP subjects, was normal whereas the catabolism of VLDL isolated from type 4 HLP subjects, was delayed when injected into type 3 HLP patients. Hazzard and Bierman (1971), using ^{14}C-vitamin A as a lipid tag for VLDL and chylomicrons, observed that the remnants of catabolism of chylomicrons and VLDL accumulated in type 3 HLP, while in type 4, their catabolism was not delayed and they were converted to LDL_2.

1.7.5 Type 4

Type 4 HLP is characterised by an elevation of the plasma

VLDL concentration during fasting. Since these particles are triglyceride-rich, the plasma triglyceride concentration is likewise elevated. As VLDL also contains approximately 15% cholesterol (Section 1.1.4), some elevation in the plasma cholesterol concentration may also be seen. The predicted plasma cholesterol increase due to VLDL elevation alone, is approximately 1/5 of the plasma triglyceride increase (Friedwald *et al.*, 1972). In order to differentiate between type 4 and combined HLP, these authors propose that LDL cholesterol may be approximated from the formula.

$$LDL_{chol} = plasma_{chol} - HDL_{chol} - plasma_{trig/5}$$

(assuming an absence of type 3 HLP). In type 4 HLP, LDL_{chol} may be lower than normal.

Type 4 HLP is often observed in association with obesity (Barter and Nestel, 1973), excessive caloric intake (Schonfeld, 1970), diabetes (Bagdade *et al.*, 1968) nephrotic syndrome (Losowsky and Kenward, 1968, Ibels *et al.*, 1976) and alcohol intake (Kudzma and Schonfeld, 1971).

Possible biochemical defects associated with type 4 HLP may involve an overproduction or a decreased clearance of circulating VLDL. VLDL overproduction might result from (1) an increased rate of apolipoprotein synthesis; (2) an increased production of triglyceride as a result of increased plasma FFA flux (as discussed in section 1.4.2); (3) increased synthesis of triglyceride from carbohydrate (lipogenesis); (4) a decreased rate of FFA utilization through alternative pathways in the liver. Decreased clearance of VLDL may

result from reduced tissue lipolytic activity (particularly lipoprotein lipase). No abnormalities in the structure of VLDL from type 4 HLP have been reported, though, in carbohydrate-induced hypertriglyceridaemia, VLDL particles are larger and contain relatively more triglyceride than normal VLDL particles (Ruderman *et al.*, 1971).

A number of studies have investigated the causes of hypertriglyceridaemia. These studies have elucidated complex interrelationships between lipid, lipoprotein and carbohydrate metabolism. Reaven *et al.* (1965, 1967) and Nestel (1966) concluded that overproduction of VLDL was an important factor in development of hypertriglyceridaemia. The former group has proposed that increased peripheral resistance to the action of insulin provokes an enhanced production of insulin with a resultant increase in the synthesis and release of triglycerides by the liver (Stern *et al.*, 1972). On the other hand, other investigators have not demonstrated an abnormal increase in production of triglycerides in type 4 HLP on carbohydrate-rich diets and have suggested that endogenous hypertriglyceridaemia may result from a reduced clearance of triglyceride from the plasma (Quarfordt *et al.*, 1970; Nestel *et al.*, 1970; Ryan and Schwartz, 1965; Harris and Felts, 1973). Post-heparin lipolytic activity is usually normal in type 4 HLP (Fredrickson *et al.*, 1963; Schreibman *et al.*, 1969) though it is reduced in type 4 HLP secondary to diabetes (Bagdade *et al.*, 1968) and hypothyroidism (Kirkeby, 1968).

It is generally agreed now that both overproduction and a relative inefficiency in clearance contribute to the development of this condition. Turnover studies describe only the equilibrium state and

do not necessarily identify the initiating cause. Thus overproduction may lead to the development of hypertriglycerideaemia which only becomes established if removal rates do not increase correspondingly (Nestel, 1973b). Recent studies on the metabolism of the B apolipoprotein (Sigurdsson *et al.*, 1976) also suggest that the underlying defect in endogenous hypertriglyceridaemia is heterogenous. In these studies, overproduction as well as a reduced fractional clearance of VLDL B apolipoprotein was observed. Saturation of removal mechanisms for VLDL and chylomicron triglyceride in type 4 and 5 HLP has been demonstrated by Brunzell *et al.* (1973).

1.7.6 Type 5

Type 5 HLP is characterised by an accumulation of both chylomicrons and VLDL in plasma during fasting. Thus the fasting plasma triglyceride is markedly elevated. It occurs most commonly as a secondary form (diabetes, alcoholism) than as a familial form.

The biochemical defect of type 5 HLP is not understood. Post-heparin lipolytic activity may be reduced as in diabetes but is generally normal (Fredrickson and Levy, 1972). The factors responsible for type 5 HLP may be similar to those discussed for endogenous hypertriglyceridaemia and may in fact represent the overloading of removal mechanisms by dietary particles when clearance has been saturated by endogenous VLDL.

1.8 LIPOPROTEINS AND ATHEROSCLEROSIS

In the past fifty years, human diseases characterised by

atherosclerotic changes in arteries have become prevalent. The most common and lethal manifestation of atherosclerosis is coronary heart disease. This disease is the most common serious disease in Australia, as in most Western cultures, and accounts for approximately 30% of all deaths.

Coronary heart disease (ischaemic heart disease) has been defined as "cardiac disability, acute or chronic, arising from reduction or arrest of blood supply to the myocardium in association with disease processes in the coronary arterial system" (Report, W.H.O., 1957). The primary event leading to myocardial infarction is occlusion of a major coronary artery or one of its subdivisions, usually by a thrombus in an atheromatous vessel. In most cases coronary heart disease occurs only when the walls of the coronary arteries have been altered by atherosclerosis.

Atherosclerosis has been defined as a "variable combination of changes in the intima of arteries (as distinct from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits with associated medial changes" (Report, W.H.O., 1958). Atherosclerosis affects the aorta and larger distributing arteries and may manifest itself in, other than coronary heart disease, cerebrovascular accidents and intermittent claudication.

Three types of arterial lesions are associated with atherosclerosis - fatty streaks, fibrous plaques and complicated lesions. The latter two are raised lesions that result in a constriction within the lumen of the artery. Fibrous plaques are localised lesions of the

arterial wall that consist primarily of an accumulation of smooth muscle cells and fibroblasts. Complicated lesions appear to be an advanced stage of atherosclerosis involving calcification and ulceration of the whole vessel wall. The relevance of fatty streaks, which have often been observed in arteries of children, to the development of raised lesions is not known. The most widely accepted hypothesis for the primary stimulation of plaque formation is damage to the endothelial cell lining resulting in changes of permeability to macromolecules, such as lipoproteins. This leads to the proliferation of smooth muscle cells which accumulate lipid and become transformed into foam cells, the characteristic cells of the early lesion. Recent reports have described avid uptake and degradation of VLDL and LDL by cultured human aortic smooth muscle cells (Albers and Bierman, 1976). Furthermore, hypoxia in these cultures leads to a decreased degradation of LDL thereby promoting lipid accumulation within the cell.

It has long been recognised that atherosclerosis is characterised by an accumulation of lipids, mainly cholesteryl esters, in the intima of the arterial wall. Smith and Slater (1973) have concluded that the major part of cholesteryl ester in the advanced atherosclerotic lesion originates directly from plasma LDL and that only a small proportion accumulates as a result of arterial cholesterol esterification. Substantial amounts of electrophoretically and immunologically intact LDL (7 times that of albumin), but not VLDL, has been found in the intima of human aorta. However, the low proportion of triglyceride found in both normal intima and lesions also suggests that VLDL does not enter the intima, possibly because of its larger size (Smith, 1974). A comparatively greater accumulation of LDL has been

reported in the intima of patients with elevated plasma cholesterol levels than in those with normal cholesterol levels (Smith and Slater, 1972). The addition of LDL has been shown to cause proliferation of aortic smooth muscle cells in culture (Dzoga *et al.*, 1971).

Cholesterol feeding in swine results in an elevation of plasma cholesterol levels and an accelerated development of atherosclerosis. In the plasma of these animals may be found cholesteryl ester rich lipoproteins (β-VLDL) resembling those found in type 3 HLP in man. These lipoproteins, as in type 3 HLP, contain significant amounts of apolipoprotein E. Also, an α-migrating species, termed HDL_C, containing apolipoproteins A-1 and E and rich in cholesteryl ester, may be found in HDL and LDL density ranges. Similar observations have been made in cholesterol fed dogs, rabbits and rats (Mahley *et al.*, 1974; Mahley *et al.*, 1975). These authors suggest a possible association between this spectrum of abnormal cholesteryl ester rich lipoproteins (β-VLDL and HDL_C) and atherogenesis.

Epidemiological studies have illustrated an association between various risk factors (environmental and genetic) and coronary heart disease (Keys, 1970; Cotton *et al.*, 1972; Welborn *et al.*, 1969; Blacket, 1973). These risk factors include age, family history, cigarette smoking, hypertension and hyperlipoproteinaemia. Types 2A, 2B, combined, 3, and 4 HLP have a high incidence of coronary heart disease (Fredrickson, 1971).

1.9 AIMS OF THE PROJECT

The purpose of the studies reported in this thesis was to

investigate and describe various metabolic inter-relationships between the plasma lipoproteins in man. These studies were particularly concerned with an elucidation of the mechanisms involved in the catabolism of VLDL to LDL in normal and hyperlipoproteinaemic subjects. As the apolipoproteins of these lipoproteins are the determinants of this relationship, methods used for the separation and quantitation of the individual apolipoproteins are discussed (Chapter 3). A system was designed for use in metabolic studies for obtaining the B apolipoprotein free of the remaining apolipoproteins in order to obtain specific activity data for the B apolipoprotein of VLDL and LDL.

Chapter 4 describes a series of *in vitro* investigations in which the conversion of VLDL to remnants and subsequently to LDL_2 was studied in relation to post-heparin lipolytic activity. The role of hepatic and extra-hepatic lipases in the process was examined.

Chapter 5 describes studies on the *in vivo* catabolism of VLDL. These studies, carried out in normal and hyperlipoproteinaemic patients, involve kinetic analysis of the B apolipoprotein specific activity-time curves of VLDL, LDL_1 and LDL_2, following injection of VLDL-^{125}I. From these curves it has been possible to measure the turnover of the B apolipoprotein in various lipoproteins and define differences in VLDL catabolism in normal and hypertriglyceridaemic subjects.

In chapter 6 are described a further series of studies on the catabolism of various VLDL and remnant subfractions in an attempt to define, on the basis of precursor-product relationships and turnover data, the probable major intermediate between VLDL and LDL_2.

Chapter 7 summarises and generally discusses the implications of the *in vitro* and *in vivo* studies in terms of documented normal and abnormal lipoprotein metabolism.

CHAPTER 2

GENERAL METHODS

Techniques and procedures used to implement the studies in this thesis are described in this chapter. In subsequent chapters, these methods are cross-referenced to the appropriate section numbers of this chapter.

2.1 CHEMICAL ESTIMATIONS

(a) *Protein:* The protein content of lipoprotein and apolipoprotein samples was determined using the method of Lowry *et al.* (1951). Folin-Ciocalteau reagent was obtained from British Drug Houses, England. Bovine albumin (Fraction V, Sigma, U.S.A.) was used as protein standard and was particularly suitable in these experiments since it has similar chromogenicity to the B apolipoprotein. Turbidity due to the presence of lipid was cleared by extracting the final assay mixture with diethyl ether or chloroform and centrifuging at 600xg. for 1 min.

High salt concentration was found to interfere with this colourimetric procedure. Lipoproteins could not be assayed in solutions of d. > 1.019 g/ml and had to be dialysed back to d. 1.006 g/ml prior to assay. Other compounds which interfere with the Lowry method, such as SDS (sodium decyl or dodecyl sulphate) or high tris (tris(hydroxylmethyl) aminomethane, Sigma, U.S.A.) concentration, were added to standards and blanks at similar concentrations as present in the unknown solutions.

(b) *Lipids:* Triglycerides and cholesterol were simultaneously estimated by a semi-automated procedure in a Technicon Auto-analyser AA-11 (as described in Technicon publication number AA11-24, 1971). This procedure assayed cholesterol colourimetrically following the Lieberman-Burchard reaction and triglycerides by fluorimetry using a modification of the method of Kessler and Lederer as described by Leon *et al.* (1970).

The auto-analyser was calibrated using known standards of cholesterol and triglyceride (triolein) and SMA reference serum. Although plasma lipids are now more routinely expressed in molar units, values in this thesis are expressed in mg/100ml plasma similar to the units to describe apolipoprotein concentrations.

(c) *Free fatty acids:* Plasma free fatty acids were extracted from plasma by the procedure of Dole and Meinertz (1960) and assayed using the colourimetric assay of Duncombe (1964). The Doles extraction procedure was preferred to the direct two-phase chloroform extraction described by Duncombe (1964) as many of the samples to be assayed contained significant amounts of post-heparin lipolytic activity. In contrast to the two-phase chloroform extraction, the addition of the plasma aliquot to Doles extraction medium immediately arrests any further lipolytic activity.

2.2 LIPOPROTEIN ISOLATION AND PURIFICATION PROCEDURES

Plasma lipoproteins were isolated and purified by ultra-centrifugation using the conditions described by Havel *et al.* (1955) and Gustafson *et al.* (1965) as shown in Table 2.1. For these procedures, 35, 40, 40.3 and 50 rotors well spun in L 2-50 and L 3-50 Beckman

preparative ultracentrifuges.

A number of various lipoprotein fractions were investigated during these studies. These fractions were isolated by the usual sequential flotation techniques. An example will be described where the required fractions were s_f 60-400, s_f 12-60, s_f 0-12 and HDL (as in section 6.2). In the metabolic studies, 10ml plasma samples obtained at each time point were centrifuged. Larger volumes were used for preparation of lipoproteins for iodination or apolipoprotein characterisation.

The plasma (10 ml) was pipetted into Spinco 40 tubes (cellulose nitrate) and overlaid with buffered saline (0.15M NaCl, 0.001M EDTA adjusted to pH 7.4 with tris) which has a salt density of 1.006 g/ml. Lipoproteins of $s_f > 400$, if present, were removed by centrifugation at 20,000 xg. for 30 min. and slicing the top 3.5 ml of the tube. The infranate (bottom 10 ml of the tube after slicing) was transferred to a separate tube, overlaid with a further 3.5 ml of buffered saline and spun for 2 hours at 100,000 xg. to isolate lipoproteins of s_f 60-400. After removing this top fraction, the infranate (which then contained particles of $s_f < 60$) was adjusted to d. 1.019 g/ml and spun at 108,000 xg. for 18 hours to float s_f 12-60 lipoproteins. The subsequent isolation of s_f 0-12 lipoprotein and HDL followed this sequential procedure using the conditions described in Table 2.1.

Each lipoprotein fraction was recentrifuged at its respective density, in a 40.3 rotor, using the same conditions for its isolation.

The efficacy of this two spin procedure in isolating lipo-

TABLE 2.1

CONDITIONS USED TO FLOAT AND PURIFY LIPOPROTEINS OF VARIOUS SIZES FROM HUMAN PLASMA BY ULTRACENTRIFUGATION.

These conditions include salt density, centrifugal force and time. Most fractions may be denoted by Svedberg flotation units (10^{-13} cm/sec/dyne/g) at d. 1.063 g/ml with the exception of HDL which floats at a salt density of 1.21 g/ml. These conditions are as defined by Havel *et al.* (1955) and Gustafson *et al.* (1965).

Lipoprotein Particles	Density[1]	Centrif. Force[2]	Time
S_f	g/ml	g.	hours
> 400	1.006	20,000	0.5
> 100	1.006	80,000	1.0
> 60	1.006	100,000	2.0
> 50	1.006	108,000	2.0
> 20	1.006	108,000	16.0
> 12	1.019	108,000	18.0
> 0	1.063	108,000	20.0
HDL	1.21	108,000	24.0

[1] The infranate densities were adjusted by addition of solutions of higher density according to the formula of Havel *et al.* (1955). Standard density solutions were prepared by the addition of KBr to buffered saline. The densities were checked by picnometry.

[2] Average centrifugal force.

proteins free of other plasma proteins was tested by immunodiffusion (Ouchterlony, 1958) against albumin, the protein of highest concentration in plasma. In almost all cases, the two spin regime was found to be sufficient to isolate lipoproteins free of albumin. In a few extremely concentrated preparations of VLDL, further washings were required to remove traces of albumin.

2.3 LIPOPROTEIN IODINATION

Lipoprotein fractions were iodinated with ^{125}I or ^{131}I using the iodine-monochloride technique of McFarlane (1958) as modified for lipoprotein iodination by Fidge and Poulis (1974). ^{125}I and ^{131}I, carrier free, for iodination (1MS.30) were obtained from The Radiochemical Centre, Amersham, England. Lipoproteins were iodinated at pH 10 using 0.4M glycine -NaOH, pH 10 as the buffer medium throughout the entire process. The amount of iodine monochloride added to the iodination mixture was calculated to give an iodine:protein ratio of less than 1. Lipoprotein bound iodine was separated from free iodine initially by filtration on sephadex G-50 (column 0.6 × 25 cm) followed by dialysis against numerous changes of buffered saline over a minimum period of 4 hours.

Intramolecular distribution of radioisotope was determined in all iodinated preparations. Lipid was extracted by the Folch procedure (Folch *et al.*, 1957). Protein in the aqueous phase of the Folch extraction was precipitated (following removal of the methanol by evaporation using a stream of nitrogen) by the addition of an equal volume of 10% (w/v) TCA (trichloroacetic acid) followed by centrifugation (2,000 xg. for 5 min.). The TCA precipitate was dissolved in 0.1M

NaOH and an aliquot counted for radioactivity. An aliquot of the TCA soluble phase (containing free iodine) and of the lipid (redissolved in ethanol) was likewise counted and so the percentage of radioisotope bound to lipid or protein or remaining free could be calculated. In preparations used in these studies the percentage radioisotope bound to protein varied from 89-96% with 3-8% being lipid bound and 1-3% remaining free.

2.4 STERILISATION OF LIPOPROTEINS FOR RE-INJECTION

Radioiodinated autologous lipoproteins for re-injection (chapters 5 and 6) were passed sequentially through 0.45 and 0.22mμ millipore filters (Millipore Corp., Mass., U.S.A.) before iodination and then finally, under sterile conditions, prior to injection. An aliquot of the sterile preparation was routinely selected for radioactivity determination and culture in thioglycolate medium to test for any bacterial contamination.

2.5 LIPOPROTEIN DELIPIDATION

Lipoproteins were dialysed against 5mM NH_4HCO_3, pH 8.0, prior to delipidation. Large preparations of apolipoproteins for subsequent column procedures (as described in Chapter 3 where 15-150 mg apolipoprotein was prepared) were delipidated using the chloroform: methanol: diethyl ether procedure as described by Herbert *et al.* (1973).

Smaller amounts of lipoprotein, such as used for separation of the B apolipoprotein by the NH_4HCO_3 procedure (described in 3.2.5)

were delipidated using a 'scaled-down' procedure of that described by Herbert *et al.* (1973) in order to obtain quantitative recoveries of protein. Aliquots containing 0.3 - 1.0 mg protein were lyophylised in their respective delipidation tubes (glass centrifuge tubes of 4 ml capacity). The dried lipoprotein was resolubilised in 100 µls 0.05M NH_4HCO_3, pH 8.0. Delipidation was performed using 1/12 the volume of organic solvents described by Herbert *et al.* (1973). Recovery of protein was improved by placing the samples at -10°C for 1 hour at each step of the delipidation process. Apolipoproteins were dried, after the final diethyl step, under a gentle stream of nitrogen. Protein recoveries using this procedure ranged from 87-107%.

2.6 PREPARATION OF UREA

Urea (Univar Chemicals, Australia) was used for column and polyacrylamide gel electrophoresis (PAGE) procedures. Prior to use a 40% (w/v) solution of urea was passed over a column (5 × 50 cm) of Rexyn I-300 (Fisher Scientific, U.S.A.) to reduce the cyanate content in order to prevent protein carbamylation. The effectiveness of this procedure was monitored by determining the conductivity of the solution after passage through the column. This was routinely found to be less than 3 µmhos. The 40% urea solution was reduced to 36% (6M) and buffered with tris for column procedures or lyophylised prior to use in PAGE techniques. It was maintained at 2°C until use to minimise further cyanate formation.

2.7 POLYACRYLAMIDE GEL ELECTROPHORESIS PROCEDURES

Two types of PAGE separations were used throughout these

studies. Polyacrylamide gels containing 6M urea were used for most apolipoprotein separations because of the excellent resolution of apolipoproteins by this technique. SDS-gels were used for molecular weight determinations and in particular the presence of apolipoprotein E in VLDL apolipoprotein preparations.

Urea-gels were prepared and operated using the method of Kane (1973). Following electrophoresis, gels were stained in 1% (w/v) Amidoschwartz 10B (Schmidt and Co., Stuttgart, West Germany) in 7% (v/v) acetic acid for 1 hour after which they were destained in 7% (v/v)acetic acid by diffusion.

SDS-gel electrophoresis was performed using the system originally described by Laemmli (1970) but more recently outlined by Weber (1975). Following electrophoresis, the gels were stained in 0.25% (w/v) coomassie blue R (Sigma, U.S.A.) in 50% (v/v) methanol containing 7% (v/v) acetic acid and destained by diffusion in 15% (v/v) methanol in 7% (v/v) acetic acid.

2.8 AMINO ACID ANALYSES

(a) *Total:* Proteins (0.5 mg) were dialysed against distilled water and lyophylised. The dried protein was hydrolysed with 6M HCl for 22 hours at 105°C. Amino acid analysis of the hydrolysate was performed on a Technicon 120B model amino acid analyser using the method of Spackman *et al.* (1958). Cysteine, if determined, was estimated as cysteic acid on a separate hydrolysate following performic acid oxidation.

(b) *Carboxy-terminals:* Carboxy-terminal amino acids were hydrolysed by incubation with carboxy-peptidare A (Worthington Biochemical Corp., New Jersey, U.S.A.) using the procedure outlined by Ambler (1972). The relative release rates of amino acids over a 2 hour incubation period were determined by quantitative estimation of amino acids on the amino acid analyser. The carboxy-terminal amino acids were also determined by hydrazinolysis (Akabori *et al.*, 1952; Schroeder, 1972). Anhydrous hydrazine was obtained from the Pierce Chemical Co., New Jersey, U.S.A. Protein samples (1 mg) were incubated *in vacuo*, at 80°C for 24 hours in 0.2 ml hydrazine.

2.9 LIPOPROTEIN LIPASE ASSAY

A radiolabelled triolein emulsion (Glyceryl tri [9,10(n)-^{3}H] oleate obtained from The Radiochemical Centre, Amersham, England) was prepared by sonication with egg yolk lecithin (Fielding, 1970). The enzyme activity was assayed using the procedure of Schotz *et al.* (1970). The concentration of albumin (bovine, fatty acid poor obtained from Gallard-Schlesinger Chemical Corp., N.Y., U.S.A.) in the assay medium was 60 mg/ml.

Milk lipase was prepared by spinning fresh cow's milk at 20,000 g for 30 min. and then lyophylising the infranate. 1 gm of lyophylised material was dissolved in 10 ml of 0.05M NH_4Cl, pH adjusted to 8.4 with NH_4OH. 0.1 ml of this preparation was used in each incubation.

2.10 CLINICAL PROCEDURES

Most of the studies of this thesis required the co-operation of consenting human volunteers to whom the nature of the studies was

fully explained. All procedures were carried out according to the conditions of the Helsinki declaration of human experimentation (appendix 1).

CHAPTER 3

ISOLATION AND QUANTITATION OF THE B AND C APOLIPOPROTEINS

3.1 INTRODUCTION

The plasma lipoproteins are complex structures. Each lipoprotein class contains all of the main lipid groups and at least one of the apolipoprotein species although the proportions of these individual components may vary from one lipoprotein class to another. The nomenclature system used for apolipoproteins in this thesis is that proposed by Alaupovic *et al.* (section 1.2.1) and is based on the premise that certain apolipoproteins, upon binding lipids, form individual lipoprotein families. At present, five lipoprotein families have been described though many more may exist. The presence of subclasses within these lipoprotein families (i.e. LP-A-1, LP-A-11) has also been suggested.

The A apolipoproteins are found primarily, but not exclusively, in HDL. The C apolipoproteins are also found in HDL though their concentration is relatively minor in comparison to that of the A apolipoproteins (Kostner *et al.*, 1974). The major apolipoprotein of LDL is the B apolipoprotein, although small quantities of A, C and D apolipoproteins may also be present (Lee and Alaupovic, 1974; Kostner *et al.*, 1974). All apolipoprotein classes have been found in VLDL, although some are present in trace and probably insignificant amounts. VLDL apolipoprotein composition is a reflection of the lipoprotein particle size with the larger particles being relatively richer in C apolipo-

proteins (particularly C-11) but containing relatively less of the B and E apolipoproteins (Kane *et al.*, 1975).

The synthesis of the B apolipoprotein is essential for the release of VLDL into the circulation (Isselbacher *et al.*, 1964; Bar-On *et al.*, 1973). The catabolism of VLDL, leading to the formation of LDL, involves losses of all of the VLDL components except the B apolipoprotein (Eisenberg *et al.*, 1973; Eisenberg and Rachmilewitz, 1975). Thus, the B apolipoprotein is the only component to remain within these macromolecules (VLDL and LDL) during their entire circulatory life. It is therefore appropriate, when studying VLDL and LDL inter-relationships, to investigate the metabolism of this apolipoprotein. The studies of chapters 4, 5 and 6, which are concerned with the transformation of VLDL to LDL and the respective production and removal rates of these lipoproteins, involve studies on the catabolism of various radioiodinated lipoprotein preparations. B apolipoprotein specific activity data was obtained in these studies and thus made possible kinetic analyses of the metabolism of this apolipoprotein in each lipoprotein fraction and its conversion from one lipoprotein species to another. The first part of this chapter (section 3.2) deals with methods in current use for the preparative isolation of the B apolipoprotein. The suitability of these procedures for routine determinations of B apolipoprotein specific activities will be discussed. Many investigators have quantitated the B apolipoprotein using radioimmunoassay procedures (Schonfeld *et al.*, 1974; Bautovich *et al.*, 1975) however these techniques are unsuitable for the purposes of the metabolic studies here as they have not been designed for the preparative isolation of the B apolipoprotein and consequently,

the direct determination of specific activities.

Apolipoproteins perform essential functions, not only as structural elements of lipoproteins but also as modulators (at least *in vitro*) of the activities of enzymes essential in lipoprotein metabolism (section 1.6.1). If *in vitro* findings are applicable *in vivo*, apolipoproteins may determine the rates of metabolism of lipoproteins. In this regard, the possible role of the C apolipoproteins in regulating the activities of various lipases and phospholipases is of particular interest. It is possible that a deficiency in one or more of these apolipoproteins may result in a defect in a lipoprotein catabolic step and so lead to an accumulation of a lipoprotein fraction in the circulation. Thus, it is important to be able to determine the concentration of these individual apolipoproteins in various lipoprotein fractions and to establish their respective turnover characteristics, both in normal and hyperlipoproteinaemic subjects. The second part of this chapter describes a densitometric method for the quantitation of the C apolipoproteins which depends on the use of individual C apolipoprotein standards. Therefore, the preparative isolation and identification of these apolipoproteins will also be described.

In this chapter, sections describing methodology actually employed for the preparative isolation of apolipoproteins, either for further characterisation or for use in the metabolic studies, is presented under section headings marked 'experimental'.

3.2 ISOLATION AND QUANTITATION OF THE B APOLIPOPROTEIN

3.2.1 Gel filtration techniques

Separation of the apolipoproteins of apo-VLDL by gel filtrat-

ion was first described by Brown *et al.* (1969). Apo-VLDL, solubilised in the presence of 0.1M sodium decyl (or dodecyl) sulphate (SDS) was resolved into two protein fractions by filtration on Sephadex G-100 in the presence of 0.001M SDS. Improved separations have recently been achieved with sephadex G-200 superfine (Herbert *et al.*, 1973) and sepharose 4-B columns (Shelburne and Quarfordt, 1974).

3.2.2 Experimental: separation of apo-VLDL on sephadex G-200

The flow rate with which sephadex columns may be run is limited by the development of compression in the gel bed and the consequent diminution in flow rate. Sachs and Painter (1972) have devised a column system whereby glass beads inside the column provide an internal support to the sephadex gel, thereby allowing the use of much higher flow rates without impairment of column elution. This system has been used by Herbert *et al.* (1973) for the rapid separation of apo-VLDL on sephadex G-200 superfine in the presence of 6M urea.

Using the procedure of Herbert *et al.* (1973) a sephadex G-200 superfine glass bead column (150 × 5 cm with 6 mm glass beads) was prepared. The column buffer was 0.2M tris-HCl, 6M urea, pH 8.2. All urea, prior to preparation of the buffers had been deionised as described in section 2.6. Glass surfaces had been previously treated with Siliclad (Clay Adams) to minimise non-specific adsorption of protein. Chromatography was carried out at 2°C to minimise protein carbamylation by urea.

Figure 3.1 illustrates a typical G-200 superfine elution profile obtained after gel filtration of apo-VLDL. Two major peaks were obtained. As the firstpeak (F1) contains the B apolipoprotein as

well as other VLDL apolipoproteins, this peak was divided arbitrarily into three fractions (A, B and C as described in figure 3.1) in an attempt to obtain a fraction containing B apolipoprotein only. Following exhaustive dialyses of the fractions against 5mM NH_4HCO_3, pH 8.0 and then lyophylisation, the protein of each fraction (except F2 which was soluble in 0.05M NH_4HCO_3, pH 8.0) was resolubilised in 0.05M tris-HCl, 0.05M SDS, pH 8.2 and monitored by electrophoresis on polyacrylamide gels (figure 3.2). The protein band remaining at the interface of the main and stacking gel was consistent with the electrophoretic behaviour of the B apolipoprotein when using this PAGE system (described by Kane, 1973). This protein was present in all fractions except F2 which contained the C apolipoproteins only. All fractions of F1 contained, as well as the B apolipoprotein, other protein components, one of which was presumably apolipoprotein E. These proteins however were mainly confined to the second half of F1 (B and C fractions). Although other proteins than the B apolipoprotein were present in the A fraction of F1 (gel F1/A), the amount present would be relatively minor in terms of the total amount of protein loaded onto the gel (~ 1-5%).

An aliquot of F1/A was exhaustively dialysed (for approximately 10 days with many changes) against distilled H_2O and lyophylised. A solution was then prepared (in 0.1M NaOH) containing a known mass, determined gravimetrically, of the dried apolipoprotein. The chromogenicity of this preparation (using the Lowry technique - section 2.1) was similar to that of the albumin standard giving almost identical standard curves. This observation has also been reported by Kane *et al.* (1975).

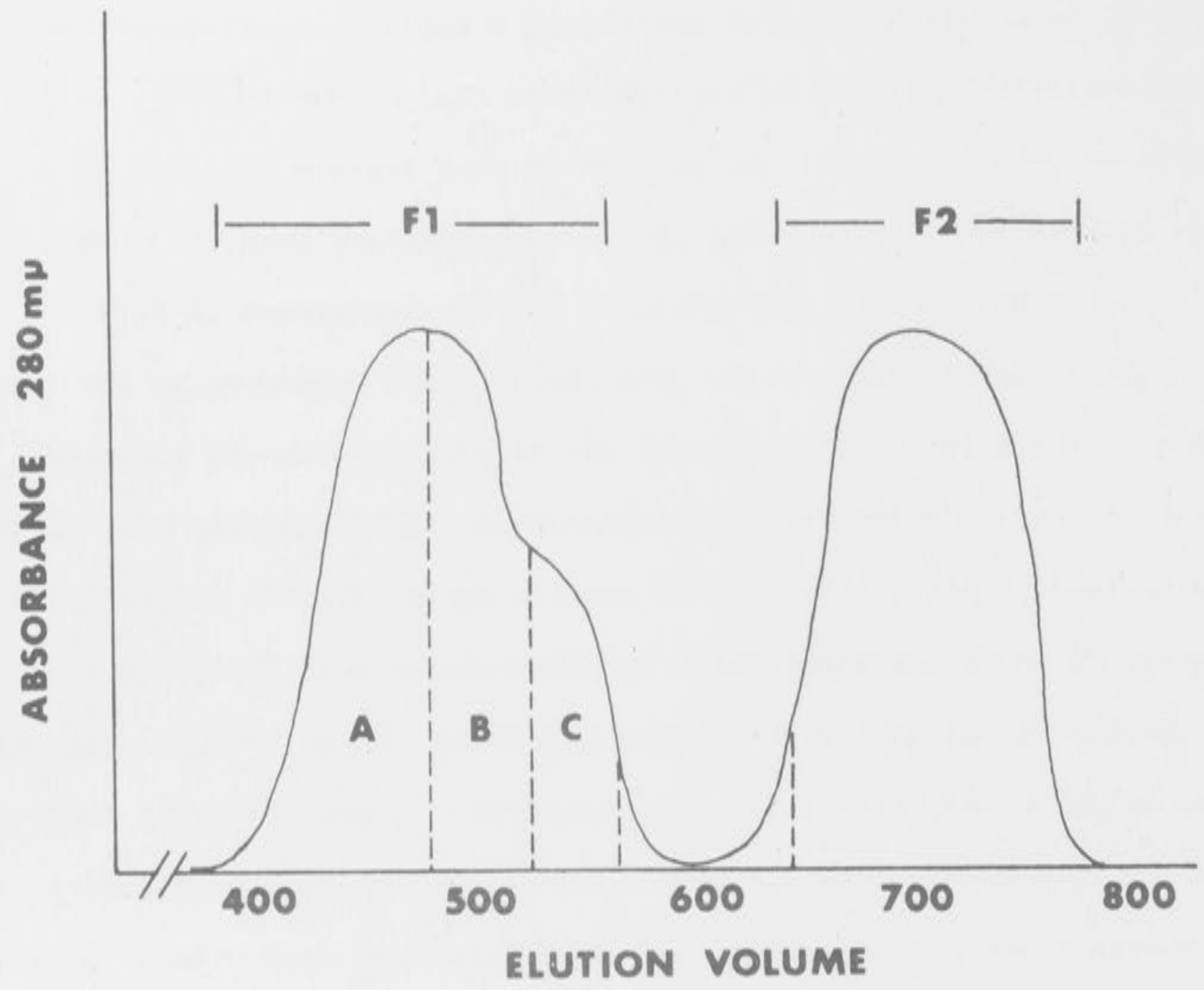


Figure 3.1 The elution profile obtained when apo-VLDL (62 mg) was chromatographed on sephadex G-200 superfine (glass bead column: 150 x 5 cm - see section 3.2.2) in the presence of 0.2M tris-HCl, 6M urea, pH_0 8.2. The column was run at a flow rate of 30 ml hour at 2 ^{0}C. Two major fractions (F1 and F2) were obtained. F1 was further subdivided into three fractions (A, B and C) as discussed in section 3.2.2.

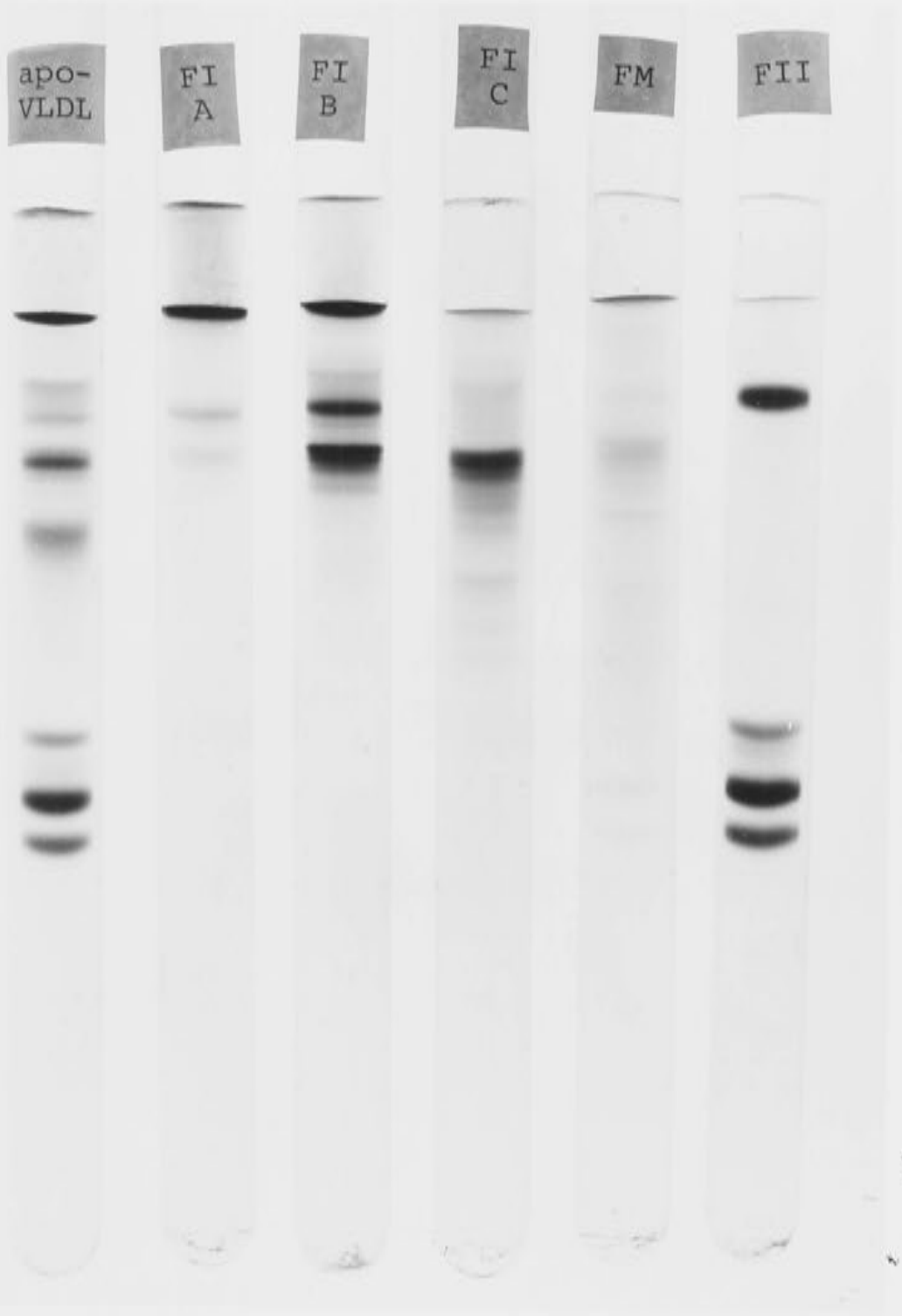


Figure 3.2. Polyacrylamide gel electrophoresis (using the system of Kane, 1973) of proteins from each fraction collected when apo-VLDL was separated by filtration on a sephadex G-200 superfine as described in section 3.2.2 and figure 3.1. 80 µg protein (in 6M urea) was loaded onto each gel. Gels were stained (with amido black) and destained as described in section 2.7.

Using the glass bead column described for the separation of apolipoproteins of VLDL, excellent resolution of the B and C apolipoproteins was obtained. However, a clear separation of the B and E apolipoproteins was not achieved. These observations are similar to those of Herbert *et al.* (1973) who originally described the column procedure.

3.2.3 Use of gel filtration techniques in B apolipoprotein specific activity determinations

Although protein recoveries from gel filtration chromatography are usually high, small quantities of apolipoprotein are often difficult to separate because of problems in detecting low concentrations of separated proteins in the relatively large elution volumes.

Sigurdsson *et al.* (1975), in order to determine the specific activities of the B apolipoprotein of VLDL, chromatographed samples of apo-VLDL on sephadex G-150 in the presence of 0.001M SDS. To obtain B apolipoprotein for further analysis, these authors collected a fraction in the maximal portion of the first peak. Amino acid analysis of this fraction was reported to be similar to that of the corresponding fraction when apo-LDL was chromatographed in an identical manner thus suggesting that the apo-VLDL fraction that was isolated contained B apolipoprotein only. In these studies however, the authors were not able to determine the specific activities of the B apolipoprotein of LDL_1 as the low plasma concentration of this lipoprotein was insufficient for routine apolipoprotein separation using gel filtration techniques.

3.2.4 Preparative isolation of the B apolipoprotein using 1,1,3,3-tetramethylurea (TMU)

Kane (1973) has described a technique, employing the organic solvent 1,1,3,3-tetramethylurea (Sigma, U.S.A.) for the selective precipitation of the B apolipoprotein from whole lipoproteins. The use and function of TMU as an organic solvent has been reviewed by Luttringhaus and Dirksen (1964). The TMU technique has recently been applied, with minor modifications, to describe the mean apolipoprotein composition of human VLDL (Kane *et al.*, 1975). With this procedure, the amino acid composition of the TMU insoluble protein of VLDL is similar to that of LDL suggesting that apolipoprotein E is not co-precipitated with the B apolipoprotein. The TMU technique, which is simple in that lipoprotein delipidation is not necessary to obtain apolipoprotein separation, requires lipoprotein solutions of approximately 600 µg/ml in buffers of ionic strength $\geqslant 0.05$ and in the pH range 6-9. Technical problems may be encountered, however, in this procedure as the separation of the TMU soluble phase is difficult without some disturbance of the B apolipoprotein-lipid pellicle.

The TMU technique has been employed in a recent report of Faergeman *et al.* (1975) where rat VLDL B apolipoprotein specific activities were determined by calculating differences between protein concentrations and radioactivity present in whole VLDL and TMU soluble VLDL protein fractions. Direct estimations of the specific activities of TMU insoluble protein carried out during the course of the project will be described in section 3.2.5.

3.2.5 Experimental: separation of VLDL and LDL apolipoproteins using 5mM NH_4HCO_3, pH 8.0

A procedure for isolating the B apolipoprotein of VLDL and LDL has been developed for the metabolic studies described in this thesis. This method depends on the relative insolubility of the B apolipoprotein in low ionic strength buffers (5mM NH_4HCO_3, pH 8.0). The differential solubility of apolipoproteins in aqueous buffers has been exploited by other investigators in determining lipoprotein apolipoprotein compositions (Lee and Alaupovic, 1974).

NH_4HCO_3 separation procedure:

Following dialysis against 5mM NH_4HCO_3, pH 8.0, 0.5 - 1.0 mg of lipoprotein-protein (either VLDL or LDL) is delipidated as previously described (section 2.5). The apolipoprotein is then incubated, at room temperature, for at least 12 hours in 0.5 ml of 5mM NH_4HCO_3, pH 8.0. The insoluble apolipoprotein is precipitated from the NH_4HCO_3 soluble apolipoproteins by centrifugation (60,000 × g.min). After removal of the soluble phase, the insoluble pellet is resuspended in a further 0.5 ml NH_4HCO_3 and then reprecipitated by centrifugation. The pellet is dried, after removal of the wash, and is then available for further analysis.

Validation of the NH_4HCO_3 separation procedure:

To test the validity of this separation procedure, the amino acid compositions of the NH_4HCO_3 insoluble apolipoprotein of samples of VLDL, LDL_1, and LDL_2 were determined (Table 3.1). A comparison of these values suggested that an identical protein had been isolated from

each lipoprotein fraction by the insolubility procedure. The composition of this fraction was similar to published reports of the amino acid composition of the B apolipoprotein of LDL (Gotto *et al.*, 1972) and TMU insoluble protein from VLDL and LDL (Kane *et al.*, 1975) as shown in Table 3.1.

The homogeneity of the NH_4HCO_3 insoluble fraction was also tested by solubilising it in 0.5M tris-Hcl, 0.2M SDS, pH 8.2 and then applying a sample to 10% polyacrylamide-SDS gels (Section 2.7). The insoluble fraction was found to be relatively free of proteins that could enter the main gel (Figure 3.3) confirming that this fraction contained predominantly the B apolipoprotein.

