PLASMA LIPOPROTEIN TRIACYLGLYCEROL METABOLISM IN SHEEP

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by

JOHN CHARLES LOUIS MAMO, B. Ag. Sc. (Hons.) (Adelaide)

Department of Animal Sciences,

Waite Agricultural Research Institute,

The University of Adelaide,

South Australia

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DEDICATION

"This thesis is dedicated to the memory of my father, the late James Benjamin Mamo (1925-1964). In 1952-53 he and my mother Maryanne Sylvia left their homeland of Malta destined for Australia so that their children may have the opportunity of a better education."

I wish to thank them.

TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

SUMMARY

This thesis examined the metabolism of plasma lipoprotein triacylglyceride in sheep (Ovis aries) under normal fed conditions, fasting and alloxan diabetes.

A number of lipoprotein analytical techniques were examined for their suitability in isolating and characterizing sheep plasma lipoproteins. Agarose gel filtration, serial ultracentrifugation, agarose gel electrophoresis and high performance liquid chromatography were used to fractionate each of the major classes of sheep plasma lipoproteins.

The plasma lipoprotein profile of fed sheep was made up of the major lipoprotein classes exhibited in other species, namely, very low density, low density and high density lipoproteins. Of these, high density lipoprotein was the major plasma component transporting 63% of total circulating lipids. Low density lipoproteins and very low density lipoproteins comprised 26% and 11% of plasma lipids respectively. The very low density lipoproteins were rich in triacylglyceride with 51% of the molecular complex being made up of this lipid. The low density lipoproteins were principally composed of cholesterol esters, whilst high density lipoproteins were essentially of phospholipid composition. As such, the plasma lipid profile in fed sheep comprised 43% phospholipids, 24% triacylglyceride, 22% cholesterol esters and 10% cholesterol.

Alloxan diabetic sheep exhibited a substantial rise in all plasma lipid components. Phospholipids were elevated 137%, triacylglyceride

356%, cholesterol esters 256% and cholesterol 106%. The hyperlipidaemia was reflected in a 58% increase in high density lipoproteins, an 89% increase in low density lipoproteins and a 12 fold elevation in very low density lipoproteins. The latter fraction represented 50% of the total plasma lipids and 89% of circulating triacylglyceride. The predominance of very low density lipoproteins in diabetic sheep plasma was considered to reflect the increased rate of hepatic triacylglyceride secretion in these animals (Mamo J.C.L., Snoswell A.M. and Topping D.L. (1983) Biochim. Biophys. Acta 753, 272-275).

The physical and chemical nature of the lipoproteins differed between fed and diabetic sheep. Very low density lipoproteins from diabetic animals contained a greater proportion of triacylglyceride and protein, though less cholesterol esters, than those particles from fed sheep. Conversely, low density lipoproteins and high density lipoproteins had a smaller triacylglyceride component and a greater cholesterol ester content. Both very low density lipoproteins and high density lipoproteins were smaller in diabetic sheep. In addition, all of the lipoprotein fractions in these animals exhibited greater rates of electrophoretic migration towards the anode, implying that the particles were glucosylated.

The steady state concentration of plasma triacylglyceride is dependent on both release and clearance from the plasma. In monogastric omnivores two enzymes are responsible for removal of plasma triacylglyceride, namely, lipoprotein lipase and hepatic lipase. The results presented in this study showed that the sheep liver contained a

lipase activity not unlike hepatic lipase reported in other species. Sheep liver lipase activity was resistant to high concentrations of sodium chloride and protamine sulphate, exhibited an alkaline pH optimum, was depressed by increasing levels of serum and was eluted in the 0.72M NaCl fraction through heparin-sepharose affinity columns.

Lipoprotein lipase and hepatic lipase activity in postheparin plasma from fed, fasted and diabetic sheep were determined. Lipoprotein lipase activity was depressed in both fasted and diabetic animals. Hepatic lipase activity was depressed in fasted animals, though conversely, activity was significantly higher in diabetic sheep.

Very low density lipoproteins from both fed and diabetic animals were incubated with postheparin plasma from fed sheep, to determine if the differences in postheparin plasma lipase activities were a reflection of physiochemical modifications in the triacylglyceride rich lipoproteins. Rates of lipolysis were nearly three fold higher in particles isolated from diabetic animals, due to a stimulation of both lipoprotein lipase and hepatic lipase mediated hydrolysis.

Postheparin plasma lipoprotein lipase and hepatic lipase were determined in ewes, fed wethers and rams. Both lipoprotein lipase and hepatic lipase were substantially higher in ewes and wethers when compared to rams. The implications of androgenic and oestrogenic control of lipase activity in relation to fat deposition were discussed. Similarly, postheparin plasma lipoprotein lipase, hepatic lipase and triacylglyceride secretion were determined in pre-ruminating and ruminating lambs designated as genetically 'lean' and 'obese'.

Triacylglyceride hydrolysis was significantly greater in 'obese' sheep than 'lean' animals maintained on the same plane of nutrition. The implications of genetic control of adiposity in terms of predetermined rates of lipolysis were considered.

The results presented in this study also report for the first time the apoprotein profile of all the major classes of sheep plasma lipoproteins, with identity based on molecular weight and conformity with the apoprotein profile of rat plasma apoproteins. Absolute confirmation of identity was hampered by the unavailability of antisera suitable for sheep apoproteins.

Very low density lipoproteins from both fed and diabetic animals contained apoproteins AI, AII, AIV, B and C. Low density lipoproteins from fed sheep contained apoproteins AI, AII, AIV, B and E, whereas the same fraction from diabetic animals contained apoproteins AI, AIII, AIV, B and E. High density lipoproteins from fed and diabetic animals contained apoproteins AI, AII, AIII, AIV and E. It was considered that the apoprotein 'A' compliment associated with very low density lipoproteins and low density lipoproteins may promote activity of hepatic lipase. In addition, apoprotein AIII correlated with particles which contained a smaller component of triacylglyceride and a greater fraction of cholesterol esters, suggesting that this protein may promote hepatic lipase and lecithin cholesterol acyl transferase activity.

Apoprotein B was quantified in all of the major lipoprotein fractions. There was nearly a five fold increase of this protein per unit of very low density lipoproteins from diabetic sheep as opposed to

fed animals, suggesting that synthesis of apoprotein B was not limiting hepatic release of very low density lipoproteins.

The results presented in this thesis suggested that the sheep liver has a substantial capacity to increase the hepatic synthesis and release of triacylglyceride rich very low density lipoproteins, in response to an increased hepatic uptake and subsequent esterification of plasma unesterified fatty acids, seen in animals under conditions of stress. These particles in diabetic sheep have undergone both physical and chemical modifications which promote the activity of lipoprotein lipase and hepatic lipase. Stimulation of these enzymes may be a reflection of an improved apoprotein compliment in particles from the latter. The decreased plasma lipolysis of very low density lipoprotein triacylglyceride in diabetic sheep, which in part was also reponsible for the large elevation of very low density lipoproteins in these animals, was due to low lipoprotein lipase activity, in response to the low levels of plasma insulin.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the Ph.D. degree.

JOHN CHARLES LOUIS MAMO

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I am indebted to Mr. Richard Fishlock, who besides having put up with me over the past three years, also provided technical advice and assistance. I very much enjoyed our many conversations, particularly the non-scientific ones! Remember Richard, if one procrastanates too long over lipoproteins, they will degenerate.

I wish also to thank Dr. Brian Siebert (C.S.I.R.O. Division of Human Nutrition) and Mrs. Abla Cuthbertson for providing me with the genetically 'lean' and 'obese' sheep used in this study.

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PUBLICATIONS

Mamo J.C.L., Topping D.L. and Snoswell A.M.- "Factors Affecting Heparin Releasable Plasma Triacylglycerol Hydrolase Activities in Merino Sheep." (1985) Proc. 7th. Int. Symp. Athero. 95.

Mamo J.C.L., Topping D.L. and Snoswell A.M.- "Preliminary Investigations Into Ovine Hepatic Triacyglycerol Hydrolase" (1985) Proc. Nutr. Soc. Aust. 10, 115.

PREFACE

Abbreviations approved by the Biochemical Journal (1985) for use without definition are used as such throughout this thesis.

Chemical compounds, their sources and degrees of purity are described in the text.

The recommendations of the Nomencalture Committee of the International Union of Biochemistry (1979, 1980, 1981) on the nomenclature and classification of enzymes have been followed as far as possible. The following enzymes are referred to by name only:

Diacylglycerol acyltransferase	EC 2.3.1.20
Glucose oxidase	EC 1.1.3.4
Lecithin-cholesterol acyltransferase	EC 2.3.1.43
Lipoprotein lipase	EC 3.1.1.34
Peroxidase	EC 1.11.1.7
Triacylglycerol lipase	EC 3.1.1.3

ABBREVIATIONS

TAG - triacylglyceride

VLDL - very low density lipoproteins

IDL - intermediate density lipoproteins

LDL - low density lipoproteins

HDL - high density lipoproteins

LPL - lipoprotein lipase

HL - hepatic lipase

LCAT - lecithin cholesterol acyl transferase

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

OVERVIEW (This literature review will only incorporate publications of interest up to the start of this study, namely 1983)