This method of obtaining the B apolipoprotein apparently free of other apolipoproteins was used to isolate the B apolipoprotein of VLDL and LDL fractions for specific activity determinations in most of the studies described in the later chapters of this thesis. After dissolving the NH_4HCO_3 insoluble fraction in 0.1M NaOH, the protein (section 2.1) and radioactivity were measured and so the specific activity of the B apolipoprotein was calculated.

To ascertain the practical efficacy of the NH_4HCO_3 technique a comparison of specific activity-time curves of VLDL B apolipoprotein from study 8 (Chapter 5) determined using the procedure outlined above or the TMU procedure is shown in Figure 3.4. The specific activities using the TMU procedure were estimated using the same procedure described by Kane *et al.* (1975) for apolipoprotein separation but with subsequent lipid removal from the lipid-protein pellicle (plus precipitate) by the

TABLE 3.1

THE AMINO ACID COMPOSITION OF THE NH_4HCO_3 INSOLUBLE FRACTION OF VLDL, LDL_1 AND LDL_2.

The insoluble fractions were obtained as described in section 3.2.5. The amino composition of these fractions are compared to previously documented values for the composition of the B apolipoprotein isolated from apo-LDL chromatographically[1] and the TMU insoluble fraction of VLDL and LDL.[2] Values are expressed as μmoles/1000 μmoles amino acid recovered.

	NH_4HCO_3 Insoluble Fractions			B Apolipoprotein — Documented		
	VLDL	LDL_1	LDL_2	apo-B[1]	VLDL[2]	LDL[2]
lysine	76	75	75	74	82	82
histidine	21	23	26	21	23	25
arginine	30	30	32	30	35	33
aspartate	107	106	104	108	102	102
threonine	70	70	65	67	67	70
serine	97	93	94	87	97	100
glutamate	119	135	121	122	133	130
proline	62	43	47	48	39	39
glycine	53	55	54	49	53	50
alanine	66	63	65	63	63	59
valine	52	50	54	51	55	53
methionine	10	10	10	16	-	-
isoleucine	51	50	52	54	53	54
leucine	114	120	120	120	115	116
tyrosine	26	30	33	34	35	35
phenyl alanine	45	46	49	51	49	50

[1] Gotto *et al.* (1972) [2] Kane *et al.* (1975)

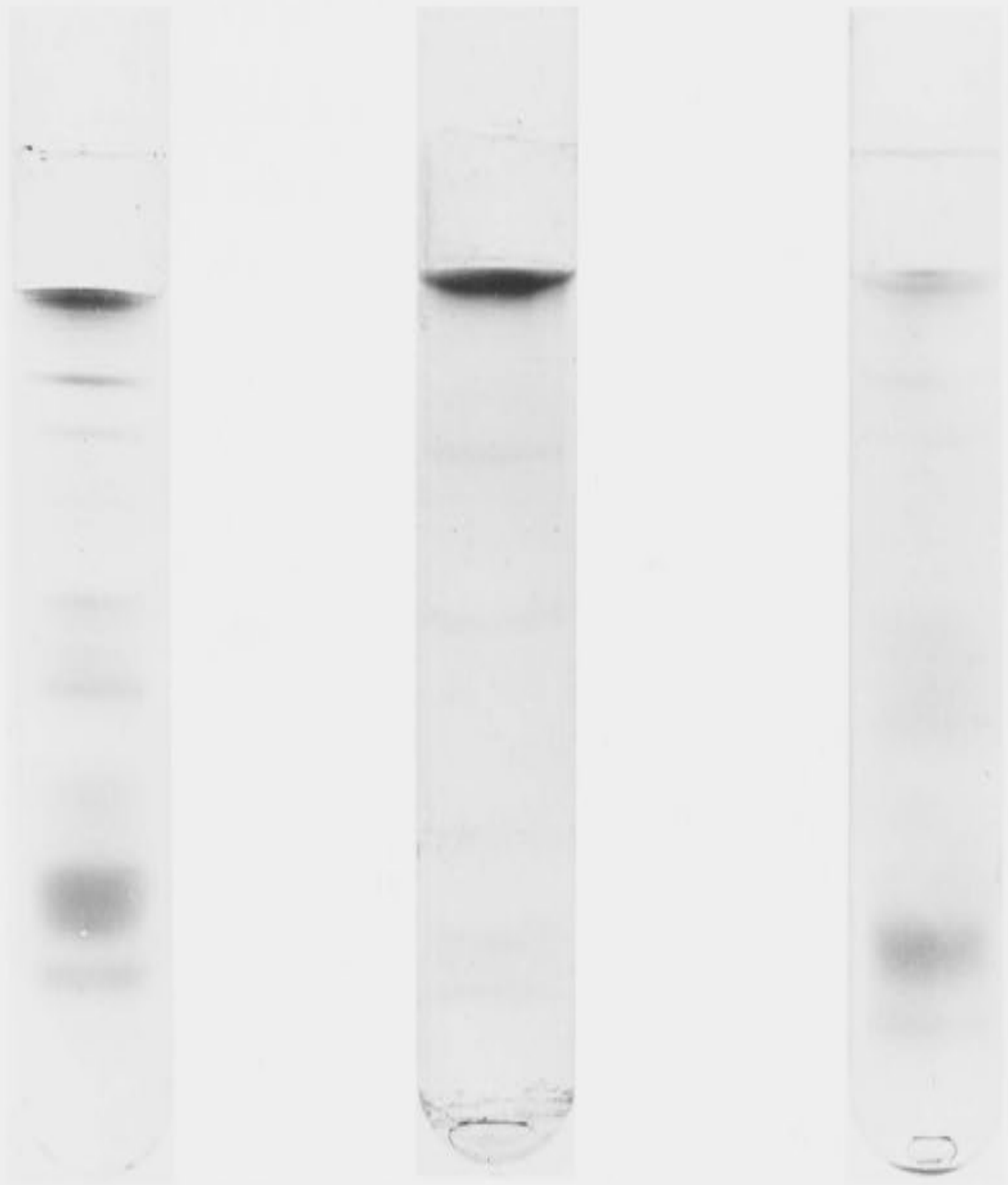


Figure 3.3. Polyacrylamide gel electrophoresis (10% acrylamide SDS gels using the system of Weber, 1975) of proteins of whole and 5mM NH_4HCO_3 soluble and insoluble fractions of apo-VLDL (see section 3.2.5). Approximately 100 µg protein was incubated for 12 hours in 0.1% (w/v) SDS (as described by Weber) and then loaded onto gels for electrophoresis. Following electrophoresis, gels were stained (with coomassie blue) and destained as described in section 2.7.

usual delipidation procedures, thereby leaving the TMU insoluble protein for specific activity determination as was described for NH_4HCO_3 insoluble protein.

As can be seen in Figure 3.4 the specific activity time curves are virtually identical using either procedure. Importantly, the bi-exponential nature of this curve is observed using both methods.

3.2.6 Conclusions

A number of procedures have been described for the isolation and/or quantitation of the B apolipoprotein of plasma lipoproteins. As the aims of the studies in the subsequent chapters were to investigate the metabolic apolipoprotein interrelationships mainly between low and very low density lipoproteins (which have in common the B apolipo-protein) a technique was required for the determination of the specific activity of this apolipoprotein. Although gel chromatographic techniques could have been employed, they required larger masses of lipoprotein-protein that, in practice, were not always available (particularly from LDL_1). Secondly, good resolution between the B and E apolipoproteins by these methods has not been definitively confirmed.

Both TMU and HN_4HCO_3 procedures depend on the relative insolubility of the B apolipoprotein and both require delipidation (either before or after apolipoprotein separation) to accurately determine the specific activity by direct measurement (which is preferable to the difference method used by Faergeman *et al.*, 1975). For the purposes of the present studies, the NH_4HCO_3 procedure was employed as the method was less critically dependent on sample concentration and was found to be technically easier in the separation of soluble and insoluble apolipo-

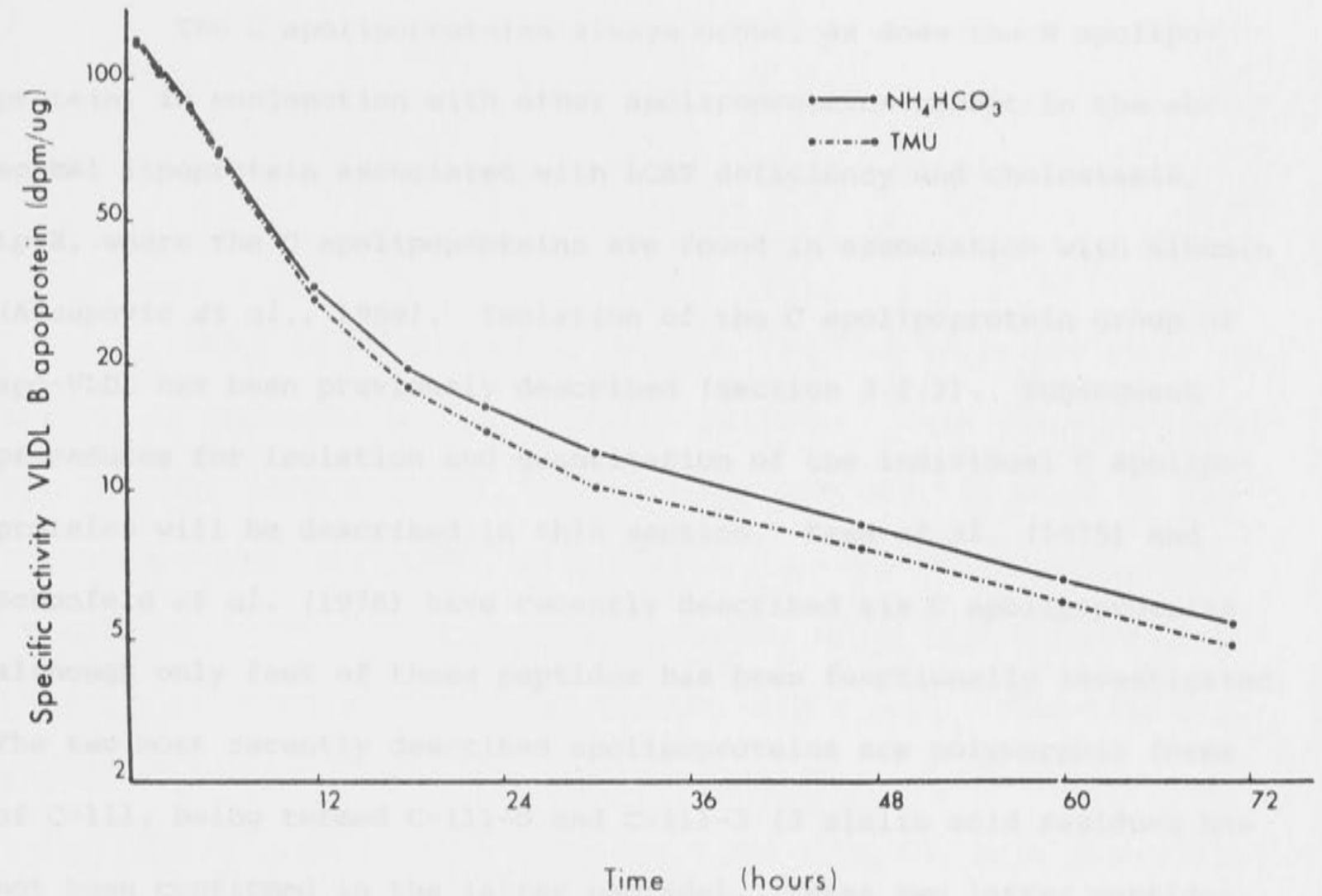


Figure 3.4 A comparison of the specific activities of VLDL B apolipoprotein determined either by the NH_4HCO_3 or TMU procedures on VLDL samples from study # 8 (described in chapter 5). The bi-exponential nature of the curve is observed with both techniques. Both apolipoprotein separation procedures are described in section 3.2.5.

protein phases.

3.3 ISOLATION AND QUANTITATION OF THE INDIVIDUAL C APOLIPOPROTEINS

The C apolipoproteins always occur, as does the B apolipoprotein, in conjunction with other apolipoproteins except in the abnormal lipoprotein associated with LCAT deficiency and cholestasis, Lp-X, where the C apolipoproteins are found in association with albumin (Alaupovic *et al.*, 1969). Isolation of the C apolipoprotein group of apo-VLDL has been previously described (section 3.2.2). Subsequent procedures for isolation and quantitation of the individual C apolipoproteins will be described in this section. Kane *et al.* (1975) and Schonfeld *et al.* (1976) have recently described six C apolipoproteins although only four of these peptides has been functionally investigated. The two most recently described apolipoproteins are polymorphic forms of C-111, being termed C-111-0 and C-111-3 (3 sialic acid residues has not been confirmed in the latter peptide). These two latter peptides are present in low, but not necessarily insignificant amounts in VLDL. C-111-3 tends to lose some of its sialic acid residues upon being delipidated and so is not always seen in this form (Kane *et al.*, 1975).

The C apolipoproteins may be separated, on the basis of charge, by ion-exchange chromatography or polyacrylamide gel electrophoresis (Shore and Shore, 1969; Brown *et al.*, 1969, 1970a, 1970b; Albers and Scanu, 1971; Kane, 1973; Herbert *et al.*, 1973). All of these methods involve the use of urea as a dissociating agent.

3.3.1 Experimental: isolation of the individual C apolipoproteins using DEAE cellulose

The small molecular weight C apolipoproteins were isolated from apo-VLDL by gel filtration chromatography as described in section 3.2.2. Preparative separation of the individual C apolipoproteins was achieved by ion exchange chromatography on DEAE cellulose columns (1.5 × 90 cm) using the procedure described by Herbert *et al.* (1973).

Prior to packing these columns, the DEAE cellulose resin was washed, in sequence, in the following solutions: 0.5M HCl; 0.01M tris-HCl, pH 8.0; 0.25M tris-HCl, pH 8.0; 0.01M tris-HCl, 6M urea, pH 8.0. The resin column was poured in the latter buffer. These columns were loaded with 30-80 mg of C apolipoprotein (in 0.01M tris-HCl, 6M urea, pH 8.0), after which progressive elution with a gradient of tris-HCl (0.01M - 0.25M) was commenced. The gradient (linear) was established using 1 litre reservoirs of 0.01M and 0.25M tris-HCl, both containing 6M urea and at pH 8.0. This tris gradient was found to achieve a better resolution of the apolipoproteins than the NaCl gradient of Brown *et al.* (1969).

The elution profile of a C apolipoprotein separation from DEAE cellulose is shown in Figure 3.5. Recoveries of protein with this system varied from 47-73%. The individual proteins peaks were collected and exhaustively dialysed against 5mM NH_4HCO_3, pH 8.0 and then lypophylised. The dried protein residue was resolubilised in various solutes as described by Herbert *et al.* (1973). Although the C apolipoprotein complex (i.e. F2 of the apo-VLDL separation by gel filtration) was soluble in 0.05M NH_4HCO_3, pH 8.0, some of the isolated apolipoproteins

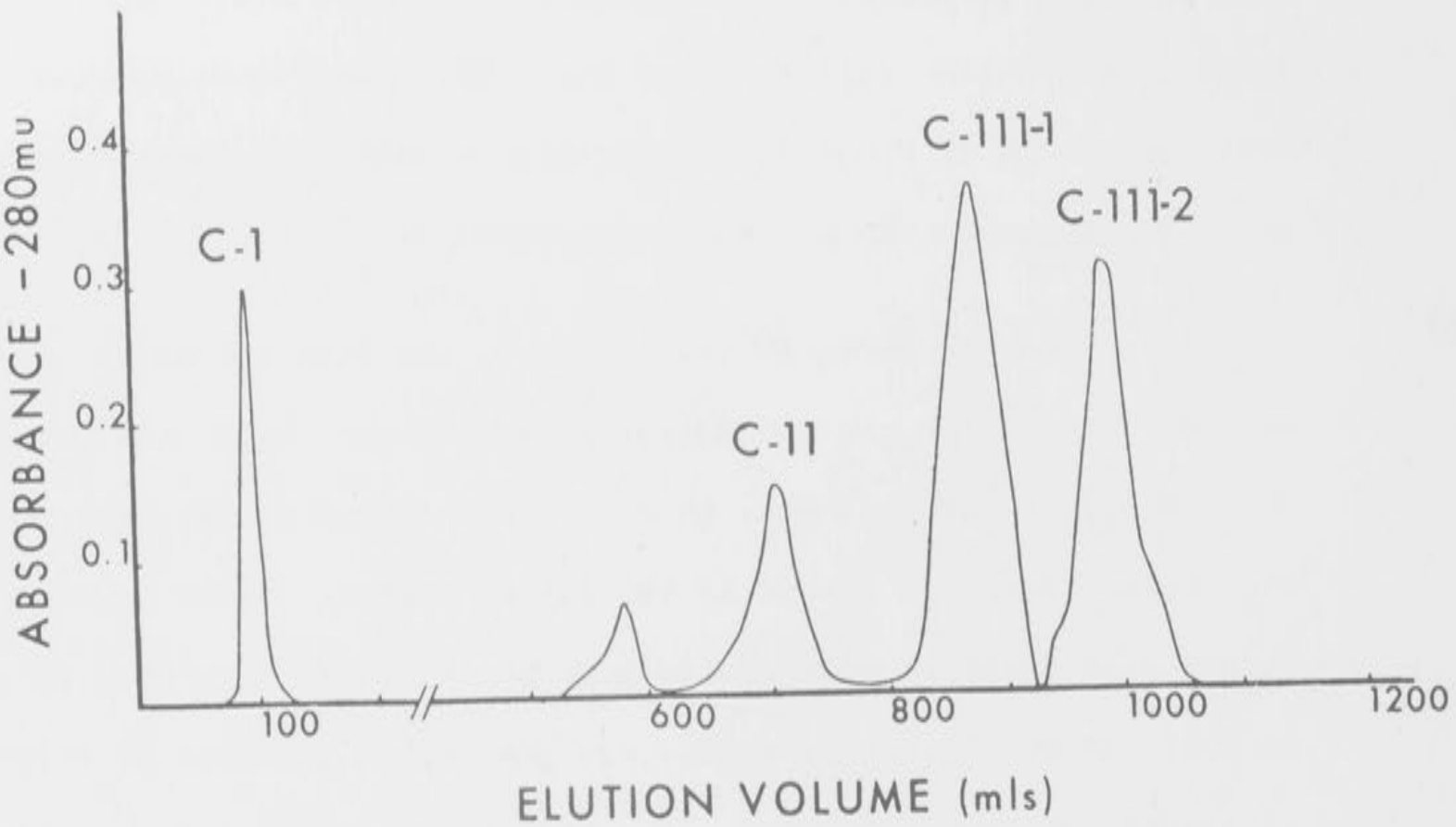


Figure 3.5 Elution profile obtained when 86 ug of C apolipoprotein were applied to DEAE cellulose (column: 1.5 × 90 cm) and eluted with a tris gradient of 0.01 - 0.25M at pH 8.2. The separation was performed in the presence of 6M urea at 2°C. The flow rate of the column was 12 ml/hour. The identity of the protein collected in each peak was confirmed as described in section 3.3.2.

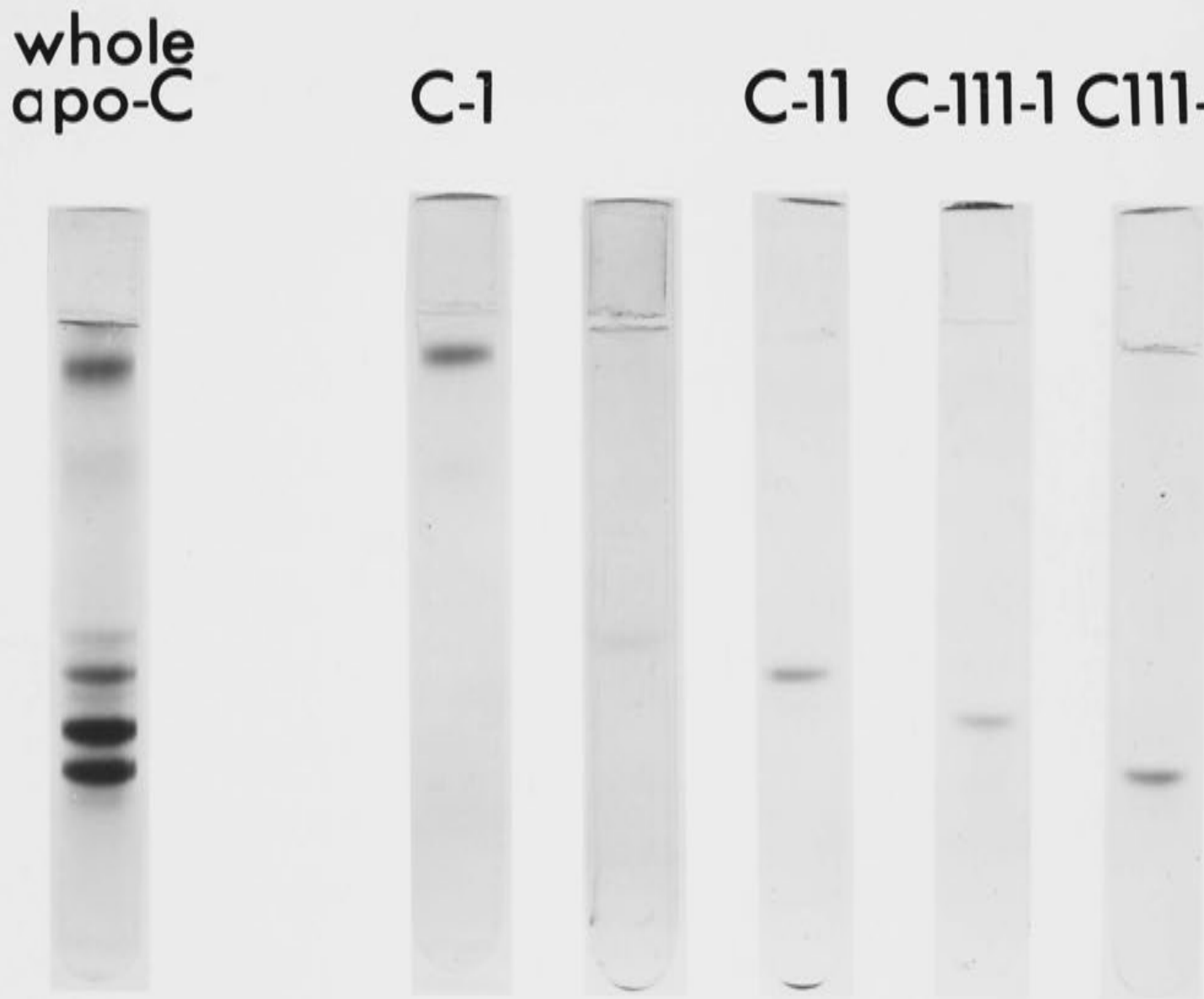


Figure 3.6. Polyacrylamide gel electrophoresis (using the system of Kane, 1973) of protein from each fraction of the separation of C apolipoproteins on DEAE cellulose as described in section 3.3.1 and figure 3.5. 40 µg of each isolated C apolipoprotein were loaded in the presence of 6M urea. The unlabelled gel was that of the unlabelled peak in figure 3.5 and probably represents a carbamylated form of C-1 as suggested by amino acid analysis. Gels were stained (with amido black) and destained as described in section 2.7.

were not. C-1 was soluble in 0.1M acetic acid, C-11 in 0.1M NH_4OH, and C-111 in 0.05M NH_4HCO_3, pH 8.0. Following solubilisation, the proteins were electrophoresed on urea-polyacrylamide gels (Figure 3.6) and shown to be single protein bands. The identity of the apolipoproteins was confirmed by a variety of techniques.

3.3.2 Experimental: identification of the isolated C apolipoproteins

The total amino acid composition of each isolated protein, as shown in Table 3.2, was determined by the procedure described in section 2.8. These values when compared to those of Herbert *et al.* (1973) confirm that similar proteins were isolated. Although not included in Table 3.2, the protein of unidentified peak in Figure 3.5 and gel of Figure 3.6 was similar in amino acid composition to C-1 except for a reduced lysine content suggesting that this protein was a carbamylated form of C-1 apolipoprotein. This band was not routinely found on other separations. Although not shown in Figure 3.5, a peak similar to C-X described by Herbert *et al.* (1973) was observed in some separations but insufficient protein was available for further analysis.

The carboxy terminal amino acid of the major C apolipoproteins was determined after carboxy-peptidase A digestion and hydrazinolysis (as described in section 2.8). As expected, the terminal amino acids released with incubations of C-1, C-11, C-111-1 and C-111-2 were serine, glutamic acid, alamine and alamine respectively.

The ability of these peptides to potentiate the activity of milk lipoprotein lipase preparations on an artificial triglyceride emulsion (section 2.9) was ascertained as shown in Table 3.3. 30µg of

TABLE 3.2

THE AMINO ACID COMPOSITION OF THE MAJOR C APOLIPOPROTEINS OF VLDL.

The C apolipoproteins were isolated by ion-exchange chromatography as described in section 3.3.1. The amino acid compositions shown [a] are compared to those of Herbert *et al.* (1973) [b]. Values[1] are expressed as μmoles/1000 μmoles amino acid recovered.

	C-1		C-11		C-111-1		C-111-2	
	[a]	[b]	[a]	[b]	[a]	[b]	[a]	[b]
lysine	143	156	79	77	61	75	78	67
histidine	6	0	0	0	16	12	16	11
arginine	23	54	18	16	30	25	25	25
aspartate	76	96	76	70	94	95	98	96
threonine	41	48	93	108	64	64	63	69
serine	130	118	111	115	136	140	130	141
glutamate	138	165	169	178	137	136	131	136
proline	16	13	53	53	29	28	39	29
glycine	45	22	49	30	51	42	56	41
alanine	98	56	86	83	126	133	126	129
valine	32	37	57	50	86	78	77	75
methionine	6	16	23	22	19	24	20	24
isoleucine	38	50	10	9	2	0	0	0
leucine	118	111	93	102	66	70	69	71
tyrosine	0	1	51	61	27	27	22	27
phenyl alanine	41	54	32	26	56	55	53	54

[1] The values obtained in this study are the mean of two observations.

TABLE 3.3

C APOLIPOPROTEIN ACTIVATION

OF LIPOPROTEIN LIPASE

A comparison of the activation of milk lipoprotein lipase (prepared as described in section 2.9) by each of the major C apolipoproteins. Lipase activity was assayed, as described in section 2.8, in the absence of C apolipoproteins (control) or following the addition of 30 µg of isolated C apolipoprotein. Values for activation (mean ± 1 standard deviation of four observations) are expressed in relation to the control activity.

C apolipoprotein addition	ACTIVATION[1]
C-1	0.46 ± 0.21
C-11	17.94 ± 2.16
C-111-1	1.27 ± 0.09
C-111-11	1.94 ± 0.42

[1] Activation = activity in presence of C apolipoprotein/control.

isolated apolipoprotein was added to each incubation (volume of 1 ml). C-11 apolipoprotein only was found to markedly stimulate the enzyme.

3.3.3 Experimental: quantitation of the individual C apolipoproteins by densitometry

Even though the individual C apolipoproteins may be isolated by ion exchange chromatography, this method is not suitable for the quantitation of small amounts of these proteins because of the low (and therefore non-quantitative) recoveries using these procedures.

Polyacrylamide gel electrophoresis has been widely used to study microgram quantities of proteins. With this procedure the quantity of protein in the stained gel band may be ascertained by estimating the amount of stain taken up by the protein in the respective band (Fishbein, 1972). This method requires that the relationship between dye and protein concentration obeys Beers Law. The concentration of dye in the band may be estimated directly by extraction of the dye from the gel (Johns, 1967) or by densitometry. As amido black stains protein metachromatically, it is of particular importance to establish the relationship between dye uptake and protein concentration when this stain is employed (Gorovsky *et al.*, 1970).

Having preparatively isolated the major C apolipoproteins it was possible to establish the relationship between apolipoprotein concentration and dye uptake (as determined by densitometry) for each C apolipoprotein on polyacrylamide gels.

As the Lowry method (Lowry *et al.*, 1951, section 2.1) for the estimation of protein mass relies partly on the presence of tyrosine

residues, it would be expected that the C-1 apolipoprotein in particular (which is almost devoid of tyrosine residues) would be underestimated by this procedure. From freeze-dried samples of isolated C apolipoproteins, solutions were prepared containing a known mass, determined gravimetrically, of each C apolipoprotein. A comparison of the actual protein concentration to that determined by the Lowry technique (using albumin as standard) is shown in Table 3.4. From this table it can be seen that the mass of 1 mg of each C apolipoprotein, when estimated colourimetrically, gave a range of values indicating that the Lowry technique may not be used for C apolipoprotein quantitation if albumin is used as a standard.

Known amounts (determined gravimetrically) of the individual C apolipoproteins were electrophoresed on urea-polyacrylamide gels using the system of Kane (1973). Following electrophoresis (as described in section 2.7) the gels were stained in 0.1% (w/v) amido black in 7% (v/v) acetic acid for precisely 60 minutes. After staining, all gels were destained by diffusion under identical conditions for 72 hours by continuous replacement of 7% acetic acid.

Gels were scanned at 601 mμ using a Varian Techtron gel scanner fitted with a glass cuvette of the bucket type. Scanning speed was 10 mm/min with a chart speed of 30 cm/min. Slit width was set at 0.2 mm. The protein mass applied to the gel was related to the area of its respective peak on the scan record. Area was determined by triangulation or planimetry.

A linear concentration was established between protein mass (over the range 5-60 ug) and peak area for each of the four peptides

TABLE 3.4

ERRORS IN THE COLOURIMETRIC ESTIMATION OF C APOLIPOPROTEIN MASS WHEN USING BOVINE ALBUMIN AS STANDARD

This table compares, for each C apolipoprotein, the mass estimated colourimetrically to that determined gravimetrically. The colourimetric estimation procedure[1] [Lowry *et al.*, (1951] employed bovine albumin as the protein standard. Values (mean ± 1 standard deviation from four observations) are expressed as the 'estimated' mass of 1 mg (gravimetric) of each C apolipoprotein.

C APOLIPOPROTEIN	'ESTIMATED' MASS mg
C-1	0.509 ± 0.014
C-11	0.829 ± 0.049
C-111-1	1.101 ± 0.050
C-111-11	1.116 ± 0.021

[1] section 2.1.

examined, as shown in Figure 3.7. This relationship applied, though with differing slopes when the proteins were loaded in urea or TMU.

Having established linearity for each C peptide within the range described, the mass of individual C apolipoproteins from test samples could be determinations by densitometric analysis of gels after electrophoresis. A standard mixture of known amounts of each of the four C apolipoproteins was prepared and applied at 3 different concentrations, to separate gels for each gel run. The clear separation of C apolipoproteins on urea-polyacrylamide gels resulted in a densitometric scan having well resolved peaks as shown in Figure 3.8.

In using this technique, it has been recognised, as have other investigators (Kane *et al.*, 1975; Schonfeld *et al.*, 1976) that the linear expression of the area-protein relationship is specific for each C apolipoprotein.

3.3.4 Documented methods for the quantitation of C apolipoproteins by densitometry

By densitometric scanning of polyacrylamide gels, Kane *et al.* (1975) have quantitated the TMU soluble proteins of VLDL, after having established the linearity of the area protein relationship for each peptide. Using this technique, these authors have described the mean apolipoprotein composition of VLDL as well as percentage variations in the apolipoprotein content of VLDL particles in various size ranges. Schonfeld *et al.* (1976), also using similar procedures, but applying apo-VLDL in 8M urea to 9% gels, have demonstrated variations in VLDL C apolipoprotein content induced by high carbohydrate diets. These

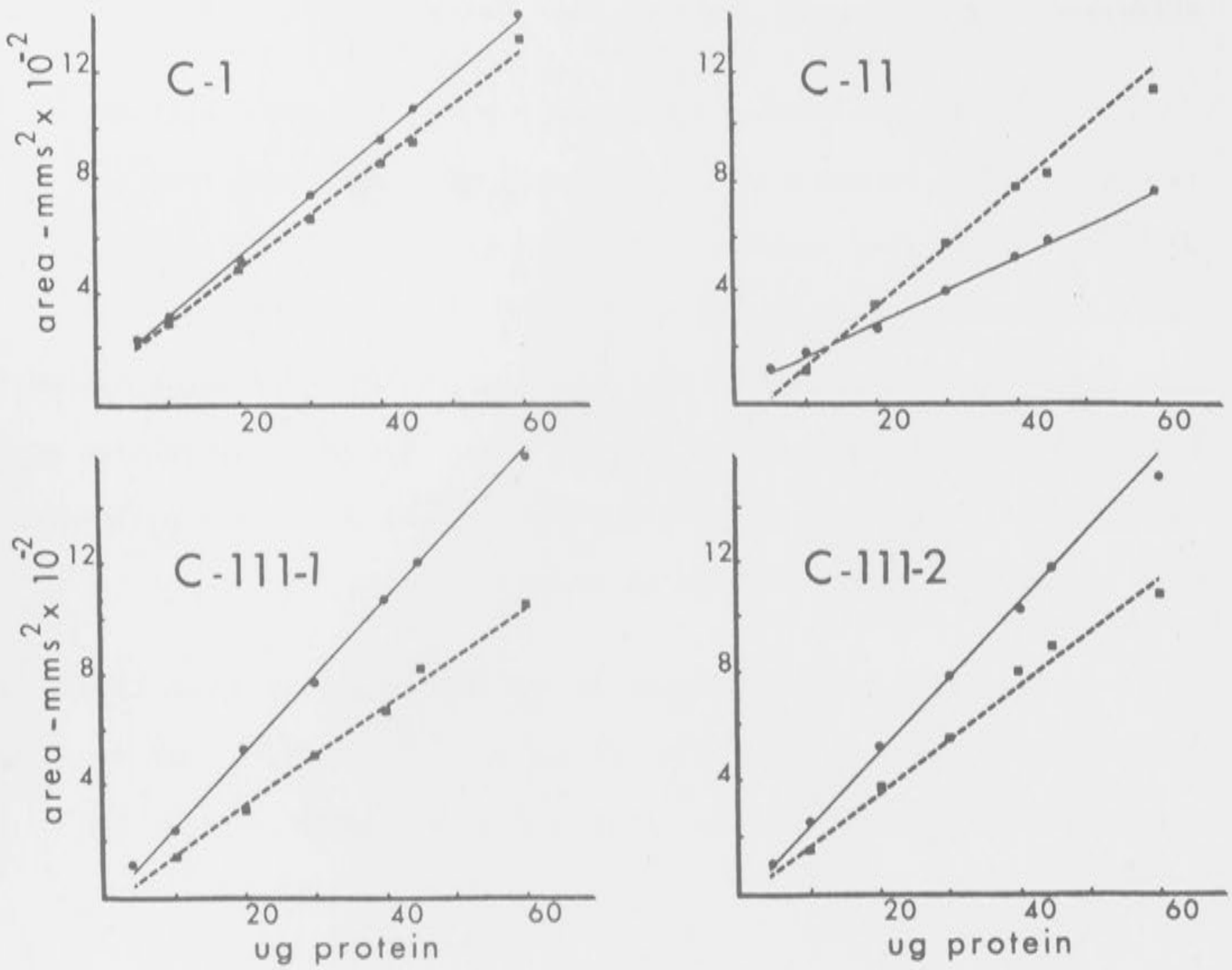


Figure 3.7 Relationship between area under the peak (from the densitometric scan) and protein mass for the major C apolipoproteins. Apolipoprotein mass was determined gravimetrically while area was measured by planimetry. Gels were scanned as described in section 3.3.3. The C apolipoproteins were loaded onto the gels in 6M urea (———) or 4.2M TMU (-----).

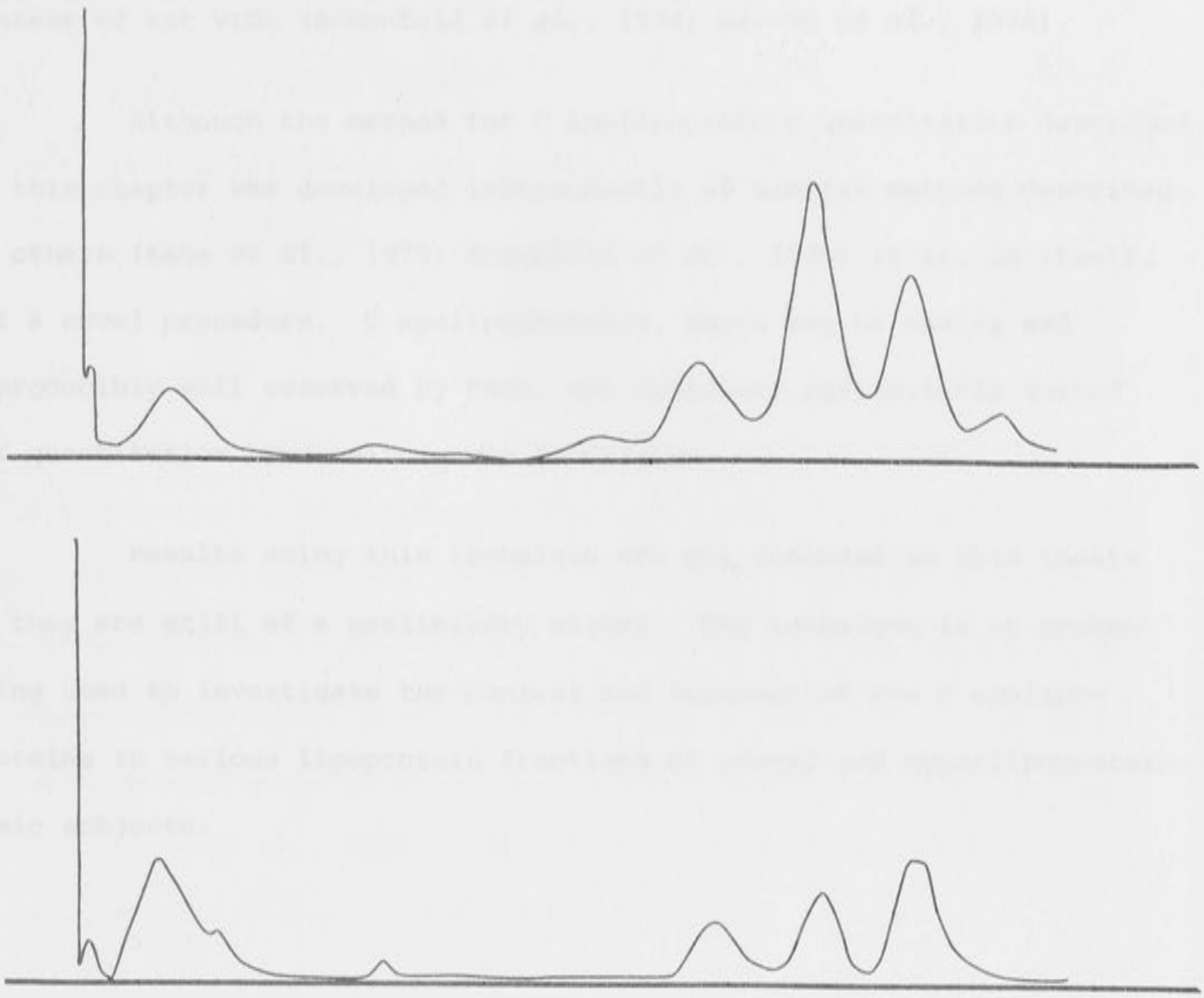


Figure 3.8 Typical densitometric scan recordings of gels electrophoresed with human apo-VLDL. The upper scan represents that of a gel loaded with apo-VLDL in 6M urea while the lower scan illustrates that obtained from a gel loaded with TMU soluble proteins of VLDL. The scan records are that of main gel only (not the stacking gel).

methods have also been used to describe changes in the apolipoprotein content of rat VLDL (Schonfeld *et al.*, 1974; Bar-On *et al.*, 1974).

Although the method for C apolipoprotein quantitation described in this chapter was developed independently of similar methods described by others (Kane *et al.*, 1975; Schonfeld *et al.*, 1976) it is, in itself, not a novel procedure. C apolipoproteins, which may be easily and reproducibly well reserved by PAGE, are obviously particularly suited for quantitation by densitometric techniques.

Results using this technique are not included in this thesis as they are still of a preliminary nature. The technique is at present being used to investigate the content and turnover of the C apolipoproteins in various lipoprotein fractions of normal and hyperlipoproteinaemic subjects.

CHAPTER 4

THE *IN VITRO* CONVERSION OF VLDL TO LDL BY LIPOLYTIC ENZYMES

4.1 INTRODUCTION

The transport of newly synthesised lipid of endogenous, or non dietary origin, is mediated by the VLDL class (Havel, 1961; Barter, 1974; Clifton-Bligh *et al.*, 1974). Of the protein constituents of VLDL of hepatic origin, the B apolipoprotein is secreted together with the lipids (Isselbacher *et al.*, Bar-On *et al.*, 1973) whereas the small molecular weight C apolipoproteins appear to be derived from circulating HDL (Havel *et al.*, 1973b). The origin of apolipoprotein E of VLDL has not been determined (section 1.2.3).

The clearance of triglyceride from the plasma into tissues is dependent upon the activity of the enzyme lipoprotein lipase (glyceryl-ester hydrolase, E.C. 3.1.1.3) (Robinson, 1970). The removal of triglyceride from VLDL is associated with the loss of surface lipids and the transfer of C apolipoproteins to HDL. This results in the formation of a population of remnant lipoproteins in which the relative proportions of B apolipoprotein and cholesteryl ester are increased (Shore and Shore, 1962; LaRosa *et al.*, 1970; Eisenberg *et al.*, 1973; Bierman *et al.*, 1973; Eisenberg and Rachmilewitz, 1975). The fate of these remnant particles may differ in various species. For instance, it is probable that in the rat, the bulk of VLDL B apolipoprotein is

removed by the liver (Fidge and Poulis, 1975; Faegerman *et al.*, 1975) whereas in man, the VLDL B apolipoprotein may be largely retained in the plasma within the LDL class. During infusion of heparin into man, virtually all of the protein that is lost from VLDL is found in the smaller lipoproteins (Homma and Nestel, 1975).

The incubation of VLDL with plasma obtained after the injection of heparin leads to a redistribution of lipid and protein among the plasma lipoproteins resembling that seen *in vivo* (Shore and Shore, 1962; Nichols *et al.*, 1968; Eisenberg *et al.*, 1973). This chapter describes a series of experiments in which the transfer of the B and C apolipoproteins from lipoproteins of very low and intermediate density ranges to lipoproteins of higher density has been studied during incubation of various radioiodinated lipoprotein fractions with post-heparin plasma (whole) or post-heparin plasma with lipolytic activity of hepatic origin only. By measuring the specific activities of the B apolipoprotein, a better understanding of the relationships between these lipoproteins was possible. Also, the relevance of hepatic or extra-hepatic lipases to these conversions was investigated.

4.2 METHODS

4.2.1 Collection of plasma for incubation studies

Plasma used in the incubations was obtained from fasted healthy young subjects before and after the administration of intravenous heparin. The plasma obtained prior to heparin was designated HEP-0. Ten units of heparin per kg body weight was then rapidly administered intravenously and blood was collected in heparinised tubes after

10 min (plasma referred to as HEP-10). Three hours later a further 100 units heparin/kg body weight was injected and blood collected 30 minutes later (plasma referred to as HEP-100). This regime provided samples possessing negligible, low or high post heparin lipolytic activity as described by Boberg (1972). All plasma was maintained at 4°C and used as soon as possible after collection.

4.2.2 Incubation procedures

Four types of experiments (A, B, C and D) are reported in this chapter. Each experiment was carried out at least twice, though single experiments only of type A and B are shown.

Procedures for lipoprotein isolation, purification and iodination have been previously described (sections 2.2 and 2.3).

Experiments A and B

The labelled substrate was in VLDL s_f 20-400 in experiment A and in VLDL s_f 100-400 in experiment B. Incubations were performed at 37°C in 3 flasks containing 50 ml of either HEP-0, HEP-10 or HEP-100 plasma. The labelled substrate (approximately 1 mg lipoprotein - protein) was added at zero time and 10 ml aliquots of incubation medium were removed at 5, 10, 15 and 30 min for lipoprotein analysis. Further lipolytic activity in the removed aliquot was effectively stopped by immediate immersion in ice. 0-2.1% of the lipolytic activity observed at 37°C was detected at this low temperature (0°C). The addition of inhibitors of lipolytic activity, e.g. NaCl, paroxon (gift from Dr G. Schrader, Bayer, Germany) were also tested but the inhibitory capacity of these agents was no greater than the chilling procedure.