1 INTRODUCTION

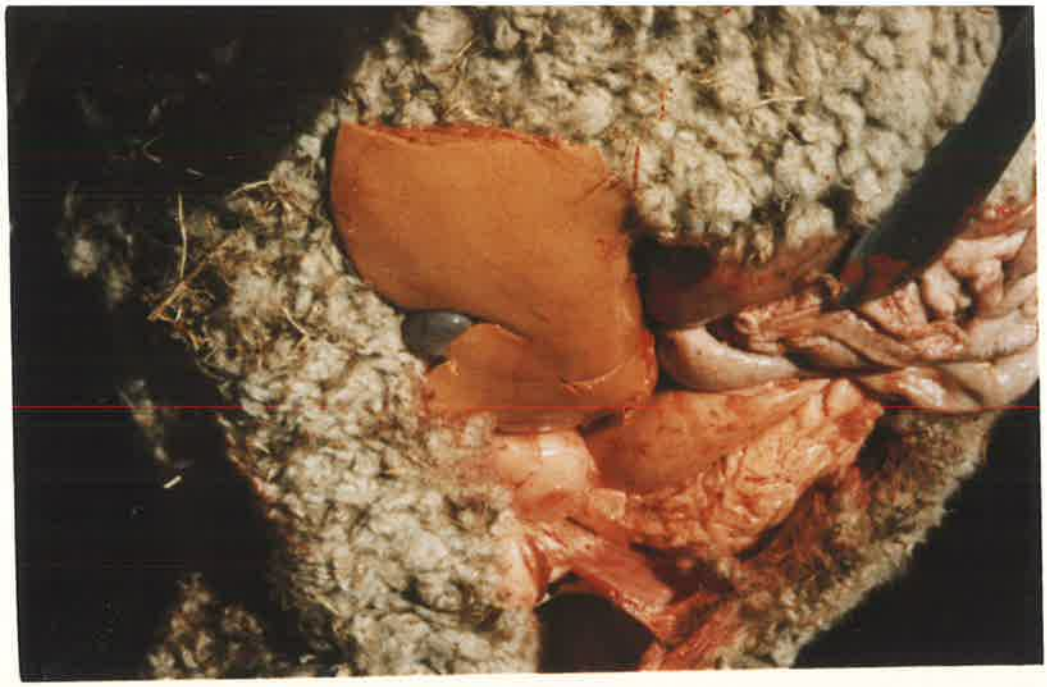
Ruminants are prone to the rapid development of livers infiltrated with vast quantities of lipid, when under conditions of metabolic stress such as fasting, pregnancy toxaemia, lactation ketosis or hypocalcaemia (Jarrett et al. 1956, Ford 1962, Jackson et al. 1964, Patterson 1966, Baird et al. 1968, Reid 1968, Schultz 1968, 1971, Taylor and Jackson 1968, Bergman 1971, Smith and Osborne-White 1973, Brumby et al. 1975, Pethick 1975, Smith and Walsh 1975, Reid et al. 1976, 1977a,b and Baird 1977). Such disorders are usually associated with either late pregnancy or early lactation, when the metabolic demands of the foetus or mammary glands far outway net energy intake. The continued accumulation of hepatic lipid is associated with a progressive breakdown of liver functions, cirrhosis of the liver and, eventually, death. Figure 1 shows stages of hepatic fat accumulation in metabolically stressed sheep. The economic burden due to the loss of livestock or at best diminished productivity is very high. It is estimated that some one million sheep die annually on properties in Australia, due to stress states associated with hepatic fat accumulation. (10% of sheep deaths on Australian properties, Year books of Australia 1975-1982). Unfortunately the symptoms associated with such disorders often appear rather spontaneously and at a time, where due to the advanced state of the disease, treatment is not possible.

All of the domestically important ruminant species namely sheep,

Figure 1

Figure 1 shows stages of hepatic fat accumulation in sheep. The top picture shows the deep pink colouration associated with normal healthy livers. The middle picture shows liver tissue samples from severely diabetic sheep (blood glucose greater than 10mM), note the yellow colouration indicative of fat accumulation. The bottom picture shows the liver from a severely pregnant toxaemic ewe with massive fat infiltration.

(top and bottom pictures kindly provided by Dr. Alan Snoswell)



cattle and goats share the same basic physiology and therefore, the tendency to develop similar metabolic disorders. There are however, a number of species differences associated with their physiology, diet, environment and metabolic demands, which affect the frequency and intensity of these manifestations. Sheep (Ovis aries) has been chosen as a ruminant animal model for this thesis, therefore, the subsequent literature review will be mainly confined to this species. As such, it must be borne in mind, that parallelisms to other ruminants, may not always be justifiable.

In recent years, a great deal of insight has been gained as to the principal causes of diseases such as pregnancy toxaemia, which give rise to the development of a 'fatty' liver. Consequently, agricultural producers, through good farm management practices, have been able to reduce their incidence. It was realized that fatty acids were mobilized from the adipose tissue under periods of stress, which in turn was reflected by an increased synthesis of lipids and subsequent accumulation of hepatic triacylglycerols (TAG). It was considered that accumulation occurred when the ability of the liver to secrete TAG, is far outweighed by its rate of synthesis.

The biochemical cascade of cellular events which lead to the development of a 'fatty' liver in ruminants, is however, far from resolved. The preferential hepatic accumulation of TAG under conditions of stress raises many questions which are not readily answered. Why are TAG the major lipid accumulating? Does the ruminant liver preferentially esterify incoming non esterified fatty acids (NEFA) as opposed to oxidizing them? If so, then why? Does the liver selectively esterify NEFA to TAG and not cholesterol-esters and/or phospholipids? Is there a

defect in the synthesis, packaging, transport or secretion of TAG rich lipoproteins? If so, is this due to a lack of lipoprotein components, such as phospholipids or cholesterol, or an inability to increase or maintain lipoprotein biosynthesis, so as to export all endogenous hepatic TAG? Is there a physical impairment which is inhibiting lipoprotein secretion? Is there a deficiency or defect in the synthesis of the apoprotein components essential for lipoprotein metabolism? Does very low density lipoprotein (the plasma TAG rich lipoprotein under fed conditions) mediate the bulk of plasma TAG in metabolically stressed ruminants, or does there exist an abnormal lipoprotein? What role do the membrane bound triacylglycerol hydrolases, namely lipoprotein lipase and hepatic lipase, have in the metabolism of TAG rich lipoproteins in metabolically stressed animals and subsequent hepatic accumulation of TAG? Is the liver TAG accumulation a result of a complex combination of cellular disorders?

It is apparent that the synthesis, secretion and metabolism of hepatic TAG in ruminant animals has been long neglected and requires urgent investigation. In part fulfillment of this need, this study was concerned with the metabolism of TAG rich lipoproteins within the plasma compartment, in metabolically stressed sheep.

This overview will examine the anabolic and catabolic processes of very low density lipoprotein-TAG metabolism, in view of its association with 'fatty' liver syndrome. Throughout this study, comparisons will be made with non-ruminant diabetes, which in man, is of great clinical significance.

2 THE USE OF ALLOXAN DIABETES AS A MODEL OF METABOLICALLY STRESSED SHEEP

Many of the naturally occuring metabolic disorders associated with 'fatty' liver syndrome which afflict ruminants are often unpredictable, making their study a difficult task. Preliminary investigations in this laboratory have shown that pregnancy toxaemia is difficult to induce artificially, after which maintenance of the animal in a stressed state is near futile. Another complication of using naturally occuring manifestations, is the inability to measure and subsequently manipulate the severity of the disease.

Although diabetes is not a naturally occuring disorder of any consequence in ruminants, it offers very many advantages as a model of 'fatty' liver syndrome. Induction of diabetes, either by surgical pancreatectomy or use of the drugs alloxan or streptozotocin, allows generation of a number of stressed sheep, in the same condition, which if required, can be maintained by exogenous insulin administration. In addition, blood or urine concentrations of glucose, or plasma insulin levels may be monitored quickly and cheaply and used as indicators of the effectiveness of the induction. By removing the pancreas or irreversibly destroying the beta cells of the Islets of Langerhans, which synthesize insulin in vivo, metabolism of glucose is severely impaired. To meet the metabolic requirements of the animal in the short term, adipose tissue TAG is mobilized and released into the plasma as NEFA.

Most of the naturally occurring manifestations which promote hepatic TAG accumulation are also associated with a reduced, if not

complete cessation of food intake, which in turn is reflected in decreased levels of plasma insulin (Bouchat et al. 1981). It appears therefore, that the biochemical process of hepatic TAG accumulation observed in diabetic sheep, would not differ substantially to that observed in naturally occuring pathological disorders.

This study makes use of alloxan induced diabetes as a model for the examination of TAG metabolism in chronically stressed sheep. Alloxan monohydrate permanently prevents the enzymatic synthesis and release of insulin from the pancreas (Rerup 1970) and unlike pancreatectomy does not interfere with other functions of this tissue, such as digestive enzymic secretions.

3 LIVER LIPID ACCUMULATION

The susceptibility of an animal to develop 'fatty' liver syndrome varies dramatically between species and possibly breed. For instance, rats are less susceptible than sheep to hepatic steatosis associated with fasting (Harrison 1953, Manns 1972) and guinea pigs are less susceptible than rats to 'fatty' livers associated with choline deficiency (Lucas and Ridout 1967).

Sheep livers infiltrated with fat are generally enlarged and paler in colour (figure 1). Both features are dependent on the degree of fat accumulation. The greater mass associated with 'fatty' livers is also in part attributable to an elevated water content (Lucas and Ridout 1967).

A healthy sheep liver is about 5% lipid by weight, of which approximately 70% is phospholipid and 30% is neutral lipid (Peters and Smith 1964). Phosphatidylcholine and phosphatidylethanolamine are the

major phospholipids (Peters and Smith 1964, Noble et al. 1971) and TAG and free cholesterol are the major neutral lipids (Peters and Smith 1964).

Studies as to the type of fat accumulating in the 'fatty' livers of varying aetiology, show that TAG are the predominant lipid component. Dryerre and Robertson (1941) first reported that neutral fat was the main class of the increased liver lipid in pregnant ewes, pregnant toxaemic ewes and abattoir wethers. This was later substantiated by Read (1976) and Henderson, Read and Snoswell (1982), who reported that in alloxan diabetic wethers and pregnant toxaemic ewes, TAG were elevated substantially and that the phospholipid concentration did not change markedly. Smith and Walsh (1975) also reported a smaller, though still significant elevation in liver cholesterol ester in pregnant and lactating ewes.

4 ROLE OF INTESTINE AND LIVER AS SOURCES OF TRIACYLGLYCEROL-RICH CONTAINING LIPOPROTEINS.

Lipoproteins are the vehicles by which hydrophobic lipids are transported in the generally aqueous environment of plasma, to tissues which utilize the constituents for oxidative metabolism, membrane homeostasis or for storage purposes. They are synthesized at two sites, namely, the intestinal epithelium and within the hepatocyte. The maintenance of synthesis and secretion of lipoprotein particles is thus essential for normal lipid metabolism. A defect in either or both of these processes results in the rapid accumulation of lipid. Normal plasma lipoproteins are generally spherical macromolecular complexes

containing a mixture of core lipids, encased by a hydrophilic layer of phospholipid, cholesterol and specific proteins (termed apoproteins) which act as recognition sites and regulators for the uptake and catabolism of the particles. Lipoproteins are most commonly differentiated by their density, lipid compostion and origin. Classes of lipoproteins and the categories by which they are defined are discussed in chapter one. The role of apoproteins in the metabolism of lipoproteins is discussed in chapter three.

In all species studied thus far, two distinct lipoprotein particles, namely chylomicrons and very low density lipoprotein (VLDL) carry the majority of circulating TAG. The contribution of either of these particles to total circulating TAG is particularly dependent on the nature of the diet and physiology of the animal concerned.

Chylomicrons are synthesized within the intestinal epithelium. The digestion of lipid, its absorption into the enterocyte (mucosal cell of the small intestine) and secretion as chylomicron particles in monogastric animals has been reviewed extensively (Johnston 1970, Hamilton 1972, Simmonds 1972, Green and Glickman 1981 and Miller and Gotto 1982) and the whole process is only briefly summarized here. The major products of the hydrolysis of dietary fats are fatty acids and monoglycerides. These pass into the enterocytes. TAG are resynthesized within the smooth endoplasmic reticulum and become chylomicron precursors. The particles pass to the Golgi apparatus, which is involved in the process of apoprotein and carbohydrate addition. The resulting chylomicron particles are then expelled from the enterocyte by reverse pinocytosis (exocytosis), into the intestinal lymphatics.

In monogastric omnivores and herbivores, the contribution of

dietary derived chylomicron-TAG to plasma TAG concentration varies considerably and is particularly dependent on the nature of the diet. For example, in adult rats maintained on a normal low fat chow diet, consuming approximately 0.5g fat per 100g of body weight daily, approximately 80% of circulating TAG are attributable to hepatically derived VLDL (Palmer et al. 1978, Risser et al. 1978, Holt and Dominguez 1980, Huang and Williams 1980, Kalopissis et al. 1980, 1982 and Agius et al. 1981). When adult rats are fed a diet containing 70% of calories as fat, intestine contributes 85% of plasma TAG (Kalopissis et al. 1980, 1982). In addition these particles are rapidly metabolised in vivo, and so the contribution to total plasma TAG levels is also critically dependent on the time of blood sampling after the previous meal. Investigations which determine the concentration of circulating plasma TAG may thus be exagerated if chylomicron particles are present, because they are the means by which dietary fat is packaged for further metabolism, and hence, represent exogenous rather than endogenous lipid. Most lipoprotein studies use subjects which have been without food for a period of time sufficient to clear any circulating chylomicron particles.

In contrast, ruminants have negligible amounts of dietary derived TAG due to the low lipid content of the diet in general and particularly, the fermentative properties of the reticulo-rumen system, (Scott 1971) as evidenced by the absence of chylomicron particles in the plasma of fed sheep (Nelson 1973 and Leat et al. 1976). The rumen microflora have the capacity to hydrolyze dietary lipids before absorption can take place. Leat and Harrison (1974) observed that ruminant lymph contained a high content of phospholipids relative to TAG

and suggested that lymph lipids were transported in VLDL rather than chylomicrons. They subsequently confirmed that 75% of ruminant lymph lipids resided in VLDL, with the maximum concentration occuring in the Sf range 150-200 (see chapter one) region and suggested that VLDL probably predominates because of the low intake of dietary fat (Harrison and Leat 1975). This was later confirmed by Gooden et al. (1979) who showed that the size of the lymph liporotein particles increased with the amount of lipid ingested.

Although lymphatic VLDL and chylomicrons are present in sheep, it is not known why few, if any are found in plasma (Nelson 1973 and Leat et al. 1976). In grazing ruminants, the low intake of dietary fat may account for the absence of these particles. However, in ruminants fed high fat concentrate diets, or protected fat diets, substantial quantities of chylomicrons occur in lymph but only small amounts in plasma (Scott and Cook 1975). A possible explanation is that lymph particles are rapidly metabolized by lung tissue (which has a very large capillary bed) and the peripheral tissues. In support of this the turnover time of chylomicron TAG is 7.5-11.5 minutes in the lactating goat (Lascelles et al. 1964) and 10-20 minutes in man (Havel and Kane 1975).

The majority of pathological conditions which lead to the development of a 'fatty' liver in sheep, are usually associated with a reduced or complete cessation of food intake. It is apparent, therefore, that for the purpose of this study, dietary derived TAG in sheep may be considered as negligible.

VLDL are synthesized principally within the hepatic sinusoids, although the intestinal epithelium may also contribute to an

indeterminate extent. The biosynthesis, assembly and secretion of lipoproteins by the liver shares many common features with the intestinal epithelium, although the origin of the lipid moiety is clearly different. A schematic representation of the subcellular biosynthetic route of lipoprotein particles in the liver is shown in figure 2. In monogastric animals hepatically derived VLDL are first formed on the smooth and rough endoplasmic reticulum (Glaumann et al. 1975) whereby the TAG and phospholipid components are derived. The rough endoplasmic reticulum is also responsible for the synthesis of the apoprotein components (De Jong and Marsh 1968 and Alexander et al 1976). After being packaged into secretory vacuoles by the golgi apparatus, fusion with the plasma membrane results in expulsion of the nascent lipoproteins by exocytosis into the space of Disse (Hamilton et al. 1967, Jones et al. 1967 and Claude 1970), which represents a localized high concentration of hepatic secretory products. The mechanism of hepatic VLDL synthesis and secretion in ruminant animals has been the subject of little investigation, however, there is no published data suggesting that the process differs from that in monogastric animals.

As a result of the digestive physiology of ruminant animals, plasma TAG concentration is in effect, a reflection of the balance between the secretion of hepatically derived VLDL-TAG and subsequent catabolism by the extrahepatic tissues. In comparison to non-ruminants, sheep (like other ruminants) have extremely low levels of circulating VLDL-TAG (and non VLDL-TAG) (Nelson 1973 and Leat et al. 1976). It is not known whether the small concentration of this lipoprotein fraction is due to a low rate of hepatic synthesis and release, or the exceptional avidity of extrahepatic tissues for VLDL-TAG. In support of

Figure 2

Figure 2 is a schematic representation of hepatic biosynthesis of lipoprotein particles. Particle formation begins on the smooth and rough endoplasmic reticulum where the lipid components are derived. The rough endoplasmic reticulum is also responsible for the synthesis of the apoproteins. These particles are then packaged into secretory vacuoles by the golgi apparatus, after which fusion with the plasma membrane results in their expulsion into the space of Disse.

(Diagram from Dolphin P.J. (1985) Can. J. Biochem. Cell Biol. 63, 850-869)

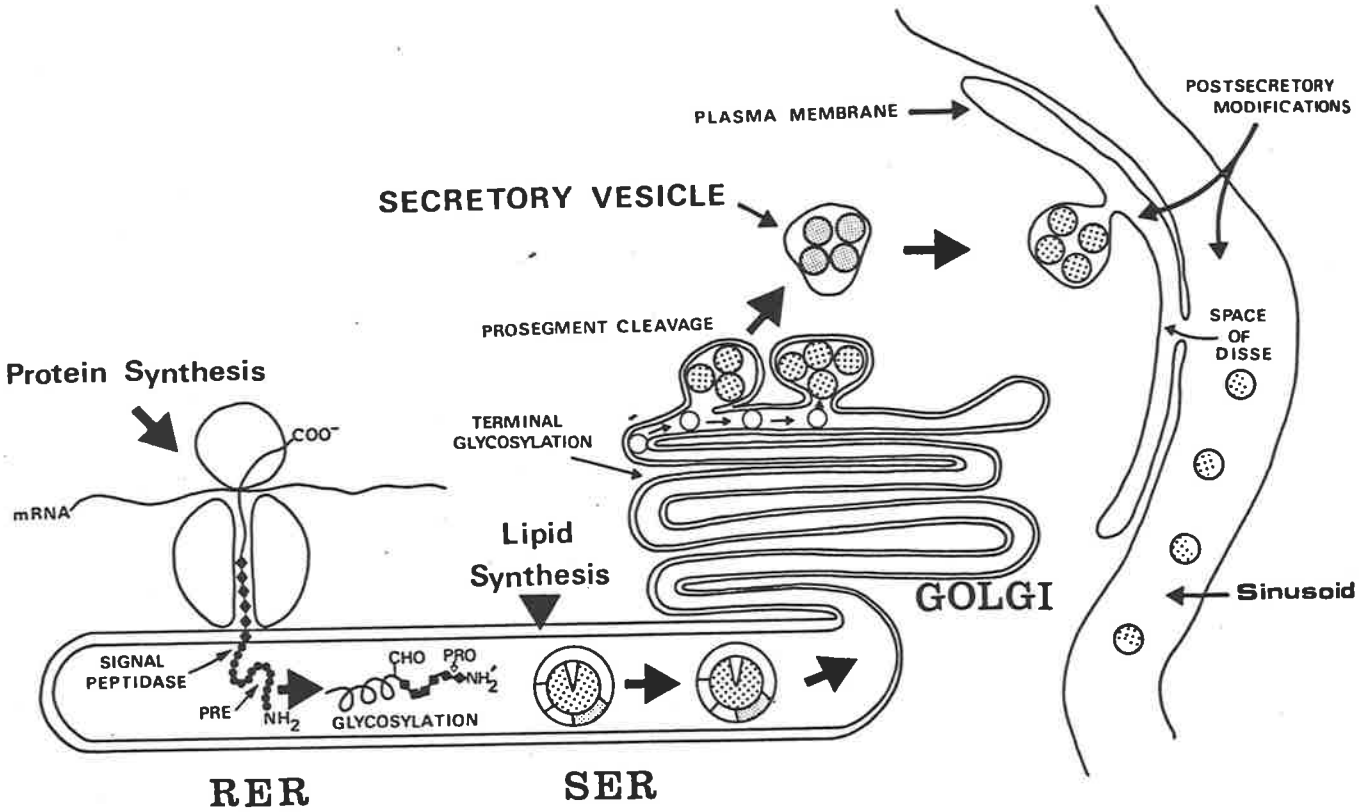

PLASMA MEMBRANE
POSTSECRETORY MODIFICATIONS
SECRETORY VESICLE
PROSEGMENT CLEAVAGE
SPACE OF DISSE
Protein Synthesis
COO⁻
TERMINAL GLYCOSYLATION
mRNA
Lipid Synthesis
GOLGI
Sinusoid
SIGNAL PEPTIDASE
CHO
PRO
NH2
PRE
NH2
GLYCOSYLATION
RER
SER

fig. 2

the latter suggestion, the rate of turnover of plasma VLDL in the lactating cow was rapid relative to that of other lipoproteins (Glascock and Welch 1974 and Palmquist and Mattos 1978). However, this would not be unexpected in view of the demand of the mammary gland for TAG fatty acids.

The synthesis, secretion and metabolism of VLDL is obviously of fundamental importance in the process of hepatic TAG accumulation in sheep, though as yet, there has been no investigation into this process.

5 THE ROLE OF FATTY ACIDS IN TRIACYLGLYCEROL METABOLISM

Fatty acids may be utilized for the alternative pathways of oxidation and esterification in the liver. They are mainly derived from either the circulating plasma NEFA, de novo fatty acid synthesis or from intrahepatic lipolytic processes. The relative contributions of these for utilization in the liver are variable according to a number of factors and are under hormonal and nutritional control (Fritz 1961, Mayes and Felts 1967, Spector 1971, Ontko 1972, Heimberg et al. 1978 and McGarry and Foster 1980).

5a LIPOGENESIS

In the fed ruminant, metabolism is dominated by the extensive microbial fermentation of dietary carbohydrate and other organic constituents to short chain fatty acids in the reticulo-rumen and to a lesser extent, the caecum (Harfoot 1978 and Noble 1978). Short chain fatty acids pass into the abomasum and are absorbed mainly inthe

reticulo-rumen and omasum. The fluid entering the duodenum contains a high proportion of these fatty acids (Bath and Hill 1967). Heath and Hill (1969) have reported that up to six grams may be absorbed from the duodenum of sheep per day under fed conditions. Three short chain acids are produced in significant amounts; acetate, propionate and butyrate, of which the first predominates. Acetate is metabolized least by ruminal epithelium and liver, and therefore, large amounts are available for post-hepatic metabolism in the fed animal (Pethick et. al 1981). Much of this is oxidized in peripheral tissues (Annison and Armstrong 1970 and Pethick et al. 1981). Surplus acetate then becomes the most important source of acetyl-CoA for the synthesis of long chain fatty acids (Hanson and Ballard 1967, 1968, Young et al. 1969, Hood et al. 1972 and Ingle et al. 1972a,b). Acetate utilization in fasted-alloxan diabetic sheep is similar to that in normal fed animals (Pethick et al. 1981).