Aliquots were also taken during incubation for FFA determination (section 2.1).

Experiment C

This study was designed to compare the rate of transfer of labelled B apolipoprotein from three lipoprotein fractions, namely, s_f 100-400, s_f 20-50 and s_f 12-20 (LDL_1) into the LDL_2 (1.019-1.063 g/ml) density range. The protocol was devised to provide equal concentrations of all lipoproteins in each incubation system by the addition of appropriate amounts of each of the 3 subfractions though each flask contained only one labelled subfraction. Incubations were carried out as above but aliquots of the incubation medium were removed at 2, 15 and 30 min.

Experiment D

Lipoprotein lipase of extra-hepatic origin is inhibited in the presence of 1M NaCl or protamine sulphate (Korn, 1955; LaRosa *et al.*, 1972). The relative importance of lipases or hepatic or extra-hepatic origin in the catabolism of VLDL to higher density lipoproteins was studied, *in vitro*, by incubating VLDL or LDL_1 fractions with post-heparin plasma in the presence or absence of 1M NaCl. The concentration of NaCl was adjusted to 1M by the addition of solid NaCl 5 min prior to the commencement of incubation. The radioiodinated fractions added were either large VLDL particles (s_f 100-400) or small VLDL remnants (s_f 12-50). These experiments were of similar design to those of experiment C so that total lipoprotein concentrations were identical in each incubation. Aliquots for lipoprotein analysis and FFA determination were

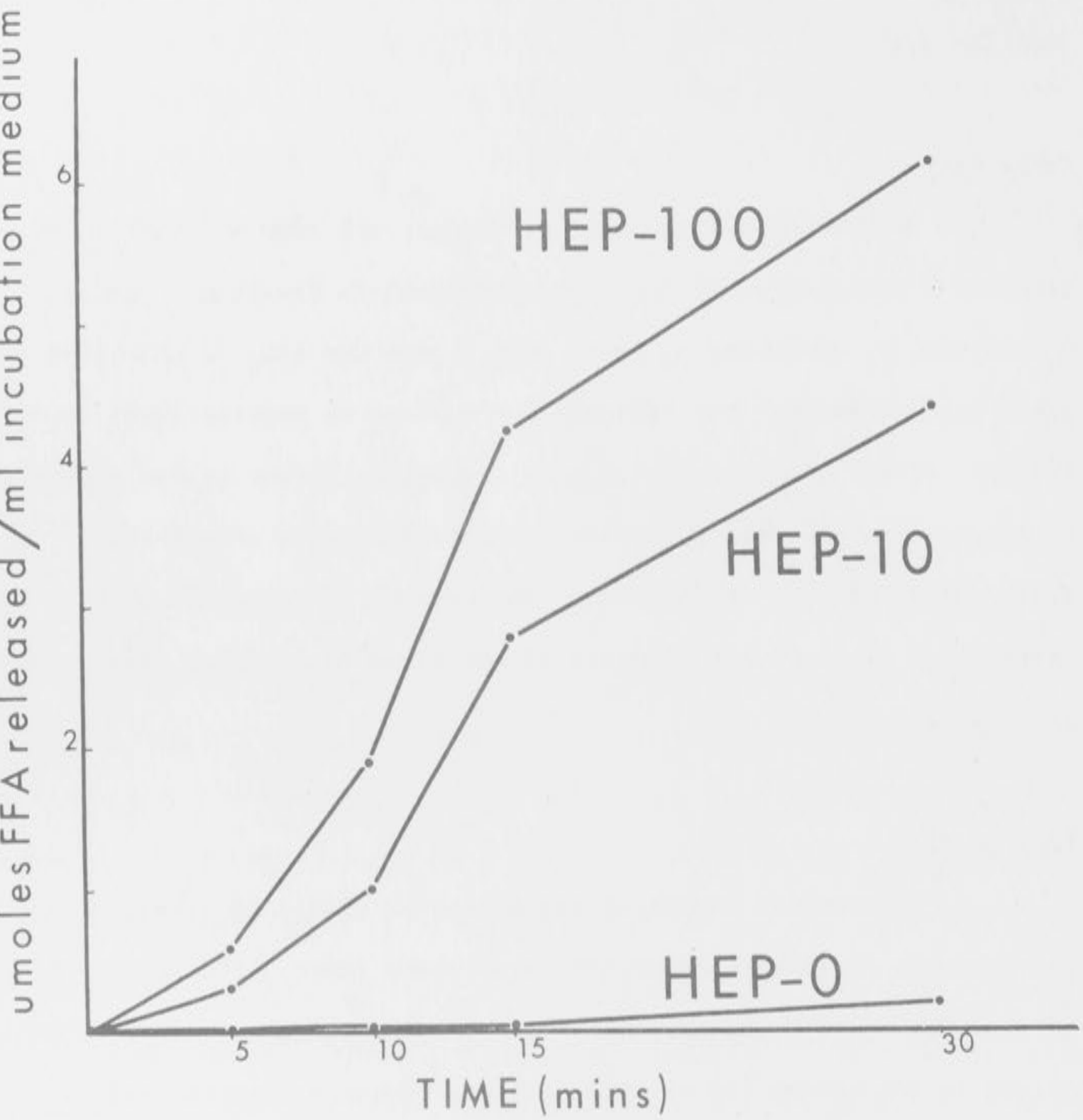


Figure 4.1 Lipolytic activity (as assessed by FFA release) of HEP-0, HEP-10 and HEP-100 plasma (see section 4.2.1). The activity was an expression of the heparin dose administered. Maximal activity was observed in plasma collected following the administration of 100 units heparin/kg (HEP-100) while there was negligible activity in plasma collected prior to heparin administration. (Heparin was obtained from Commonwealth Serum Labs., Melbourne, Aust.).

taken at 5 and 30 min. For sake of uniformity, prior to ultracentrifugation, the concentration of NaCl was adjusted to 1M (if necessary) in all aliquots and the salt density to 1.21 g/ml by the addition of solid KBr. In this way, all major apolipoproteins were floated in the first ultracentrifugation. The salt density in the supernatent was then reduced to 1.006 g/ml by dialysis and VLDL, LDL_1, LDL_2 and HDL were isolated at their respective densities as described in section 2.2.

Plasma aliquots (from all experiments) were kept on ice until lipoprotein fractionation was commenced. Lipoproteins were isolated into VLDL, LDL_1, LDL_2 and HDL fractions as previously described (section 2.2). Aliquots of each lipoprotein fraction were assayed for total radioactivity in order to determine the redistribution of radioactivity among the plasma lipoproteins. Appropriate quenching corrections were incorporated for the salt density of the aliquot being counted. Following the purification spin, the lipoprotein fractions (VLDL, LDL_1 and LDL_2 only) were dialysed against 5mM NH_4HCO_3 pH 8.0 and delipidated as described in section 2.5. The apolipoproteins were then separated using the NH_4HCO_3 procedure (section 3.2.5) and the specific activities of the insoluble (B apolipoprotein) and soluble fractions were determined.

4.3 RESULTS

4.3.1 s_f 20-400 labelled fraction in pre- and post-heparin plasma

In experiment A, radioiodinated VLDL (s_f 20-400) was incubated in HEP-0, HEP-10 and HEP-100 plasma. The rate of lipolytic activity in each incubation, as assayed by FFA release, is illustrated in figure 4.1.

When radioiodinated VLDL was incubated in HEP-0 plasma, there was an immediate and substantial loss of radioactivity from the VLDL fraction of which most was recovered in the HDL fraction (Table 4.1). This pattern of redistribution continued though at a very slow rate over the next 30 minutes. By contrast, in the presence of lipolytic activity (HEP-10 and HEP-100 incubations), there was further substantial redistribution of radioactivity with time which appeared to be a function of the rate of lipolysis, being more pronounced in the HEP-100 than in the HEP-10 incubation. Radioactivity was transferred not only into HDL but also into LDL_1 and to a lesser extent into LDL_2.

In Table 4.2 are shown the specific radioactivities of the separated B and soluble apolipoproteins in experiment A. There was an increase in the specific radioactivity of the B apolipoprotein in LDL_1 in HEP-10 and HEP-100 incubations at 30 min and also in LDL_2 in the HEP-100 incubation but no increases were observed in the absence of lipolytic activity. The specific radioactivity of the soluble proteins was increased in LDL_1 at 30 min in both the HEP-10 and HEP-100 incubations. The apparent fall in VLDL soluble apolipoprotein specific radioactivity probably reflected exchange with unlabelled C apolipoproteins of HDL.

4.3.2 S_f 100-400 labelled fraction in pre- and post-heparin plasma

The findings in experiment B were similar to those in experiment A (Tables 4.3 and 4.4), except that there was a greater initial percent transfer of radioactivity to the LDL_1 fraction and a proportionately smaller transfer to HDL in the absence of lipolytic activity. This probably reflected the relative labelling of the B and C apolipoproteins

TABLE 4.2

SPECIFIC ACTIVITIES OF THE B AND C APOLIPOPROTEINS OF THE VLDL, LDL_1 and LDL_2 LIPOPROTEIN FRACTIONS DURING INCUBATION IN EXPERIMENT A

B and soluble apolipoproteins were separated by incubation in 5mM NH_4HCO_3, pH 8.0 as described in section 3.2.5

Plasma Medium	Time (min)	VLDL		LDL_1		LDL_2	
		B	sol	B	sol	B	sol
		counts/min/µg protein					
HEP-0	5	5469	2167	N.D.	N.D.	4.3	134
	10	4325	1997	407	482	4.3	144
	15	5077	1710	475	349	4.4	140
	30	4764	1046	472	497	4.8	156
HEP-10	5	4407	1321	486	284	7.1	180
	10	6018	1131	611	385	7.7	140
	15	6724	1120	589	294	7.7	199
	30	6966	N.D.*	1628	671	9.6	179
HEP-100	5	7750	1690	440	293	10.6	183
	10	7046	1094	501	293	12.5	174
	15	N.D.*	N.D.*	701	374	18.0	176
	30	N.D.*	N.D.*	1771	540	27.2	180

*N.D.: Not determined due to insufficient protein for estimation

TABLE 4.1

REDISTRIBUTION OF RADIOACTIVITY AMONG LIPOPROTEIN FRACTIONS DURING INCUBATION IN EXPERIMENT A

VLDL-^{125}I (s_f 20-400) was incubated in human plasma possessing various activities of post-heparin lipolytic activity (see section 4.2.1). Incubations were performed at 37°C and aliquots taken at various times for lipoprotein separation by ultracentrifugation

Plasma Medium	Time (min)	LIPOPROTEIN FRACTION[1]			
		VLDL	LDL_1	LDL_2	HDL
		% total radioactivity			
HEP-0	5	73.9	6.1	2.5	14.0
	10	73.1	4.2	2.7	16.2
	15	70.3	4.4	2.1	18.6
	30	68.5	4.5	3.0	20.1
HEP-10	5	72.3	3.7	2.3	18.3
	10	66.4	4.1	3.7	21.6
	15	60.7	4.8	3.4	26.6
	30	36.2	15.7	4.9	37.5
HEP-100	5	67.0	5.8	3.2	20.1
	10	56.1	7.0	4.0	28.6
	15	48.2	9.3	5.2	32.0
	30	16.5	31.9	8.9	36.7

[1] VLDL s_f 20-400; LDL_1 s_f 12-20; LDL_2 s_f 0-12; HDL d. 1.063 - 1.21 g/ml

TABLE 4.3

REDISTRIBUTION OF RADIOACTIVITY AMONG LIPOPROTEIN FRACTIONS DURING INCUBATION IN EXPERIMENT B

This experiment was similar to experiment A except the labelled addition was VLDL-^{125}I subfraction s_f 100-400

Plasma Medium	Time (min)	LIPOPROTEIN FRACTION[1]			
		VLDL	LDL_1	LDL_2	HDL
		% total radioactivity			
HEP-0	5	67.9	18.6	4.0	6.4
	10	69.2	14.7	4.1	8.7
	15	69.7	15.6	3.7	8.2
	30	67.3	16.4	4.2	9.0
HEP-10	5	68.5	15.6	5.4	8.0
	10	60.0	19.8	8.2	11.1
	15	55.3	22.7	6.4	11.1
	30	38.5	30.4	12.2	13.0
HEP-100	5	53.3	20.8	10.2	12.1
	10	47.3	26.8	8.2	13.0
	15	40.2	26.9	14.1	13.7
	30	21.6	29.8	26.0	16.8

[1] VLDL s_f 20-400; LDL_1 s_f 12-20; LDL_2 s_f 0-12
HDL d. 1.063 - 1.21 g/ml

TABLE 4.4

SPECIFIC ACTIVITIES OF THE B AND C APOLIPOPROTEINS OF THE VLDL, LDL_1 and LDL_2 LIPOPROTEIN FRACTIONS DURING INCUBATION IN EXPERIMENT B

B and soluble apolipoproteins were separated by incubation in 5mM NH_4HCO_3, pH 8.0 as described in section 3.2.5

Plasma Medium	Time (min)	VLDL		LDL_1		LDL_2	
		B	sol	B	sol	B	sol
		counts/min/µg protein					
HEP-0	5	5489	843	1851	474	19	177
	10	5319	993	2408	434	23	143
	15	6101	1132	2490	506	15	169
	30	6230	1435	2280	494	18	178
HEP-10	5	4831	1640	2287	520	25	197
	10	6102	1139	2487	500	33	202
	15	5902	941	3094	486	44	229
	30	5436	936	3007	568	98	234
HEP-100	5	5873	1456	2952	554	51	228
	10	4686	1378	2895	584	65	250
	15	4050	1071	3947	652	103	302
	30	4489	N.D.*	5156	491	230	271

*N.D.: Not determined.

in the VLDL: the initial specific radioactivity of the C apolipoprotein was higher in the preparation used in experiment A and hence the transfer of label to HDL was greater.

In the presence of lipolytic activity the percent reduction in VLDL radioactivity at 30 minutes was similar in the 2 experiments. The percent increase in the specific radioactivity of LDL_2 B apolipoprotein was greater in experiment B because the specific radioactivity in LDL_1 B apolipoprotein (the presumed precursor) was initially higher than in experiment A. The increase in B apolipoprotein specific radioactivity in both LDL_1 and LDL_2 was greater with the HEP-100 than the HEP-10 incubations. No change occurred in the specific radioactivity of the soluble fraction of LDL_1 during lipolysis, although it fell in VLDL possibly due to continuing exchange with the much larger mass of HDL C apolipoprotein.

4.3.3 Comparison of the conversion of VLDL and LDL_1 subfractions to LDL_2

Experiment C was designed to define more precisely the role of LDL_1 in the VLDL-LDL_2 relationship. Two VLDL subfractions (s_f 100-400 and s_f 20-50) and LDL_1 (s_f 12-20) were incubated with HEP-0 and HEP-100 plasma. The experiment was carried out twice.

The data shown in Table 4.5 are results of incubations from 2 experiments with HEP-100 plasma. The HEP-0 data was essentially the same as described in experiments A and B. When the s_f 100-400 fraction contained the label (study 1), substantial labelling occurred in both the LDL_1 and LDL_2 fractions. In study 2, the labelled fraction was the s_f 20-50 class which may represent a remnant or intermediate of VLDL catabolism (Eisenberg *et al.*, 1973). The labelling of both the LDL_1

TABLE 4.5

SPECIFIC ACTIVITIES OF THE B APOLIPOPROTEIN

DURING INCUBATION IN EXPERIMENT C

Two independent studies are shown. Incubations were in HEP-100 plasma

Experiment	Study	Labelled addition	Time (min)	LIPOPROTEIN FRACTION[1]		
				VLDL	LDL_1	LDL_2
				counts/min/μg protein		
C-A	1	100-400	2	1258	79	7
			15	1249	101	10
			30	1274	172	17
	2	20-50	2	1949	259	17
			15	1473	609	24
			30	843	879	35
	3	12-20	2	-	1376	49
			15	-	1101	56
			30	-	803	85
C-B	1	100-400	2	2139	91	19
			15	1923	165	23
			30	1810	223	40
	2	20-50	2	1430	707	16
			15	1185	1222	26
			30	527	1750	80
	3	12-20	2	-	1331	64
			15	-	1287	64
			30	-	1043	143

[1] VLDL s_f 20-400; LDL_1 s_f 12-20; LDL_2 s_f 0-12

and LDL_2 fractions was greater and more rapid in study 2 than in study 1. One probable explanation for this is that the B apolipoprotein specific radioactivity of the s_f 20-50 fraction was higher in study 2 than in study 1, thus inferring that the s_f 100-400 fraction is initially degraded to lipoproteins in the s_f 20-100 range before being transformed into the s_f 12-20 or LDL_1 class.

The most rapid transfer of radioactivity to LDL_2 occurred in the presence of labelled s_f 12-20 lipoprotein (study 3). This is again consistent with a sequential transformation of VLDL (s_f 100-400) into a series of smaller lipoproteins. An alternative possibility is for LDL_2 or at least its B apolipoprotein to be derived simultaneously from several species of larger lipoproteins of various sizes. The data in Table 4.5 make this unlikely for 2 reasons. Firstly, in the incubations involving the s_f 20-50 and s_f 12-20 labelled additions, there was a rapid dilution of the labelled fraction from larger non-labelled lipoproteins. Secondly, since the mass of B apolipoprotein in VLDL s_f 100-400 greatly exceeded that in the other 2 fractions, any transfer of unlabelled B apolipoprotein directly from the s_f 100-400 class to LDL_2 would have prevented the rapid rise in LDL_2 B apolipoprotein specific radioactivities that was seen in studies 2 and 3.

4.3.4 Comparison of the conversion of VLDL and intermediate lipoproteins to LDL_2 by whole and 1M NaCl inhibited post-heparin lipolytic activity

Plasma post-heparin lipolytic activity contains lipases from a number of sources. In experiment D, lipases of extra-hepatic origin were inhibited by the presence of 1M NaCl. The degree of inhibition, of FFA

TABLE 4.6

RELEASE OF FREE FATTY ACIDS DURING INCUBATION

IN EXPERIMENTS D-A AND D-B

Labelled Fraction	NaCl conc. (molar)	Time (min)	µmoles FFA released/ml inc. med.	
			D-1	D-2
100-400	0.15	5	1.76	0.41
		30	7.29	2.10
	1.0	5	0.75	0.18
		30	3.30	1.31
12-50	0.15	5	1.81	0.37
		30	7.40	2.47
	1.0	5	1.06	0.14
		30	3.48	0.91

0.5 ml aliquots of incubation medium were added to 10 ml of Doles extraction medium. The extracted FFA were assayed colourimetrically (section 2.1)

release was 36-62% of the control (Table 4.6). At the same time, the presence of 1M NaCl strikingly altered the redistribution of radioactivity among the lipoprotein fractions when labelled s_f 100-400 was added to HEP-100 plasma (Table 4.7). In contrast to the rapid transfer of radioactivity to LDL_1 and LDL_2 fractions seen in the absence of 1M NaCl, its presence inhibited the transfer of radioactivity to LDL_1 and LDL_2 fractions. This was also reflected in the B apolipoprotein specific radioactivities in VLDL, LDL_1 and LDL_2 (Table 4.8). However, when the s_f 12-50 fraction was labelled, some transfer of radioactivity from VLDL to LDL_1 occurred in the presence of 1M NaCl although considerably less than with 0.15 1M NaCl. In contrast, lipolytic transfer of s_f 12-50 radioactivity did not proceed beyond LDL_1 in the absence of extra-hepatic lipases. Hepatic lipolytic activity therefore appeared to promote some conversion of s_f 12-50 to s_f 12-20 lipoproteins but failed to catabolise the latter to LDL_2. This was also evident from the specific radioactivity data. This led to a greater retention of radioactivity in LDL_1 and a greater proportionate increase in the specific activity of this fraction than was observed in the uninhibited system.

4.4 DISCUSSION

In these studies, we have utilized an *in vitro* system for studying the interaction of VLDL with heparin induced lipolytic activity with respect to the fate of the B and C apolipoproteins of VLDL. This system has certain advantages in that all metabolic products remain available for analysis. There are considerable limitations in extrapolating *in vitro* observations to *in vivo* events. An *in vitro* system excludes many factors which may influence lipoprotein interrelationships

TABLE 4.7

PERCENT REDISTRIBUTION OF RADIOACTIVITY AMONG LIPOPROTEIN FRACTIONS DURING INCUBATION IN EXPERIMENT D (2 STUDIES)

Experiment	Labelled addition	NaCl conc. (molar)	Time (min)	LIPOPROTEIN FRACTION[1]				
				VLDL	LDL_1	LDL_2	HDL	D > 1.21
				% total radioactivity				
D-A	100-400	0.15	5	34.7	15.6	16.8	25.0	7.9
			30	9.0	24.4	30.0	27.9	8.8
		1.0	5	47.8	7.5	8.9	25.1	10.7
			30	51.9	6.5	9.7	23.9	7.8
	12-50	0.15	5	32.7	30.4	19.4	14.3	3.3
			30	6.6	21.3	50.5	17.4	4.1
		1.0	5	55.0	15.2	12.1	14.2	3.6
			30	45.5	21.5	13.7	15.4	3.9
D-B	100-400	0.15	5	53.4	5.5	5.9	18.8	16.5
			30	47.6	11.3	7.0	17.7	16.1
		1.0	5	52.5	5.6	3.9	16.5	21.5
			30	55.6	5.5	4.2	17.7	17.0
	12-50	0.15	5	55.2	19.1	13.0	8.9	3.8
			30	37.3	19.1	29.0	10.8	3.8
		1.0	5	58.1	16.7	12.2	8.9	3.8
			30	53.4	22.9	13.1	6.9	3.2

[1] VLDL s_f 20-400; LDL_1 s_f 12-20; LDL_2 s_f 0-12; HDL d. 1.063 - 1.21 g/ml

TABLE 4.8

SPECIFIC ACTIVITIES OF THE B APOLIPOPROTEIN OF

VLDL, LDL_1 and LDL_2 DURING INCUBATION

IN EXPERIMENT D (2 STUDIES)

Experiment	Labelled addition	NaCl conc. (molar)	Time (min)	LIPOPROTEIN FRACTION[1]		
				VLDL	LDL_1	LDL_2
				counts/min/µg B apolipoprotein		
D-A	100-400	0.15	5	10038	1616	42
			30	3800	2225	163
		1.0	5	16105	724	5
			30	17144	802	7
	12-50	0.15	5	4614	7097	404
			30	1153	8004	618
		1.0	5	12508	3672	2
			30	11081	5669	15
D-B	100-400	0.15	5	4887	282	52
			30	2954	552	91
		1.0	5	5899	236	43
			30	3725	216	54
	12-50	0.15	5	7698	2775	316
			30	N.D.	4323	685
		1.0	5	14795	2434	293
			30	10746	3582	294

[1] VLDL s_f 20-400; LDL_1 s_f 12-20; LDL_2 s_f 0-12

such as lipoprotein influx and removal and the possible inhibitory effect on the LCAT system of post-heparin plasma.

As a consequence of post-heparin lipolytic activity, radioactivity associated with VLDL B apolipoprotein was transferred to LDL_1 and LDL_2 (as confirmed by the specific activity data). Some VLDL radioactivity, presumably in C apolipoproteins was transferred to HDL. Although there were clear increases in the specific radioactivity of the B apolipoprotein in both LDL_1 and LDL_2, a proportionately greater increase was observed in LDL_1 than LDL_2. These relationships appear to be dependent upon lipolytic activity.

The data of experiment C suggest that, *in vitro* at least, the catabolism of VLDL to LDL_2 proceeds via an intermediate lipoprotein. The catabolism of VLDL triglyceride has previously been described as a 'cascade-type' degradation with the larger VLDL particles being converted to lipoproteins of decreasing size and increasing density (Barter and Nestel, 1972). In experiment C, conversion of LDL_1 to LDL_2 was noted only in the presence of lipolytic activity. Eisenberg *et al.* (1973) have suggested from *in vivo* studies of VLDL catabolism following heparin administration that the conversion of LDL_1 to LDL_2 is independent of the lipolytic process. In their studies, little conversion of LDL_1 to LDL_2 occurred despite increased plasma lipolytic activity. On the other hand, Homma and Nestel (1975) demonstrated substantial conversion of LDL_1 to LDL_2 during constant infusion of heparin into type 3 hyperlipoproteinaemic subjects.

The frequency distribution of diameters of VLDL remnants (after exposure of VLDL to post-heparin lipolytic activity) is quite narrow and, in the rat, extends from approximately 160 to 400 Å (Eisenberg

and Rachmilewitz, 1975). However, when rat VLDL is catabolised *in vivo* by a technique that excludes the liver from the circulation, the average diameter is larger (350 to 550 Å) (Mjøs *et al.*, 1975). Catabolism of VLDL may therefore proceed further, perhaps, under artificially high concentrations of lipolytic activity (as in post-heparin plasma) than occurs under *in vivo* conditions. The present studies suggest that post-heparin lipolytic activity, in high concentrations, rapidly degrades human VLDL to LDL_1 (s_f 12-20) and beyond to LDL_2. This does not rule out the possibility that, when post-heparin lipolytic activity is largely absent from plasma, as occurs normally, VLDL catabolism may partly cease at the formation of the VLDL remnant which may then be removed from the circulation without complete conversion to LDL_2. Evidence for this has been obtained in hypertriglycerdemic individuals in whom as much as 2/3 of VLDL B apolipoprotein may bypass conversion to LDL_2 (to be described in chapter 5).

The role, if any, for the hepatic lipase in VLDL catabolism is uncertain. It has been suggested that the function of the hepatic lipases may be to facilitate uptake of small quantities of triglyceride from VLDL and chylomicron remnants (Redgrave, 1970; Bergman *et al.*, 1971) and possibly convert these remnants to LDL_2 (Felts *et al.*, 1975). The relative hydrolytic activities of hepatic and extra-hepatic lipases on VLDL indicate that VLDL serves as a much poorer substrate for hepatic lipases than extra-hepatic lipases (LaRosa *et al.*, 1972; Krauss *et al.*, 1973). The present studies suggest that the hepatic lipase is capable of some conversion of small VLDL to LDL_1 at least *in vitro* but that it does not participate measurably in the conversion of LDL_1 to LDL_2.

The data on VLDL soluble apolipoproteins is more limited because of the heterogeneity of this group of peptides. Nevertheless, it was worth noting that very little radioactivity was transferred from VLDL to LDL_1 during the process of lipolysis despite the presence of soluble apolipoproteins in LDL_1. On the other hand, exchange between VLDL soluble apolipoproteins and similar proteins, some presumably from HDL, appeared to occur throughout the incubation even in the absence of lipolysis. It has been previously demonstrated that the catabolism of VLDL is associated with a net transfer of C apolipoproteins from VLDL to HDL (LaRosa *et al.*, 1970; Eisenberg *et al.*, 1972). The present results suggest that this movement may be partially bidirectional and are at variance with the findings of Eisenberg and Rachmilewitz (1975) using rat lipoproteins and rat post-heparin plasma which suggested a unidirectional movement of C apolipoproteins from VLDL to HDL during catabolism of VLDL.

CHAPTER 5

IN VIVO STUDIES OF VLDL AND LDL METABOLISM

5.1 INTRODUCTION

Abnormalities of lipid metabolism may be more meaningfully described in terms of lipoprotein metabolism. Investigations of the metabolism of plasma lipoproteins has increasingly focussed on the apolipoproteins which appear to be the functional components of these macromolecules.

The plasma concentration of lipoproteins, as for any metabolite, is determined by the relative rates of influx and efflux. A rise in the concentration must therefore be due to an increased input into, and/or a decreased outflow from the plasma.

The following two chapters describe a series of *in vivo* studies, carried out in man, of VLDL and LDL metabolism determined from the turnover of their common apolipoprotein, the B apolipoprotein. These studies utilize the methodology developed for the separation of the B apolipoprotein from the soluble apolipoproteins described in chapter 3 (section 3.2.5). Little has previously been reported on this subject because of difficulties in measuring the specific activity of the B apolipoprotein, particularly in VLDL. It has therefore not been possible to characterise, completely, the relationship between VLDL and LDL apolipoprotein turnover, although it was known that VLDL B apolipoprotein was at least

partly transferred to LDL as a result of VLDL catabolism (Gitlin *et al.*, 1958; Bilheimer *et al.*, 1972; Eisenberg *et al.*, 1973; Eaton *et al.*, 1976).

The current view, in the rat, is that a substantial fraction of VLDL B apolipoprotein is removed from the circulation as a 'remnant' that is probably larger than LDL. At the same time, the question of the origin of LDL remains open, since it is possible that at least some LDL may be formed independently of VLDL catabolism (Fidge and Poulis, 1975; Faergeman *et al.*, 1975; Fidge, personal communications). Findings in man are also at a preliminary stage. It has been suggested that LDL is totally derived from VLDL and also, that in normotriglyceridaemic individuals, at least, all VLDL is catabolised to LDL (Sigurdsson *et al.*, 1975).

The catabolism of VLDL triglyceride is thought to proceed via a step-wise conversion of large (and triglyceride-rich) VLDL particles to smaller particles of higher density and lower triglyceride content (Barter and Nestel, 1972). During the initial stages of VLDL catabolism, lipoproteins of intermediate size and density (termed remnant or intermediate lipoproteins) are formed (Shore and Shore, 1962; Bilheimer *et al.*, 1972; Eisenberg *et al.*, 1973) some of which, as seen in the rat, may be removed without prior conversion to LDL (Fidge and Poulis, 1975; Faergeman *et al.*, 1975). As discussed in chapter 4, it has been suggested that the lipolytic process that converts VLDL to these intermediate lipoproteins may not extend to the formation of LDL (Eisenberg *et al.*, 1973). Excessive accumulation of a subclass of intermediate lipoproteins may therefore reflect the failure of VLDL catabolism to proceed

beyond a specific point. It has been postulated that type 3 HLP, which is characterised by the accumulation of a cholesterol-rich intermediate lipoprotein, develops in this way (section 1.7.4).

This chapter describes the precursor-product relationships found between VLDL, LDL_1 and LDL_2 in ten studies, carried out in normal and hyperlipoproteinaemic subjects. Turnover rates of VLDL B apolipoprotein were determined by compartmental analysis. The likelihood of LDL_1 being the true intermediate lipoprotein between VLDL catabolism and LDL formation was examined from the relationships of the relative turnover rates of VLDL, LDL_1 and LDL_2. The turnover of LDL_2 was calculated from the relative rates of change in the specific activities of VLDL and LDL_2.

5.2 METHODS

5.2.1 Dietary procedures for maintenance of steady-state conditions

The clinical details of each subject are shown in the result section (section 5.4). All subjects were maintained on a fat-free diet for the first two days of study. However, dietary carbohydrate intake was not increased to replace the fat in order to prevent a possible increase in hepatic VLDL production. Thus, during this period, the subjects received approximately 65% of their normal eucaloric intake. In practice, this regime led to a constant concentration of plasma VLDL B apolipoprotein during the first two days of study. For the remaining study period, dietary fat intake was kept less than 25% of the normal total calories. Prior to, during and following the injection of the radioiodinated lipoproteins, potassium

iodide was administered (orally) twice daily to minimise radioactive iodine uptake by the thyroid.

5.2.2 Preparation of radioiodinated lipoproteins for re-injection

Four days prior to study, 100 ml blood was collected, into heparinised tubes, from the fasting subject for preparation of VLDL (s_f 20 - 400) and LDL_2 (s_f 0-12) (studies 3 and 5 only) by ultracentrifugation as previously described (section 2.2). VLDL was iodinated with ^{125}I and LDL_2 with ^{131}I as described in section 2.3 and prepared for reinjection by Millipore filtration (section 2.4). Sterility was checked bacteriologically.

5.2.3 Injection of autologous lipoprotein preparations and the subsequent collection of blood samples

Approximately 3-5 mg of autologous VLDL protein labelled with 20-70 µC of ^{125}I was injected into each subject. In studies 4 and 5, the subject was also injected with approximately 15 µC of LDL-^{131}I containing 3 mgs of LDL protein. The vials containing the labelled lipoprotein were weighed before and after the injection sample was removed in order to determine accurately the injected dose (except study 1). Following injection, blood samples (approx. 22 ml) were drawn at regular intervals over the period of study (3-7 days). Studies were usually carried out over a three day period, by which most of the radioactivity associated with VLDL and LDL_1 had disappeared. VLDL turnover was calculated from observations over the first 50 hours by which time less than 5% of the injected ^{125}I dose remained in this fraction.

5.2.4 Determination of B apolipoprotein specific activities in VLDL, LDL_1 and LDL_2

VLDL, LDL_1 and LDL_2 were isolated and purified from 10 ml plasma samples as described in section 2.2. The lipoprotein preparations were delipidated (section 2.5) and the B apolipoprotein isolated, using the NH_4HCO_3 procedure for specific activity determination (section 3.2.5).

Plasma concentrations of B apolipoprotein (in VLDL or LDL_2) were corrected for any losses during ultracentrifugation or delipidation. The intravascular mass of VLDL B apolipoprotein was calculated assuming the plasma volume to be 4.5% body weight.

5.3 CALCULATIONS

This section describes the rationale behind the different calculations and interpretations made from the data of specific activities of the B apolipoprotein in VLDL, LDL_1 and LDL_2.

5.3.1 Analyses of specific activity-time plots for precursor-product relationships

Zilversmit (1943), in a theoretical consideration of a situation where a compound B is derived from compound A, concluded that "at any time, the slope of the specific activity-time curve of B is proportional to the difference between the specific activities of A and B". If this criterion is met, the relationship is compatible with a precursor-product relationship between A and B although it does not necessarily prove it. The precursor A could be in rapid equilibrium with a further compound X and therefore the relationship between X and B would meet the

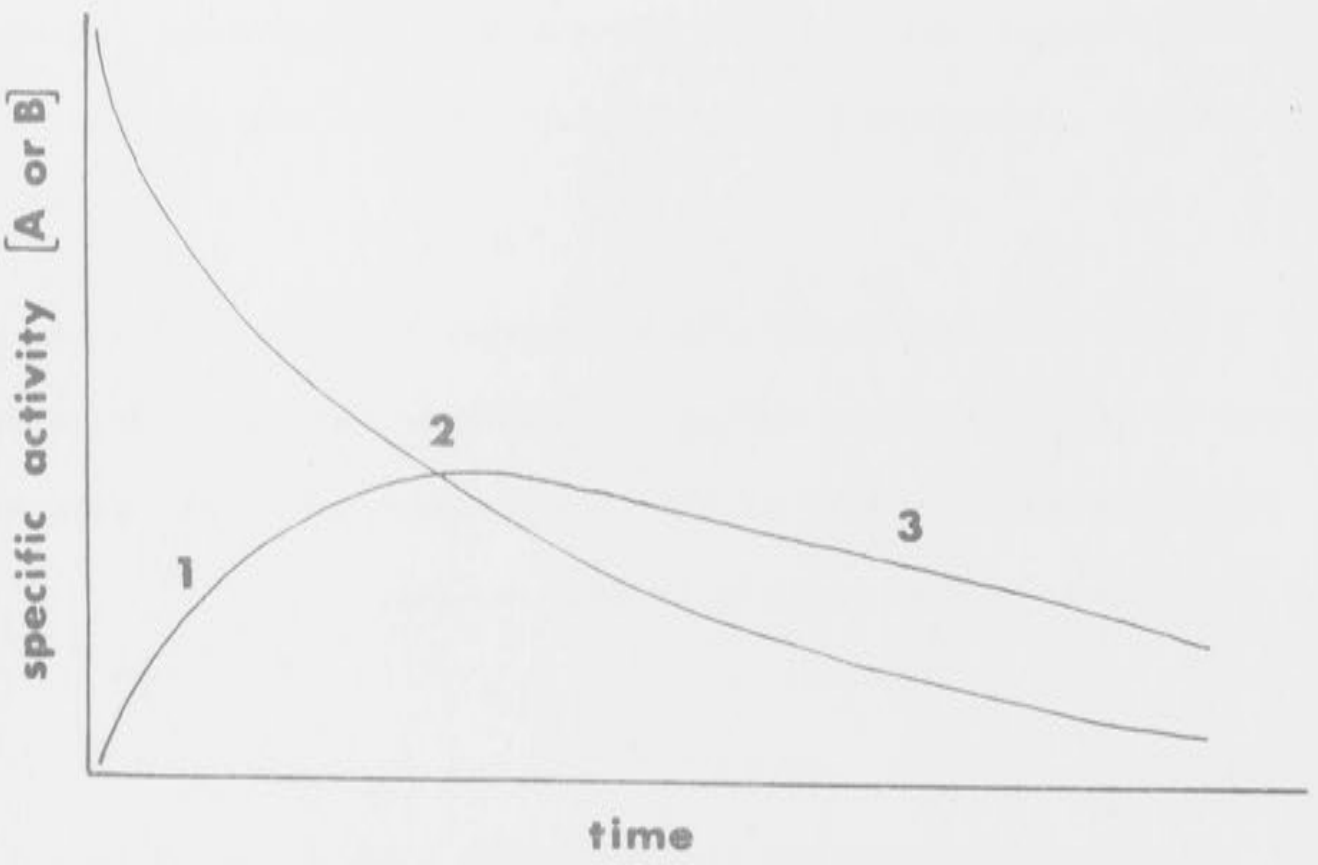


Figure 5.1 A plot of the specific activities of a precursor (A) and product (B) against time. Position 1 demonstrates the slope of B(+ve) before it intersects A at position (2) when the slope of B becomes zero. In Position 3 the slope of B is -ve. The slope of B must be an expression of the difference in the specific activities of A and B if B is derived solely from A. (Zilversmit *et al.*, 1943).

above criterion yet X would not be the precursor to B (Zilversmit, 1960).

The above stated criterion therefore demands that the slope of the specific activity-time curve of B will be positive while A is greater than B as demonstrated in figure 5.1 (position 1). However, as the specific activities of A and B approach each other, the difference in specific activities approaches zero. This therefore demands that, at the point of cross-over of A and B, the slope of B be zero (as shown in position 2), B having reached peak specific activity. Thereafter, the specific activity of B will be greater than that of A and the slope of B will become negative (position 3). If B is not entirely derived from A, the fraction of B that is derived from A will equal the ratio of the B and A specific activities at the peak of B. In this situation, the specific activity of A will cross B after the peak of B.

Using this criterion, the specific activity-time curves of VLDL, LDL_1 and LDL_2 were examined to indicate the possible precursors of LDL_1 and more importantly LDL_2, as well as the relationships between the various VLDL and LDL_1 fractions investigated in chapter 6.

5.3.2 Calculation of turnover rates by kinetic analysis

5.3.2.1 Single pool

The kinetics of VLDL B apolipoprotein appeared to conform more closely to a 2-pool than a 1-pool model as shown in figure 5.4 and subsequently in the experimental data in figures 5.6 to 5.15. However, for purposes of comparison with the only previous reports of VLDL B apolipoprotein turnover (Sigurdsson *et al.*, 1975, 1976) analyses

were also performed using the single pool model which was used by Sigurdsson *et al.*

All analyses for turnover rates assume the pool or pools being investigated are in a 'steady state' with respect to the particular compound being investigated (as devised for these studies in section 5.2.1). If the concentration of the compound remains constant, its rate of entry into the pool will equal the rate at which it is being removed.

If a compound is being metabolised within a single pool (in a steady state), the fraction of that pool that is being removed (and so replaced) per unit time is referred to as the fractional catabolic rate (k). The total amount of metabolite that is leaving the pool (which is equivalent to that entering the pool) per unit time is referred to as the 'turnover rate', 'synthetic rate' or more recently 'turnover'.

The fractional catabolic rate may be determined from analysis of the disappearance of a radioactive tracer, introduced into the pool, expressed as a specific activity-time plot. The specific activity of the compound is dependent upon two equivalent functions - its dilution by input of new (unlabelled) metabolite and its removal from the pool. It is assumed that, in the removal of the compound, there is no discrimination in removal of labelled or unlabelled compound from the pool.

The specific activity (SA) at any time (t) may be defined by the expression

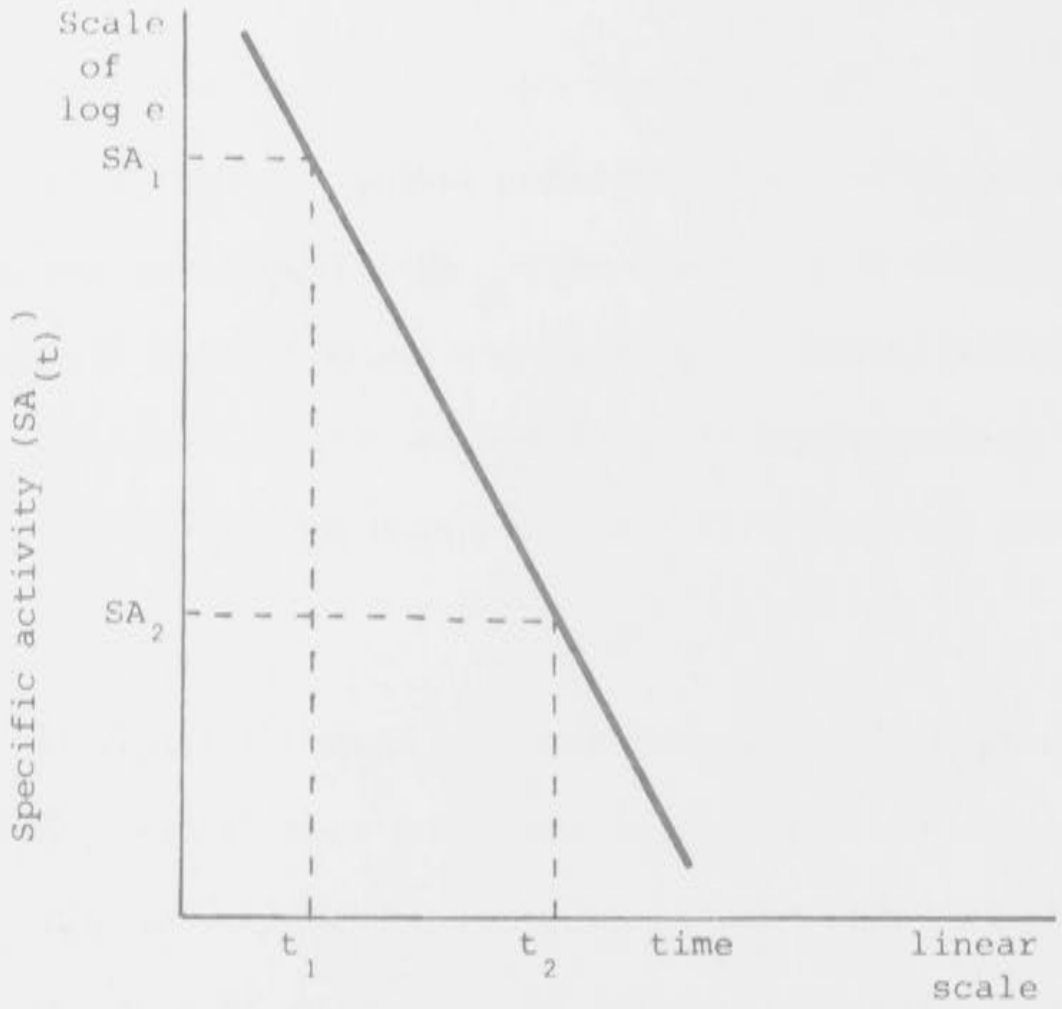


Figure 5.2 A plot of the expression

$$SA_{(t)} = SA_{(o)} e^{-kt}$$

where $SA_{(t)}$ is the specific activity at a given time (t), e is the natural base of logarithms and k (the slope of the expression) is a constant. The value of k may be determined as follows:

$$k = \frac{\ell n\ SA_1 - \ell n\ SA_2}{t_2 - t_1}$$

$$SA_{(t)} = SA_{(o)} e^{-kt}$$

where e is the base for natural logarithms. Such an expression, if plotted on semi-logarithmic scale (to the base e) will be linear with a slope equal to k (figure 5.2). Therefore, from figure 5.2, if two time points (t_1 and t_2) are selected with their concomitant specific activities (SA_1 and SA_2) then k may be expressed as

$$k = \frac{\ell n\ SA_1 - \ell n\ SA_2}{t_2 - t_1}$$

For ease of calculation, it is more convenient to calculate this value using 'half times' ($t_{\frac{1}{2}}$) i.e. the time required for the specific activity at any time to decline by half. Therefore, in the above equation if $SA_2 = \frac{SA_1}{2}$ when $t_2 - t_1 = t_{\frac{1}{2}}$ then

$$k = \frac{\ell n\ SA_1 - \ell n \frac{SA_1}{2}}{t_{\frac{1}{2}}}$$

$$= \frac{\ell n\ 2}{t_{\frac{1}{2}}}$$

$$= \frac{0.693}{t_{\frac{1}{2}}}$$

(Shipley and Clark, 1972).

As k is an expression of the fraction of the pool turning over per unit time, the total turnover may be calculated if the total mass of metabolite in the pool is known (turnover = k × pool mass). As the plasma concentration of B apolipoprotein was known, the plasma pool size could be calculated assuming the plasma volume to be 4.5% body weight. The

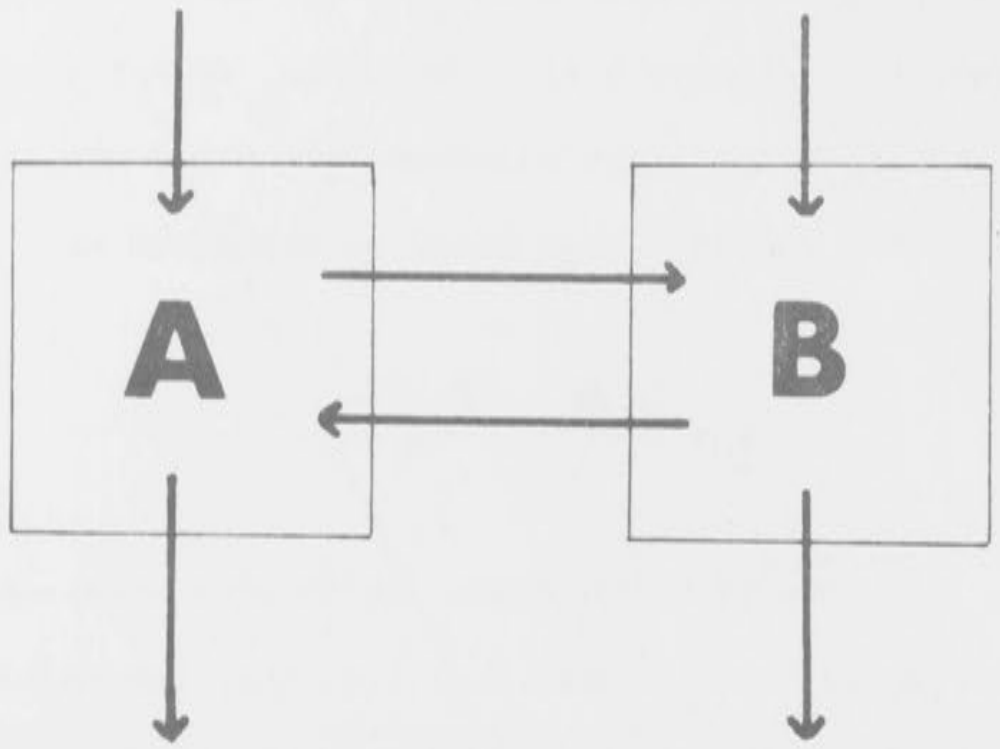


Figure 5.3 The two pool model proposed by Gurpide *et al.* (1964). This model allows for independent rates of entry and exit from each pool and independent equilibration rates between each pool.

value of k (or $t_{\frac{1}{2}}$) was determined from the line of best fit for B apolipoprotein specific activities over the first 12 to 24 hours.

5.3.2.2 Two pool

As previously mentioned, the disappearance curves for VLDL B apolipoprotein (Figures 5.6 to 5.15) were clearly not mono-exponential over a 50-hour period. As these curves could be resolved by conventional 'curve-peeling' techniques into two exponentials, a 2-pool model for kinetic analyses was used.

The 2-pool model proposed by Gurpide *et al.* (1964) which describes the relationship between two pools, A and B, allows for independent entry and exit from both pools (Figure 5.3). As will be discussed later, the catabolism of VLDL B apolipoprotein may occur extravascularly in tissues, as well as in the generally accepted manner by conversion to LDL within the plasma compartment. It therefore seemed appropriate to select a model that incorporated efflux of material from the second pool B. This is in contrast to the 2-pool model of Matthews (1957) which has been used for kinetic analysis of LDL disappearance curves (Langer *et al.*, 1972; Hurley and Scott, 1970; Simons *et al.*, 1975). The Gurpide model has been applied for the kinetic analyses of cholesterol turnover (Goodman and Noble, 1968).

The two pool model, as proposed by Gurpide *et al.* (1964), is illustrated in Figure 5.3. Pools A and B are in equilibrium with each other and both have independent rates of input and removal. With this system, the natural logarithm of the specific activity in pool A, when expressed as a function of time, will describe a curve which may be

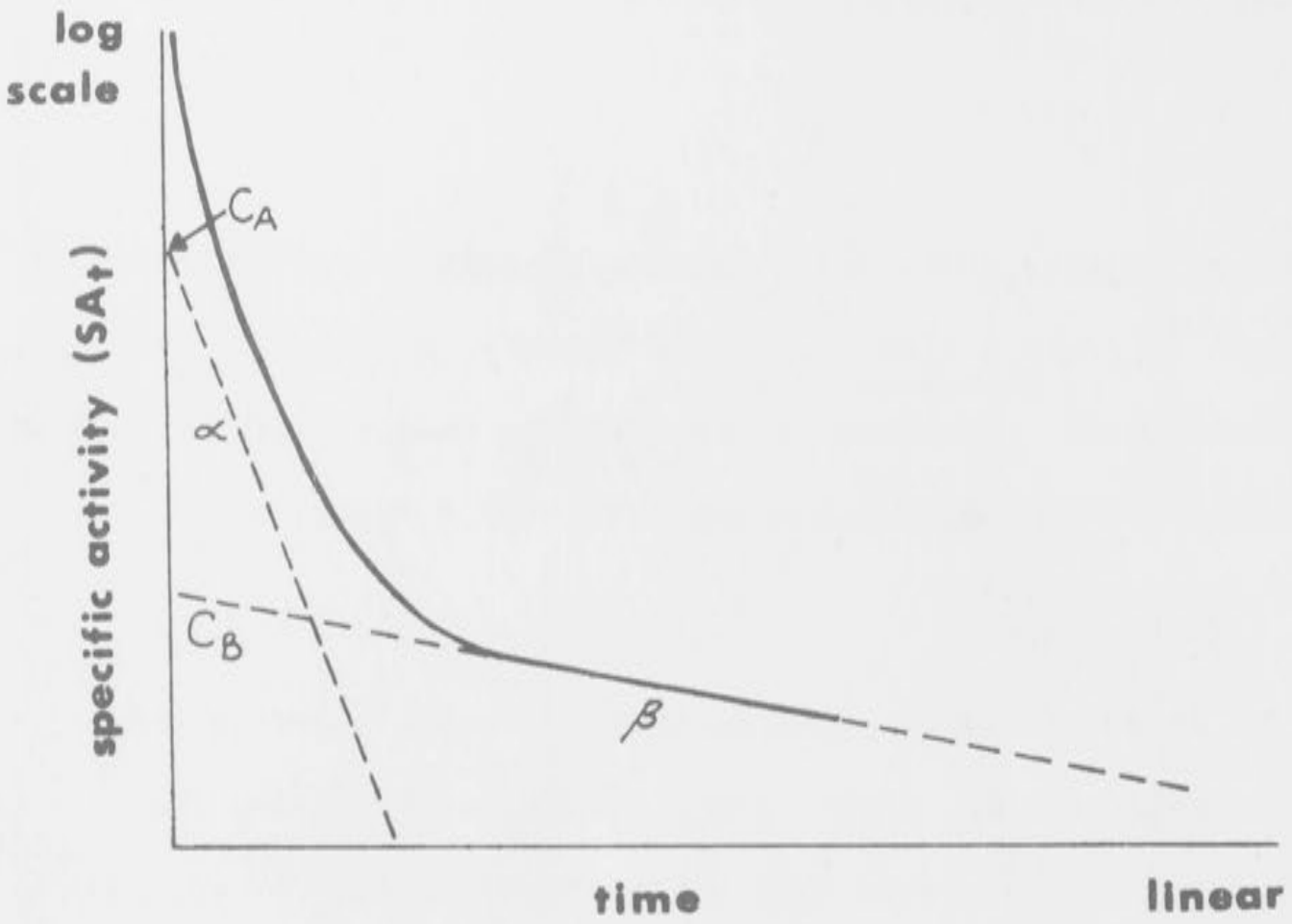


Figure 5.4 Plot of the expression

$$SA_{(t)} = C_A e^{-\alpha t} + C_B e^{-\beta t}$$

which may be resolved into two exponentials, $C_A e^{-\alpha t}$ and $C_B e^{-\beta t}$, where $SA_{(t)}$ is the specific activity at a given time (t). C_A and C_B are the respective intercepts. α and β are the rate constants of the 2 exponentials. β is the extension of the terminal phase of the specific activity curve and α is obtained by "curve-peeling".

resolved into 2 exponentials as illustrated in Figure 5.4. The expression for this curve is the sum of the two exponentials:

$$SA_{(t)} = C_A e^{-\alpha t} + C_B e^{-\beta t} \quad .$$

The constants C_A and C_B may be found at $t_{(o)}$ as illustrated where the respective exponentials intercept the ordinate axis. α and β (equivalent to k) are calculated, from their respective exponential as for the 1-pool model from the $t_{\frac{1}{2}}$ (i.e. $0.693/t_{\frac{1}{2}}$).

The mass of metabolite in pool A (M_A) may be calculated from the equation

$$M_A = \frac{R_A}{C_A + C_B}$$

where R_A is the amount of radioactivity added to pool A.

The turnover of metabolite in pool A, may also be determined from the equation

$$PR_A = \frac{R_A \cdot \alpha \cdot \beta}{\beta C_A + \alpha C_B}$$

where PR_A = the turnover in Pool A (other than material recycled through pool B) (Gurpide *et al.*, 1964; Goodman and Noble, 1968). It is worth noting that this equation does not require knowledge of pool sizes.

Although the mass in pool A may be calculated, that in pool B cannot be unless it is assumed that there is no loss from pool B, an assumption that would be unjustified for these studies in view of the recent findings of VLDL catabolism by cultured cell preparations (e.g. Goldstein and Brown, 1974).

5.3.2.3 Non-compartmental analysis

An alternative procedure for determining turnover is to use the Stewart-Hamilton equation (as discussed by Shipley and Clarke, 1972) where

$$PR_A = \frac{R_A}{\int_0^\infty SA \,.\, dt}$$

where PR_A = turnover in pool A (as above) and R_A is the dose of radioactivity added to pool A.

This formula is derived from the obvious premise that the amount of tracer ultimately lost from a labelled pool must equal the amount originally introduced (R). The integral of the specific activity-time curve ($\int_0^\infty SA \,.dt$) was determined graphically by measuring the area under the curve (by planimetry). This non-compartmental method of analysis was used as a verification of values obtained by compartmental analysis, as well as providing an index of the turnover of the LDL_1 fraction when labelled from VLDL.

The turnover as determined in terms of PR_A applies only to pool A and not to the input-output of the whole system i.e. to turnover in all compartments. However if all the input or all the output occurs through pool A into which the label is introduced, then turnover in pool A equals that in the whole system. VLDL are secreted directly from the liver and the gut into the plasma and it seems likely therefore that VLDL influx occurs only through pool A which includes the plasma VLDL. The calculated turnover of VLDL is therefore likely to be equal to VLDL turnover in the whole system.

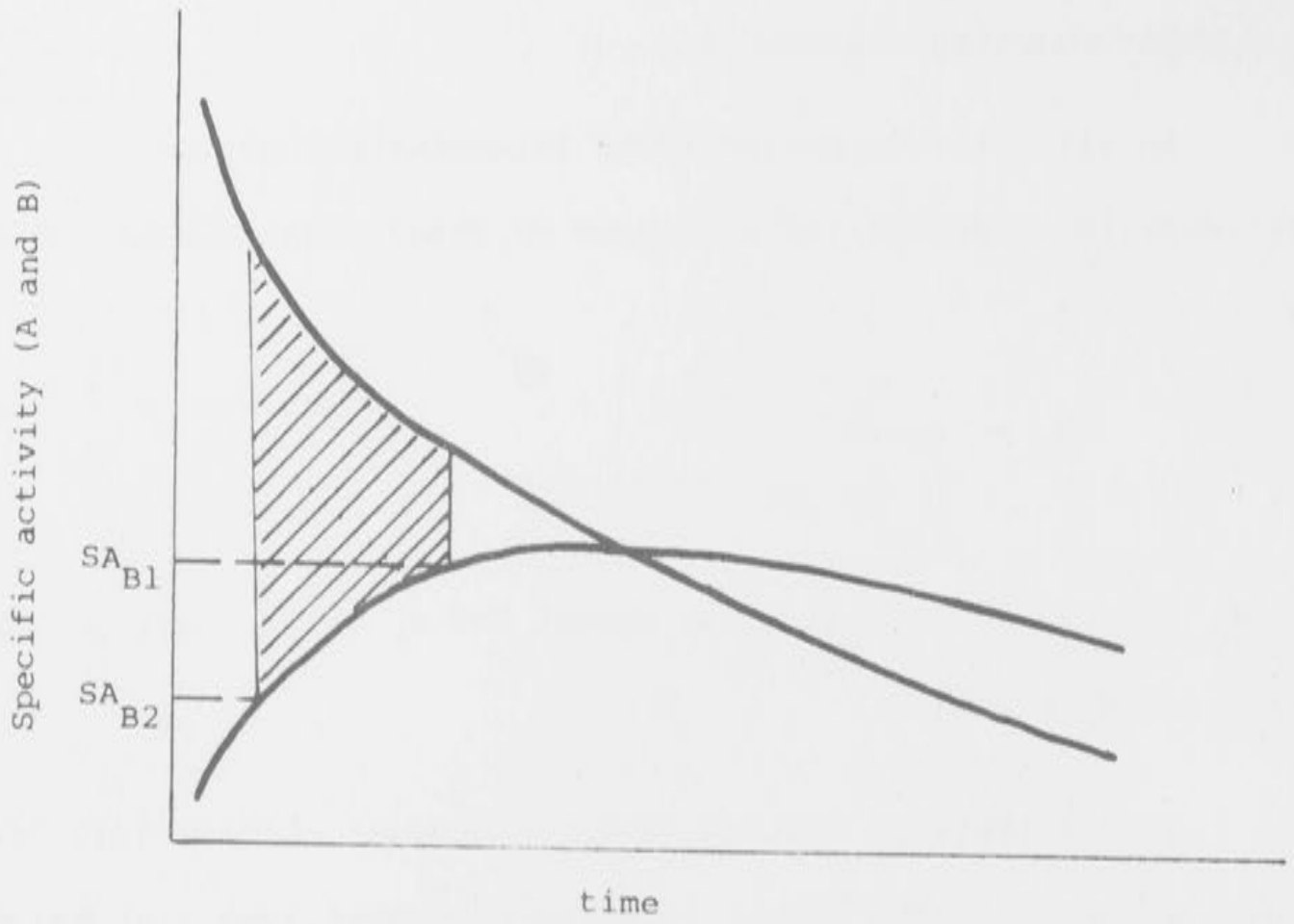


Figure 5.5 A plot of a precursor-product relationship between A and B analysed graphically for the determination of the fractional catabolic rate (k) of the product (section 5.3.3).

5.3.3 Determination of fractional catabolic rate of a product

In instances where the specific activity time curves of precursor and product are available, the fractional catabolic rate (k) of the product may be determined graphically (Zilversmit, 1960). As shown in Figure 5.5,

$$k_B = \frac{SA_{B1} - SA_{B2}}{\text{area shaded}}$$

This mathematical model requires that the product is derived solely from the precursor, but does not require that all of the precursor be converted to the product.

This model was used to calculate LDL_2 turnover. The specific activity-time curves of VLDL and LDL_2 B apolipoprotein were plotted on arithmetic paper. The curves, as shown in Figures 5.6 to 5.15 intersected at various times between 18 and 36 hours after injection of VLDL-^{125}I generally at or near the peak specific radioactivity of LDL_2. This may be regarded as demonstrating that all LDL_2 is derived from VLDL though it does not require that all VLDL is converted to LDL_2.

To verify this model, in two subjects, LDL_2 turnover was calculated by the above procedure (following injection of VLDL-^{125}I) and also simultaneously by the more conventional 2-pool analysis determined from the LDL_2 B apolipoprotein specific activities obtained over a 14-day period following injection of LDL_2-^{131}I.

To calculate turnover, by the first technique, the pool size must be known. However since only the plasma pool size can be directly measured and since it is known that approximately 20% of the LDL pool

may be found in the liver (Sniderman *et al.*, 1974) this method may underestimate the real turnover of LDL_2.

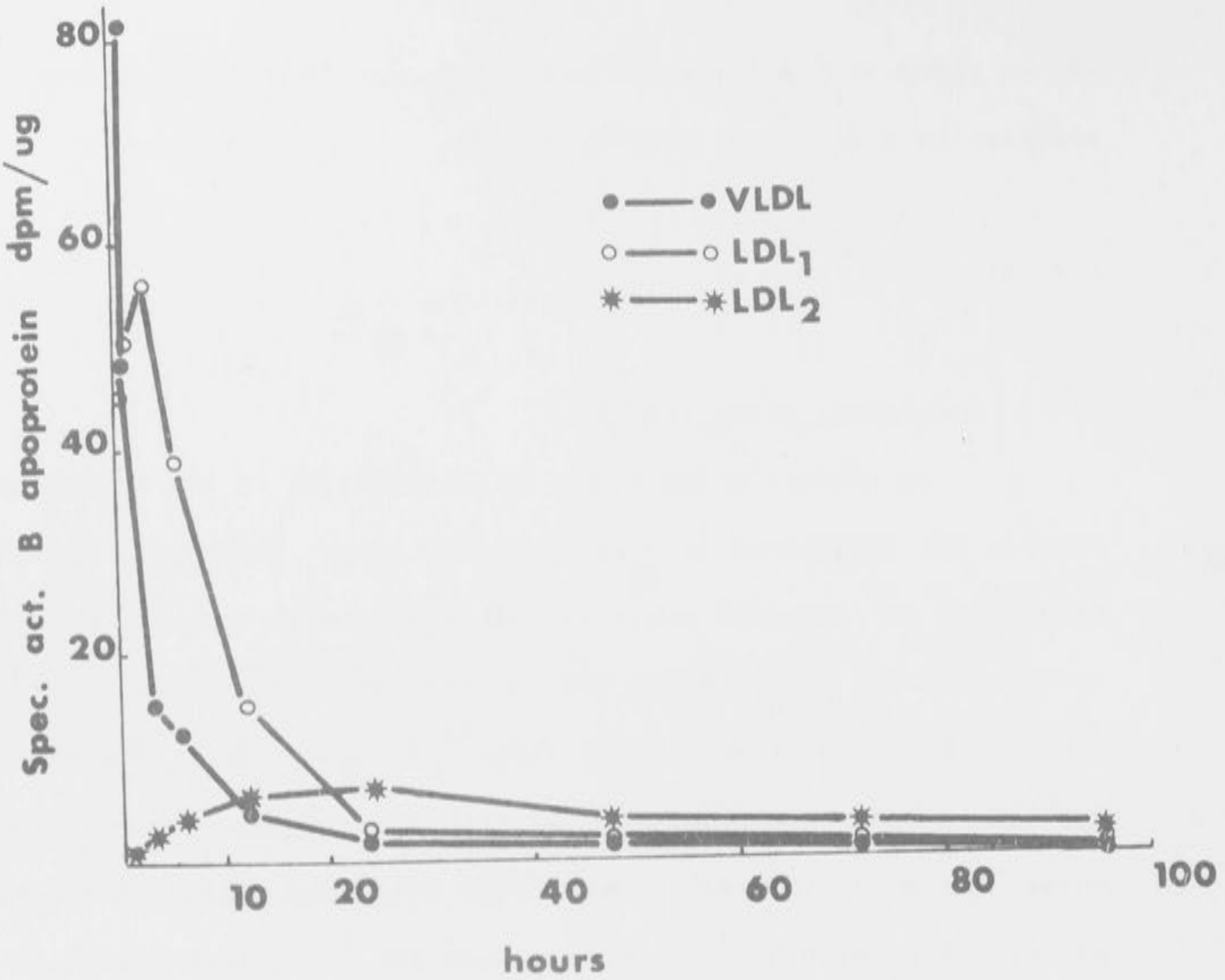
5.4 RESULTS

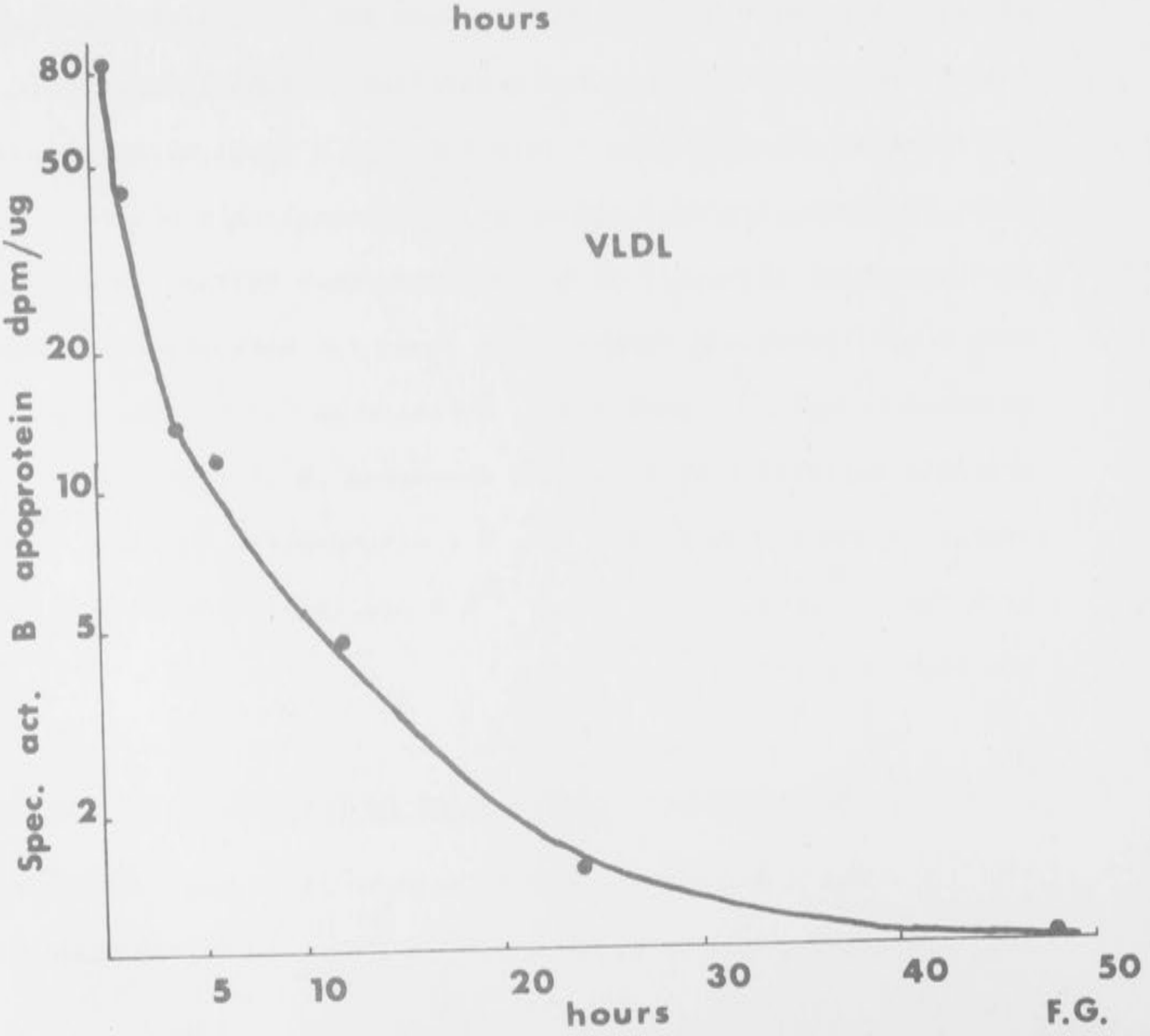
5.4.1 Individual study results

In these studies the specific activity of the B apolipoprotein was determined in VLDL, LDL_1 and LDL_2. Throughout the text, references to 'specific activity' are always with respect to this apolipoprotein. Ten studies are reported in this chapter, each involving the injection of autologous VLDL-^{125}I (s_f20 - 400). The clinical details, kinetic parameters and appropriate specific activity-time curves for each study are shown on the following pages. The specific activity-time curves for VLDL, LDL_1 and LDL_2 are shown to illustrate the intercepts of the specific activities of these lipoprotein fractions. The specific activity-time curves for VLDL B apolipoprotein (over the first 50 hours) are also shown, on semi-logarithmic plots to illustrate the biexponential nature of the disappearance curves. Three values for VLDL B apolipoprotein turnover are given for most studies. These were obtained using the 1-pool model, 2-pool model or by area under the specific activity-time curve (as discussed in section 5.3.2). In studies 4 and 5, values for LDL_2 B apolipoprotein turnover determined from the disappearance of LDL_2-^{131}I B apolipoprotein (2-pool analysis) are also included.

5.4.2 Precursor-product relationships between VLDL, LDL_1 and LDL_2

The precursor-product relationships between VLDL, LDL_1 and LDL_2 are illustrated in Figures 5.6 to 5.15. In all studies (except


80
60
40
20
Spec. act. B apoprotein dpm/ug
VLDL
LDL1
LDL2
10
20
40
60
80
100
hours


80
50
20
10
5
2
Spec. act. B apoprotein dpm/ug
VLDL
5
10
20
30
40
50
hours
F.G.

Study # 1 *SUBJECT*: F.G.

Age: 74 years Weight: 52.2 kg

Plasma lipids Cholesterol: 209 mg/100 ml

Triglyceride: 90 mg/100 ml

Lipoprotein phenotype: Normal

VLDL B APOLIPOPROTEIN

Plasma concentration = 4.2 mg/100 ml

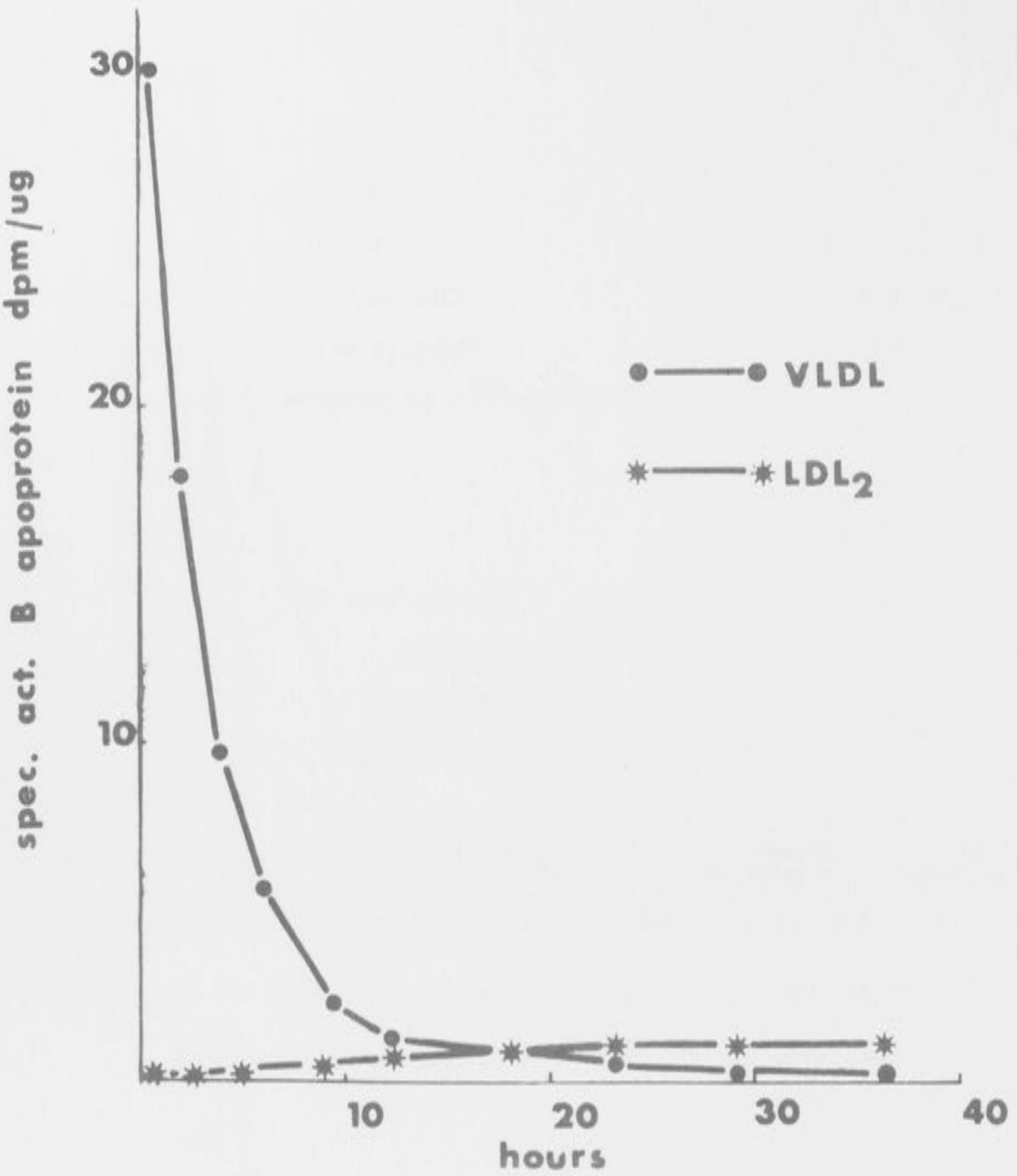
1-pool analysis k = 3.9/day

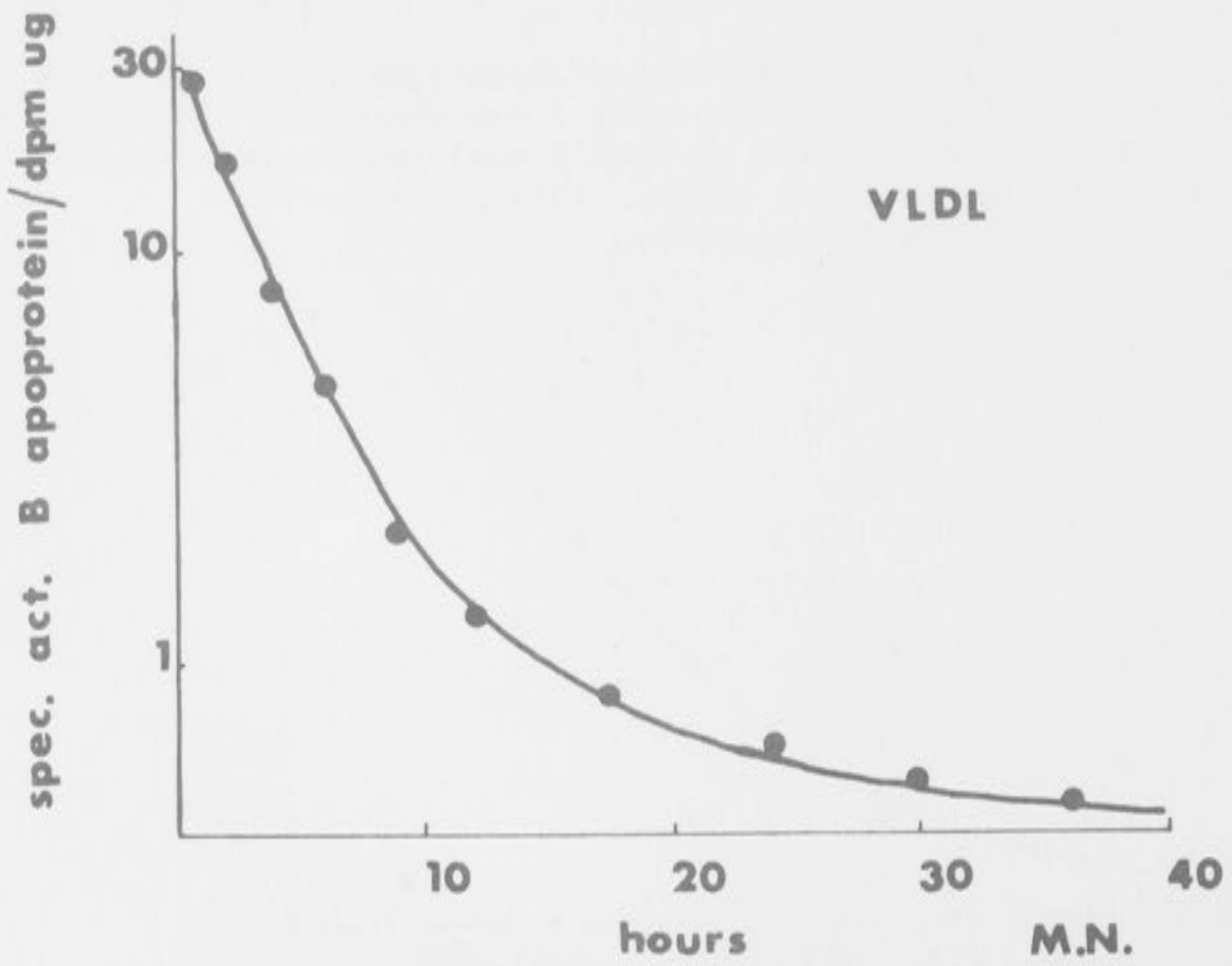
turnover = 99mg/day

turnover/kg = 7.0 mg/day

The first study demonstrated that the 1-pool model described only a small portion of the entire VLDL B apolipoprotein specific activity curve. A 2-pool analysis was not performed as the dose of B apolipoprotein-^{125}I administreed in VLDL-^{125}I was not determined.

Figure 5.6 (facing page). The specific activity-time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 1 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph. This curve could be resolved into two single exponentials.


spec. act. B apoprotein dpm/ug
30
20
10
VLDL
LDL2
10
20
30
40
hours


spec. act. B apoprotein/dpm ug
30
10
1
VLDL
10
20
30
40
hours
M.N.

Study #2 *SUBJECT:* M.N.

Age: 21 years Weight: 56.0 kg

Plasma lipids Cholesterol: 110 mg/100 ml

Triglyceride: 62 mg/100 ml

Lipoprotein phenotype: Normal

VLDL B APOLIPOPROTEIN

Plasma concentration = 1.7 mg/100 ml

1-pool analysis k = 8.4

turnover = 359 mg/day

turnover/kg = 6.4

LDL_2 B APOLIPOPROTEIN

Plasma concentration = 47 mg/100 ml

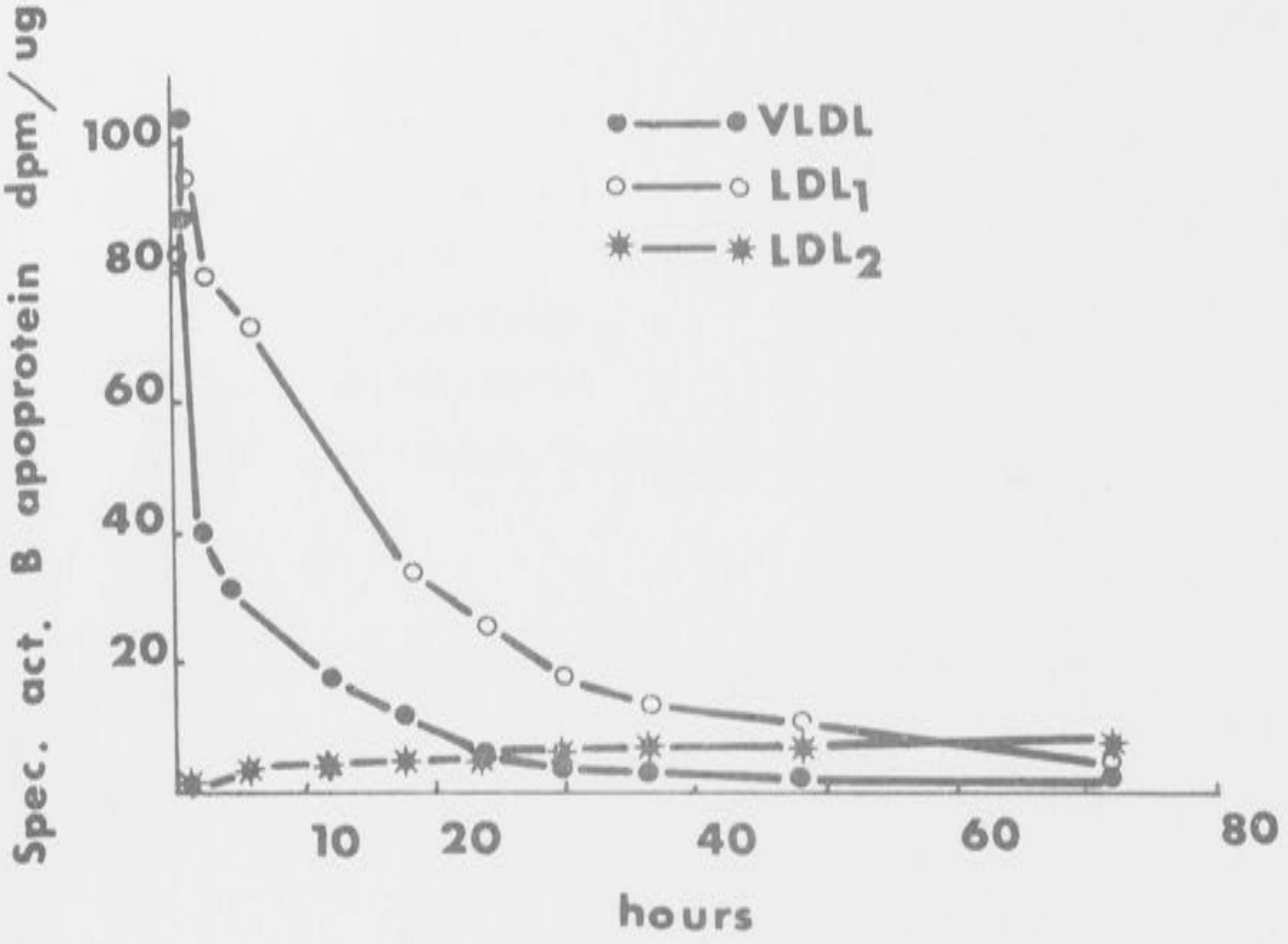
k = 0.33/day

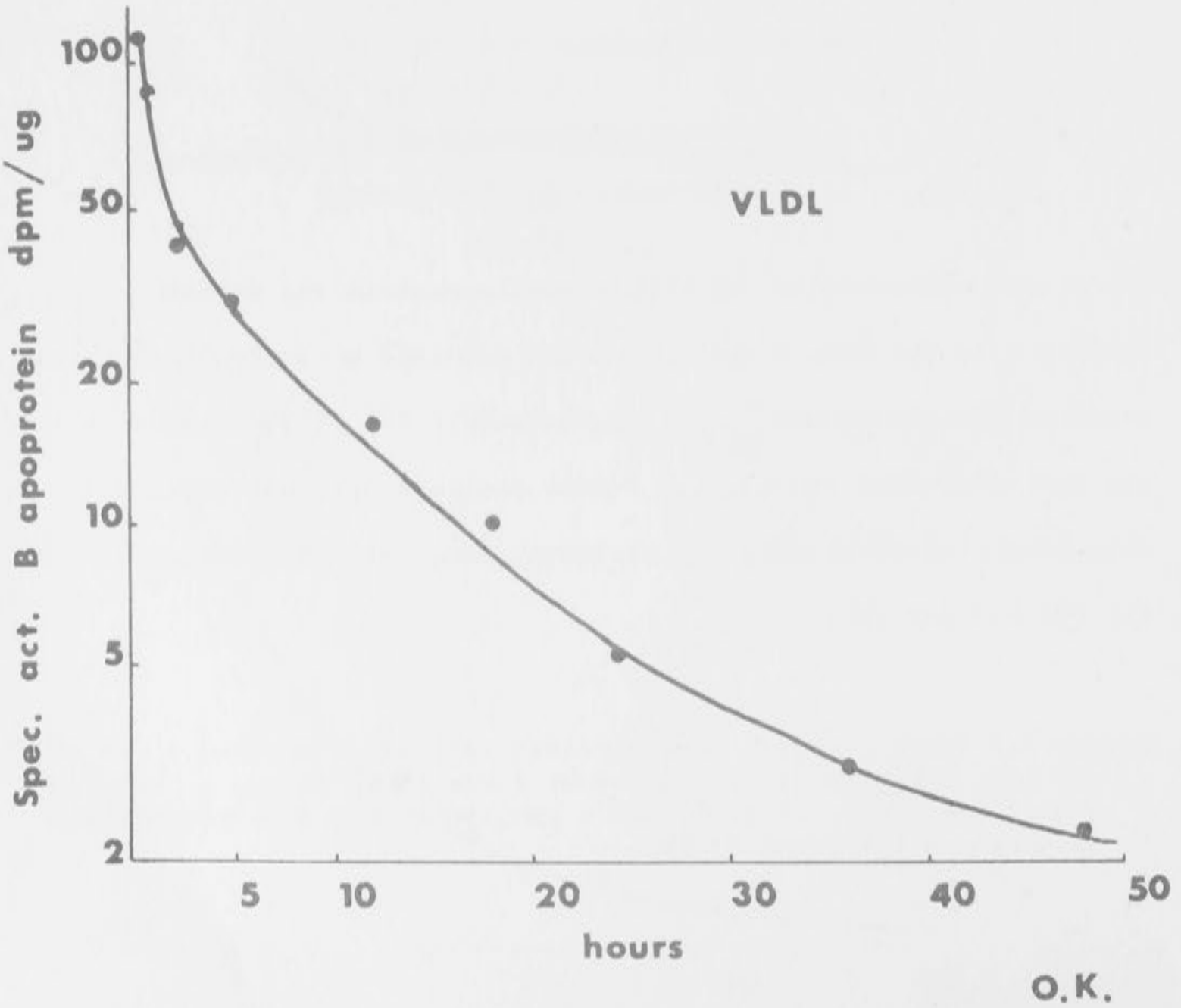
turnover = 405 mg/day

turnover/kg = 5.5 mg/day

The plasma concentration of VLDL B apolipoprotein was extremely low in this subject. As the VLDL B apolipoprotein specific activity-time curves remained monoexponential until approximately 95% of the radioactivity was lost from this fraction, a 1-pool analysis only was performed. In this study, although LDL_1 was isolated, there was insufficient protein for further analysis.

Figure 5.7 (facing page). The specific activity time curves for VLDL and LDL_2 B apolipoprotein in study 2 are shown in the upper graph. A semilogarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.


Spec. act. B apoprotein dpm/ug
100
80
60
40
20
10
20
40
60
80
hours
VLDL
LDL1
LDL2


Spec. act. B apoprotein dpm/ug
100
50
20
10
5
2
5
10
20
30
40
50
hours
VLDL

O.K.

Study #3 *SUBJECT:* O.K.