The other major short chain fatty acids, (propionate and butyrate) are also involved in lipogenesis through utilization of their metabolites, that is, glucose and 3-hydroxybutyrate respectively. Almost all propionate which reaches the liver is metabolized via the tricarboxylic acid (Krebs) cycle, some of which is oxidized to carbon dioxide, but the majority of which is converted to oxaloacetate and used for glucose synthesis (Leng et al. 1967, Leng 1970 and Smith and Walsh 1975). Indeed approximately half of a fed ruminant's carbohydrate requirements are met by this means. Glucose produced by this pathway is only a minor source for fatty acid synthesis, but nonetheless it is very important in lipogenesis as a source of reducing equivalents, in the form of NADPH, for esterification of long chain fatty acids (Yang and

Baldwin 1973a,b).

Butyrate is metabolized predominantly in the rumen epithelium (and to a lesser degree the liver) to 3-hydroxy-butyrate (Pennington 1952, Katz and Bergman 1969, Weigand et al. 1972 and Baird et al. 1975). This contributes to fatty acid synthesis, particularly in the lactating mammary gland (Bell 1979).

The appearance of these short chain acids in the blood after feeding, gives rise to an increase in insulin secretion (Bassett 1975 and Brockman 1978). This hormone has been shown to enhance lipogenesis from both glucose and acetate (Khachadurian et al. 1966, Bartos and Skarda 1970, Baldwin and Smith 1971, Yang and Baldwin 1973a and Vernon 1979) and also to inhibit catecholamine-stimulated lipolysis in ruminant adipose tissue in vitro. Administration of exogenous insulin in ruminants has been shown to produce substantial decreases in the plasma concentration of NEFA (Kochen et al. 1959, Annison 1960, Trenkle and Kuhlmeier 1966, West and Passey 1967, Bergman 1968, Luthman and Jonson 1972, Hertelendy and Kipnis 1973 and Bauman 1976) and glycerol (Bergman 1968), and in the net output of these substrates from adipose tissue in sheep.

The dietary supply of short chain fatty acids is obviously crucial for lipogenesis in ruminant tissues and has been reviewed extensively elsewhere (Leng 1970 and Church 1976). In ruminants, as in other animals, lipid synthesis occurs in most tissues of the body. In the healthy fed non-lactating ruminant, more than 90% of lipogenesis occurs in adipose tissue alone (Payne and Masters 1971, Hood et al. 1972, Ingle et al. 1972b and Martin et al. 1973).

5b NON ESTERIFIED FATTY ACID METABOLISM

When the metabolic energy requirement of an animal exceeds its net metabolic intake, adipose tissue TAG is mobilized so as to meet the deficiency. In fasted sheep and fasted-pregnant ewes, net NEFA release from adipose tissue increased following an increase in the rate of lipolysis (Adrouni and Khachadurian 1968 and Pethick et. al. 1983). Adipose TAG are hydrolyzed to NEFA by the enzyme hormone sensitive lipase and released into the plasma where they bind with albumin. Under such conditions this tissue becomes the major source of plasma NEFA. Adipose tissue is the major site of TAG storage and is not a direct contributor to the plasma component of this lipid fraction. In other studies with fasted sheep it was shown that an inverse relationship exists between the circulating levels of acetate and NEFA (Bassett 1974 and Bell and Thompson 1979). In the latter study, changes in plasma glycerol paralleled those of NEFA. Such changes are consistent with an increase in the rate of lipolysis and diminishing levels of circulating insulin seen in fasted ruminants (Bouchat et al. 1981). In non-ruminants, glucagon augments the lipolytic effects of insulin, but efficiency, glucagon is only weakly lipolytic in ruminants (Christie 1979) and as such, is probably not an important regulator of adipose tissue mobilization in these animals.

The sheep liver is the most important individual organ for the removal of NEFA from circulating blood plasma (Bergman et al. 1971) though other tissues such as skeletal muscle, cardiac muscle and kidney avidly metabolize NEFA and under certain conditions may increase their uptake. Approximately 25% of plasma NEFA clearance can be directly

attributable to the liver in conscious fed sheep (Bergman et al 1971). The rate of uptake remains constant in a variety of metabolic stress states (Katz and Bergman 1969, Thompson and Darling 1975, Thompson et al. 1975, 1978) and is directly proportional to the plasma concentration (Katz and Bergman 1969 and Thompson and Darling 1975 and Thompson et al. 1975). Hepatic NEFA uptake is not under hormonal or metabolic regulation, but rather is a function of plasma concentration (Woodside and Heimberg 1972). The sheep liver is also selective in the uptake of individual NEFA (Thompson et al. 1975, 1978) and appears to be similar in qualitative terms to that demonstrated for the perfused rat liver, (Soler-Argilaga 1973), being directly proportional to the degree of unsaturation and inversely related to carbon chain length. The hepatic uptake of NEFA in alloxan diabetic sheep has not been reported, though there is no evidence suggesting the process should differ from that in normal animals.

NEFA taken up by the liver can be totally oxidized to carbon dioxide and water via the tricarboxylic acid cycle or partially oxidized to form the ketone bodies (acetoacetate and beta-hydroxybutyrate), rather than be esterified to form complex lipids. The factors which determine which of these alternate pathways will predominate are poorly understood. In rats, in the absence of added substrate, perfused livers from fed animals will produce more carbon dioxide and less ketone bodies than livers from fasted or alloxan diabetic rats (Heimberg et al. 1962 and Morris 1963a). However, when NEFA are added to the medium, a larger fraction of NEFA will be oxidized completely or partially to ketone bodies by livers from fasting or alloxan diabetic animals, and a smaller proportion will be esterified

and secreted as TAG, than will livers from normal fed animals (Havel et al. 1962, Heimberg et al. 1966, 1967, 1969, Morris 1963a, 1963b, Mayes and Felts 1967 and Van Harken et al. 1967).

There are few communications which have dealt with the oxidation of fatty acids in the ruminant liver. However, the capacity for sheep liver to oxidize NEFA appears to be limited (Koundakjian and Snoswell 1970), due principally to low levels of hepatic carnitine, a key factor in beta-oxidation. These studies showed that in sheep liver mitochondria, palmitic and stearic acids were oxidized at a rate of only 30% of that observed in rat liver mitochondria.

Fed sheep have relatively high circulating levels of ketone bodies when compared to non-ruminants (Baird et al. 1968), much of which is derived from the metabolism of dietary derived butyrate produced in the rumen epithelium (Katz and Bergman 1969). In the same study, fasted pregnant and non pregnant ewes had much higher levels of circulating ketone bodies, even though the intestinal contribution was severely reduced. In fasted animals there is no doubt that ketogenesis increases and that the liver assumes the major role in this process (Pethick and Lindsay 1982a, 1982b). Nevertheless, Krebs (1966) suggested that hepatic ketogenesis in the ruminant animal may still be limited by the relative availability of acetyl CoA and particularly the tricarboxylic acid cycle intermediate oxaloacetate. Hyperketoneaemia initiated by an increased rate of ketogenesis is exacerbated by a reduced capacity for ketone body utilization in some tissues, including skeletal muscle (Pethick and Lindsay 1982b), kidney and heart (Varnam et al. 1978).

Bergman et al. (1971) reported that in fed sheep, despite considerable uptake of radiolabelled NEFA by the sheep liver, little

appeared in the VLDL-TAG fatty acids. Ballard et al. (1969) suggested that the low rates of lipogenesis observed in the ruminant liver may be due to low levels of oxaloacetate, which is committed to gluconeogenesis. However, as the capacity for sheep liver to oxidize NEFA is somewhat small, a large hepatic influx of NEFA would suggest a dramatic increase in the process of esterification to complex lipids. It is known that an increased supply of fatty acids in perfused rat liver (Kohout et al. 1971 and Topping and Mayes 1982) and isolated rat or chicken hepatocytes (Mooney and Lane 1981 and Davis and Boogaerts 1982) results in an increased rate of TAG synthesis and lipoprotein secretion. This stimulation appears to be coordinated with an increased activity of the final enzyme involved in TAG synthesis, namely diacylglycerol acyltransferase (Haagsman and van Golde 1981). Few such studies have reported rates of ruminant hepatic NEFA esterification, particularly under stressed conditions. Presumably, TAG are the major product of the esterification process, as suggested by their dramatic rate of hepatic accumulation. Furthermore, a TAG molecule is the most efficient means (on an energy/mole basis) of storing NEFA, and hence would serve best at packaging hepatic NEFA. Fatty acids which enter the esterification pathway are either retained within the liver cell for the formation of membrane phospholipids and for storage in TAG droplets, or they are secreted in the form of lipoproteins. It is apparent that metabolism of fatty acids proceeds under homeostatic regulation.

To my knowledge there has been no published data suggesting that the process of hepatic esterification in ruminant animals differs from other species.

It is evident that both the output of VLDL-TAG and the accumulation

of TAG in the liver are functions of NEFA concentration in the serum and the period of time to which the organ is exposed.

The ruminant liver produces little fatty acid de novo, principally because it is unable to use glucose as the source of acetyl CoA (a key intermediate in fatty acid synthesis) (Ballard et al. 1968). This is not unlikely in view of the extremely low carbohydrate supply derived from the diet, and conforms with other features of its carbohydrate metabolism. The process of gluconeogenesis in ruminant livers accounts for almost all of the animal's carbohydrate requirements (Leng 1965, Lindsay 1970 and Bergman 1973). Thus, hepatic TAG synthesized from the esterification of de novo fatty acids in the ruminant animal, may be considered as negligible.

6 HEPATIC TRIACYLGLYCEROL SECRETION

In the chronically stressed sheep, hepatic TAG accumulation will result if the rate of release of the lipoprotein particles which effect transport of this lipid is limited.

The extent of TAG output from the liver in vivo in different metabolic conditions has generally been assessed by one of two methods. In the first, doses of a radioactively labelled TAG precursor (NEFA or glycerol) are given intravenously and the specific activity and total radioactivity of the liver and the plasma lipids determined at intervals thereafter. The values obtained have been interpreted in terms of model systems constructed on the basis of estimated fatty acid fluxes in the liver, through pathways often based on a number of assumptions. Problems in interpretations associated with this technique have been

reviewed previously (Baker and Schotz 1967). The second method depends on the fact that the plasma TAG concentration is a result of a balance between rates of TAG entry and removal from the circulatory system. Removal can be prevented by the use of surface active substances, the most common of which is the non-ionic detergent Triton WR1339 (oxyethylated-tert-octylphenol polymethylene polymer) which associates with the circulating VLDL-TAG, in such a way as to prevent normal removal mechanisms from operating. Measurement of the rate of increase in plasma TAG, then provides a measure of the rate of TAG efflux. Since the removal of all TAG fatty acids in the plasma is blocked by the administration of such detergents, the method can only provide a measure of hepatic TAG release when the intestinal contribution is negligible.