Age: 51 Weight: 55.5 kg

Plasma lipids Cholesterol: 202 mg/100 ml

Triglyceride: 110 mg/100 ml

Lipoprotein phenotype: Normal

VLDL B APOLIPOPROTEIN

Plasma concentration = 5.2 mgs/100 ml

2-pool analysis α = 22.1/day β = 1.1/day

turnover = 637 mg/day

turnover/kg = 11.5 mg/day

Mass of pool A = 18 mg

Intravascular mass = 131 mg

1-pool analysis k = 7.9/day

turnover = 1041 mg/day

Area under curve turnover = 976 mg/day

LDL_2 B APOLIPOPROTEIN

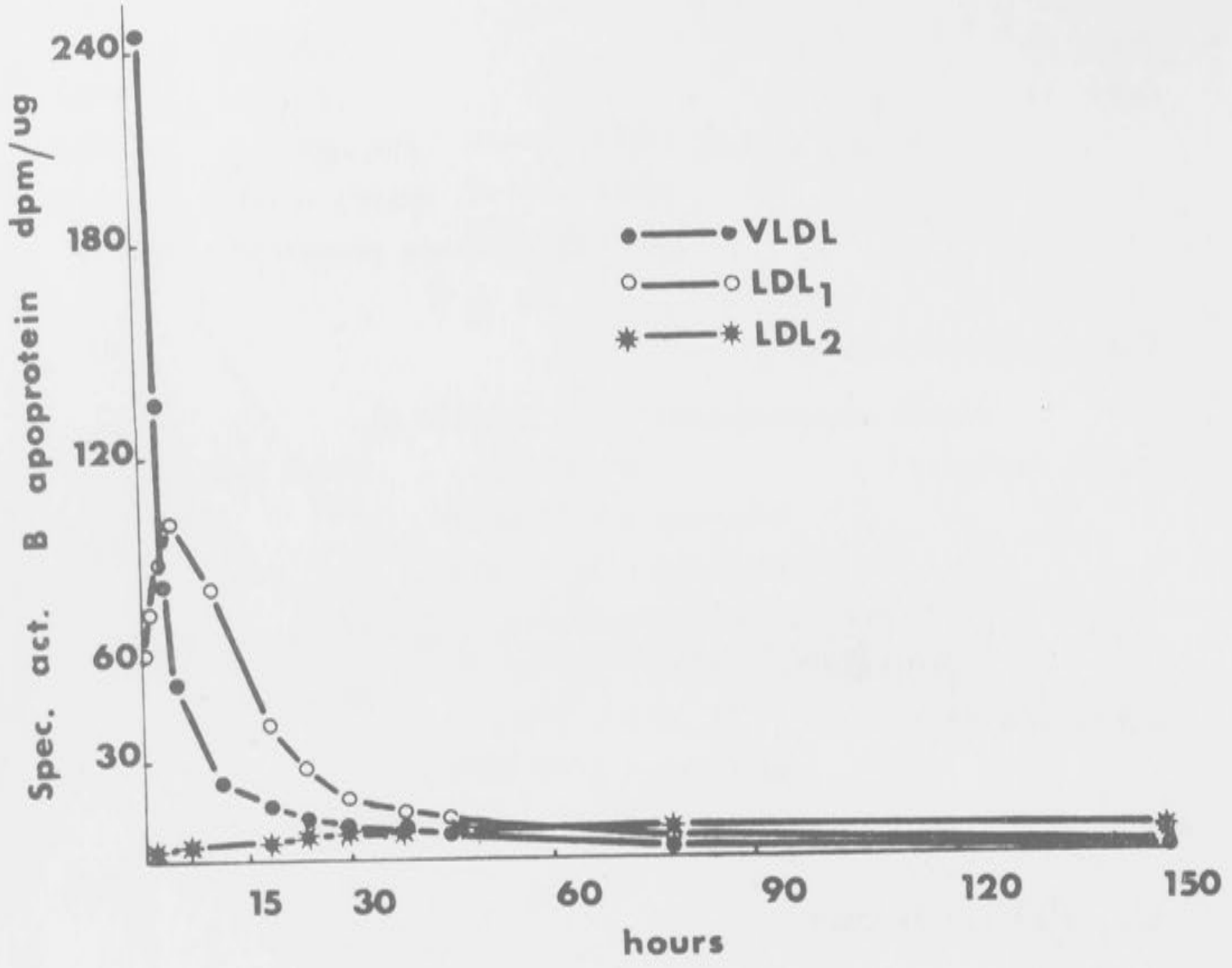
Plasma concentration = 80.1 mg/100 ml

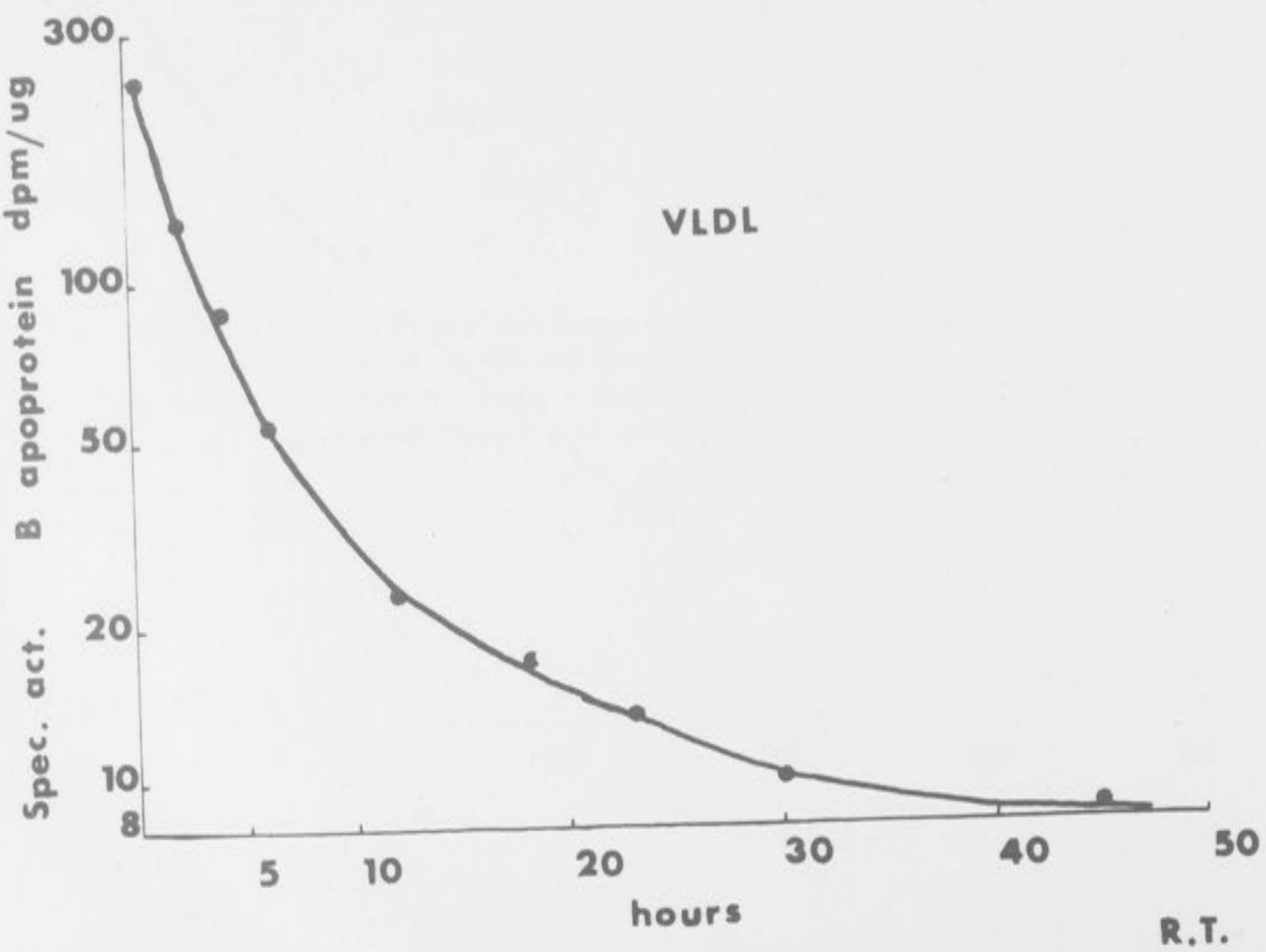
k = 0.29 /day

turnover = 579 mg/day

turnover/kg = 10.3 mg/day

Figure 5.8 (facing page). The specific activity time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 3 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.


Spec. act. B apoprotein dpm/ug
240
180
120
60
30
VLDL
LDL1
LDL2
15
30
60
90
120
150
hours


Spec. act. B apoprotein dpm/ug
300
100
50
20
10
8
VLDL
5
10
20
30
40
50
hours
R.T.

Study #4

Age: 25 years

SUBJECT: R.T.

Weight: 68.9 kg

Plasma lipids

Cholesterol: 295 mg/100 ml

Triglyceride : 150 mg/100 ml

Lipoprotein phenotype: 2A

VLDL B APOLIPOPROTEIN

Plasma concentration = 10.77 mgs/100 ml

2-pool analysis

α = 12.9/day β = 1.1/day

turnover = 1092 mg/day

turnover/kg = 15.8 mg/day

Mass of pool A = 553.8 mg

Intravascular mass = 333.9 mg

1-pool analysis

k = 3.86/day

turnover = 1291 mg/day

Area under curve

turnover = 2015 mg/day

LDL_2 B APOLIPOPROTEIN

Plasma concentration = 135.6 mg/100 ml

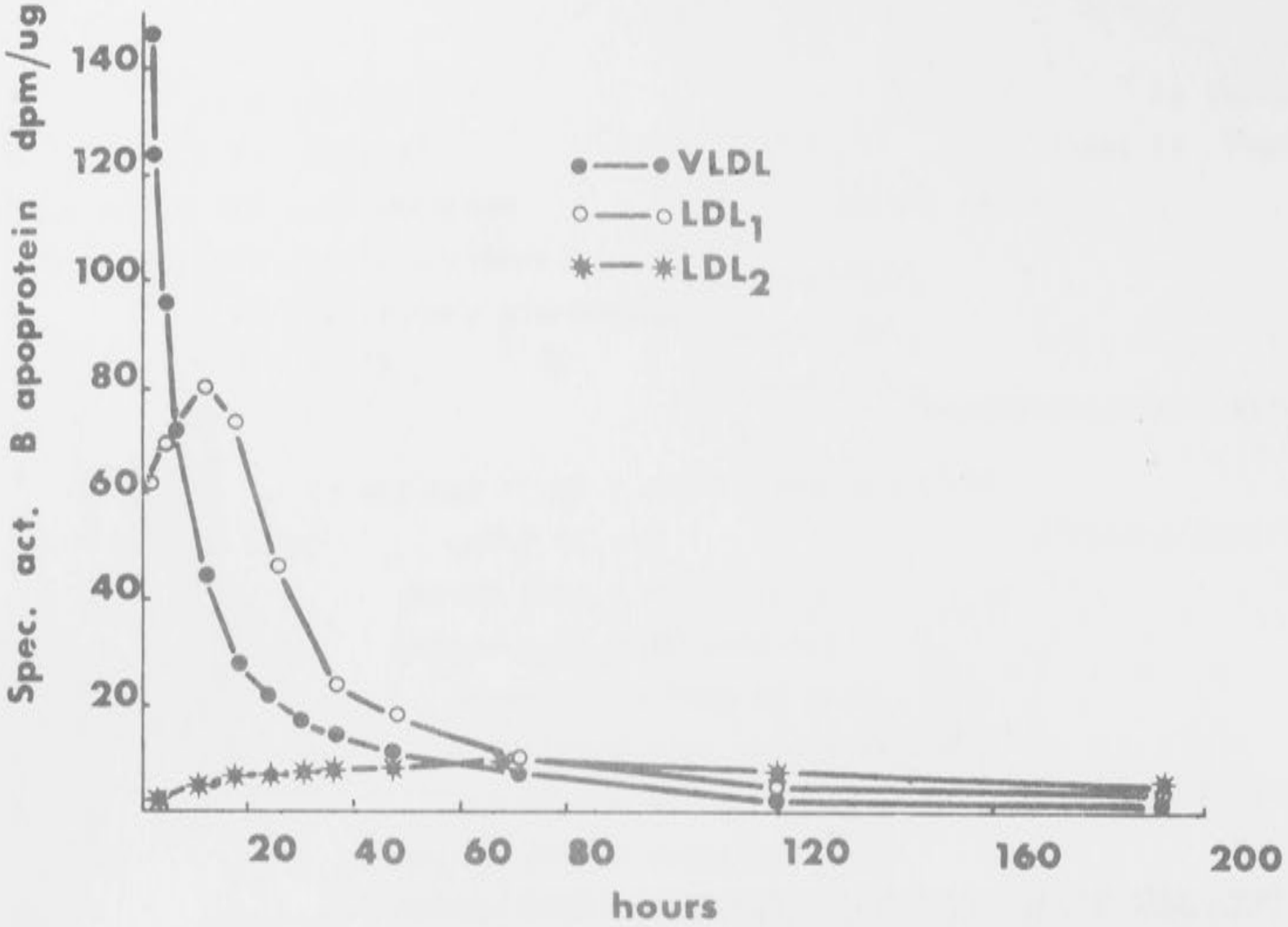
k = 0.29/day

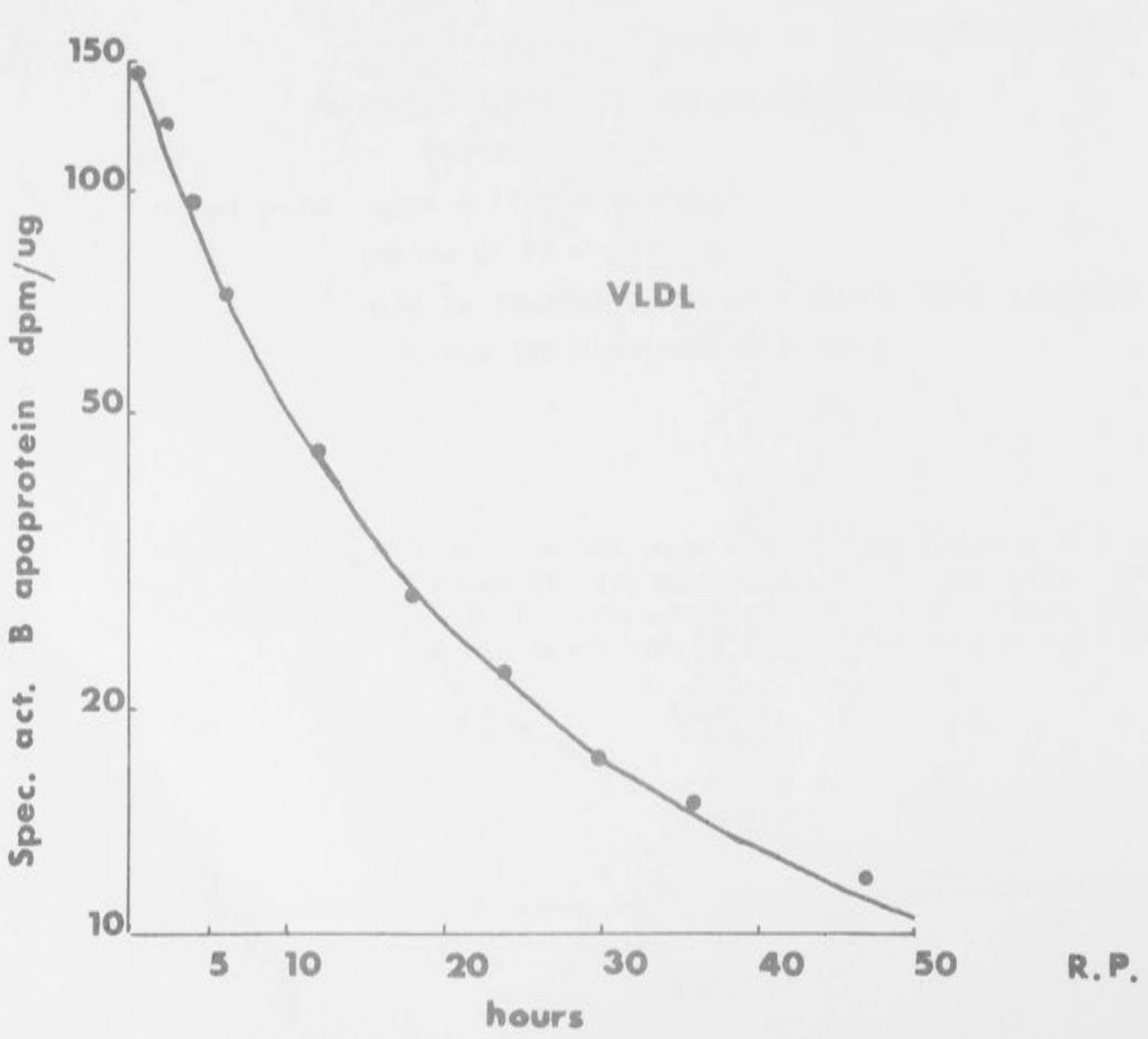
turnover = 1177 mg/day (using areas)

turnover/kg = 17.30 mg/day

turnover (calculated from disappearance of LDL-^{131}I B apolipoprotein) = 976 mgs/day

Figure 5.9 (facing page). The specific activity-time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 4 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.


Spec. act. B apoprotein dpm/ug
140
120
100
80
60
40
20
VLDL
LDL1
LDL2
20
40
60
80
120
160
200
hours


Spec. act. B apoprotein dpm/ug
150
100
50
20
10
VLDL
5
10
20
30
40
50
hours

R.P.

Study #5 *SUBJECT:* R.P.

Age: 58 years Weight: 72.5 kg

Plasma lipids Cholesterol: 294 mg/100 ml

Triglyceride : 252 mg/100 ml

Lipoprotein phenotype: 2B

VLDL B APOLIPOPROTEIN

Plasma concentration = 12.7 mgs/100 ml

2-pool analysis α = 4.3/day β = 0.9/day

turnover = 1238 mg/day

turnover/kg = 17.1 mg/day

Mass of pool A = 641 mg

Intravascular mass = 415 mg

1-pool analysis k = 3.0/day

turnover = 1255 mg/day

Area under curve turnover = 1323 mg/day

LDL_2 B APOLIPOPROTEIN

Plasma concentration = 131.9 mg/100 ml

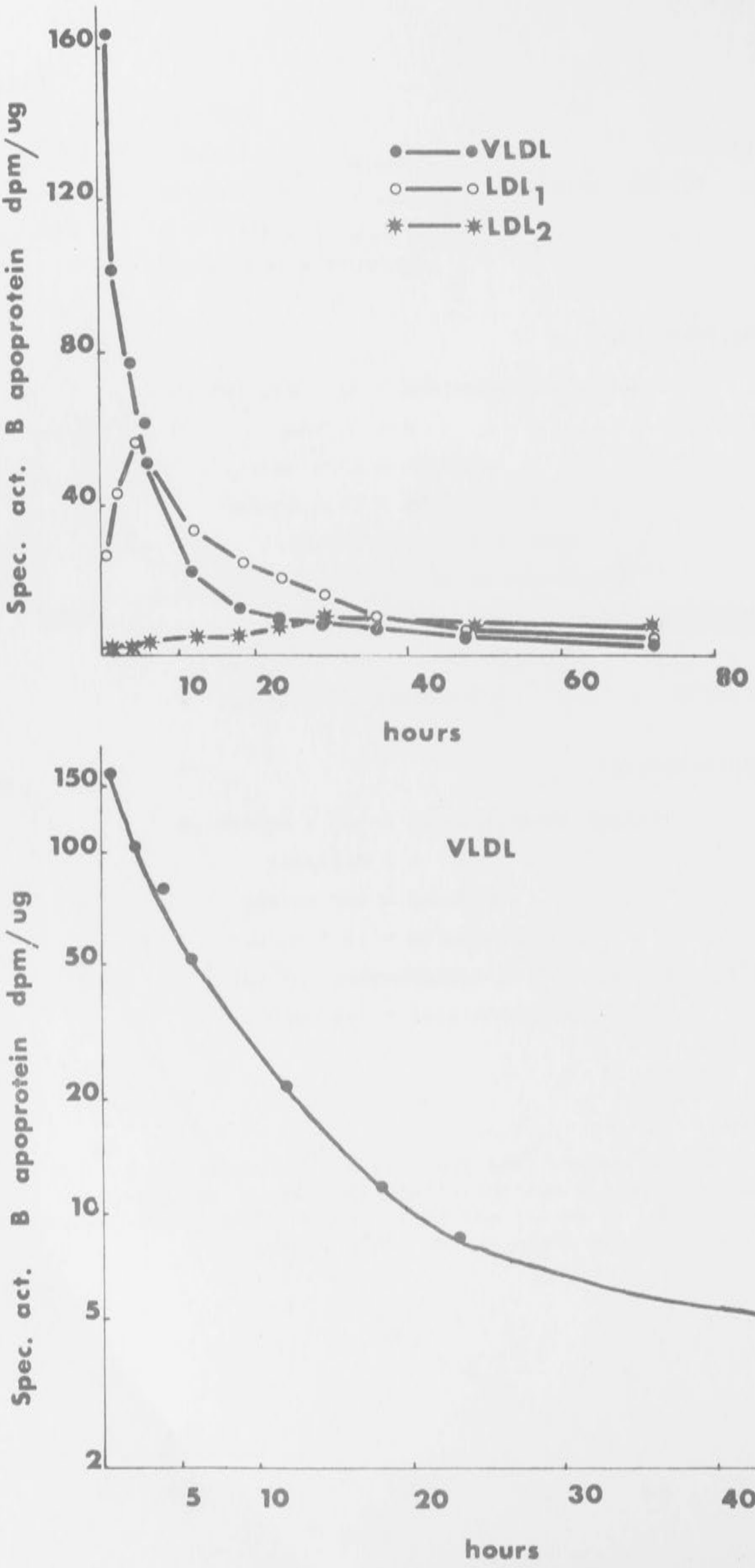
k = 0.23/day

turnover = 990 mg/day

turnover/kg = 13.7 mg/day

turnover (calculated from disappearance of LDL-^{131}I B apolipoprotein) = 748 mg/day

Figure 5.10 (facing page). The specific activity-time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 5 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.


160
120
80
40
Spec. act. B apoprotein dpm/ug
VLDL
LDL1
LDL2
10
20
40
60
80
hours
150
100
50
20
10
5
2
VLDL
Spec. act. B apoprotein dpm/ug
5
10
20
30
40
50
hours
P.F.

Study # 6 *SUBJECT:* P.F.

Age: 40 years Weight: 85.0 kg

Plasma lipids Cholesterol: 281 mg/100 ml

Triglyceride: 324 mg/100 ml

Lipoprotein phenotype: 2B

VLDL B APOLIPOPROTEIN

Plasma concentration = 12.7 mgs/100 ml

2-pool analysis α = 4.6/day β = 0.4/day

turnover = 1671 mg/day

turnover/kg = 19.7 mg/day

Mass of pool A = 485 mg

Intravascular mass = 654 mg

1-pool analysis k = 3.5/day

turnover = 1680 mg/day

Area under curve turnover = 1693 mg/day

LDL_2 B APOLIPOPROTEIN

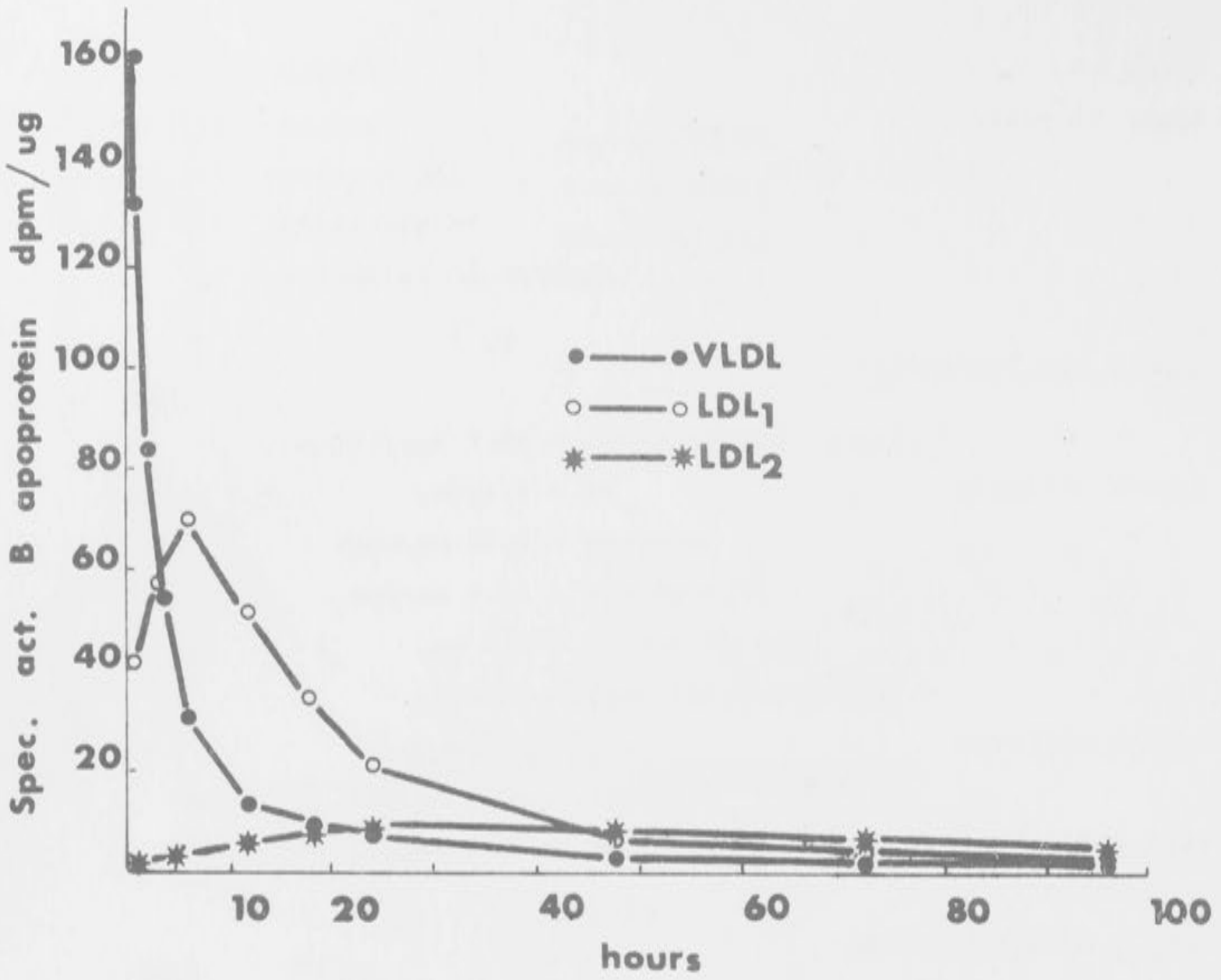
Plasma concentration = 112.0 mg/100 ml

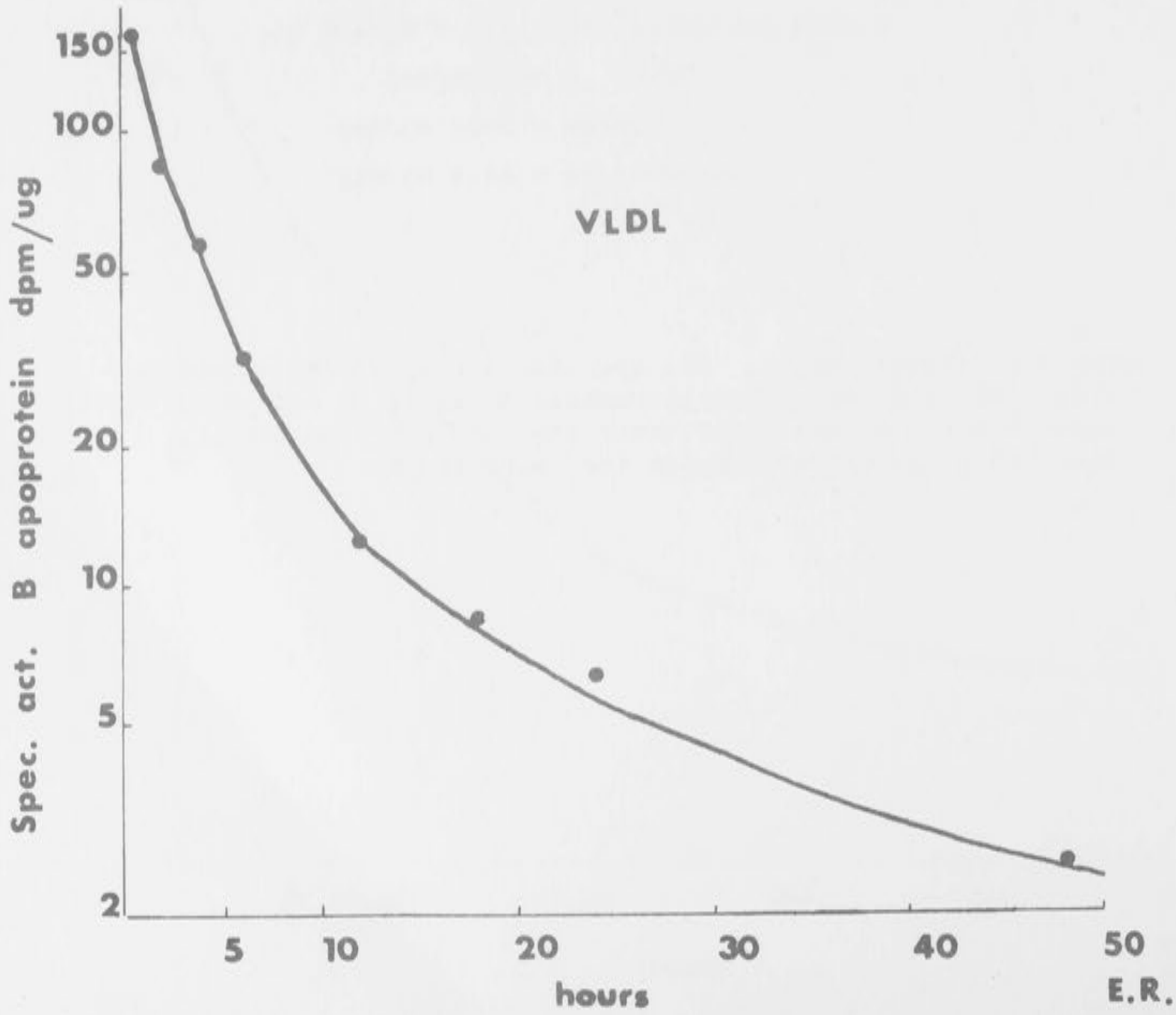
k = 0.36/day

turnover = 1542 mg/day

turnover/kg = 18.1 mg/day

Figure 5.11 (facing page). The specific activity time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 6 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.


Spec. act. B apoprotein dpm/ug
160
140
120
100
80
60
40
20
VLDL
LDL1
LDL2
10
20
40
60
80
100
hours


Spec. act. B apoprotein dpm/ug
150
100
50
20
10
5
2
VLDL
5
10
20
30
40
50
hours
E.R.

Study # 7 *SUBJECT:* E.R.

Age: 69 years Weight: 61 kg

Plasma lipids Cholesterol: 323 mg/100 ml

Triglyceride: 268 mg/100 ml

Lipoprotein phenotype: 4

VLDL B APOLIPOPROTEIN

Plasma concentration = 13.0 mgs/100 ml

2-pool analysis α = 8.5/day β = 0.7/day

turnover = 3345 mg/day

turnover/kg = 54.8 mg/day

Mass of pool A = 732 mg

Intravascular mass = 355 mg

1-pool analysis k = 8.3/day

turnover = 3033 mg/day

Area under curve turnover = 3983 mg/day

LDL_2 B APOLIPOPROTEIN

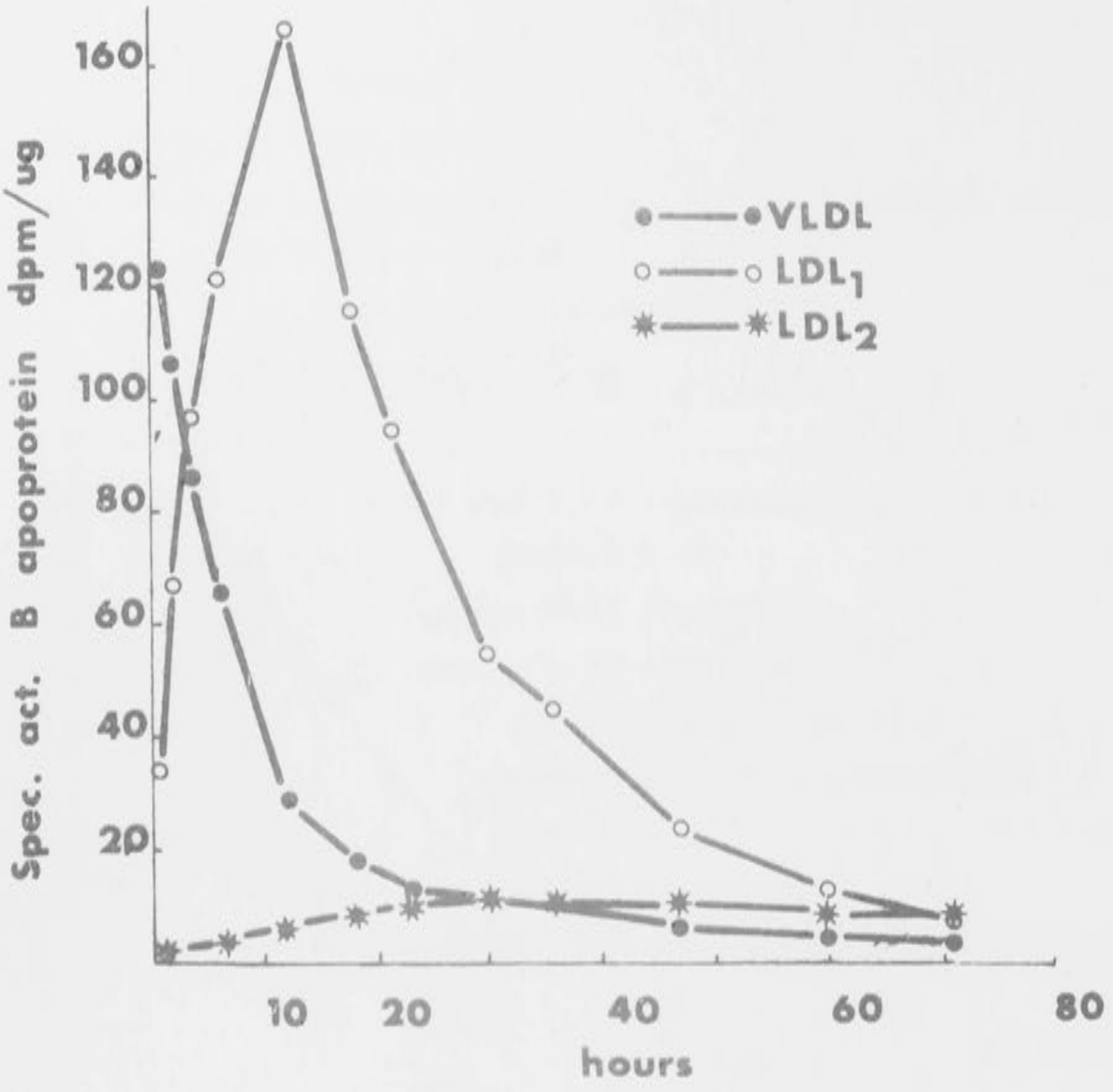
Plasma concentration = 97.0 mg/100 ml

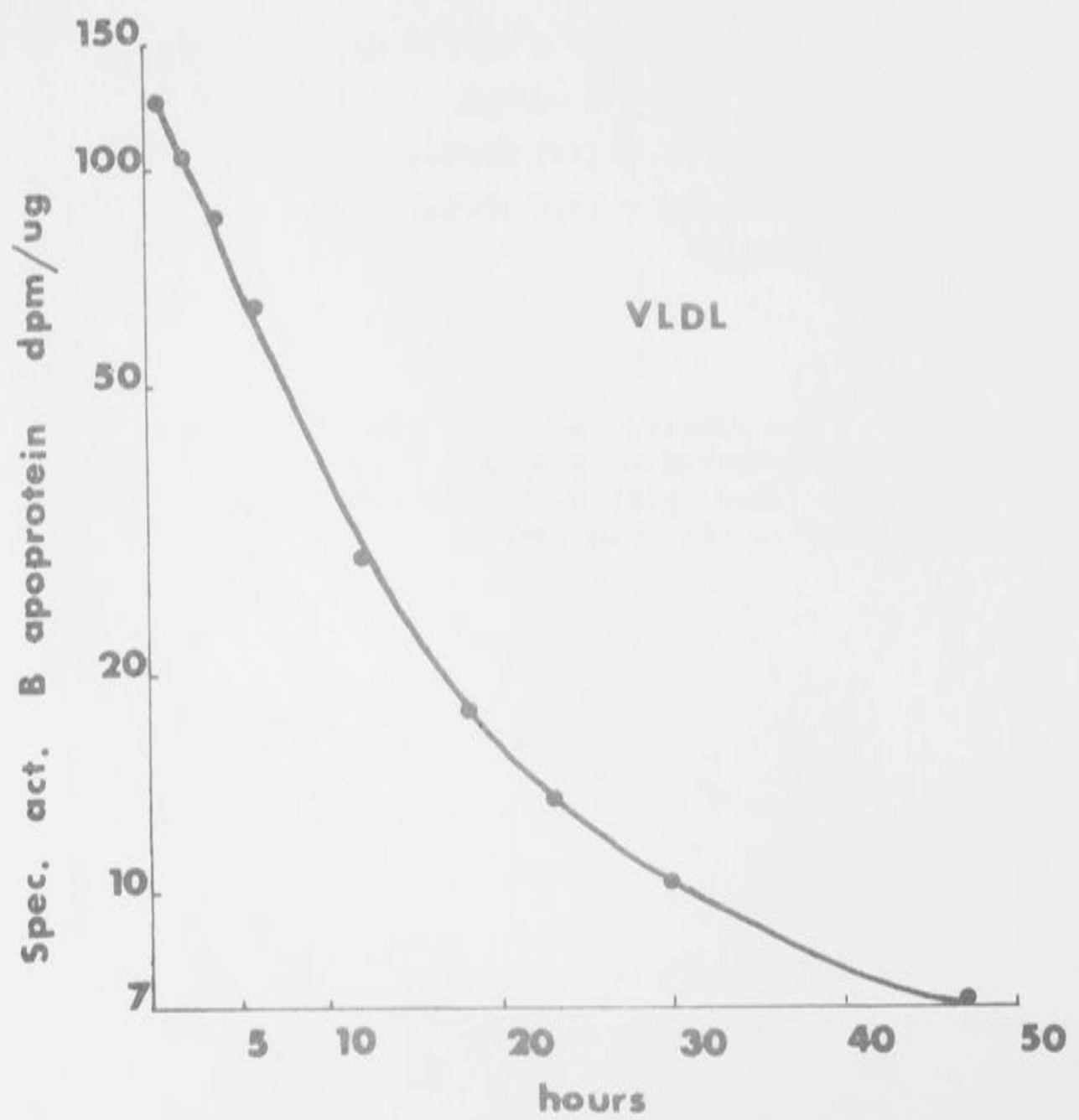
k = 0.43/day

turnover = 1144 mg/day

turnover/kg = 18.8 mg/day

Figure 5.12 (facing page). The specific activity time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 7 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.


Spec. act. B apoprotein dpm/ug
160
140
120
100
80
60
40
20
VLDL
LDL1
LDL2
10
20
40
60
80
hours


Spec. act. B apoprotein dpm/ug
150
100
50
20
10
7
VLDL
5
10
20
30
40
50
hours

D.H.

Study #8 *SUBJECT:* D.H.

Age: 62 years Weight: 82.2 kg

Plasma lipids Cholesterol: 213 mg/100 ml

Triglyceride: 405 mg/100 ml

Lipoprotein phenotype: 4

VLDL B APOLIPOPROTEIN

Plasma concentration = 25.5 mgs/100 ml

2-pool analysis α = 4.2/day β = 0.8/day

turnover = 2323 mg/day

turnover/kg = 28.3 mg/day

Mass of pool A = 1061 mg

Intravascular mass = 942 mg

1-pool analysis k = 2.8/day

turnover = 2613 mg/day

Area under curve turnover = 2380 mg/day

LDL_2 B APOLIPOPROTEIN

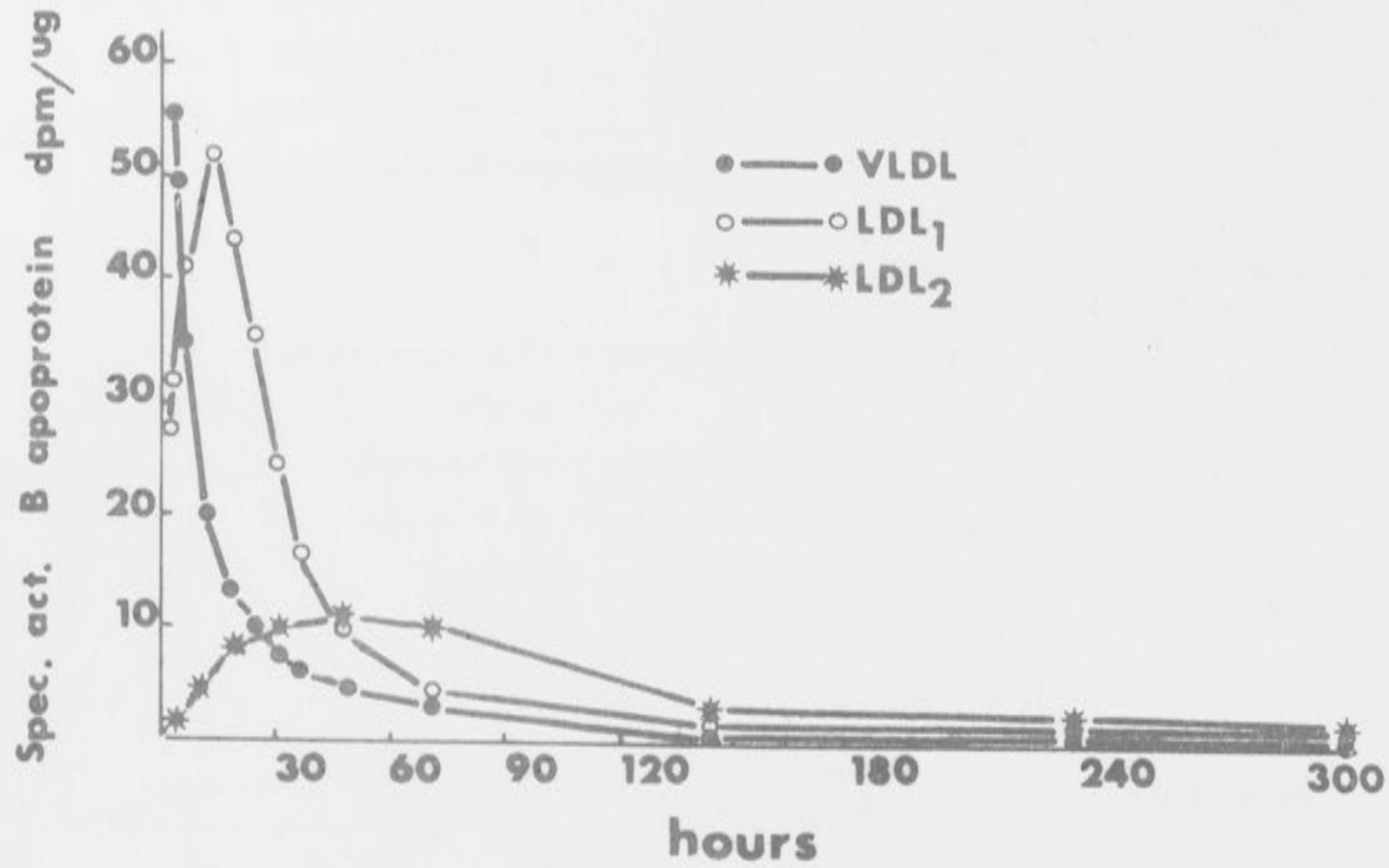
Plasma concentration = 77.1 mg/100 ml

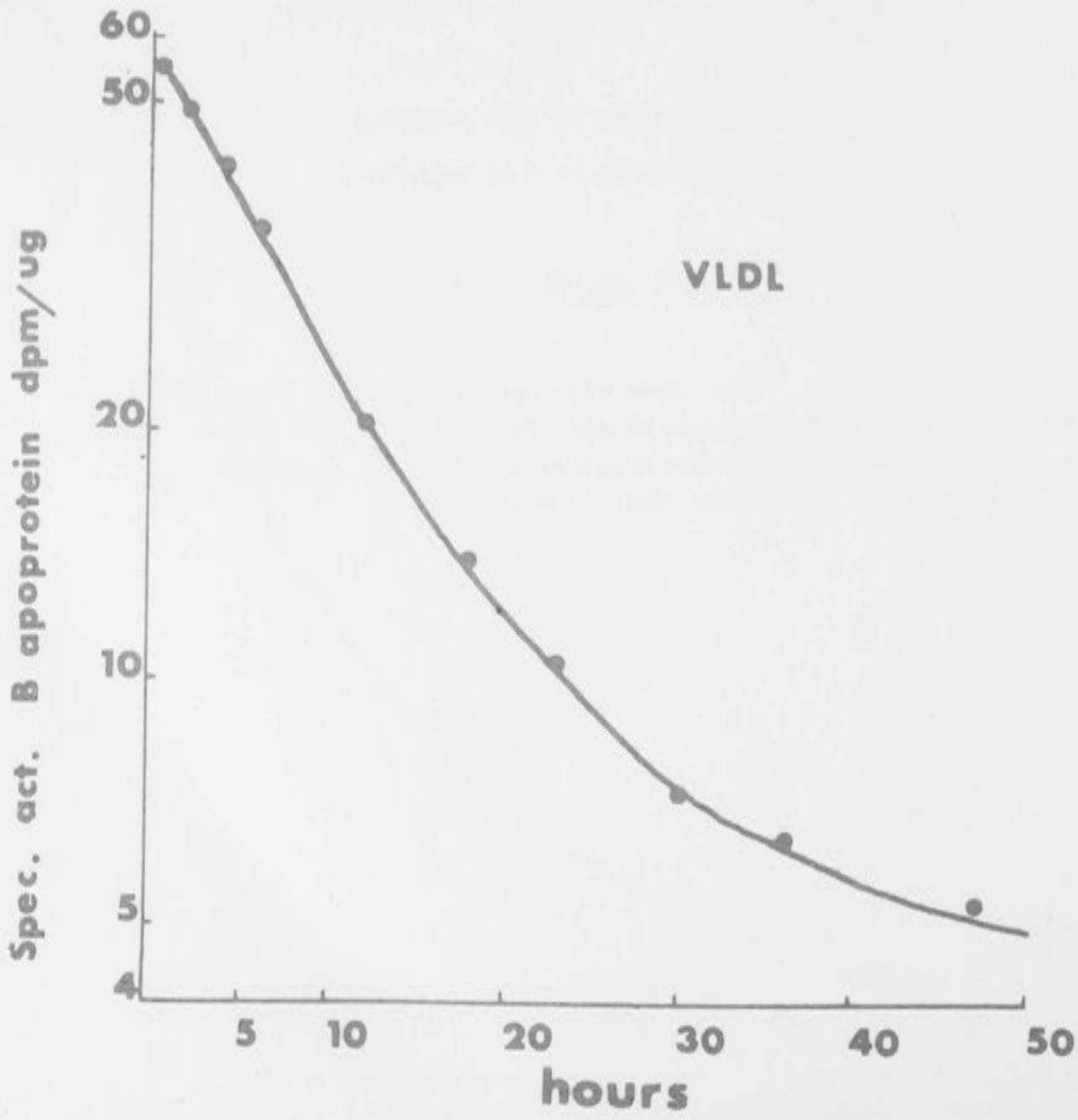
k = 0.27/day

turnover = 772 mg/day

turnover/kg = 9.4 mg/day

Figure 5.13 (facing page). The specific activity time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 8 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.


Spec. act. B apoprotein dpm/ug
60
50
40
30
20
10
VLDL
LDL1
LDL2
30
60
90
120
180
240
300
hours


Spec. act. B apoprotein dpm/ug
60
50
20
10
5
4
VLDL
5
10
20
30
40
50
hours

J.K.

Study #9

Age: 44 years

SUBJECT: J.K.

Weight: 80.6 kg

Plasma lipids

Cholesterol: 356 mg/100 ml

Triglyceride: 765 mg/100 ml

Lipoprotein phenotype: 4-5*

VLDL B APOLIPOPROTEIN (s_f 20-400 only)*

Plasma concentration = 35.6 mgs/100 ml

2-pool analysis

α = 2.78/day β = 0.5/day

turnover = 1928 mg/day

turnover/kg = 23.9 mg/day

Mass of pool A = 1544 mg

Intravascular mass = 1326 mg

1-pool analysis

k = 1.9/day

turnover = 2468 mg/day

Area under curve

turnover = 1917

LDL_2 B APOLIPOPROTEIN

Plasma concentration = 102.4 mg/100 ml

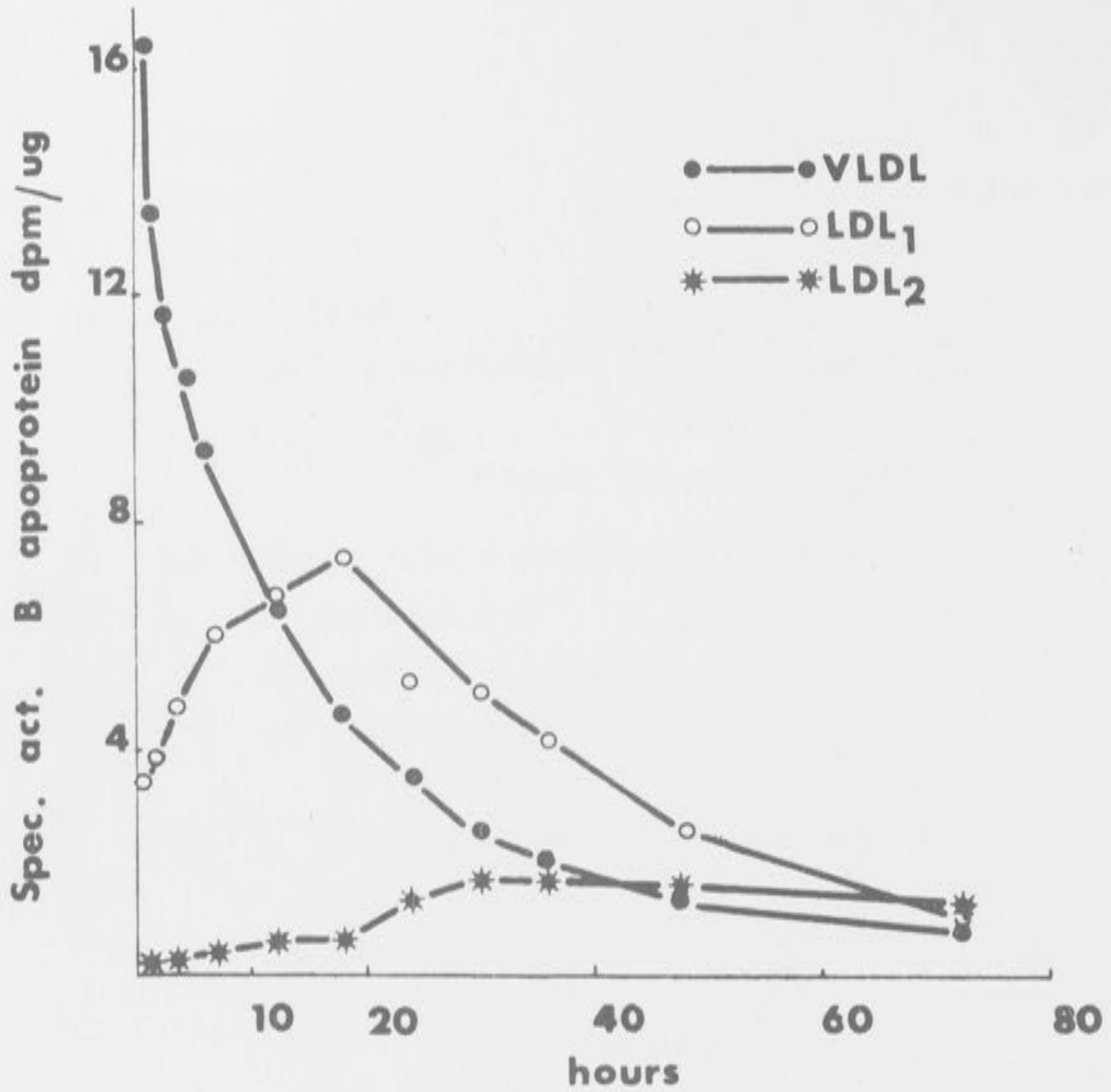
k = 0.37/day

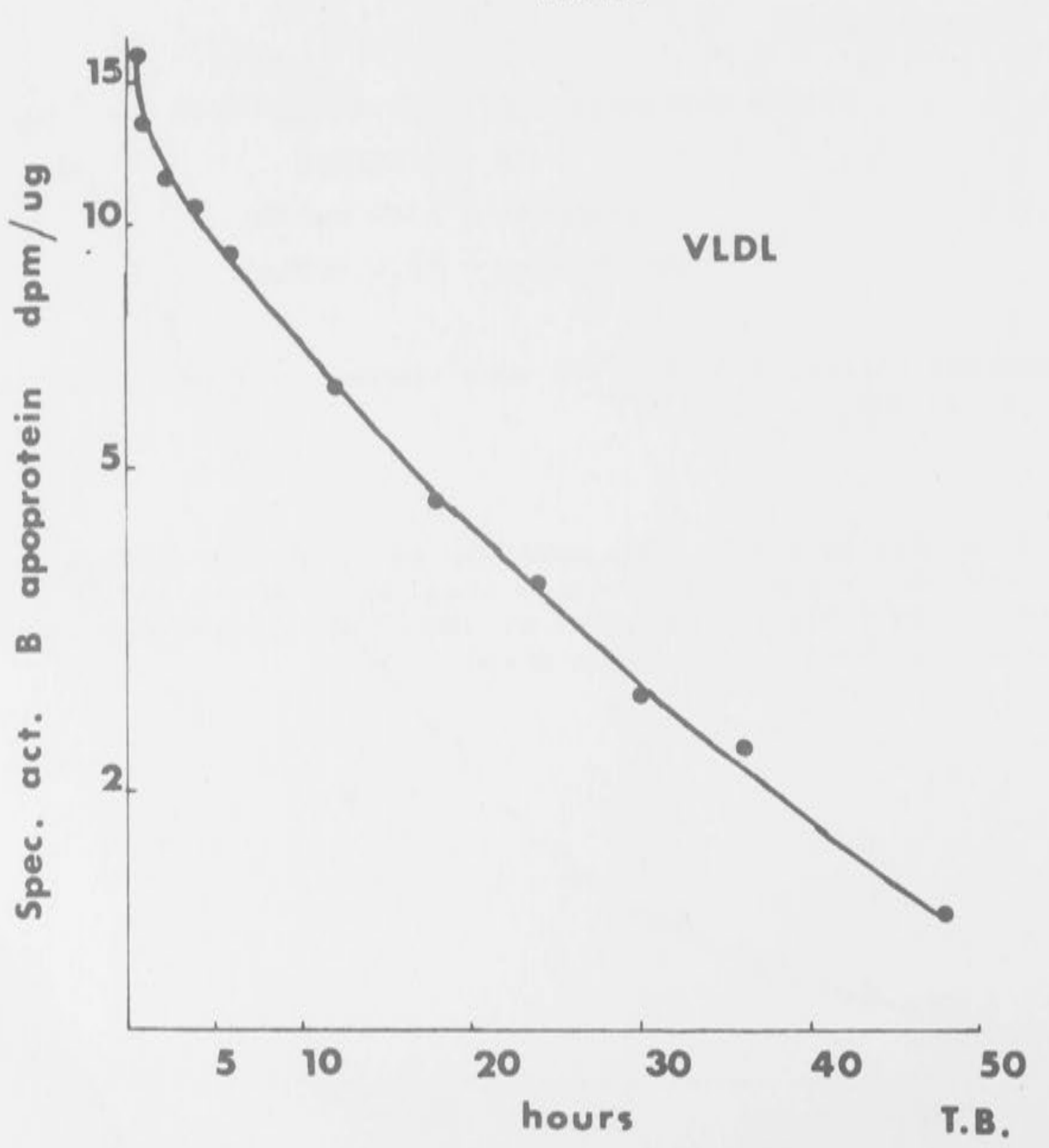
turnover = 1368 mg/day

turnover/kg = 17.0 mg/day

* Lipoprotein particles of $s_f > 400$ were removed prior to isolation of VLDL (s_f 20-400)

Figure 5.14 (facing page). The specific activity time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 9 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.


Spec. act. B apoprotein dpm/ug
16
12
8
4
VLDL
LDL1
LDL2
10
20
40
60
80
hours


Spec. act. B apoprotein dpm/ug
15
10
5
2
VLDL
5
10
20
30
40
50
hours
T.B.

Study #10 *SUBJECT*: T.B.

Age: 36 Weight: 90.2

Plasma lipids Cholesterol: 707 mg/100 ml

Triglyceride: 4,050 mg/100 ml

Lipoprotein plenotype: 5*

VLDL B APOLIPOPROTEIN (s_f 20-400 only)*

Plasma concentration	=	53.0 mg/100 ml			
2-pool analysis	α	=	3.1/day	β	= 0.9/day
turnover	=	3,060 mg/day			
turnover/kg	=	33.9 mg/day			
Mass of pool A	=	2,611 mg			
Intravascular mass	=	2,147 mg			
1-pool analysis	k	=	1.1/day		
turnover	=	2,381 mg/day			
Area under curve	turnover	=	3,163 mg/day		

LDL_2 B APOLIPOPROTEIN

Plasma concentration	=	78.2 mg/100 ml
k	=	0.34/day
turnover	=	1,074 mg/day
turnover/kg	=	11.9 mg/day

* Lipoproteins of $s_f > 400$ were removed prior of isolation of VLDL (s_f 20-400).

Figure 5.15 (facing page). The specific activity time curves for VLDL, LDL_1 and LDL_2 B apolipoprotein in study 10 are shown in the upper graph. A semi-logarithmic plot of VLDL B apolipoprotein specific activity is shown in the lower graph.

study 2 where LDL_1 was not analysed), the LDL_1 specific activity curve intersected that of VLDL in a manner suggesting a precursor-product relationship between these lipoprotein fractions. The specific activity of LDL_1 rose rapidly to intersect the VLDL specific activity-time curve between 30 min and 6 hours after injection of VLDL-^{125}I except in study 10 (type 5 HLP) where the cross-over was delayed until 18 hours after injection, presumably because of the greatly expanded VLDL pool in this subject. In 6 of the 9 studies, LDL_1 specific activity reached its peak shortly after intercepting that of the precursor. This observation would be consistent with the immediate precursor of the s_f 12-20 fraction being a smaller particle than newly secreted VLDL i.e. a lipoprotein that had undergone partial catabolism.

The specific activity of LDL_2 rose more slowly than that of LDL_1 and consequently it did not, over the time scale shown, have a precise peak. In all studies, both VLDL and LDL_1 specific activity curves intercepted the curve of LDL_2 at its peak suggesting that either or both lipoprotein fractions could serve as the precursor to LDL_2. LDL_2 specific activities intersected those of VLDL within 12 to 60 hours of injection and those of LDL_1 within 20 to 80 hours.

These findings strongly imply that, in man, all LDL_2 is derived ultimately from VLDL although it is not clear from a mere consideration of the respective specific activity-time curves whether LDL_1 is an obligatory intermediate in this process.

5.4.3 VLDL B apolipoprotein kinetics

In every study, the specific activity-time curve for VLDL was

TABLE 5.1

RESULTS FROM KINETIC ANALYSES OF THE TURNOVER OF VLDL B APOLIPOPROTEIN.

This table summarises the findings of studies #1 - 10. Analyses were performed as described in section 5.3.

Study #	Lipoprot. Phenotype	B Apolipo. Conc. (mg/100 ml)	B Apolipoprotein Turnover 1-pool (mg/day)	2-pool (mg/day)	Area (mg/day)	Rate Constants α^* (/day)	β^* (/day)	$k^\ddagger$ (/day)	B Apolipoprotein Mass Pool A (mg)	Intravascular (mg)
1	N	4.2	99	-	-	-	-	3.9	-	99
2	N	1.7	359	-	-	-	-	8.4	-	44
3	N	5.2	1041	637	751	22.2	1.1	7.9	181	131
4	2A	10.8	1291	1092	1052	12.9	1.0	3.9	554	334
5	2B	12.7	1255	1238	1323	4.3	0.9	3.0	641	415
6	2B	12.7	1680	1671	1693	4.6	0.4	3.5	654	485
7	4	13.0	3033	3345	3983	8.5	0.7	8.3	732	355
8	4	25.5	2613	2323	2380	4.2	0.8	2.8	1061	942
9	4	35.6	2468	1928	1917	2.8	0.5	1.9	1544	1326
10	5	53.0	3401	3060	3163	3.1	0.9	1.1	2611	2147
Mean ± 1 SD†			2098±897	1912±953	2034±1097	7.8±6.7	0.8±0.2	4.1±2.7	997±764	767±677

* Rate constants for each exponential (2-pool analyses).

‡ Fractional catabolic rate (1-pool analyses).

† Studies #3 - 10 only.

multi-exponential over at least 50 hours (as shown by the semi-logarithmic plots in Figures 5.6 to 5.15). The first five points on these curves encompassing the first 12 hours almost described a monoexponential function in most subjects. However, when the 50-hour curve was resolved into 2 exponential functions by the curve peeling technique, the first and faster exponential of the 2-pool model conformed closely, if not precisely, to a monoexponential function. However, in study 2 where the subject has a VLDL B apolipoprotein concentration of only 1.7 mg/100 ml, the specific activity-time curve appeared monoexponential until 95% of the radioactivity had left the 20-400 fraction (Figure 5.7) and it seemed more appropriate to calculate the turnover from a single pool analysis only.

As shown in Table 5.1 (summarising the data of studies 1-10) the calculated values for turnover, using either the 1 or 2-pool model were in most cases similar. It should be borne in mind that for calculations of the 1-pool model, it is assumed that the pool is confined to the plasma compartment only. The 2-pool model requires no knowledge of pool sizes to determine the turnover of pool A. The mass of pool A (as determined by compartmental analysis described in section 5.3.2.2) was in all studies higher (average 30%) than the intravascular mass of VLDL B apolipoprotein suggesting that some of the metabolism of s_f 20-400 VLDL might occur in the extravascular space.

The turnover of VLDL B apolipoprotein in pool A was also assessed from the area under the specific activity-time curve (as described in section 5.3.4). This alternative calculation gave values that were quite similar for all subjects confirming the suitability of the 2-pool model.

The relationship between the intravascular mass of VLDL B apolipoprotein and the turnover (derived by 2-pool analysis) suggested a saturable system (Figure 5.16). In the 7 subjects with normal or moderately raised levels of triglyceride, turnover and pool size were directly related indicating that expansion of the pool was a function of increased production. The 3 subjects with the highest triglyceride level, 2 of whom also showed chylomicronaemia (though particles of $s_f > 400$ have been excluded from all assays and calculations) showed an increase in pool size that was out of proportion to the increase in turnover, suggesting saturation of removal mechanisms.

5.4.4 LDL_1 B apolipoprotein turnover

As mentioned in section 5.4.1, in every study, the LDL_1 specific activity curve intersected the VLDL curve in a manner suggestive of a precursor-product relationship. An index of the turnover rates of LDL_1 in relationship to those of VLDL were gained by comparing the respective areas under the specific activity-time curves of the two lipoprotein fractions (Table 5.2). In all cases, the area under the LDL_1 curve exceeded that for VLDL (average 80% greater). Since turnover is inversely related to the area under the curve, LDL_1 turnover was clearly less than that of VLDL. If some of the label injected with the VLDL lipoproteins was removed by a process other than through the LDL_1 fraction, then the estimated turnover of the latter fraction would be even less. It therefore seems highly likely that a substantial fraction of VLDL catabolism bypasses the LDL_1 fraction, as will be further illustrated by results in chapter 6.

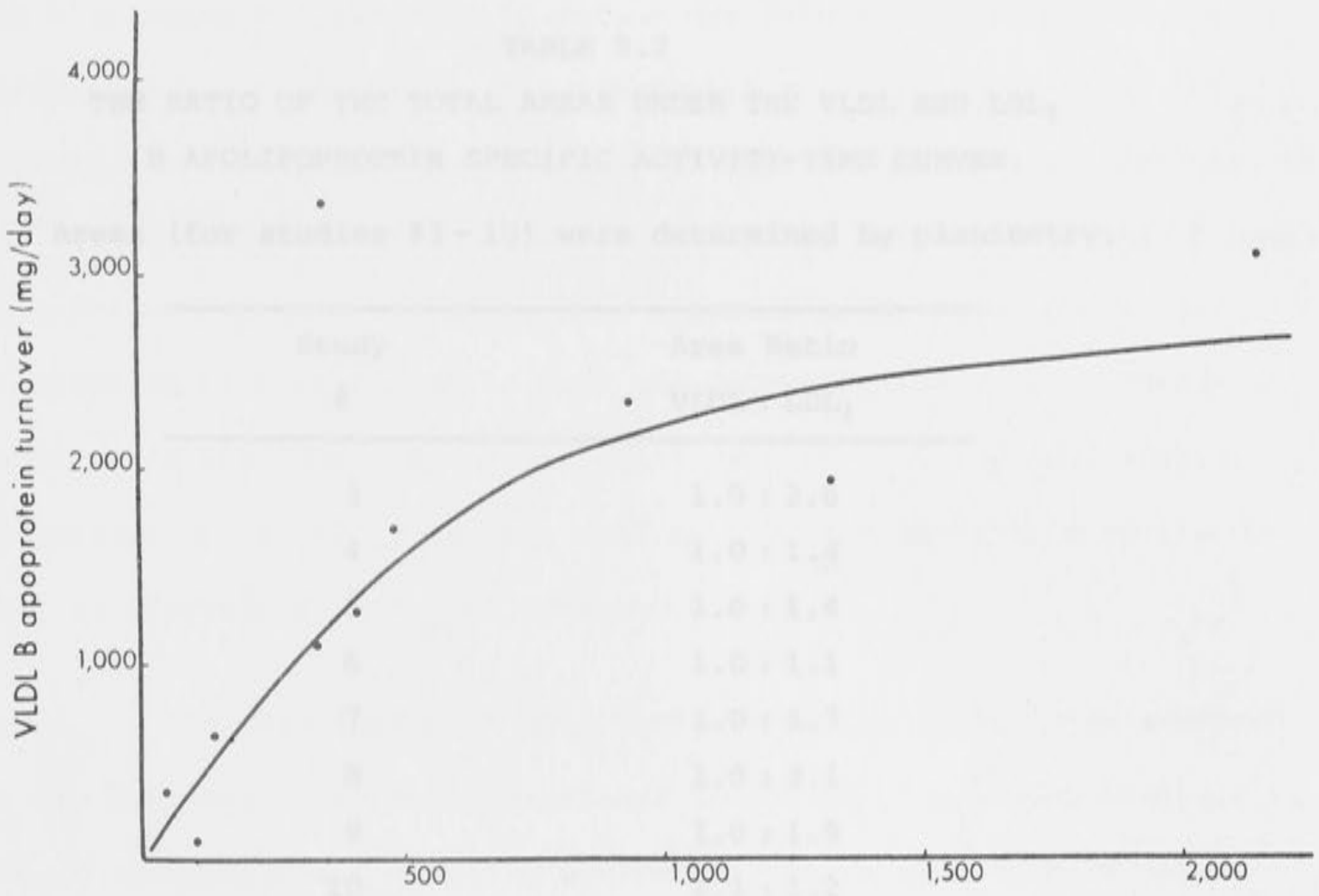


Figure 5.16 The relationship between VLDL B apolipoprotein turnover (2-pool analyses) and intravascular VLDL B apolipoprotein mass (studies #1-10).

TABLE 5.2

THE RATIO OF THE TOTAL AREAS UNDER THE VLDL AND LDL_1 B APOLIPOPROTEIN SPECIFIC ACTIVITY-TIME CURVES.

Areas (for studies #3 - 10) were determined by planimetry.

Study #	Area Ratio VLDL : LDL_1
3	1.0 : 2.6
4	1.0 : 1.4
5	1.0 : 1.4
6	1.0 : 1.1
7	1.0 : 1.7
8	1.0 : 3.1
9	1.0 : 1.9
10	1.1 : 1.2
Mean ± 1 SD	1.0 : 1.8 ± 0.7

5.4.5 Conversion of VLDL to LDL_2

The specific radioactivity of the B apolipoprotein in LDL_2 was also measured primarily to define the nature of the VLDL to LDL relationship. In addition, the fractional rate of labelling of LDL_2 protein was calculated from the rate of rise in LDL specific activity and the rate of fall in VLDL specific activity during the first 18 hours. As described in section 5.3.3, this model requires that LDL_2 B apolipoprotein be derived solely from the corresponding apolipoprotein in VLDL. This was indicated by the cross-over of VLDL and LDL_2 specific activities occurring at or very near to the peak specific activity of LDL_2 as previously mentioned (section 5.4.1).

The calculation of LDL_2 turnover by this method was compared in two subjects with the conventional technique of a 2-pool analysis of LDL_2 B apolipoprotein specific activities over a period of 14 days after the intravenous administration of LDL_2-^{131}I. In both subjects, the present method (i.e. as described in section 5.3.4) gave values that were 20% less than by 2-pool analysis. However, the 2-pool method may overestimate turnover when compared with the possibly more appropriate 3-pool model (Goodman *et al.*, 1973). On the other hand, the present method may underestimate LDL_2 turnover since the calculation utilises only the mass of intravascular LDL_2 B apolipoprotein whereas it is known that some LDL_2 B apolipoprotein metabolism occurs extravascularly (section 1.5.3).

This much more rapid estimate of LDL_2 turnover was therefore compared in all subjects (except study 1) with the turnover of VLDL s_f 20-400 lipoproteins (Table 5.3). The LDL_2 turnover ranged from 5.5 to 18.7 mg/kg/day which is in line with findings reported by others with

TABLE 5.3

COMPARISON OF TURNOVER OF B APOLIPOPROTEIN IN VLDL AND LDL_2.

Study	Lipo-protein Phenotype	LDL B Apolipoprotein Data				VLDL Apolipo-protein B Turnover	LDL/VLDL Turnover
		Concen-tration	Turnover Rate	Turnover			
#		mg/100 ml	k/day	mg/day	mg/day/kg	mg/day	%
2	N	47	0.33	405	5.5	359	113
3	N	80	0.29	579	10.3	637	91
4	IIA	135	0.29	1177	17.3	1092	107
5	IIB	131	0.23	990	13.7	1238	80
6	IIB	112	0.36	1542	18.1	1671	92
7	IV	97	0.43	1144	18.7	3345	34
8	IV	77	0.27	772	9.4	2323	33
9	IV	102	0.37	1368	17.0	1928	70
10	V	78	0.34	1074	11.9	3060	35
Mean ± 1 SD		94 ± 30	0.32 ± .06	996 ± 384	13.5 ± 4.6	1739 ± 1030	

the 2-pool model (Bilheimer *et al.*, 1975). There was little difference in LDL_2 turnover between the 4 subjects with hypertriglyceridaemia alone and the others. However, whereas LDL_2 turnover was substantially less than VLDL turnover in the 4 hypertriglyceridaemic subjects, the 2 measurements were similar in the other 5 subjects. This suggests that, at higher concentrations of VLDL, a considerable fraction of VLDL B apolipoprotein turnover is catabolised by a route other than through LDL_2.

5.5 DISCUSSION

5.5.1 LDL formation from VLDL

The existence, in man, of precursor-product relationships between VLDL and LDL has been well documented with respect to both the triglyceride and protein moieties (Gitlin *et al.*, 1958; Havel, 1961; Farquhar *et al.*, 1965; Quarfordt *et al.*, 1970; Bilheimer *et al.*, 1972; Eisenberg *et al.*, 1973; Sigurdsson *et al.*, 1975; Eaton *et al.*, 1976). Some of these studies, involving VLDL labelled in its protein moiety with ^{125}I, ^{131}I or ^{35}Se have demonstrated the transfer of the B apolipoprotein of VLDL to LDL. However, as B apolipoprotein specific activity data was not obtained in the quoted studies (with the exception of Sigurdsson *et al.*, 1975) it was not possible to determine the extent to which LDL was derived from VLDL and conversely the amount of VLDL that was catabolised to LDL. Eisenberg *et al.* (1973) and Eaton *et al.* (1976) have unequivocally demonstrated, by use of heparin administration, that the VLDL catabolic process leads to the transfer of the B apolipoprotein from VLDL to LDL.

Sigurdsson *et al.* (1975), recently described precursor-product relationships between VLDL and LDL (s_f 0-20) utilising the specific

activities of the B apolipoprotein in these two lipoprotein fractions (following the injection of VLDL-^{125}I into man). The method used by these authors for the determination of B apolipoprotein specific activities has previously been discussed (section 3.2.3). From the nature of the cross-over of the specific activity-time curves of the B apolipoprotein of VLDL and LDL (Figure 5.17a) these authors have proposed that LDL B apolipoprotein is solely derived from VLDL.

In the studies presented in this chapter, the LDL_1 fraction (s_f 12-20) was also isolated as a distinct lipoprotein species. Bilheimer *et al.* (1972) have suggested that LDL_1 may serve as the intermediate between VLDL and LDL_2 (s_f 0-12). As shown in Figures 5.6 - 5.15, precursor-product relationships were found suggesting firstly, that LDL_1 is derived solely from VLDL and secondly, that LDL_2 is derived from either VLDL or LDL_1, so that eventually it is derived from VLDL. Thus these studies agree with those of Sigurdsson *et al.* (1975), in showing that in man LDL_2 is solely derived from VLDL. However, both in the monkey (Illingworth, 1975) and in the rat (Fidge, personal communications) some LDL may be formed independently of VLDL catabolism and this may yet be demonstrated in man under certain circumstances.

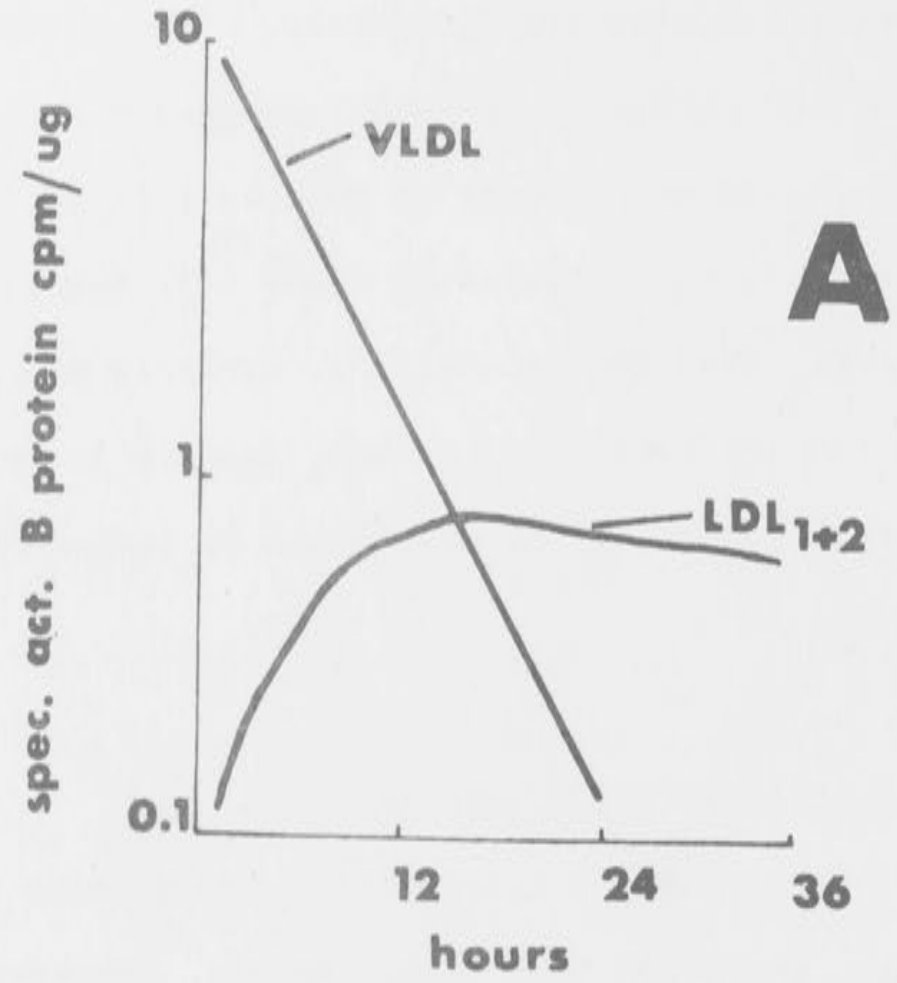
Sigurdsson *et al.* (1975) have the LDL_1 fraction (s_f 12-20) in the analysis of LDL specific activity. These authors did not isolate LDL_1 separately as the protein content of this pool was too small to measure by their technique. To compare the results of this chapter with those of Sigurdsson *et al.*, the specific activity of the LDL fraction (s_f 0-20) has been recomputed to include both the LDL_1 and LDL_2 fractions, taking into account the differences in pool size of these two fractions. The values so obtained together with those for VLDL, from

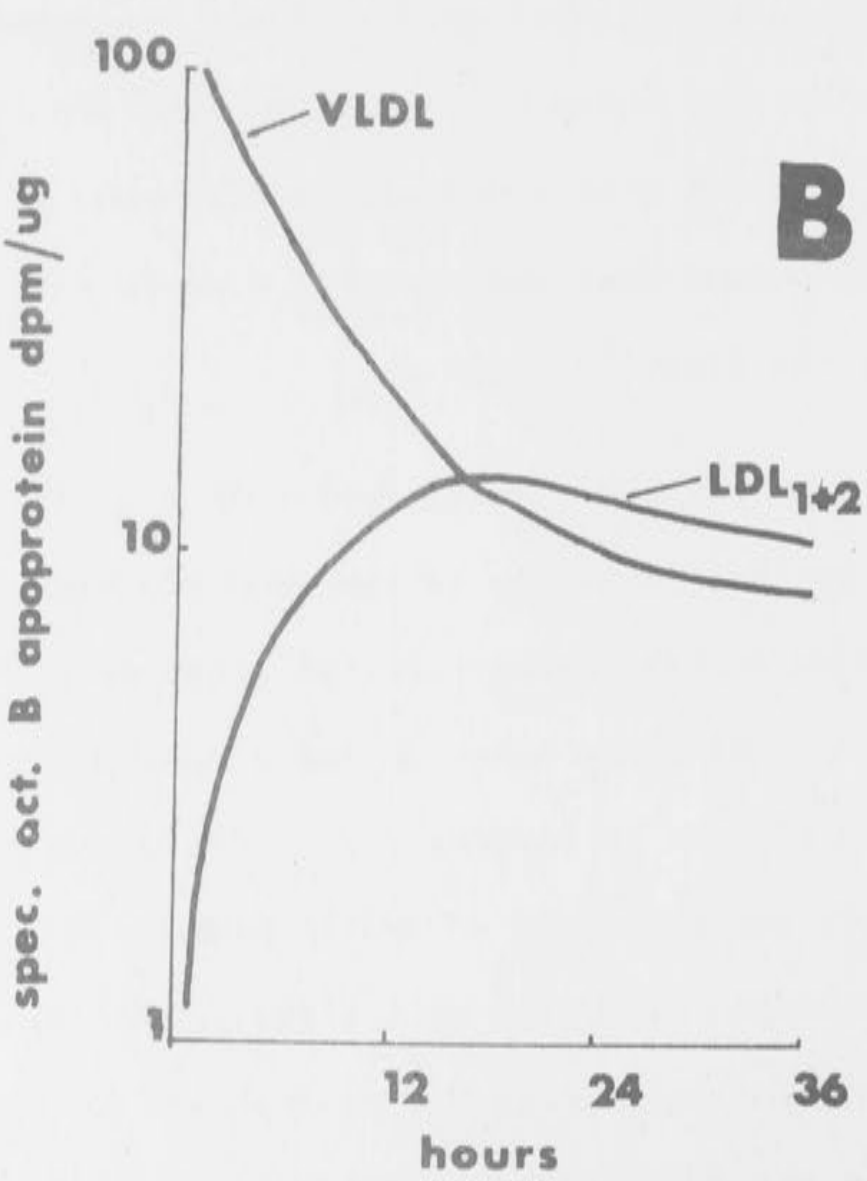
a representative study (# 8), are shown in Figure 5.17b. Although the VLDL disappearance curves are dissimilar, the specific activities of LDL cross the VLDL curves in an identical manner. However, the influence of the LDL_1 fraction on the rate of labelling of LDL is evident when the LDL_2 specific activity curve of study 8 is compared (Figure 5.17c). Although the LDL_1 fraction is small, it markedly alters the rate of labelling of the LDL fraction. As LDL_1 appears to be a metabolically distinct lipoprotein class, it should not be included in the LDL class of lipoproteins.

5.5.2 VLDL B apolipoprotein turnover

In every study, the specific activity-time curve during the first 50 hours, conformed to a 2-pool model. Sigurdsson *et al.* (1975) had also noted the curvilinear nature of the log-linear plot of the specific activity-time curve but concluded that the initial 24-hour segment was consistent with a monoexponential rate of removal. The present studies showed that was possibly true in only some subjects and then only over the first 12 hours.

The 2-pool characteristics of VLDL B apolipoprotein turnover could have arisen artefactually if the specific radioactivity values were distorted by contaminating labelled proteins with turnover kinetics that differed from those of the B apolipoprotein. This possibility has been dealt with in section 3.2.5. In summary however, the presence of only trace amounts of apolipoprotein E in SDS gels of NH_4HCO_3 insoluble proteins, the similarity in the amino acid composition of the insoluble proteins derived from VLDL and LDL and the similarity in the turnover rates derived from analysis of NH_4HCO_3 and TMU insoluble protein strongly suggest that contamination of the VLDL B


10
1
0.1
spec. act. B protein cpm/ug
VLDL
LDL 1+2
A
12
24
36
hours


100
10
1
spec. act. B apoprotein dpm/ug
VLDL
LDL 1+2
B
12
24
36
hours

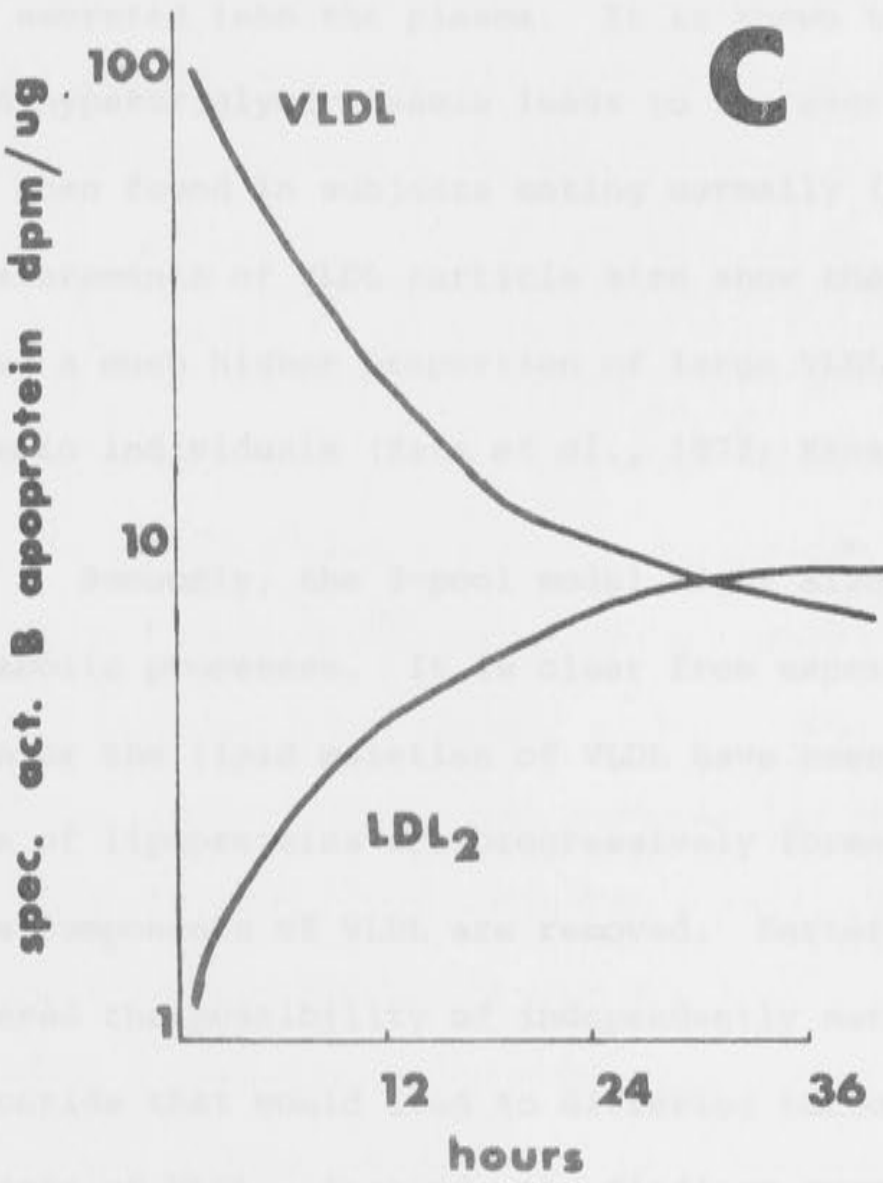


Figure 5.17 A comparison of the precursor-product relationships between VLDL and LDL (s_f 0-20) B apolipoprotein as observed by Sigurdsson *et al.* (1976) (A) or in these studies (B) - study #8. Differences in the rate of labelling of s_f 0-20 and s_f 12-20 B apolipoprotein from VLDL may be seen by comparing figures B and C (results of study #8).

apolipoprotein was minimal.

A 2-pool model is consistent with some of the known metabolic characteristics of VLDL. Firstly, more than one population of particles may be secreted into the plasma. It is known that carbohydrate-induced hypertriglyceridaemia leads to the secretion of VLDL that are larger than found in subjects eating normally (Ruderman *et al.*, 1971), and measurements of VLDL particle size show that hyperlipidaemic subjects may have a much higher proportion of large VLDL than fasting normolipidaemic individuals (Sata *et al.*, 1972; Kane *et al.*, 1975).

Secondly, the 2-pool model might also reflect heterogeneity of catabolic processes. It is clear from experiments in which the protein or the lipid moieties of VLDL have been labelled that smaller species of lipoproteins are progressively formed as triglyceride and surface components of VLDL are removed. Barter and Nestel (1970, 1972) considered the possibility of independently metabolized pools of VLDL triglyceride that would lead to differing turnover rates for different subclasses of VLDL. Instead, the findings suggested a step-wise transformation of larger to smaller lipoproteins with LDL representing the final step. However, the catabolism of the B apolipoprotein moiety of VLDL is likely to differ from that of triglyceride since the small remnants of VLDL metabolism appear to contain all the B apolipoprotein of the parent VLDL but very much less triglyceride (Eisenberg *et al.*, 1973; Eisenberg and Rachmilewitz, 1975). Experiments in the rat show that catabolism of VLDL B apolipoprotein may indeed proceed by more than one route; less than 10% of labelled B apolipoprotein in injected VLDL was reported to have been recovered in LDL whereas the uptake of label in the liver was substantial (Fidge and Poulis, 1974; Faergeman

et al., 1975). The rate and extent of uptake of the B apolipoprotein in rat liver resembles that of the cholesteryl esters (Mjøs *et al.*, 1975) which remain as the major core component of the partially catabolized VLDL. This provides strong evidence that at least in the rat, some remnant particles are removed by the liver while others are destined for conversion to LDL.