Electron microscopy studies of sheep liver hepatocytes have revealed a fenestrated membrane surrounding the hepatic sinusoi d (David 1964, Grubb and Jones 1971 and Gemmell and Heath 1972) and it was considered that this may inhibit the passage of the very large VLDL molecules, particularly if these were enlarged in metabolically stressed sheep. Studies in this laboratory using Triton WR1339 to measure hepatic TAG release had shown that fasted and alloxan diabetic wethers have increased hepatic secretion of TAG associated with an elevation in the plasma concentration of this lipid (Mamo et al. 1983). It did not appear therefore that the basal lamina surrounding the hepatic sinusoid did not completely inhibit passage of VLDL molecules. Conversely the increased secretion rate could be a 'pressure-induced' effect as a result of massive hepatic VLDL synthesis, or alternatively hepatic TAG may be released in abnormal particles in chronically stressed sheep, which are smaller than normal VLDL. Subsequently, Wright et al. (1983) claimed that the basal lamina

surrounding the sinusoid was in fact a sample preparation artifact. The increased hepatic release of TAG however, is not sufficient to prevent accumulation of this lipid in situ. It appears therefore that the synthesis of VLDL may be the rate limiting process, being outweighed by the rate of TAG production. In similar studies in goats under various physiological conditions, there was no apparent difference in the rate of hepatic TAG release between fed and fasted animals (Fiser et al. 1974). In that study, goats were fasted for two days prior to Triton administration. Results from this laboratory (not published) have shown that a forty eight hour fast is not sufficient to effect a change in liver TAG release in sheep. This is not surprising in view of the time required to digest food in the ruminant animal and thus induce a 'state' equivalent to fasting. A greater period of food deprivation may have been needed to examine any changes in the rate of hepatic release. In addition, the number of animals per treatment used in this study was not sufficient to statistically eliminate individual variation. In contrast, similar Triton studies with fasting and streptozotocin induced diabetic rats (Otway and Robinson 1967 and Bobek et al. 1981), and in isolated perfused rat livers from diabetic animals (Heimberg et al. 1966, 1967 and Van Harken et al. 1967), hepatic secretion of VLDL-TAG was reduced and could account for accumulation of this lipid in the liver of these animals. The decreased release of TAG in fasted and alloxan diabetic rats, may be due to a combination of an increased hepatic capacity to oxidize fatty acids under these conditions (Heimberg et al. 1966, 1967, Van Harken et al. 1967, 1969 and Harano et al. 1969), a reduced rate of hepatic de novo synthesis and a lowered dietary supply of lower chain acids, coupled with a possible decrease in the rates of esterification

(Fredrickson et al. 1958) and inhibition of secretion of VLDL. The contrasting results are somewhat inconclusive, and the role of VLDL in hepatic TAG accumulation can only be speculated upon. Heimberg et al. (1974) in a review on factors involved in the regulation of VLDL secretion and its relationship with ketogenesis in the perfused rat liver, concluded that the livers capacity to secrete VLDL-TAG is less than its ability to take up and esterify NEFA. When the uptake of fatty acids exceeds that necessary to maintain maximal rate of secretion of VLDL, TAG accumulates in the liver. Though this theory encompasses the paradoxical changes in the rate of hepatic VLDL-TAG secretion observed in metabolically stressed rats and sheep, it is apparent that the processes regulating VLDL-TAG synthesis and release, differs in these two species.

There have been a number of suggestions as to limitations into hepatic synthesis and secretion of VLDL. Brumby et al. (1975) said that since TAG accumulation was accompanied by decreases in the percentages of phospholipid and cholesterol in the liver, availability of one or both of these constituents may have limited lipoprotein synthesis. Conversely, Heimberg et al. (1974) postulated that the amounts of phospholipid and cholesterol secreted in VLDL are dependent on TAG secretion, and are thus regulated by factors which affect the latter. Alternatively, lipoprotein synthesis may be limited by the availability of apoproteins, since in cows, there is a marked decrease in the volume of rough endoplasmic reticulum in hepatocytes after starvation (Brumby et al. 1975). In support of this, Pelech et al. (1983) showed that incoming NEFA stimulated TAG and phosphatidyl choline biosynthesis, but not apoproteins in rat hepatocytes.

The mechanism of the regulatory control of insulin, or perhaps more importantly, the molar ratio of insulin/glucagon on VLDL-TAG synthesis and secretion has been widely investigated but remains an unresolved contentious issue. It has been reported that TAG secretion in perfused livers from insulin deficient rats have a blunted response to NEFA (Woodside and Heimberg 1972 and Assimacopoulos et al. 1974). Similarly in rats, hyperinsulinaemic animals have been reported to have increased TAG production (Steiner and Vranic 1982 and Steiner et al. 1984). There have also been several reports that insulin directly stimulates hepatic VLDL-TAG secretion in vitro (Topping and Mayes 1972, 1982, Tulloch et al. 1972 and Beynen et al. 1981), though in contrast, some authors consider that this process is inhibited by insulin (Nikkila et al. 1977 and Durrington et al. 1982). Similarly, in studies from isolated hepatocytes cultured on fibronectin media free of insulin, it was found that this hormone was found to promote fatty acid and cholesterol biosyntheis (Geelen et al. 1980), but inhibit the secretion of TAG, phospholids and apoproteins B and E (Durrington et al. 1982 and Patsch et al. 1983). Insulin has also been reported to either stimulate (Topping and Mayes 1982) or have no effect (Edwards et al. 1979) on the secretion of VLDL-cholesterol. Glucagon inhibits hepatic lipogenesis, stimulates lipolysis and inhibits VLDL secretion (Heimberg et al. 1969, Kempen 1980 and Beynen et al. 1981). Bird and Williams (1982) suggested that a higher hepatic TAG release in essential fatty acid deficient rats may have been due to a higher plasma insulin/glucagon ratio, resulting from a reduction in plasma glucagon concentration.

7 METABOLISM OF VERY LOW DENSITY LIPOPROTEINS

Previous investigations in sheep have shown that fasted and diabetic animals have highly elevated plasma TAG concentrations, and that this elevation is due to an increased hepatic output of VLDL-TAG (Mamo et al. 1983). TAG concentration is also elevated in diabetic rats (Topping and Targ 1975) and man (Albrink et al. 1963, New et al. 1963) in spite of depressed synthesis. The plasma TAG pool however, is also critically dependent on the activities of two enzymes, lipoprotein lipase and hepatic lipase (discussed in chapter two). Both enzymes are bound to the capillary endothelium of those cells utilizing TAG. Lipoprotein lipase is found in tissues which utilize TAG fatty acids for oxidative purposes such as heart (Twu et al. 1976), lung (Gal et al. 1982) and skeletal muscle (Ehnholm et al. 1977) or resynthesis of TAG for storage or secretion purposes (adipose tissue or mammary glands) (Jansen et al. 1978 and Clegg 1981a). Hepatic lipase is bound to liver plasma membranes and those of steroidogenic organs which utilize lipoprotein cholesterol (Jansen and De Greef 1981). This enzyme hydrolyzes TAG and phospholipids (Ehnholm et al. 1975b) but is distinct from lipoprotein lipase in that it it is reasonably active in the absence of apoproteins. Ovine hepatic lipase has not been previously reported, though recently the presence of this enzyme in bovine liver has (Cordle et al. 1983). There have been few reports published which have examined the activity of lipoprotein lipase in chronically stressed sheep or its mode of control. Vernon et al. (1981) reported a decrease in lipoprotein lipase activity in pregnant ewes with gestation and a subsequent increase in activity, after 95-135 days postlactation. It is currently difficult to perceive

the role of these enzymes in TAG metabolism and their association with hepatic TAG accumulation in chronically stressed sheep.

The hepatic TAG accumulation may also in part be due to an increased rate of plasma TAG uptake by this organ. In support of this it has been reported that the rate of uptake of washed chylomicrons and synthetic neutral fat emulsions in isolated fasted perfused rat livers was greater than livers from fed control animals (Heimberg et al. 1962).

8 OBJECTIVES OF STUDY

The majority of currently available published literature pertaining to TAG metabolism is for non-ruminants. Presumably this is a result of their applicability as models of corresponding human metabolic disorders. However, due to the differences in the diet and digestive physiology of ruminant animals, the subsequent activity of the biochemical pathways of lipoprotein TAG metabolism is quite different, as evidenced by the paradoxical rates of hepatic TAG release observed in chronically stressed sheep and rats. It is therefore, not valid to extrapolate data derived from monogastric studies to ruminants.

It is apparent that the process of hepatic TAG synthesis, its packaging and secretion as lipoproteins and subsequent metabolism by extrahepatic tissue has been long neglected. Bell (1979), in his review on lipid metabolism in the liver and other tissues of ruminant animals, has reconciled this by stressing the urgent requirement for research of TAG metabolism in ruminants.

In part fulfilment of this need, this thesis aims to establish suitable methods for the isolation, separation and characterization of

the major ovine lipoproteins and to determine the role of each of the major lipoproteins in lipid transport, particularly TAG.

To ascertain which lipoprotein fraction is mediating the hypertriacylglyceridaemia observed in metabolically stressed sheep, changes in the lipoprotein profile and their composition in alloxan diabetic animals will be determined. In addition, transmission electron microscopy will be utilized to examine each of the major classes of ovine lipoproteins isolated from fed and diabetic sheep, in an attempt to identify any changes in the physical properties of the lipoprotein particles.

Suitable methods for the identification and isolation of lipoprotein lipase from adipose tissue and hepatic triacylglycerol hydrolase in sheep will be established. Should the presence of the latter enzyme be verified, an examination of the characteristics usually attributed to this enzyme will be done.

The role of the two lipases in hepatic TAG accumulation and plasma hypertriacylglyceridaemia will be determined, by measuring postheparin plasma activity in fed, fasted and alloxan diabetic wethers.

Rams and ewes have significantly different degrees of adiposity. This may be due to modulation of triacylglycerol hydrolase activities by androgenic/oestrogenic control mechanisms. Thus the activities of lipoprotein lipase and hepatic lipase will be determined in both sexes.

In addition, to examine if genetic variation may also in part affect the expression of lipase activities, postheparin plasma from genetically 'lean' and genetically 'obese' sheep will be examined for triacylglycerol hydrolase activities. Correlations of activities will be made with the TAG secretion rate observed in these groups.

Apoproteins are the means by which the catabolism of lipoprotein particles, namely their binding, hydrolysis and uptake by tissues is regulated. As such, this study will quantitate the apoprotein B (the major protein component of the VLDL-TAG in monogastric omnivores) of each of the major ovine lipoprotein fractions in normal and alloxan diabetic animals, and determine qualitative changes in the total apoprotein profile of each lipoprotein class, in an attempt to correlate these with changes in the metabolism of VLDL-TAG.

1 CHAPTER ONE

1.1 INTRODUCTION

The first report on the appearance of distinct lipoproteins in serum appeared in 1929 (Macheboeuf 1929a,b). In 1941, motivated by studies on atherosclerosis, Blix et al. separated classes of lipoproteins according to their electrophoretic mobility in a solid support media and Gofman et al. (1949), showed that the plasma lipids were bound in a stable union to certain proteins, using an ultracentrifuge. These proteins were designated as lipoproteins. Lipoprotein formation, composition, secretion and metabolism have since enjoyed extensive investigation, as a result of lipid abnormalities associated with disease conditions such as diabetes, renalopathy, cirrhosis of the liver and particularly, ischaemic heart disease and the process of atherogenesis.

This overview will briefly summarize the major classes of lipoproteins and the physical parameters by which they are distinguished. For more extensive reviews refer to (Hatch and Lees 1968, Forte and Nichols 1972, Eisenberg and Levy 1975, Jackson et al. 1976, Morrisett et al. 1977, Osborne and Brewer 1977, Smith et al. 1978, Edelstein et al. 1979, Miller and Gotto 1982, Mills et al. 1984 and Dolphin 1985).

1.1.1 LIPOPROTEIN STRUCTURE AND FUNCTION

The plasma lipoproteins of animal species encompass a

macromolecular complex of lipids (essentially TAG, cholesterol and phospholipids) and one or more specific proteins, referred to as apoproteins (or apolipoproteins). Their main function is to transport the hydrophobic lipids of dietary or endogenous origin within the hydrophylic environment of the plasma. A number of tissues can then utilize the constituent TAG-fatty acids for oxidative metabolism (such as heart and skeletal muscle), for storage (in adipose tissue), or simply maintenance of cellular function and membrane integrity. In addition, the cholesterol component may serve as a precursor for bile acid and steroid synthesis. The plasma lipoproteins also transport other lipid soluble substances including vitamins (McCormick et al. 1960), drugs (Chen and Danon 1979) and toxins (Chen et al. 1979).

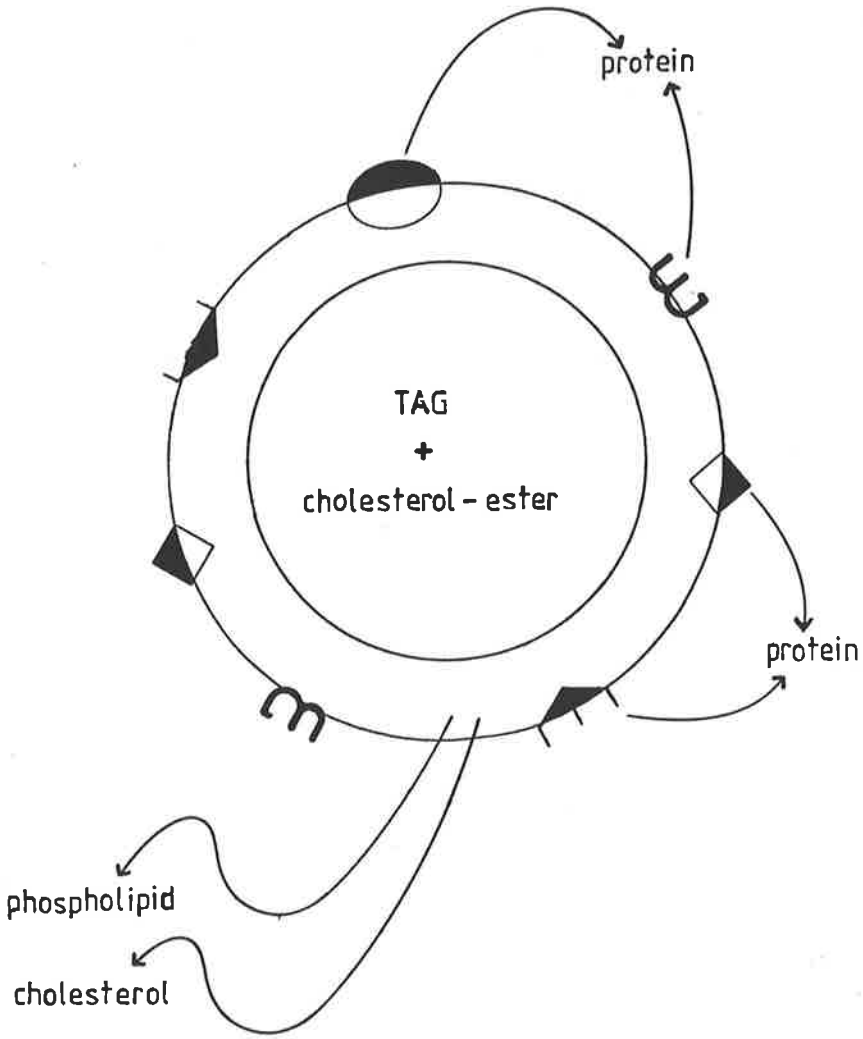
The functions of the particular apoproteins is not completely understood although much progress has been made in recent years. They confer many of the specific properties possessed by the individual lipoprotein classes in which they occur. For example, particular apoproteins regulate the activity of the major enzymes involved in lipoprotein metabolism in plasma, and are necessary for the secretion of TAG-rich lipoproteins by both liver and intestine (Gotto et al. 1971, Malloy and Kane 1982). In addition, the apoproteins play an important structural role in the lipoproteins. The composition and function of each of the apoproteins is discussed in chapter three.

Lipoproteins are now considered to be composed of a hydrophobic lipid core made up of TAG and esters of cholesterol, with the more hydrophylic cholesterol and phospholipids forming a surface interfacial monolayer (figure 1.1) (Shen et al. 1977). The apoproteins are believed

Figure 1.1

Figure 1.1 depicts a model of the structure of plasma lipoproteins. The more hydrophobic lipids, triacylglyceride and cholesterol esters are thought to occupy the core of the molecular complex and are surrounded by an amphiphilic shell of phospholipid and cholesterol. Specific proteins also occupy the outer surface of the particle but some may also bind with the inner lipid components.

fig. 1.1


protein
TAG
+
cholesterol - ester
protein
phospholipid
cholesterol

to occupy the outer part of a shell on the surface of the particles, where they are adjacent to the polar head groups of phospholipids. Amphipathic helical regions within the domain of the apoproteins permit binding and orientation of the apoprotein within the surface monolayer (Assman and Brewer 1974 and Segrest et al. 1974). With this arrangement, the non-polar residues occupy one face of the helix and point towards the hydrophobic interior of the lipoprotein, probably interacting with the first few carbons of the fatty acyl chains of the phospholipids. The acidic residues of the apoproteins (glutamic and aspartic acids) occupy the opposite face and are orientated towards the aqueous environment. The basic amino acids (lysine and arginine) occupy a position in the helix at the borders of the polar and non polar faces.

The major lipoproteins all contain cholesterol, cholesterol esters, TAG, phospholipids and protein, but are distinguishable from each other by the proportions of these constituents and the nature of the protein moiety. These differences allow the separation of lipoprotein classes by a variety of physiochemical parameters such as density, particle size, electric charge, interactions with supporting media in zone electrophoresis, interactions with macro molecular reagents, antigenicity and information derived from plasma chemical analysis. The methods involved in applying these parameters include, zone electrophoresis, double diffusion immunological methods, immuno-electrophoresis, chromatography, combinations of preparative ultracentrifugation or precipitation with chemical analysis, electron microscopy and membrane filtration.

The selection of an analytical method of maximum value is a balance of the factors of expense, technical difficulty, quantitative accuracy

and capacity for numbers of samples. Whichever the method chosen, only a limited amount of information can be derived from any one technique and so it is therefore usual to integrate data from two or more techniques.

The study of plasma lipoproteins is complicated by the constant changes occuring within individual particles. In certain situations the lipoprotein is metabolized as a whole, but frequently different lipoprotein constituents have different metabolic fates (Streja et al. 1977, Steiner and Ilse 1981 and Steiner and Reardon 1982, 1983). Individual lipoprotein components undergo exchange between lipoprotein classes and in the case of unesterified cholesterol and phospholipids, with cell membranes also (Bell 1978). Specific exchange proteins are involved in cholesterol ester, TAG and phospholipid transfer.* There are also the metabolic transformations mediated by the enzymes lecithin cholesterol acyltransferase (this enzyme mediates the transfer of the fatty acid from the beta position of lecithin, to cholesterol, to form cholesterol esters and lysolecithin (Glomset et al. 1962)), lipoprotein lipase and hepatic lipase. Nevertheless, differences between lipoprotein classes, both in lipid and protein composition are greater than those within classes and so the variations in physiochemical properties remain the basis of analytical methods for their study.

1.1.2 ROLE OF PLASMA LIPOPROTEINS

1.1.2.1 MONOGASTRIC ANIMALS

Lipoproteins have been examined in a number of species under a number of metabolic and pathological conditions. The vast majority of

* Note; sheep have low cholesteryl transfer activity
[Ha and Barter,1982,Comp. Biochem. Physiol.71B,265-269]

the currently available literature is, however, confined to monogastric animals, as a consequence of intensive research into the processes of atherogenesis.

The major classes of human plasma lipoproteins and their physical-chemical characteristics by which they are defined are shown in table 1.1. Lipoproteins are now classically defined by their densities, a reflection of the ratio of protein and lipid associated with each particle. For human lipoproteins the percentage of the particle weight that is lipid is approximately 95%, 90%, 75% and 50% for chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) respectively. As the particle decreases in density, the percent content of TAG and cholesterol ester decreases and the relative amount of phospholipid increases. Thus, of the lipid components chylomicrons contain approximately 9% phospholipid, VLDL-20%, LDL-30% and HDL-50%. The phospholipid component is located on the outer monolayer of the lipoprotein surface. Hence, the increased proportion of phospholipid in the smaller particles is a logical consequence of the increased surface/volume ratio.

Lipoprotein density is probably the most unusual property of these particles as it is lower than that of any other naturally occurring macromolecule. All lipoproteins can be floated by centrifugation after adjustment of solution density (defined as the density of the solution of salts and other small molecules but, excluding the contributions made by proteins and lipoproteins) to values between 1.006 and 1.250 g/ml. No other plasma macromolecules float at these densities unless adsorbed to lipoproteins. On this basis the lipoproteins have been divided into six major classes:

Table 1.1

Table 1.1 lists the major classes of human plasma lipoproteins and the physiochemical characteristics attributed to them.