A third contributing factor to multiexponential kinetics might occur from the distribution of VLDL B apolipoprotein in more than one pool with each pool having different turnover characteristics. While it is highly probable that the catabolism of VLDL takes place largely within the plasma, lipoproteins resembling VLDL have been identified in human lymph (Ockner *et al.*, 1968, 1969; Windmueller and Levy, 1968). Catabolism of VLDL remnants has been described in several tissues (Stein *et al.*, 1974) and VLDL B apolipoprotein can be degraded in a variety of cultured cells (Bierman *et al.*, 1973; Goldstein and Brown, 1974). The presence of VLDL-like particles in the intestine (Tytgat *et al.*, 1971; Jones and Ockner, 1971) represents another possible pool of VLDL that might exchange with the intravascular pool. In this regard, it is also important to note the recent demonstration by Rachmilewitz *et al.*, (1967) of the capacity of the human intestine to synthesise the B apolipoprotein.

One can only speculate about the physiological or anatomical dimensions of the 2 pools. Pool A may include in addition to plasma, the lymph and possibly tissues such as liver and gut which give rise to VLDL. Pool B may include cells like fibroblasts, arterial walls and lymphocytes. Human lymphocytes have recently been shown to bind and degrade VLDL B apolipoprotein (Vijayogopal and Nestel, personal communications).

The multiexponential nature of the specific activity curves might therefore reflect several factors including heterogeneity of turnover rates among several populations of VLDL particles, alternative catabolic processes and catabolism within several tissue or lipoprotein pools.

5.5.3 LDL turnover in relation to VLDL turnover

The values obtained for LDL turnover by the present technique were in reasonable agreement in 2 subjects with the turnover calculated from reinjected labelled LDL and were in the range reported for LDL turnover by others (Bilheimer *et al.*, 1975). Of special interest was the similarity between VLDL B apolipoprotein and LDL B apolipoprotein turnover in the 5 subjects without hypertriglyceridaemia, suggesting the total conversion of VLDL particles to LDL (Table 5.3). However, alternate pathways may be available for VLDL catabolism in hypertriglyceridaemic subjects. In 3 of the 4 hypertriglyceridaemic subjects, as little as one third of VLDL B apolipoprotein turnover appeared in LDL_2. Since the turnover of LDL was similar in those with and without hypertriglyceridaemia, whereas the turnover of VLDL B apolipoprotein was much higher in the hypertriglyceridaemic subjects, it is possible that LDL turnover cannot be readily increased to accommodate the higher flux of VLDL B apolipoprotein. A proportion of the latter is then catabolized elsewhere possibly in the liver, as occurs in the rat. Sigurdsson *et al.* (1976) have also reported normal values for LDL turnover in hypertriglyceridaemic subjects many of whom presumably showed increased VLDL turnover. Thus, in normotriglyceridaemic subjects it appears that almost all VLDL is catabolised to LDL_2, as proposed by Sigurdsson *et al.* (1975). However, in hypertriglyceridaemic subjects only a portion

of VLDL is totally catabolised to LDL. This latter observation has also been proposed from a computer-based analysis of VLDL catabolism by Hall *et al.* (1974, abstract only).

5.5.4 VLDL B apolipoprotein turnover and hypertriglyceridaemia

The curvilinear relationship between the mass and turnover of VLDL B apolipoprotein with its suggestion of a saturable system is similar to the kinetics obtained by labelling the triglyceride moiety of VLDL. The studies of Reaven *et al.* (1965) and of Nestel (1966) have shown a direct correlation between the turnover and mass of VLDL triglyceride up to a VLDL triglyceride concentration of about 200 mg/100 ml. At higher levels, the expansion in pool size could not be explained solely by increased production and was therefore attributed to diminishing removal capacity. The relative importance of over-production and reduced removal in the pathogenesis of hypertriglyceridaemia has not been resolved with measurements of triglyceride turnover (Nestel, 1973). Similar conclusions have been reported by Sigurdsson *et al.* (1976).

CHAPTER 6

DEFINITION OF THE INTERMEDIATE LIPOPROTEIN CLASS

6.1 INTRODUCTION

The immediate products of VLDL catabolism are referred to as 'remnant' or 'intermediate' lipoproteins. The nature of these remnants has been investigated extensively *in vitro* (Shore and Shore, 1962; Bierman *et al.*, 1973; Mjøs *et al.*, 1975; Eisenberg and Rachmilewitz, 1975); however, few studies have attempted to characterise, *in vivo*, the metabolism of this lipoprotein species.

VLDL is defined as that lipoprotein class in which triglyceride of endogenous origin is released into the circulation (section 1.4.2). These lipoproteins are said to be in the s_f 20-400 range. The immediate product of VLDL catabolism was initially considered to be the LDL_1 class (Bilheimer *et al.*, 1972; section 1.5.2) though recent analytical ultracentrifugation studies have suggested that the intermediate class may also include lipoproteins in the s_f 20-60 range (Eisenberg *et al.*, 1973).

This chapter describes a limited number of *in vivo* studies, extending from those of chapter 5, devised to determine the flotation characteristics of the intermediate lipoprotein class. As described in the previous chapter (section 5.4.4) the turnover of LDL_1 B apolipoprotein was markedly less than that of VLDL B apolipoprotein in most

of the subjects investigated (Table 5.2) suggesting that LDL_1 was not the sole or major intermediate lipoprotein in the conversion of VLDL to LDL_2.

Four studies are described in this chapter. In the first study (# 8), the turnover of VLDL and LDL_1 B apolipoprotein were both directly determined following the injection of VLDL-^{125}I and LDL_1-^{131}I. This study was carried out to confirm, from direct observation, that LDL_1 turnover was not equal to VLDL turnover. The final three studies (# 11, # 12 and # 13) investigated the turnover of various fractions in the s_f 12-400 range in an attempt to define, from the nature of the precursor product relationships and turnover values, the probable intermediate lipoprotein class.

6.2 METHODS AND RESULTS

The new studies reported in this chapter employed the same experimental protocol as described in chapter 5.

6.2.1 Comparison of the turnover of the B apolipoprotein of VLDL LDL_1

If LDL_1 were the major intermediate lipoprotein between VLDL and LDL_2, then the turnover of both VLDL and LDL_1 should be similar. However, since the pool size of LDL_1 is very much less than that of VLDL, its fractional catabolic rate (k) would need to be very much greater. This is illustrated for studies # 3-10 in Table 6.1. It is evident that the fractional catabolic rate would need to be exceptionally high.

TABLE 6.1

THE THEORETICAL FRACTIONAL CATABOLIC RATES REQUIRED FOR LDL_1 IF ALL VLDL HAD BEEN CATABOLISED TO LDL_1 (E.G. STUDIES #3 - 10).

Study #	VLDL Turnover mg B/day	LDL_1 Pool Size mg B	F.C.R. k/day
3	637	118	5.4
4	1092	132	8.3
5	1238	26	47.6
6	1671	54	30.9
7	3345	103	32.5
8	2323	133	17.5
9	1928	219	8.8
10	3060	313	9.8

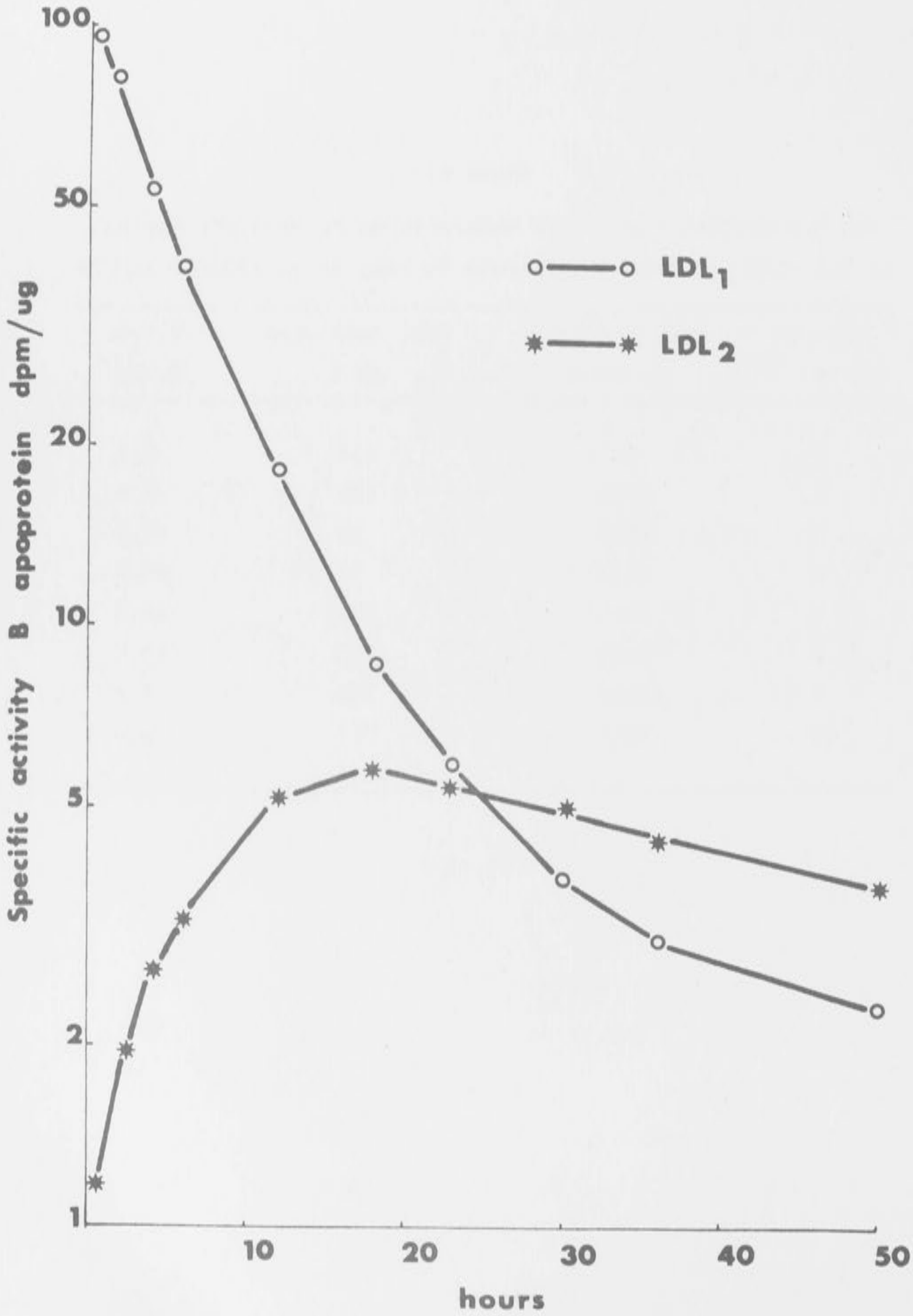


Figure 6.1 The specific activity-time curves (on a log scale) for LDL_1 and LDL_2 B apolipoprotein following the administration of LDL_1-^{131}I in study #8 (see Chapter 5).

In studies # 3-10, the turnover of LDL_1 B apolipoprotein was shown to be less than that of VLDL (as determined by comparing areas under the respective specific activity-time curves). The proportion of VLDL that might be catabolised to LDL_1 was measured directly in study # 8 by injecting simultaneously VLDL-^{125}I and LDL_1-^{131}I. In figure 6.1, the LDL_1 and LDL_2 B apolipoprotein specific activity (^{131}I) - time curves are shown. The disappearance of LDL_1 B apolipoprotein was predominantly monoexponential up to 20 hours (by which time only 5% of the injected ^{131}I radioactivity remained in LDL_1); however, over a 50-hour period, the plot could easily be resolved into two exponentials. The turnover, determined using both 1 and 2-pool models gave similar rates (462 and 444 mg/day respectively). However VLDL B apolipoprotein turnover was approximately five times greater (2323 mg/day), suggesting that a substantial proportion of VLDL particles must by-pass the LDL_1 fraction. The precursor-product relationship between LDL_1 and LDL_2 (as in figure 6.1) likewise suggested that not all LDL_2 was derived from LDL_1, as the peak of the LDL_2 specific activity curve occurred before the crossover with LDL_1. Thus, since LDL_1 alone was not the sole product of VLDL catabolism and unique precursor to LDL_2, other lipoprotein fractions were investigated.

6.2.2 Precursor-product relationships between s_f 100-400 and s_f 12-100 lipoproteins

In study # 11, the VLDL subfraction s_f 100-400 was injected into a type 4 hyperlipoproteinaemic subject. The B apolipoprotein specific activity-time curves (on a log scale) of the s_f 100-400 and s_f 12-100 fractions are shown in figure 6.2. The disappearance of the

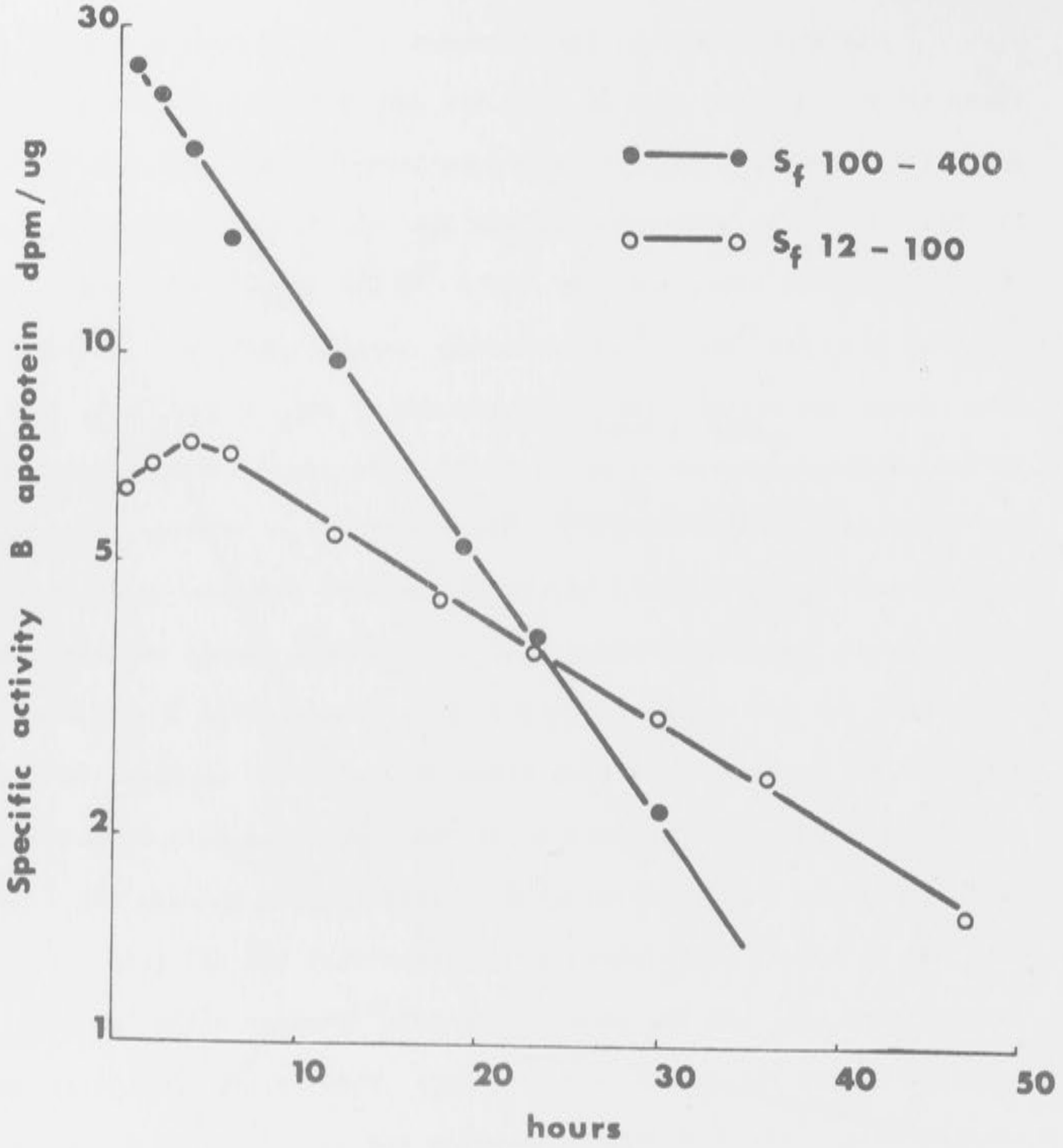


Figure 6.2 The specific activity-time curve (on a log scale) for the B apolipoprotein of s_f 100-400 and s_f 12-100 fractions following the administration of s_f 100-400-^{125}I in study #11. This study was performed on a female subject exhibiting, at the time of study, the type 4 HLP phenotype (triglyceride: 410 mg/100 ml plasma; cholesterol: 290 mg/100 ml plasma).

B apolipoprotein in the s_f 100-400 fraction conformed to a single exponential function in contrast to the previous findings with the s_f 20-400 fraction. However, the specific radioactivity curve of the s_f 12-100 fraction did not intercept the s_f 100-400 curve in the manner of a precursor-product relationship. At its peak, the specific radioactivity of the s_f 12-100 class was only 55% of the s_f 100-400 specific radioactivity, suggesting that nearly half of the s_f 12-100 lipoproteins entered the plasma independently. This also seemed likely in view of the relatively small turnover of 371 mg/day in this subject's s_f 100-400 lipoproteins and the finding that the B apolipoprotein pool size of the s_f 12-100 lipoproteins was 5 times as great as that of the s_f 100-400 fraction.

It therefore seemed likely that the major VLDL remnant was smaller than s_f 100 but larger than s_f 20. As previously mentioned it has been suggested on the basis of studies employing the analytical ultracentrifuge that the appropriate flotation range of the remnant might be s_f 12-60 (Eisenberg *et al.*, 1973).

6.2.3 Precursor-product relationships between s_f 60-400 and s_f 12-60 lipoproteins

In study # 12, s_f 60-400 lipoproteins labelled with ^{125}I and s_f 12-60 lipoproteins labelled with ^{131}I were simultaneously injected into a type 4 hyperlipoproteinaemic subject. The B apolipoprotein specific activity-time curves (^{125}I) for the s_f 60-400 and s_f 12-60 fractions are shown, on a log scale, in figure 6.3. These curves describe a precursor-product relationship between these two lipoprotein fractions, the peak specific activity of the product (s_f 12-60) occurring

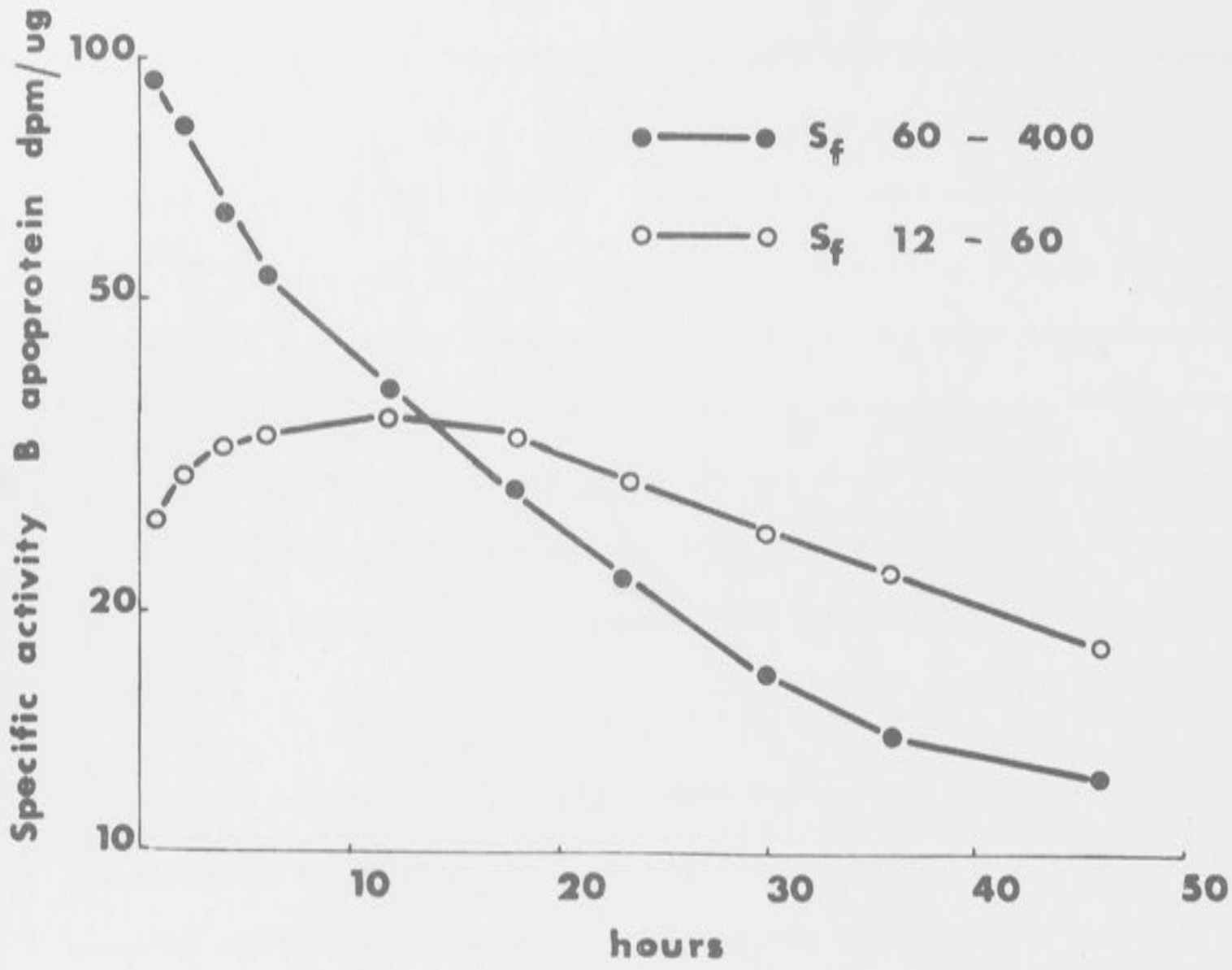


Figure 6.3 The specific activity-time curve (on a log scale) for the B apolipoprotein of s_f 60-400 and s_f 12-60 fractions following the administration of s_f 60-400-^{125}I in study #12. The study was performed on a male subject exhibiting, at the time of study, the type 4 HLP phenotype (triglyceride: 320 mg/100 ml plasma; cholesterol: 265 mg/100 ml plasma).

at the cross-over of the two curves. The s_f 60-400 specific activity curve could be resolved into two exponentials whereas, although not shown, the disappearance of the ^{131}I labelled B apolipoprotein in the s_f 12-60 fraction was monoexponential.

The turnover of the two lipoprotein fractions, measured directly (using both isotopes) were of a similar order (605 and 544 mg/day respectively) which, when taken together with the precise precursor-product characteristics of their respective specific radioactivity curves (determined from the s_f 60-400 labelling), suggested that the true VLDL remnant lies in the s_f 12-60 flotation range.

The s_f 60-400 study was repeated in another subject with type 2B hyperlipoproteinaemic phenotype (study # 13). The kinetics of the specific radioactivity-time curve conformed to a 2-pool model as in subject # 12 and showed a distinct precursor-product relationship with the s_f 12-60 fraction (figure 6.4). The turnover of the s_f 60-400 and s_f 12-60 fractions was also compared by the area under the specific activity-time curve method. In subject # 12 the respective values, obtained from the injection of s_f 60-400 lipoproteins, were 583 and 514 mg/day, which were similar to those derived from a 2-pool analysis of the injected ^{125}I labelled s_f 60-400 lipoproteins and the 1-pool analysis of the injected ^{131}I labelled s_f 12-60 lipoproteins. In subject # 13 the values for the turnover of the s_f 60-400 and s_f 12-60 B apolipoprotein were also similar, being 521 and 462 mg/day respectively.

6.3 DISCUSSION

Bilheimer *et al.* (1972) suggested that the conversion of VLDL

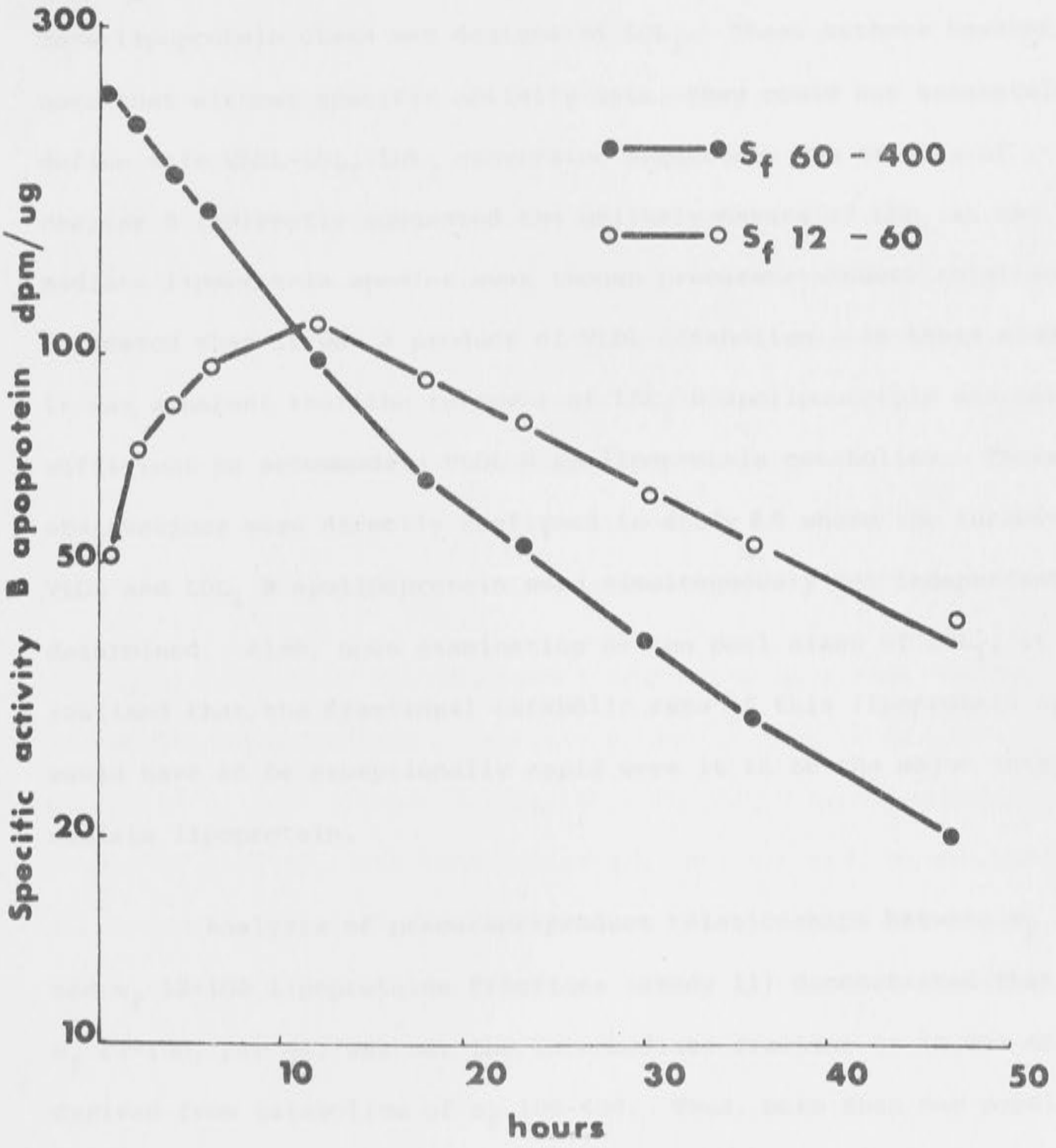


Figure 6.4 The specific activity-time curves (on a log scale) for the B apolipoprotein of s_f 60-400 and s_f 12-60 fractions following the administration of s_f 60-400-^{125}I in study #13. The study was performed on a male subject exhibiting, at the time of study, the type 2B HLP phenotype (triglycerides: 426 mg/100 ml plasma; cholesterol: 341 mg/100 ml plasma).

to LDL_2 occurs via an intermediate lipoprotein in the s_f 12-20 range. This lipoprotein class was designated LDL_1. These authors however did note that without specific activity data, they could not accurately define this VLDL-LDL_1-LDL_2 conversion sequence. The studies of chapter 5 indirectly suggested the unlikely nature of LDL_1 as the intermediate lipoprotein species even though precursor-product relationships indicated that it was a product of VLDL catabolism. In these studies, it was apparent that the turnover of LDL_1 B apolipoprotein was not sufficient to accommodate VLDL B apolipoprotein catabolism. These observations were directly confirmed in study # 8 where the turnover of VLDL and LDL_1 B apolipoprotein were simultaneously but independently determined. Also, upon examination of the pool sizes of LDL_1, it was realised that the fractional catabolic rate of this lipoprotein species would have to be exceptionally rapid were it to be the major intermediate lipoprotein.

Analysis of precursor-product relationships between s_f 100-400 and s_f 12-100 lipoproteins fractions (study 11) demonstrated that the s_f 12-100, *per se*, was not the intermediate fraction as it was not solely derived from catabolism of s_f 100-400. Thus, more than one population of triglyceride-rich lipoproteins may enter the circulation (presumably from the liver or the gut). In contrast, the studies with s_f 60-400 and s_f 12-60 lipoproteins (studies # 12 and 13) suggested that the intermediate lipoprotein class was in the s_f 12-60 range. Data obtained from Schlieren patterns of lipoproteins in the analytical ultracentrifuge have indicated a discrete lipoprotein species in the s_f 12-60 range (Jensen *et al.*, 1970; Quarfordt *et al.*, 1971; Eisenberg *et al.*, 1973).

As these results are limited in number, it is not possible to comment on variations that may occur in the 'cut-off point' of the intermediate lipoprotein. It is likely that in some hyperlipoproteinaemic subjects e.g. type 5 phenotypes larger intermediate lipoproteins are present derived from chylomicrons. It is of interest to note also that the disappearance of the s_f 100-400 fraction (study # 11) was monoexponential in contrast to the biexponential disappearance observed in the s_f 60-400 fractions. Since this study also shows that more than one population of newly synthesised VLDL may enter the circulation, the biexponential nature of the disappearance of s_f 20-400 represents at least the influx of more than one population of particles.

Remnant or intermediate lipoproteins have been implicated in the pathogenesis of atherosclerosis primarily because of the high incidence of this disease associated with type 3 hyperlipoproteinaemia. In animal studies where atherosclerosis has been induced by cholesterol feeding, remnant particles (β-VLDL) rich in apolipoprotein E are seen to accumulate. Thus it is important to define the characteristics of the intermediate lipoprotein species. The studies in this chapter suggest that it is found in the flotation range s_f 12-60 though further studies are required for confirmation.

CHAPTER 7

SYNOPSIS

An association between elevated plasma concentrations of cholesterol and triglyceride and the incidence of coronary heart disease has been well documented (Keys, 1970; Carlson and Bottiger, 1972; Goldstein *et al.*, 1973). These lipids are present in plasma as components of lipoproteins and recent evidence suggests that it is lipoprotein, rather than lipid, concentration that is a risk factor in the pathogenesis of atherosclerosis. For instance, hypertriglyceridaemia is associated with 6 of the 7 HLP phenotypes (types 1, 2B, combined, 3, 4 and 5). Of these, however, only types 2B, combined, 3 and 4 have been associated with an increased risk of atherogenesis. Furthermore, with respect to type 4 HLP, which is characterised by excess VLDL, the relevance of hypertriglyceridaemia alone as a risk factor is presently being questioned (Brunzell *et al.*, 1976; Rhoads *et al.*, 1976). In these studies, the risk of coronary heart disease did not appear to be increased by hypertriglyceridaemia alone. Interestingly, examination of the findings of Carlson and Ericsson(1975), who have examined serum lipoprotein concentrations in survivors of myocardial infraction, shows that among patients with hypertriglyceridaemia only 20% had an elevation of VLDL alone. In the others hypertriglyceridaemia was associated with hypercholesterolaemia as in types 2B, combined or type 3 HLP or in a hitherto neglected entity in which there was an accumulation of triglyceride enriched LDL (42% of

all subjects). The increased LDL triglyceride concentration implied an accumulation of a type of intermediate density lipoprotein. The importance of the intermediate lipoprotein in atherogenesis is indicated by the high incidence of the disease in association with type 3 HLP. Atherosclerosis may therefore develop much more readily in the presence of excess small lipoproteins extending from LDL through to cholesterol rich VLDL remnants than with larger lipoproteins such as VLDL. This has been demonstrated more clearly in experimental pathology where cholesterol induced atherosclerosis in several species including the dog, rat, rabbit and pig (see section 1.8) is associated with the accumulation of two unusual lipoproteins: HDL_c and β-VLDL. Both appear to be remnants of VLDL and chylomicron catabolism and are rich in apolipoprotein E.

In this regard, the findings presented in this thesis are relevant in that a number of aspects of the metabolism of these lipoproteins have been investigated. These include (a) the nature of the intermediate lipoprotein species; (b) the metabolic interrelationships between low and very low density lipoproteins including the intermediate species; (c) alternate pathways that may operate for the removal of the intermediate lipoprotein from the circulation.

A number of the analyses and interpretations included in this thesis have been facilitated by having B apolipoprotein specific activity data. The B apolipoprotein remains common to these lipoproteins from their entry into the circulation until their eventual removal (normally as LDL). It was therefore appropriate to investigate the metabolism of this apolipoprotein for the purposes of the present studies. The B apo-

lipoprotein was isolated, for specific activity determination, by a procedure based on the relative insolubility of this apolipoprotein (when delipidated with organic solvents) in low ionic strength buffers. The method, using 5mM NH_4HCO_3, pH 8.0, isolated the B apolipoprotein (as the NH_4HCO_3 insoluble fraction) from the remaining soluble apolipoproteins of VLDL and LDL (chapter 3). The adequacy of this technique in isolating the B apolipoprotein was confirmed by a number of procedures including amino acid analysis, SDS-PAGE and comparison with the TMU procedure of Kane (chapter 3). The NH_4HCO_3 procedure was employed in all of the metabolic studies included in this thesis. The main findings to emerge from these studies are presented below.

1. Role of lipases in the transformation of VLDL to LDL

An *in vitro* system employing normal human plasma and containing varying concentrations of post-heparin lipolytic activity was used to investigate the catabolism of ^{125}I labelled VLDL (chapter 4). These studies demonstrated that, *in vitro* at least, the sequential transformation of VLDL to LDL_2 was dependent upon lipolytic activity. No conversion of VLDL or intermediate lipoproteins to LDL_2 was observed in the absence of lipolytic activity. These findings are partly at variance with those of Eisenberg *et al.* (1973), discussed previously (chapter 4), which appeared to show that the conversion of the intermediate lipoproteins to LDL_2 was less dependent upon lipolytic activity than the initial transformation of VLDL to the intermediate lipoprotein. However, this conclusion was based on the findings in a hypertriglyceridaemic subject, and as shown by studies on these patients in

chapter 5, as much as 70% of the intermediate lipoprotein may be catabolised by a process other than conversion to LDL_2. The roles of the hepatic and extrahepatic lipases (which make up the total lipolytic activity of post-heparin plasma) in the conversion of VLDL to LDL was also examined under *in vitro* conditions (chapter 4). Hepatic lipase alone was not capable of converting VLDL to LDL. On the other hand some conversion of s_f 20-50 lipoproteins to s_f 12-20 did take place *in vitro* even when lipoprotein lipase activity was inhibited. This suggests that the hepatic lipase system may have a role in the removal of circulating lipoproteins, possibly of the intermediate class.

2. Kinetics of VLDL B apolipoprotein catabolism

The turnover and catabolic fate of the B apolipoprotein of VLDL (s_f 20-400) was studied in ten normal or hyperlipoproteinaemic subjects (chapter 5). The disappearance of VLDL B apolipoprotein, when plotted on a semi-logarithmic scale, was multiexponential but conformed to a 2-pool model over the first 50 hours of catabolism. The 2-pool model was interpreted as reflecting heterogeneity of VLDL catabolism as a consequence of (a) input of more than one population of VLDL into the circulation (some evidence for this was obtained from the studies of chapter 6); (b) heterogeneity of catabolic processes; (c) the possible presence of separate and discrete plasma and tissue VLDL pools. The mass of the primary pool (A) exceeded the intravascular content of VLDL B apolipoprotein by 30% (on average) indicating extravascular metabolism of VLDL. It was speculated that pool A may include plasma, liver, gut and lymph while pool B may include cells in connective tissue such as fibroblasts and in arterial walls.

3. VLDL B apolipoprotein production and removal

From a kinetic analysis of VLDL B apolipoprotein disappearance by a number of methods (section 5.3) the turnover (i.e. production rate) of VLDL B apolipoprotein was ascertained. The turnover of VLDL B apolipoprotein was highest in hypertriglyceridaemic subjects suggesting VLDL over-production as a major factor in this disorder. However, a comparison of the intravascular VLDL B apolipoprotein content and VLDL B apolipoprotein turnover demonstrated a 'saturable system' indicating that in marked hypertriglyceridaemia, defective removal of VLDL also contributes to the disorder. These findings are similar to those reported by Sigurdsson *et al.* (1976) and are in agreement with previous studies investigating VLDL triglyceride turnover in relation to the development of hypertriglyceridaemia (Reaven *et al.*, 1965, 1967; Nestel, 1966).

4. s_f 12-60 lipoproteins as the intermediate lipoprotein class

The *in vivo* studies of chapter 6, although limited in number, suggest that the intermediate lipoprotein lies in the flotation range s_f 12-60. This conclusion was reached following analysis of precursor-product relationships and turnover data of various lipoprotein fractions (chapter 6). Calculations of the turnover of LDL_1 (s_f 12-20) which has hitherto been regarded as the major intermediate lipoprotein class showed that only a proportion of VLDL is converted to LDL (chapters 5 and 6). These studies demonstrated that newly synthesised VLDL may be secreted as several populations of particles within the flotation range of s_f 60-400.

5. Origin of LDL

Precursor-product relationships between VLDL, LDL_1 and LDL_2 (10 studies, chapter 5) confirmed previous suggestions that LDL was derived from VLDL and did not have an independent origin. The studies of Sigurdsson *et al.* (1975) have also shown that, in man, LDL was solely derived from VLDL. LDL turnover was determined in most studies (chapter 5) using a technique based on the relative rate of labelling of LDL_2 from its precursor, VLDL (section 5.3.3). The values obtained with this technique were in reasonable agreement with those obtained from analysis of the disappearance of LDL-^{131}I (studies # 4 and 5). In comparison with the wide range of values observed for VLDL turnover, LDL turnover appears to vary less among individuals. LDL over-production has been clearly demonstrated only in the very rare type 2A homozygote (section 1.7.2). The type 2A HLP phenotype as seen commonly in the general population is mostly the result of defective LDL removal from the circulation. LDL overproduction may result from increased VLDL catabolism thus requiring an increase in VLDL (and so triglyceride) production and secondly, through independent secretion of LDL into the circulation. There is so far no evidence for the former in 2A HLP homozygotes but the latter process has been suggested for the rat and monkey (Fidge, personal communications; Illingworth, 1975) and possibly may be found in man under certain circumstances.

6. Direct removal, in hypertriglyceridaemic subjects, of the intermediate lipoprotein from the circulation

In the studies of chapter 5, the turnover of B apolipoprotein

in VLDL and LDL_2 was similar in subjects without hypertriglyceridaemia. These findings are in agreement with the proposal of Sigurdsson *et al.* (1975) that, in normotriglyceridaemia at least, all VLDL is catabolised to LDL. However, in three of the four hypertriglyceridaemia subjects, the turnover of LDL B apolipoprotein was as little as one third that of VLDL suggesting that in these subjects, not all VLDL was converted to LDL (similar values for LDL turnover, in hypertriglyceridaemic subjects, have also been reported by Sigurdsson *et al.*, 1976). From these observations it was suggested that, in hypertriglyceridaemia a proportion of VLDL B apolipoprotein may be catabolised elsewhere without being converted to LDL_2. The studies of chapter 6 (studies # 12 and 13) suggest that this occurs after VLDL have been converted to the s_f 12-60 class.

In figure 7.1 is shown a possible model for the metabolism of the B apolipoprotein-rich lipoproteins in man. This model incorporates the findings of this thesis into previously described concepts of lipoprotein metabolism. The newly secreted VLDL particle (i.e. prior to any catabolism) lies in the s_f 60-400 flotation range (as suggested by the studies of chapter 6). The form 'VLDL' may be more appropriate to this fraction (s_f 60-400) than to the s_f 20-400 fraction which contains some products of VLDL catabolism. The transformation of VLDL to the intermediate lipoprotein is a lipase mediated process. The lipases involved in this conversion are probably located in extra-hepatic tissues. The intermediate lipoprotein species, in accordance with the suggestions of Eisenberg *et al.* (1973) and the findings in chapter 6 occupies the s_f 12-60 range. The fate of this

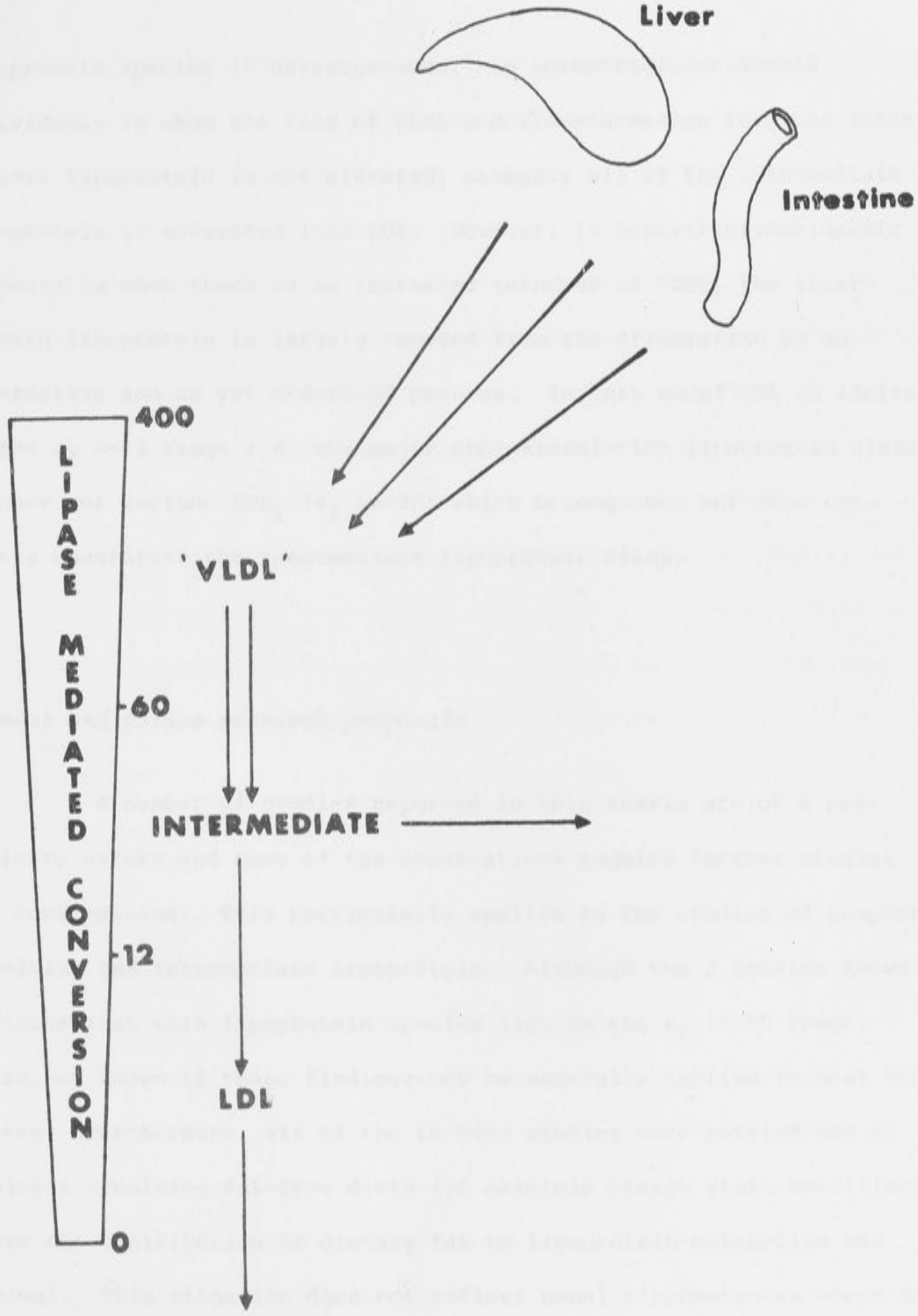


Figure 7.1 A model for the metabolism of B apolipoprotein-rich lipoproteins in man.

lipoprotein species is heterogeneous: in normotriglyceridaemic individuals in whom the flux of VLDL and transformation into the intermediate lipoprotein is not elevated, probably all of the intermediate lipoprotein is converted into LDL. However, in hypertriglyceridaemic subjects in whom there is an increased turnover of VLDL, the intermediate lipoprotein is largely removed from the circulation by an alternative and as yet undefined process. In this model LDL is limited to the s_f 0-12 range i.e. the major cholesterol-rich lipoprotein class. It does not include LDL_1 (s_f 12-20) which belongs to, but does not solely constitute the intermediate lipoprotein class.