Lipoprotein properties	Chylomicrons	VLDL	IDL	LDL	HDL2	HDL3	VHDL	NEFA-Albumin
Hydrated density (g/ml)	< 0.93	0.94-1.006	1.006-1.019	1.019-1.063	1.063-1.125	1.125-1.160	1.160-1.21	1.330
Flotation rate* Sf(1.063)	> 400	20-400	12-20	0-12	-	-	-	-
(1.20)	-	-	-	-	3.5-9.0	0.0-3.5	-	-
Diameter (nm)	120-1000	20-75	20-30	10-20	7-10	5-8	5-7	5
Molecular weight (daltons)	1-10 billion	5-10 million	3.5-7.5 million	2-4.5 million	350,000	200,000	150,000	70,000
Electrophoretic mobility (relative to albumin)	origin	pre-beta	pre-beta:beta	beta	pre-alpha1	pre-alpha1:alpha1	alpha1:beta	albumin
Mean chemical composition (%)								
Triacylglyceride	85-95	50-60	18-25	7-16	3-9	2-5	5	-
Cholesterol-ester	2-5	10-15	20-25	30-38	20-25	25-30	3	-
Phospholipid	6-9	12-20	20-28	24-32	28-40	22-29	28	-
Cholesterol	1-4	4-8	8-15	10-16	2-6	2-6	1	-
Protein	1-2	5-15	18-24	17-25	31-43	49-58	60-65	99+
Apoprotein profile (total)	B	B		B				
	CI, CII, CIII	CI, CII, CIII		C (trace)	CI, CII, CIII	CI, CII, CIII		
	AI, AII, AIV	AI, AII, AIII		A (trace)	AI	AI, AII, AIII		
	E	E		E (trace)	E			

Table 1.1

* Flotation rate (Svedberg units) at solvent density = (x.xx)

1) Chylomicrons are the largest lipoproteins (80-600nm). They are synthesized by the intestine in response to dietary fat. Chylomicrons are predominantly TAG with small amounts of free and esterified cholesterol and a protein content of only 1-2.5%. Their composition also varies with their size and whether they are isolated from lymph or plasma. Smaller particles contain a greater proportion of relatively polar lipoprotein surface constituents and less TAG. Chylomicrons enter the circulation via the lymphatic system and are rapidly catabolized (plasma half life of 10-20min in man (Havel and Kane 1975)) by the enzyme lipoprotein lipase, which is attached to endothelial surfaces, to form TAG-depleted particles called remnants. The cholesterol rich remnants are rapidly cleared by the liver. Chylomicrons have a density of less than 0.94g/ml and remain at the origin on gel electrophoresis, as they are generally too large to migrate into the pores of the stabilizing medium.

2) Very low density lipoproteins (VLDL): The very low density lipoprotein component of the lipoprotein spectrum covers a broad region of continuously varying composition. The principal variable is TAG, which when present in greater amounts progressively increases particle size and decreases density. VLDL have a size range of 30-90nm, the smaller particles containing relatively more phospholipids, protein and cholesterol. VLDL have a density greater than 0.94g/ml but less than 1.006g/ml. They are predominantly of hepatic origin and transport the bulk of endogenous plasma TAG. In terms of their electrophoretic mobility, they are termed pre-beta, because their mobility is a little greater than that of beta-globulins. Within the plasma compartment VLDL

are sequentially hydrolyzed to NEFA by endothelial TAG lipases, generating a series of smaller cholesterol-enriched lipoproteins including intermediate density lipoprotein and low density lipoprotein (Steiner and Streja 1977a, 1977b).

3) Low density lipoproteins (LDL) are particles of density between 1.006-1.063g/ml. The particle diameter is 20-25nm. There are, however, at least two subclasses. The less dense variety which has a density of 1.006-1.019g/ml is referred to as intermediate density lipoprotein (IDL), or low density lipoprotein-1 (LDL1) in older literature. The second fraction with a density range of 1.020-1.063g/ml is termed low density lipoprotein (LDL), or LDL2. The entire LDL fraction (1.006-1.063g/ml) may be considered much more homogeneous with respect to size and composition than chylomicrons or VLDL, though some heterogeneity does exist. Nearly 50% of the weight of LDL consists of cholesterol and its ester, with smaller amounts of phospholipid and neutral TAG. Approximately 20% of the molecule is protein. LDL are known as the beta-lipoproteins as they migrate at a similar rate to beta-globulins upon electrophoresis. Turnover studies have shown a half life of LDL in plasma of 3-5 days (Gitlin et al. 1958). Elevated levels of LDL are correlated with increased risk of atherosclerosis (Stamler 1979). For the purposes of this study the LDL fraction shall be considered as the lipoprotein fraction containing particles of density 1.006-1.063g/ml.

4) High density lipoproteins (HDL) are of density 1.063-1.20g/ml and appear to arise from several sources including the liver and intestine. In addition HDL, or HDL precursors, appear to be produced within the

plasma compartment from phospholipid-protein discs, generated as a result of lipolytic processing of chylomicrons (Tall and Small 1978). HDL, the smallest of the lipoproteins (8-12nm), are involved in a process referred to as 'reverse cholesterol transport', a postulated pathway whereby HDL acquire cholesterol from peripheral tissues and transport the cholesterol, directly or indirectly, to the liver for excrection (Mahley 1982). The HDL have been divided into three subfractions namely HDL1, 2 and 3. HDL1 is a minor component identifiable only with the analytical ultracentrifuge and is of uncertain physiological significance. It may contaminate LDL floated in a buffer of density 1.063g/ml, and in fact, HDL1 has by many investigators been considered as an LDL subfraction. HDL1 has alpha 2 mobility on electrophoresis. HDL2 and HDL3 which are the major fractions in human plasma consist of approximately 50% protein, 30% phospholipids and smaller amounts of cholesterol, cholesterol esters and TAG. HDL2 are richer in lipids, poorer in protein and float more rapidly in a buffer of density 1.21g/ml than HDL3. Not only is the lipid:protein ratio of HDL2 greater than that of HDL3, but there are also differences in their apoprotein composition (chapter three). HDL3 can also be formed from HDL2 during ultracentrifugation and during storage of plasma (Levy and Fredrickson 1965). Metabolic turnover studies have shown a half life in plasma of 4-5 days for the whole lipoprotein fraction of density 1.063-1.210g/ml (Furman et al. 1964). As the HDL fraction is heterogeneous, it's electrophoretic pattern is spread over the alpha, and to a lesser degree, the beta range. The recent observations suggesting that a negative correlation exists between HDL and accelerated vascular disease in man (Gordon et al. 1977), have focused attention on this lipoprotein

class and its role in cholesterol metabolism. (For review see Heiss et al. 1980).

5) Very high density lipoproteins (VHDL) have a very small lipid compliment. Consequently they mediate only a very small proportion of circulating lipids. They can be generally described as a phospholipid core encased by protein. Their density is greater than 1.20g/ml but less than 1.25g/ml. Immunologically they resemble HDL.

6) Plasma non esterified fatty acids are complexed to albumin by non-covalent forces. They are transported rapidly through plasma and have a half life of less than five minutes. This fraction is not identifiable by the usual lipoprotein techniques and must be measured by chemical methods.

In addition to the major lipoprotein fractions already described, there are a number of lipoproteins which are not found in normal individuals. Rather, these particles are the result of abnormalities in the metabolic processes of lipoprotein anabolism/catabolism. As these particles are usually attributable to particular metabolic disorders, they are beyond the scope of this review.

1.1.2.2 SHEEP PLASMA LIPOPROTEINS

Lipoproteins from domesticated ruminant species, and in particular sheep and goats, have not received the same degree of interest as those from monogastric animals.

In 1955 Morris and Courtice (1955) separated sheep and plasma

lipoproteins into slow and fast moving components on paper electrophoresis. Similarly, Perk and Lobl (1959, 1960) separated two electrophoretic lipoprotein components for both cattle and sheep plasma respectively. However, in these studies the contribution of each of the bands did not agree with the results of Campbell (1963) and Kirkeby (1966), who found that in sheep, 37% and 46% respectively of their lipoproteins, was in the slow moving fraction. Alexander and Day (1973), who examined the distribution of serum lipoproteins in selected verterbrates, also showed that 44% of sheep liproteins were in the slow moving band, on agarose plates.

The first report of the major lipoprotein components of sheep plasma was based on their rates of flotation by Mills and Taylor (1971). They found that sheep plasma contained lipoproteins with a high modal Sf rate and that VLDL were virtually absent.

Nelson (1973) isolated and separated the major classes of sheep lipoproteins based on hydrated density. In essence, he found that in fed sheep the chylomicron and VLDL fraction accounted for only 0.2% of circulatory lipids. In addition, this study also showed that agarose gel electrophoresis of sheep plasma, failed to detect a pre-beta band (VLDL), however, a beta (LDL) and two alpha (HDL) bands were observed.

Leat et al. (1976) examined the plasma lipoprotein profile of sheep in order to determine the contribution of each lipoprotein fraction to the hyperlipidaemia observed in suckling lambs. Isolation of sheep lipoproteins according to the hydrated density parameters attributed to human plasma lipoproteins reiterated the virtual absence of chylomicron and VLDL particles in the fed adult, with less than 5% of circulating lipids in this fraction. The major lipoprotein fractions (VLDL, LDL and

HDL) were similar in composition to that seen in man, though the contribution of each fraction to total plasma lipids was markedly different. HDL, LDL and VLDL accounted for 65%, 20% and less than 5% respectively of plasma lipids in sheep. In man, VLDL, LDL and HDL make up approximately 20%, 50% and 30% respectively of the plasma lipoproteins. The hyperlipidaemia observed in the suckling lamb was reflected by an increase in all of the lipoprotein fractions. VLDL was elevated to 7-15% of total lipoproteins.

1.1.3 AIMS OF CHAPTER ONE

From the published data currently available, it appears that sheep lipoproteins are not unlike those in man. However, although their densities and lipid composition are similar, the contribution of each of the fractions to circulating plasma lipids are quite different for the two species.

Metabolically stressed sheep have highly elevated levels of plasma TAG, which in turn is reflected in an accumulation of this lipid in the liver. Research into the hepatic secretion and subsequent metabolism of TAG is of fundamental importance if we are to elucidate the process of this accumulation. As lipoproteins are the mediators of all plasma lipids, their metabolism reflects the anabolic/catabolic destination of lipids.

The first part of this thesis establishes suitable methods for the isolation and separation of the major lipoprotein fractions in sheep plasma. The study reexamines the plasma lipoprotein profile (and their chemical constituents) in fed sheep for only the third time, and the

plasma lipoprotein profile from chronically stressed sheep, (which are hypertriacylglyceridaemic), for the first time.

1.2 METHODS AND MATERIALS

1.2.1 ANIMALS USED

Adult Merino sheep (Ovis Aries) were obtained from the flock maintained at the Waite Agricultural Research Institute, University of Adelaide. All animals were weight (35-60kg) and age (1-3 years) matched. Each sheep was housed individually and maintained on a diet of 900g lucerne chaff and 200g pellet supplements per day. (Charlicks,Adelaide, sheep pellets) fed at 9am each day. Water was available ad libitum

Fasted sheep were studied after 72h of complete food deprivation with water available ad-libitum.