Current and future research proposals

A number of studies reported in this thesis are of a preliminary nature and many of the observations require further studies for confirmation. This particularly applies to the studies of chapter 6 involving the intermediate lipoprotein. Although the 2 studies shown indicate that this lipoprotein species lies in the s_f 12-60 range, it is not known if these findings may be generally applied to most HLP states. Furthermore, all of the *in vivo* studies were carried out on subjects receiving fat-free diets (to maintain steady state conditions) where the contribution of dietary fat to lipoprotein metabolism was minimal. This situation does not reflect usual circumstances where many of the lipoprotein catabolic processes involve dietary particles. The catabolism of chylomicrons has been well characterised with respect to the triglyceride and cholesterol moieties but little is known of the fate of their apolipoprotein components and so also remains to be

investigated. The turnover studies of the type described in this thesis could also be usefully employed to investigate other aspects of lipoprotein metabolism, e.g. the mechanism of action of hypolipidaemic drugs and the effects of dietary changes (particularly the mechanism of action of polyunsaturated fat intake in reducing plasma cholesterol concentrations). Studies on the turnover of individual C apolipoproteins and their presence in various lipoprotein classes (employing the technique for quantitation of C apolipoproteins - chapter 3) in normal and diseased states may give some insight into possible defects leading to the development of some of the hyperlipoproteinaemias. Individual C apolipoproteins appear to activate or inhibit lipoprotein lipase and lecithin: cholesteryl acyl transferase, enzymes which are crucial to normal catabolism of lipoproteins. Simultaneous measurements of B apolipoprotein and C apolipoprotein turnover would define the anabolic and catabolic phases of lipoprotein interconversion.

In conclusion, perhaps the most significant finding to eventuate from these studies was the realisation of the complexity and heterogeneity of VLDL catabolism. The catabolism of triglyceride-rich lipoproteins may no longer be regarded solely as an intravascular phenomenom but rather, as in the case of LDL catabolism, to involve a number of tissues including probably the liver. The possible significance, of the extravascular component of VLDL catabolism and the removal of intermediate species directly from the circulation in the pathogenesis of atherosclerosis remains to be ascertained.

REFERENCES

Akabori, S., K. Ohno and K. Narita (1952). *Bull. Chem. Soc. Jap.* 25: 214-218.

Akanuma, Y. and J. Glomset (1968). *J. Lipid Res.* 9:620-626.

Alaupovic, P. (1971). *Protides Biol. Fluids, Proc. Colloq.* 19:9-19.

Alaupovic, P., D.M. Lee and W.J. McConathy (1972). *Biochim. Biophys. Acta* 260:689-707.

Alaupovic, P., D. Seidel, W.J. McConathy and R.H. Furman (1969). *FEBS Lett.* 4:113-116.

Albers, J.J. and E.L. Bierman (1976). *Biochim. Biophys. Acta* 424:422-429.

Albers, J.J. and A.M. Scanu (1971). *Biochim. Biophys. Acta* 236:29-37.

Albers, J.J., P. Wahl and W.R. Hazzard (1974). *Biochem. Genet.* 11:475-486.

Ambler, P.P. (1972). *Meth. Enzymol.* 25:143-154.

Anderson, N.G. and B. Fawcett (1950). *Proc. Soc. Exp. Biol.* 74:768-771.

Assman, G., B.G. Brown and R.W. Mahley (1975). *Biochemistry* 14:3996-4003.

Bachorik, P.S., J.N. Livingston, J. Cooke and P.O. Kwiterovich (1976). *Biochem. Biophys. Res. Commun.* 69:927-935.

Bagdade, J.D., D. Porte, Jr. and E.L. Bierman (1968). *Diabetes* 17:127-132.

Bagnall, T.F. (1972). *Clin. Chim. Acta* 42:229-233.

Bailey, J.M. (1973). In *Atherogenesis: Initiating Factors*, CIBA Foundation Symp. 12, Elsevier, Amsterdam, pp.63-92.

Baker, H.N., A.M. Gotto, Jr. and R.L. Jackson (1975). *J. Biol. Chem.* 250:2725-2738.

Ballard, F.J., R.W. Hanson and D.S. Kronfield (1969). *Fed. Proc.* 28: 218-231.

Bar-On, H., A.I. Kook, O. Stein and Y. Stein (1973). *Biochim. Biophys. Acta* 306:106-114.

Bar-On, H., P.S. Roheim and H.A. Eder (1974). *J. Clin. Invest.* 53:4a.

Barter, P.J. (1974). *J. Lipid Res.* 15:11-19.

Barter, P.J. and J.I. Lally (1976). *Proc. Aust. Biochem. Soc.* 9:22.

Barter, P.J., P.J. Nestel and K.F. Carroll (1972). *Metabolism* 21:117-124.

Barter, P.J. and P.J. Nestel (1972). *J. Clin. Invest.* 51:174-180.

Barter, P.J. and P.J. Nestel (1973). *Metabolism* 22:779-785.

Bautovich, G.J., L.A. Simons, P.F. Williams and J.R. Turtle (1975). *Atherosclerosis* 21:217-234.

Bergman, E.N., R.J. Havel, B.M. Wolfe and T. Bohmer (1971). *J. Clin. Invest.* 50:1831-1839.

Bhattacharyya, A.K., W.E. Connor and A.A. Spector (1972). *J. Clin. Invest.* 51:2060-2070.

Biale, Y. and E. Shafrir (1969). *Clin. Chim. Acta* 23:413-419.

Bierman, E.L. and J.J. Albers (1975). *Biochim. Biophys. Acta* 388:198-202.

Bierman, E.L., S. Eisenberg, O. Stein and Y. Stein (1973). *Biochim. Biophys. Acta* 329:163-169.

Bierman, E.L., T.L. Hayes, J.N. Hawkins, A.M. Ewing and F.T. Lindgren (1966). *J. Lipid Res.* 7:65-72.

Bilheimer, D.W., S. Eisenberg and R.I. Levy (1971). *Circulation* 43/44, Supplt. II:56.

Bilheimer, D.W., S. Eisenberg and R.I. Levy (1972). *Biochim. Biophys. Acta* 260:212-221.

Bilheimer, D.W., J.L. Goldstein, S.M. Grundy and M.S. Brown (1975). *J. Clin. Invest.* 56:1420-1430.

Blacket, R.B. (1973). *Med. J. Aust.* 1:969-973.

Blanchette-Mackie, E.J. and R.O. Scow (1971). *J. Cell Biol.* 51:1-25.

Blaton, V., R. Vercaemst, N. Vandecasteele, H. Caster and H. Peeters (1974). *Biochemistry* 13:1127-1135.

Bloch, K., B.N. Berg and D. Rittenberg (1943). *J. Biol. Chem.* 149:511-517.

Blomstrand, R.J., J. Gürtler and B. Werner (1964). *Acta Chem. Scand.* 18:1019-1021.

Boberg, J. (1972). *Acta Med. Scand.* 191:97-102.

Boberg, J., L.A. Carlson and V. Freyschuss (1972). *Eur. J. Clin. Invest.* 2:123-132.

Borensztajn, J., S. Otway and D.S. Robinson (1970). *J. Lipid Res.* 11: 102-110,111-117.

Brewer, H.B., Jr., S.E. Lux, R. Ronan and K.M. John (1972). *Proc. Natl. Acad. Sci. US* 69:1304-1308.

Brewer, H.B., Jr., R. Schulman, P. Herbert, R. Ronan and K. Wehrly (1974). *J. Biol. Chem.* 249:4975-4984.

Brown, H.B., L.A. Lewis and I.H. Page (1973). *Atherosclerosis* 17:181-196.

Brown, M.S., S.E. Dana and J.L. Goldstein (1974). *J. Biol. Chem.* 249: 789-796.

Brown, M.S. and J.L. Goldstein (1974). *Science* 185:61-63.

Brown, W.V., R.I. Levy and D.S. Fredrickson (1969). *J. Biol. Chem.* 244: 5687-5694.

Brown, W.V., R.I. Levy and D.S. Fredrickson (1970a). *Biochim. Biophys. Acta* 200:573-575.

Brown, W.V., R.I. Levy and D.S. Fredrickson (1970b). *J. Biol. Chem.* 245:6588-6594.

Brunzell, J.D., W.R. Hazzard, D. Porte and E.L. Bierman (1973). *J. Clin. Invest.* 52:1578-1585.

Brunzell, J.D., H.G. Schrott, A.T. Motulsky and E.L. Bierman (1976). *Metabolism* 15:313-320.

Carlson, L.A. and L.-E. Böttiger (1972). *Lancet* 1:865-868.

Carlson, L.A. and M. Ericsson (1975). *Atherosclerosis* 21:417-433,435-450.

Carlson, L.A., L. Orö and J. Östman (1968). *J. Athero Res.* 8:667-677.

Chen, C.-H. and F. Aladjem (1974). *Biochem. Biophys. Res. Commun.* 60: 549-554.

Chesterton, C.J. (1968). *J. Biol. Chem.* 243:1147-1151.

Clifton-Bligh, P., N.E. Miller and P.J. Nestel (1974). *Clin. Sci. Mol. Med.* 47:547-557.

Cotton, S.G., J.M. Nixon, R.G. Carpenter and D.W. Evans (1972). *Brit. Heart J.* 34:458-464.

Courtice, F.C. and B. Morris (1955). *Quart. J. Exp. Physiol. Cog. Med. Sci.* 40:138-148.

David, J.S.K., L.A. Soloff and A.G. Lacko (1976). *Life Sci.* 18:701-706.

Dietschy, J.M. and W.G. Gamel (1971). *J. Clin. Invest.* 50:872-880.

Dietschy, J.M. and J.D. Wilson (1970). *New Engl. J. Med.* 282:1128-1138, 1179-1183,1241-1249.

Dole, V.P. and H. Meinertz (1960). *J. Biol. Chem.* 235:2595-2599.

Dolphin, P.J. and D. Rubinstein (1974). *Biochem. Byophys. Res. Commun.* 57:808-814.

Duncombe, W.G. (1964). *Clin. Chim. Acta* 9:122-125.

Dzoga, K., R.W. Wissler and D. Vesselinovitch (1971). *Circulation* 43/44, Supplt. II:6.

Eaton, R.P., M. Berman and D. Steinberg (1969). *J. Clin. Invest.* 48: 1560-1579.

Eaton, R.P., C. Crespin and D.M. Kipnis (1976). *Diabetes* 25:44-50.

Edelstein, C., C. Noyes and A.M. Scanu (1974). *FEBS Lett.* 38:166-170.

Ehnholm, C., W. Shaw, H. Greten and W.V. Brown (1975). *J. Biol. Chem.* 250:6756-6761.

Eisenberg, S., D.W. Bilheimer, R.I. Levy and F.T. Lindgren (1973). *Biochim. Biophys. Acta* 326:361-377.

Eisenberg, S., D. Bilheimer, F. Lindgren and R.I. Levy (1972). *Biochim. Biophys. Acta* 260:329-333.

Eisenberg, S. and D. Rachmilewitz (1975). *J. Lipid Res.* 16:341-351.

Faergeman, O., O.D. Mjøs and R.J. Havel (1974). *Clin. Res.* 22:128a.

Faergeman, O., T. Sata, J.P. Kane and R.J. Havel (1975). *J. Clin. Invest.* 56:1396-1403.

Farquhar, J.W., R.C. Gross, R.M. Wagner and G.M. Reaven (1965). *J. Lipid Res.* 6:119-134.

Felts, J.M., H. Itakura and R.T. Crane (1975). *Biochem. Biophys. Res. Commun.* 66:1467-1475.

Fidge, N.H. and I.C. Calder (1972). *Chem. Phys. Lipids* 9:255-265.

Fidge, N.H. and P. Poulis (1974). *Clin. Chim. Acta* 52:15-26.

Fidge, N.H. and P. Poulis (1975). *J. Lipid Res.* 16:367-378.

Fielding, C.J. (1970). *Biochim. Biophys. Acta* 206:109-117.

Fielding, C.J. (1972). *Biochim. Biophys. Acta* 280:569-578.

Fielding, C.J. and P.E. Fielding (1971). *FEBS Lett.* 15:355-358.

Fielding, C.J. and J.M. Higgins (1974). *Biochemistry* 13:4324-4329.

Fielding, C.J., V.G. Shore and P.E. Fielding (1972). *Biochem. Biophys. Res. Commun.* 46:1493-1498.

Fine, M.B. and R.H. Williams (1960). *Amer. J. Physiol.* 199:403-406.

Fishbein, W.N. (1972). *Anal. Biochem.* 46:388-401.

Folch, J., M. Lees and G.H. Sloane-Stanley (1957). *J. Biol. Chem.* 226: 497-509.

Forte, G.M., A.V. Nichols and R.M. Glaeser (1968). *Chem. Phys. Lipids* 2:396-408.

Forte, T., E. Gong and A.V. Nichols (1974). *Biochim. Biophys. Acta* 337: 169-183.

Fredrickson, D.S. (1971). *Brit. Med. J.* 2:187-192.

Fredrickson, D.S. and R.S. Gordon, Jr. (1958). *Physiol. Rev.* 38:585-630.

Fredrickson, D.S. and R.I. Levy (1972). In *The Metabolic Basis of Inherited Disease*, edited by J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson, McGraw-Hill, New York, pp.545-614.

Fredrickson, D.S., R.I. Levy and R.S. Lees (1967). *New Engl. J. Med.* 276:34-44, 94-103, 148-156, 215-225, 273-281.

Fredrickson, D.S., S.E. Lux and P.N. Herbert (1972). *Advan. Exp. Med. Biol.* 26:25-56.

Fredrickson, D.S., K. Ono and L.L. Davis (1963). *J. Lipid Res.* 4:24-33.

Friedwald, W.T., R.I. Levy and D.S. Fredrickson (1972). *Clin. Chem.* 18: 499-502.

Ganessan, D. and H.B. Bass (1975). *FEBS Lett.* 53:1-4.

Ganessan, D., R.H. Bradford, P. Alaupovic and W.J. McConathy (1971). *FEBS Lett.* 15:205-209.

Garfinkel, A.S., P. Nilsson-Ehle and M.C. Schotz (1976). *Biochim. Biophys. Acta* 424:264-273.

Garner, C.W., Jr., L.C. Smith, R.L. Jackson and A.M. Gotto (1972). *Circulation* 46, Supplt. II:246.

Gidez, L.I., P.S. Roheim and H.A. Eder (1967). *J. Lipid Res.* 8:7-15.

Gitlin, D., D.G. Cornwell, D. Nakasato, J.L. Oncley, W.L. Hughes and C.A. Janeway (1958). *J. Clin. Invest.* 37:172-184.

Glauman, H. and G. Dallner (1968). *J. Lipid Res.* 9:720-729.

Glickman, R.M. and K. Kirsch (1974). *Biochim. Biophys. Acta* 371:255-266.

Glomset, J.A. (1962). *Biochim. Biophys. Acta* 65:128-135.

Glomset, J.A. (1968). *J. Lipid Res.* 9:155-167.

Glomset, J.A. (1972). In *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism*, edited by G.J. Nelson, Wiley-Interscience, New York, pp.745-787.

Glomset, J.A., E.T. Janssen, R. Kennedy and J. Dobbins (1966). *J. Lipid Res.* 7:638-648.

Glomset, J.A. and K.R. Norum (1973). *Adv. Lipid Res.* 11:1-65.

Goldrick, R.B. and D.J. Galton (1974). *Clin. Sci. Mol. Med.* 46:469-479.

Goldstein, J.L. and M.S. Brown (1974). *J. Biol. Chem.* 249:5153-5162.

Goldstein, J.L. and M.S. Brown (1975). *Arch. Pathol.* 99:181-184.

Goldstein, J.L., H.G. Schrott, W.R. Hazzard, E.L. Bierman, A.G. Motulsky, E.D. Campbell and M.J. Levinski (1973). *J. Clin. Invest.* 52:1544-1568.

Goodman, De W.S. (1962). *J. Clin. Invest.* 41:1886-1896.

Goodman, De W.S. and R.P. Noble (1968). *J. Clin. Invest.* 47:231-241.

Goodman, De W., R.P. Nobel and R.B. Dell (1973). *J. Lipid Res.* 14:178-188.

Goodman, De W.S. and T. Shiratori (1964). *J. Lipid Res.* 5:307-313.

Gorovsky, M.A., K. Carlson and J.L. Rosenbaum (1970). *Anal. Biochem.* 35:359-370.

Gotto, A.M., W.V. Brown, R.I. Levy, M.E. Birnbaumer and D.S. Fredrickson (1972). *J. Clin. Invest.* 51:1486-1494.

Greten, H. (1972). *Exposés Ann. Biochem. Médicale* 31:161-168.

Greten, H., R.I. Levy and D.S. Fredrickson (1969). *J. Lipid Res.* 10: 326-330.

Greten, H., A.D. Sniderman, J.G. Chandler, D. Steinberg and W.V. Brown (1974). *FEBS Lett.* 42:157-160.

Gulbrandsen, C.L., R.B. Wilson and R.S. Lees (1971). *Circulation* 44, Supplt. II:10.

Gurpide, E., J. Mann and E. Sandberg (1964). *Biochemistry* 3:1250-1255.

Gustafson, A., P. Alaupovic and R.H. Furman (1965). *Biochemistry* 4: 597-605.

Hahn, P.F. (1943). *Science* 98:19-20.

Hall, M., D.W. Bilheimer, R.D. Phair and R.I. Levy (1974). *Circulation* 49 *Supplt.* III:114.

Harlan, W.R., Jr., D.S. Winesett and A.J. Wasserman (1967). *J. Clin. Invest.* 46:239-247.

Harris, K. and J.M. Felts (1973). *Biochim. Biophys. Acta* 316:288-295.

Havel, R.J. (1961). *Metabolism* 10:1031-1034.

Havel, R.J., A. Eder and J.H. Bragdon (1955). *J. Clin. Invest.* 34:1345-1353.

Havel, R.J., J.M. Felts and C.M. van Duyne (1962). *J. Lipid Res.* 3:297-308.

Havel, R.J., C.J. Fielding, T. Olivecrona, V.G. Shore, P.E. Fielding and T. Egelrud (1973a). *Biochemistry* 12:1828-1833.

Havel, R.J. and R.S. Gordon, Jr. (1960). *J. Clin. Invest.* 39:1777-1790.

Havel, R.J. and J.P. Kane (1973). *Proc. Natl. Acad. Sci. US* 70:2015-2019.

Havel, R.J., J.P. Kane, E.O. Ballasse, N. Segal and L.V. Basso (1970a). *J. Clin. Invest.* 49:2017-2035.

Havel, R.J., J.P. Kane and M.L. Kashyap (1973b). *J. Clin. Invest.* 52: 32-38.

Havel, R.J., V.G. Shore, B. Shore and D.M. Bien (1970b). *Circ. Res.* 27: 595-600.

Hay, R.V., L.A. Pottenger, A.L. Reingold, G.S. Getz and R.W. Wissler (1971). *Biochem. Biophys. Res. Commun.* 6:1471-1477.

Hazzard, W.R. and E.L. Bierman (1971). *Clin. Res.* 19:476.

Henderson, L., P. Herbert, H. Windmueller and R. Krauss (1974). *Circulation* 50, Supplt. 3:114.

Herbert, P., R.I. Levy and D.S. Fredrickson (1971). *J. Biol. Chem.* 246: 7068-7069.

Herbert, P.N., R.S. Shulman, R.I. Levy and D.S. Fredrickson (1973). *J. Biol. Chem.* 248:4941-4946.

Herbert, P.N., H.G. Windmueller, T.P. Bersot and R.S. Shulman (1974). *J. Biol. Chem.* 249:5718-5724.

Homma, Y. and P.J. Nestel (1975). *Atherosclerosis* 22:551-563.

Hurley, P.J. and P.J. Scott (1970). *Atherosclerosis* 11:51-76.

Ibels, L.S., M.F. Reardon and P.J. Nestel (1976). *J. Lab. Clin. Med.* 87:648-658.

Illingworth, D.R. (1975). *Biochim. Biophys. Acta* 388:38-51.

Illingworth, D.R. and O.W. Portman (1972). *Biochim. Biophys. Acta* 280: 281-289.

Illingworth, D.R., O.W. Portman and L.E. Whipple (1974). *Biochim. Biophys. Acta* 369:304-314.

Isselbacher, K.J., R. Scheig, G.R. Plotkin and J.B. Caufield (1964). *Medicine* 43:347-361.

Jackson, R.L., J.T. Sparrow, H.N. Baker, J.D. Morrisett, O.D. Taunton and A.M. Gotto, Jr. (1974). *J. Biol. Chem.* 249:5314-5320.

Jansen, G.R., C.F. Hutchison and M.E. Zanetti (1966). *Biochem. J.* 99: 323-340.

Jensen, L.C., T.H. Rich and F.T. Lindgren (1970). *Lipids* 5:491-493.

Johns, E.W. (1967). *Biochem. J.* 104:78-82.

Johnston, J.M. (1968). In *Handbook of Physiology*, Section 6, Volume 3, American Physiol. Soc., Washington, p.1353.

Jones, A.L. and R.K. Ockner (1971). *J. Lipid Res.* 12:580-589.

Karmen, A., H.M. Whyte and De W.S. Goodman (1963). *J. Lipid Res.* 4:312-321.

Kane, J.P. (1973). *Anal. Biochem.* 53:350-364.

Kane, J.P., E.G. Richards and R.J. Havel (1970). *Proc. Natl. Acad. Sci. US* 66:1075-1082.

Kane, J.P., T. Sata, R.L. Hamilton and R.J. Havel (1975). *J. Clin. Invest.* 56:1622-1634.

Kessler, J.I., J. Stein, D. Dannacker and P. Narcessian (1970). *J. Biol. Chem.* 245:5281-5288.

Keys, A. (1970). *Circulation* 41, Supplt. 1:1-211.

Khachadurian, A.K. (1964). *Amer. J. Med.* 37:402-407.

Khoo, J.C., D. Steinberg, J.J. Huang and P.R. Vagelos (1976). *J. Biol. Chem.* 251:2822-2890.

Kinnunen, P.J.K. and C. Ehnholm (1976). *FEBS Lett.* 65:354-357.

Kirkeby, K. (1968). *Acta Endocrin.* 59:555-563.

Klauda, H.C. and F.W. Quackenbush (1971). *Lipids* 6:964-965.

Kook, A.I. and D. Rubinstein (1973). *Can. J. Biochem.* 51:490-494.

Korn, E.D. (1955). *J. Biol. Chem.* 215:1-14, 15-26.

Kostner, G.M. (1974a). *Biochim. Biophys. Acta* 336:383-395.

Kostner, G.M. (1974b). *Scand. J. Clin. Lab. Invest.* 33 Supplt. 137:19-21.

Kostner, G. and P. Alaupovic (1971). *FEBS Lett.* 15:320-324.

Kostner, G. and A. Holasek (1972). *Biochemistry* 11:1217-1223.

Kostner, G.M., J.R. Patsch, S. Sailer, H. Braunsteiner and A. Holasek (1974). *Eur. J. Biochem.* 45:611-621.

Krauss, R.M., H.G. Windmueller, R.I. Levy and D.S. Fredrickson (1973). *J. Lipid Res.* 14:286-295.

Kwiterovich, P.O., R.I. Levy and D.S. Fredrickson (1970). *Circulation* 42 Supplt. III:11.

Kudzma, D.J. and G. Schonfeld (1971). *J. Lab. Clin. Med.* 77:384-395.

Laemmli, U.K. (1970). *Nature* 227:680-685.

Langer, T., W. Strober and R.I. Levy (1972). *J. Clin. Invest.* 51:1528-1536.

LaRosa, J.C., R.I. Levy, P. Herbert, S.E. Lux and D.S. Fredrickson (1970). *Biochem. Biophys. Res. Commun.* 41:57-62.

LaRose, J.C., R.I. Levy, H.G. Windmueller and D.S. Fredrickson (1972). *J. Lipid Res.* 13:356-363.

Lee, D.M. and P. Alaupovic (1974). *Atherosclerosis* 19:501-520.

Lees, R.S., D.E. Wilson, G. Schonfeld and S. Fleet (1973). *Prog. Med. Genet.* 9:237-290.

Leon, L.P., R.L. Rush and J. Turrell (1970). In *Advances in Automated Analysis*, Technicon International Congress, Technicon Inst. Corp., Tarrytown, N.Y., U.S.A.

Levy, R.I. and D.S. Fredrickson (1965). *J. Clin. Invest.* 44:426-441.

Levy, R.I., R.S. Lees and D.S. Fredrickson (1966). *J. Clin. Res.* 45:63-77.

Lindgren, F.T., H.A. Elliott and J.W. Gofman (1951). *J. Phys. Chem.* 55: 80-93.

Lindgren, F.T., A.V. Nichols, T.L. Hayes, N.F. Freeman and J.W. Gofman (1959). *Ann. N.Y. Acad. Sci.* 72:826-844.

Losowsky, M.S. and D.H. Kenward (1968). *J. Lab. Clin. Med.* 71:736-843.

Lossow, W.J., S.N. Shah and I.L. Chaikoff (1966). *Biochim. Biophys. Acta* 116:172-174.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). *J. Biol. Chem.* 193:265-275.

Luttringhaus, A., and H.W. Dirksen (1964). *Angew. Chem. Internat.* 3:260-269.

Macheboeuf, M.A. (1929). *Bull. Soc. Chim. Biol.* 11:268-293, 485-503.

Mahley, R.W. and K.H. Weisgraber (1974). *Biochemistry* 13:1964-1969.

Mahley, R.W., K.H. Weisgraber and T. Innerarity (1974). *Circ. Res.* 35: 722-733.

Mahley, R.W., K.H. Weisgraber, T. Innerarity, H.B. Brewer and G. Assmann (1975). *Biochemistry* 14:2817-2823.

Marcel, Y.L. and C. Vezina (1973). *J. Biol. Chem.* 248:8254-8259.

Marsh, J.B. (1974). *J. Lipid Res.* 15:544-550.

Marsh, J.B. (1976). *J. Lipid Res.* 17:85-93.

Matthews, C.J. (1957). *Phys. Med. Biol.* 2:36-53.

Mayes, P.A. and J.M. Felts (1968). *Biochem. J.* 105:18c-20c.

McConathy, W.J. and P. Alaupovic (1973). *FEBS Lett.* 37:178-182.

McConathy, W.J. and P. Alaupovic (1976). *Biochemistry* 15:515-520.

McElroy, W.T., W.L. Seifert and J.D. Spitzer (1960). *Proc. Soc. Exp. Biol. Med.* 104:20-23.

McFarlane, A.S. (1958). *Nature* 182:53.

Miller, A.L. and L.C. Smith (1973). *J. Biol. Chem.* 248:3359-3362.

Mjøs, O.D., O. Faergeman, R.L. Hamilton and R.J. Havel (1975). *J. Clin. Invest.* 56:603-615.

Naito, C. and J.M. Felts (1970). *J. Lipid Res.* 11:48-53.

Nestel, P.J. (1966). *Clin. Sci.* 31:31-38.

Nestel, P.J. (1973a). In *Atherosclerosis III*, edited by G. Schettler and A. Weizel, Springer-Verlag, Berlin, pp.502-511.

Nestel, P.J. (1973b). *Prog. Biochem. Pharmacol.* 8:125-160.

Nestel, P.J. and W. Austin (1969). *Life Sci.* 8:157-164.

Nestel, P.J., K.F. Carroll and N. Havenstein (1970). *Metabolism* 19:1-18.

Nestel, P.J. and E.A. Couzens (1966). *J. Lipid Res.* 7:487-491.

Nestel, P.J., R.J. Havel and A. Bezman (1963). *J. Clin. Invest.* 42: 1313-1321.

Nestel, P.J. and E.A. Monger (1967). *J. Clin. Invest.* 46:967-974.

Nestel, P.J. and D. Steinberg (1963). *J. Lipid Res.* 4:461-469.

Nestruck, A.C. and D. Rubinstein (1975). *Fed. Proc.* 34:688.

Nichols, A.V. (1967). *Advanc. Biol. Med. Phys.* 11:109-158.

Nichols, A.V. and E.L. Gong (1971). *Biochim. Biophys. Acta* 231:175-184.

Nichols, A.V. and L. Smith (1965). *J. Lipid Res.* 6:206-210.

Nichols, A.V., E.H. Strisower, F.T. Lindgren, G.L. Adamson and E.L. Coggiola (1968). *Clin. Chim. Acta* 20:277-283.

Nikkari, T., P.H. Schreibman and E.H. Ahrens, Jr. (1974). *J. Lipid Res.* 15:563-573.

Nilsson, A. and D.B. Zilversmit (1972). *J. Lipid Res.* 13:32-38.

Nilsson-Ehle, P. and P. Belfrage (1972). *Biochim. Biophys. Acta* 270:60-64.

Nordby, G. and K.R. Norum (1974). *Europ. J. Clin. Invest.* 4:388.

Noels, S.-P. and D. Rubinstein (1974). *J. Lipid Res.* 15:301-308.

Osuga, T. and O.W. Portman (1971). *Amer. J. Physiol.* 220:735-741.

Ochterlony, Ö. (1958). *Prog. Allergy* 5:1-78.

Phair, R.D., M.G. Hammond, J.A. Bowden, M. Fried, W.R. Fisher and M. Berman (1975). *Fed. Proc.* 34:2263-2270.

Quarfordt, S.H., A. Frank, D.M. Shames, M. Berman and D. Steinberg (1970). *J. Clin. Invest.* 49:2281-2297.

Quarfordt, S.H. and De W. Goodman (1967). *J. Lipid Res.* 8:264-273.

Quarfordt, S., R.I. Levy and D.S. Fredrickson (1971). *J. Clin. Invest.* 50:754-761.

Rachmilewitz, D., J.L. Albers and D.R. Saunders (1976). *J. Clin. Invest.* 57:530-533.

Rachmilewitz, D., O. Stein, P.S. Roheim and Y. Stein (1972). *Biochim. Biophys. Acta* 270:414-425.

Reaven, G.M., D.B. Hill, R.C. Gross and J.W. Farquhar (1965). *J. Clin. Invest.* 44:1826-1833.

Reaven, G.M., R.L. Lerner, M.P. Stern and J.W. Farquhar (1967). *J. Clin. Invest.* 46:1756-1769.

Redgrave, T.G. (1970). *J. Clin. Invest.* 49:465-471.

Rehnborg, C.S. and A.V. Nichols (1964). *Biochim. Biophys. Acta* 84:596-603.

Reichl, D., A. Postiglione, N.B. Myant, J.J. Pflug and M. Press (1975). *Clin. Sci. Mol. Med.* 49:419-426.

Reichl, D., L.A. Simons, N.B. Myant, J.J. Pflug and G.L. Mills (1973). *Clin. Sci. Mol. Med.* 45:313-329.

Reinke, R.T. and J.D. Wilson (1967). *Clin. Res.* 15:242.

Report, W.H.O. (1957). *Wld. Hlth. Org. Techn. Rep.* Ser. No. 117.

Report, W.H.O. (1958). *Wld. Hlth. Org. Techn. Rep.* Ser. No. 143.

Rhoads, G.G., C.L. Gulbrandsen and A. Kagan (1976). *New Engl. J. Med.* 294:293-298.

Ritter, M.C., A.W. Kruski and A.M. Scanu (1974). *Fed. Proc.* 33:2046.

Robinson, D.S. (1970). *Compr. Biochem.* 19:51-116.

Robinson, D.S. and P.M. Harris (1959). *Quart. J. Exp. Physiol.* 44:80-90.

Robinson, D.S., P.M. Harris and C.R. Ricketts (1959). *Biochem. J.* 71:286-292.

Roheim, P.S., L.I. Gidez and H.A. Eder (1966). *J. Clin. Invest.* 45:297-300.

Roheim, P.S., D.E. Haft, L.I. Gidez, A. White and H.A. Eder (1963). *J. Clin. Invest.* 42:1277-1285.

Roheim, P.S., H. Hirsch, D. Edelstein and D. Rachmilewitz (1972). *Biochim. Biophys. Acta* 278:517-529.

Rose, H.G., P. Kranz, M. Weinstock, J. Juliano and J.I. Haft (1974). *Atherosclerosis* 20:51-64.

Ruderman, N.B., A.L. Jones, R.M. Krauss and E. Shafrir (1971). *J. Clin. Invest.* 50:1355-1368.

Ryan, W.G. and T.B. Schwartz (1965). *Metabolism* 14:1243-1254.

Sachs, D.H. and E. Painter (1972). *Science* 175:781-782.

Salen, G. and S.M. Grundy (1973). *J. Clin. Invest.* 52:2822-2835.

Sardet, C., H. Hansma and R. Ostwald (1972). *J. Lipid Res.* 13:624-639.

Sata, T., R.J. Havel and A.L. Jones (1972). *J. Lipid Res.* 13:757-768.

Scanu, A.M., C. Edelstein and R.H. Wolf (1974). *Biochim. Biophys. Acta* 351:341-347.

Scanu, A. and J.L. Granada (1966). *Biochemistry* 5:446-455.

Scanu, A., J. Toth, C. Edelstein, S. Koga and E. Stiller (1969). *Biochemistry* 8:3309-3316.

Scanu, A.M. and C. Wisdom (1972). *Ann. Rev. Biochem.* 41:703-730.

Schoefl, G.I. and J.E. French (1968). *Proc. Roy. Soc.* B 169:153-165.

Schonfeld, G. (1970). *J. Lab. Clin. Med.* 75:206-211.

Schonfeld, G., C. Felski and M.A. Howard (1974). *J. Lipid Res.* 15:457-464.

Schonfeld, G., C.L. Gulbrandsen, R.B. Wilson and R.S. Lees (1972). *Biochem. Biophys. Acta* 270:426-432.

Schonfeld, G., B. Pfleger and R.S. Lees (1974). *J. Clin. Res.* 53:1458-1467.

Schonfeld, G., S.W. Weidmar, J.L. Witztum and R.M. Bowen (1976). *Metabolism* 15:261-275.

Schotz, M.C. and A.S. Garfinkel (1965). *Biochim. Biophys. Acta* 106:202-205.

Schotz, M.C. and A.S. Garfinkel (1972). *Biochim. Biophys. Acta* 270:472-478.

Schotz, M.C., A.S. Garfinkel, R.J. Huebotter and J.E. Stewart (1970). *J. Lipid Res.* 11:68-69.

Schroeder, W.A. (1972). *Meth. Enzymol.* 25:138-143.

Schumaker, V. and G. Adams (1970). *J. Theoret. Biol.* 26:89-91.

Scow, R.O. and T. Egelrud (1976). *Biochim. Biophys. Acta* 431:538-549.

Shefer, S., S. Hauser, I. Bekersky and E.H. Mosbach (1970). *J. Lipid Res.* 11:404-411.

Shelburne, F.A. and S.H. Quarfordt (1974). *J. Biol. Chem.* 249:1428-1433.

Shipley, R.A. and R.E. Clark (1972). *Tracer Methods for in vivo Kinetics*, Academic Press, New York.

Shiratori, T. and De W.S. Goodman (1965). *Biochim. Biophys. Acta* 106: 625-627.

Shore, B. and V. Shore (1957). *Arch. Biophys. Biochem.* 71:1-10.

Shore, B. and V. Shore (1962). *J. Athero. Res.* 2:104-114.

Shore, B. and V. Shore (1969). *Biochemistry* 8:4510-4516.

Shore, B. and V. Shore (1974). *Biochem. Biophys. Res. Commun.* 58:1-7.

Shore, B., V. Shore and R.G. Hart (1974). *Biochemistry* 13:1579-1585.

Shore, V.G. and B. Shore (1973). *Biochemistry* 12:502-507.

Shrago, E., J.A. Glennon and E.S. Gordon (1971). *Metabolism* 20:54-62.

Shreibman, P.H., D.E. Wilson and R.A. Arky (1969). *New Engl. J. Med.* 281:981-985.

Shulman, R.S., P.N. Herbert, K. Wehrly and D.S. Fredrickson (1975). *J. Biol. Chem.* 250:182-190.

Sigurdsson, G., A. Nicoll and B. Lewis (1975). *J. Clin. Invest.* 56: 1481-1490.

Sigurdsson, G., A. Nicoll and B. Lewis (1976). *Europ. J. Clin. Invest.* 6:151-158, 167-177.

Simmonds, W.J., A.F. Hofman and E. Theodore (1967). *J. Clin. Invest.* 46:874-890.

Simon, J.B. and J.L. Boyer (1971). *Biochim. Biophys. Acta* 218:549-551.

Simons, L.A., D. Reichl, N.B. Myant and M. Mancini (1975). *Atherosclerosis* 21:283-298.

Sinclair, R.G. (1929). *J. Biol. Chem.* 82:117-136.

Skipski, V.P. (1972). In, *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism*, edited by G.J. Nelson, New York, Wiley-Interscience, pp.471-583.

Slack, J. and G.L. Mills (1970). *Clin. Chim. Acta* 29:15-25.

Smith, E.D. (1974). *Adv. Lipid Res.* 12:1-49.

Smith, E.B. and R.S. Slater (1972). *Lancet* 1:463-469.

Smith, E.B. and R.S. Slater (1972). In *Atherogenesis: Initiating Factors*, CIBA Foundation Symp. 12, Elsevier, Amsterdam, pp.39-62.

Smith, R., J.R. Dawson and C. Tanford (1972). *J. Biol. Chem.* 247:3376-3381.

Sniderman, A.D., T.E. Carew, J.G. Chandler and D. Steinberg (1974). *Science* 183:526-528.

Spackman, D.H., W.H. Stein and S. Moore (1958). *Anal. Chem.* 30:1190-1206.

Sperry, W.M. (1935). *J. Biol. Chem.* 111:467-478.

Stein, O., D. Rachmilewitz, L. Sanger, S. Eisenberg and Y. Stein (1974). *Biochim. Biophys. Acta* 360:205-216.

Stein, O., Y. Stein, De W.S. Goodman and N. Fidge (1969). *J. Cell Biol.* 43:410-431.

Stein, Y., M.C. Glangeaud, M. Fainaru and O. Stein (1975). *Biochim. Biophys. Acta* 380:106-118.

Stein, Y. and B. Shapiro (1959). *Amer. J. Physiol.* 196:1238-1241.

Stern, M., J. Olefsky, J. Farquhar and G. Reaven (1972). *Clin. Res.* 20: 557.

Stewart, J.E. and M.C. Schotz (1971). *J. Biol. Chem.* 246:5749-5753.

Stewart, J.E. and M.C. Schotz (1974). *J. Biol. Chem.* 249:904-907.

Stewart, J.E., C.F. Whelan and M.C. Schotz (1969). *Biochem. Biophys. Res. Commun.* 34:376-381.

Sundler, R., B. Åkesson and Å. Nilsson (1973). *Biochem. Biophys. Res. Commun.* 55:961-968.

Silvén, C. and B. Borgström (1968). *J. Lipid Res.* 9:596-601.

Technicon Publications (1971). No. AA11-24, Technicon Inst. Corp., Tarrytown, N.Y., U.S.A.

Thompson, G.R. and N.B. Myant (1976). *Atherosclerosis* 23:371-377.

Tytgat, G.N., C.E. Rubin and D.R. Saunders (1971). *J. Clin. Invest.* 50: 2065-2078.

Utermann, G., M. Jaeschke and J. Menzel (1975). *FEBS Lett.* 56:352-355.

Utermann, G., H.J. Menzel and K.H. Langer (1974). *FEBS Lett.* 45:29-32.

Vogel, W.C., J.D. Brunzell and E.L. Bierman (1971). *Lipids* 6:805-814.

Weber, K. (1975). In, *The Proteins*, edited by H. Neurath and R.L. Hill, Academic Press, New York, pp.179-223.

Weinstein, D.B., T.E. Carew and D. Steinberg (1976). *Biochim. Biophys. Acta* 424:404-421.

Welborn, T.A., G.N. Cumpston, K.J. Cullen, D.H. Curnow, M.G. McCall and N.H. Stenhouse (1969). *Amer. J. Epidem.* 89:521-536.

Wheeler, H.O. (1972). *Arch. Int. Med.* 130:533-541.

Windmueller, H.G., P.N. Herbert and R.I. Levy (1973). *J. Lipid Res.* 14:215-223.

Windmueller, H.G. and R.I. Levy (1967). *J. Biol. Chem.* 242:2246-2254.

Windmueller, H.G. and R.I. Levy (1968). *J. Biol. Chem.* 243:4878-4884.

Windmueller, H.G. and A.E. Spaeth (1972). *J. Lipid Res.* 13:92-105.

Wing, D.R., C.J. Fielding and D.S. Robinson (1967). *Biochem. J.* 104:45c.

Zakim, D., R.H. Herman and W.C. Gordon, Jr. (1969). *Biochem. Med.* 2: 427-437.

Zieve, F.J. and L. Zieve (1972). *Biochem. Biophys. Res. Commun.* 47: 1480-1485.

Zilversmit, D.B. (1960). *Amer. J. Med.* 29:832-848.

Zilversmit, D.B., C. Entenman and M.C. Fishler (1943). *J. Gen. Physiol.* 26:325-331.

APPENDIX

The studies reported in this thesis were performed according to the Declaration of Helsinki (part of which is shown below)

Basic Principles

1. Clinical research must conform to the moral and scientific principles that justify medical research, and should be based on laboratory and animal experiments or other scientifically established facts.

2. Clinical research should be conducted only by scientifically qualified persons and under the supervision of a qualified medical man.

3. Clinical research cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the subject.

4. Every clinical research project should be preceded by careful assessment of inherent risks in comparison to foreseeable benefits to the subject or to others.

5. Special caution should be exercised by the doctor in performing clinical research in which the personality of the subject is liable to be altered by drugs or experimental procedure.

Non-therapeutic Clinical Research

1. In the purely scientific application of clinical research carried out on a human being it is the duty of the doctor to remain the protector of the life and health of that person on whom clinical research is being carried out.

2. The nature, the purpose, and the risk of clinical research must be explained to the subject by the doctor.

3a. Clinical research on a human being cannot be undertaken without his free consent, after he has been fully informed; if he is legally incompetent the consent of the legal guardian should be procured.

3b. The subject of clinical research should be in such a mental, physical, and legal state as to be able to exercise fully his power of choice.

3c. Consent should as a rule be obtained in writing. However, the responsibility for clinical research always remains with the research worker; it never falls on the subject, even after consent is obtained.

4a. The investigator must respect the right of each individual to safeguard his personal integrity, especially if the subject is in a dependent relationship to the investigator.

4b. At any time during the course of clinical research the subject or his guardian should be free to withdraw permission for research to be continued. The investigator or the investigating team should discontinue the research if in his or their judgment it may, if continued, be harmful to the individual.