Alloxan diabetes was induced by an intravenous injection of sterile alloxan (2,4,5,6-Tetraoxypyrimidine;5,6-Dioxyuracil mono-hydrate), 50mg/kg body weight, into the jugular vein one week prior to experimentation. A diabetic condition was confirmed by blood glucose concentration in excess of 10mM and all animals were hyperketoaemic.

1.2.1.1 COLLECTION AND PRESERVATION OF BLOOD PLASMA

Blood was drawn from the jugular vein into heparinized or lithium-EDTA (ethylenediaminetetra-acetic acid tetra-sodium salt) tubes, to prevent coagulation.

Plasma was collected immediately by centrifuging at 3,000 r.p.m. for 15min at 4°C. The plasma was held on ice and used immediately for lipoprotein isolation.

Ellman's reagent (5,5'-dithiobis(2 nitro-benzoic acid)) at a

concentration of 1.5 mM was used to inhibit lecithin cholesterol acyl transferase and phenylmethyl-sulphonyl fluoride (PMSF) (2mM) was used to inhibit proteolytic enzymes. Sodium azide and thimerosol (sodium ethylmercurothiosalicylate-mercury-((o-carboxyphenyl)-thio)-ethyl sodium salt) were also included as bacteriocides and EDTA (1mM) was added to inhibit autooxidation:

Solution A: 0.75g of Thimerosol and 1.3g Sodium azide dissolved in 100ml of water, and adjusted to pH8.

Solution B was freshly prepared and contained 0.595g of Ellmans reagent in 10 ml of 0.2M sodium bicarbonate solution.

Solution C: 0.35g of PMSF in 10 ml of 2-propanol.

To 10ml of plasma, 100ul of solutions A, B,and C were added. EDTA was added to give a concentration of 0.37mg/ml plasma.

1.2.2 DETERMINATION OF BLOOD GLUCOSE

Blood or plasma glucose concentration was determined essentially according to the method of Bergmeyer and Bernt (1974).

Solution A: 0.33M Perchloric acid solution.

Solution B: 1.38g Na_2HPO_4, 0.727g$NaH_2PO_4.2H_2O$, 0.05048g 2,2'-Azino-di-(3-ethylbenzthiazoline)-6-sulphate (ABTS), 933 International Units Glucose Oxidase and 150 International Units of Peroxidase in 100ml water.

0.1ml of blood or plasma was deproteinized by mixing with 1ml of solution A. After centrifuging at 1500 r.p.m. for 5min, 20ul of the clear supernatant was mixed thoroughly with 1ml of solution B and allowed to stand at room temperature for 30min. The absorbance was read at 420nm. Glucose standards ranging 0-10mm were included in each assay.

1.2.3 ADJUSTMENT OF PLASMA SOLVENT DENSITY

The amount of salt (NaCl or KBr), either solid, or in a concentrated solution, needed to bring about a specified adjustment of plasma density was determined according to the method of Radding and Steinberg (1960) as described by Mills et al. (1984).

Plasma was maintained at 20°C in a circulating water bath. Density was determined by weighing in duplicate at constant temperature.

1.2.4 SEPARATION AND PURIFICATION OF PLASMA LIPOPROTEINS

1.2.4.1 TIME COURSE STUDIES

2g of sudan black was added to each 20ml of plasma and mixed thoroughly for 2h at 4°C. The solution was then filtered through fibreglass discs.

Total plasma lipoproteins were isolated from plasma essentially according to the method of Rudel et al (1974). The solvent density of sheep plasma was raised to 1.225g/ml by the addition of solid potassium bromide. 8ml of stained plasma solution was then placed in SW41 (12ml) nitrocellulose, ultraclear or polyallomer tubes and carefully overlayered with 4ml of a buffered salt solution of density 1.225g/ml using a peristaltic pump, at a flow rate of approximately 0.5ml/min. The buffered solution was prepared by the addition of solid KBr to a 1.006g/ml solution described by Scanu and Granda (1966) which contained 1.42g anhydrous Na_2PO_4, 7.27g NaCl, 0.1g EDTA made up to 1l, pH 7.6. The

tubes were centrifuged at 40,000 r.p.m.in a SW41 swinging bucket rotor at 20°C, for either 18h, 24h or 40h in a Beckman L5 65 ultracentrifuge. The centrifuge was stopped with the brake off.

By monitoring the profile of the sudan black stained lipoproteins, a measure of their rate of migration to the top of the tubes was determined.

Duplicate tubes from each time period were carefully removed and stabilized in a centrifuge tube brace. The top and bottom of each tube were pressed tight against inert silicone rubber septa. The sudan black stained lipoprotein profile was monitored by puncturing the base of each tube using a fine gauge needle and expelling the contents by volume displacement using a saturated KBr solution, at a peristaltic pump flow rate of 0.25ml/min. The contents were monitored continuously (Zeiss PMQII spectrophotometer) at 600nm using a flow through cell (volume 250ul). Prestained lipoproteins were discarded and not retained for any further analyses.

1.2.4.2 COLLECTION OF TOTAL PLASMA LIPOPROTEINS.

SW41 ultracentrifuge tubes were loaded with 8 ml of plasma (not stained) solution with the density adjusted to 1.225g/ml and overlayered with the 1.225g/ml buffered salt solution as described above. Tubes were centrifuged in an SW41 swinging bucket rotor for 40h at 20°C, at 40,000 r.p.m.. The centrifuge was stopped with the brake off, so as not to disturb the lipoprotein buffer interface and prevent mixing. Tubes were carefully removed from the rotor and the top 2ml containing the concentrated lipoproteins removed either with a tube slicer or via a

500ul glass syringe (S.G.E.). The top portion of the tube was washed several times in a small volume of 1.006g/ml buffer solution. The resulting lipoprotein concentrate will for the purposes of this study be referred to as the "d1.225g/ml lipoprotein concentrate".

1.2.4.3 ESTIMATION OF TOTAL PLASMA LIPOPROTEINS

Sheep plasma lipoproteins isolated by ultracentrifugation at density 1.225g/ml were placed in dialysis tubing (5mm), which were extensively prewashed in double distilled water, dried, and weighed.

Each dialysis bag contained 1.5ml of lipoprotein concentrate and was dialysed for 24h against 3 X 4 litres of double distilled water at 4°C. The bags were then suspended in air and dried at 60°C for 3h. The dialyses bags were reweighed and the amount of lipoproteins determined by difference in bag weight.

1.2.4.4 AGAROSE GEL FILTRATION

Aliquots of the d1.225g/ml lipoprotein concentrates ranging 2-25ml (20-200mg lipoprotein) were applied without further manipulation, to agarose columns (Bio Gel A-5m, 200-400 mesh, 2.5cm X 100cm), and eluted with either 0.1M NaCl, 0.2M potassium phosphate, 0.01%EDTA, ph7.4 or 0.15M NaCl, 0.1%EDTA, ph7.0 at a flow rate of approximately 30ml/h. The eluate was monitored at 280nm and collected in 2.5ml fractions. The contents of tubes containing individual lipoprotein peaks were pooled and concentrated ten fold by pressure dialysis through XM50 membranes (Amicon Corporation) in a stirred 10ml Amicon filtration cell. Magnetic

stirring was maintained at approximately 10r.p.m. and pressure was achieved by high purity nitrogen. Temperature was maintained at 4°C.

1.2.4.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Up to 5ml of d1.225g/ml lipoprotein concentrate was dialyzed against 3 X 1L of 0.25M tris-phosphate pH7.6 for 18h at 4°C, to remove halides.

Aliquots of the dialyzed d1.225g/ml lipoprotein concentrate were chromotographed on Toyo Soda high performance gel-filtration columns. The high performance liquid chromatography (HPLC) was carried out using an ETP Kortec liquid chromatograph system equipped with a high pressure pump (model K25), an ETP Kortec (model K95) variable wavelength spectrophotometer and a Spectra Physics (model SP2470) integrating recorder, linked to a Pharmacia Frac-100, selective fraction collector. The chromatography columns were a Toyo Soda Company PWH guard column followed by a Toyo Soda G3000-SW (600mm) and G5000-PW (600mm) in series. HPLC was carried out at room temperature after applying up to 200ug of the lipoprotein concentrate mixture to the columns and eluting it in a buffer consisting of 0.25M tris-phosphate, pH 7.6 under an argon atmosphere.

Material eluted from the HPLC columns was monitored at 280nm and was collected in 0.5ml fractions. The contents of the tubes containing individual lipoprotein peaks were pooled for further analysis.

Lipoprotein molecular weight estimations were determined by interpolating the retention volume of each lipoprotein fraction with the retention volume of proteins of known molecular weight and diameter.

Protein standards included;

	Molecular weight (daltons)	Stokes radius (Å)
Blue dextran	2,000,000	-
Thyroglobulin	669,000	85.0
Ferritin	440,000	61.0
Catalase	232,000	52.2
Aldolase	158,000	48.1
Albumin	67,000	35.5
Ovalbumin	43,000	30.5
Chymotrypsinogen	25,000	20.9
Ribonuclease	13,700	16.4

1.2.4.6 SERIAL CENTRIFUGATION OF PLASMA LIPOPROTEINS

Five diluent solutions were required for the isolation of the major classes of lipoprotein. Each diluent contained EDTA (1mM), azide (2mM) and Thimerosol (0.2mM);

0.196 M NaCl solution, density=1.006g/ml at 20°C

0.844 M NaBr solution, density=1.063g/ml at 20°C

2.973 M NaBr solution, density=1.210g/ml at 20°C

4.778 M NaBr solution, density=1.320g/ml at 20°C

7.593 M NaBr solution, density=1.479g/ml at 20°C

The density of each solution was determined by weighing in duplicate at constant temperature.

1.2.4.6.1 ISOLATION OF VLDL

8 ml of plasma was placed in SW41 tubes and carefully overlayered with 4ml of 1.006g/ml solution as previously described. The tubes were spun for 24h in an SW Beckman 41 rotor at 40,000 r.p.m. at 20°C. For tubes containing fed sheep plasma, 1ml of the top fraction was removed and for tubes containing plasma from alloxan diabetic wethers a 2ml fraction was taken. In addition, another 1ml of the salt solution underlying the concentrated lipoprotein fraction was also removed to ensure complete recovery. Samples were drawn using a 500ul glass syringe. The top of the tube was then sliced and washed twice with 500 ul of the 1.0063g/ml solution so as to remove any adhering lipoprotein.

Unless stated otherwise, the VLDL concentrate was washed by overlayering with 1.006g/ml solution and centrifuging under the same conditions as specified above.

1.2.4.6.2 ISOLATION OF LDL

The resulting infranatant after VLDL isolation was mixed thoroughly. It's solvent density was adjusted to 1.063g/ml by the addition of solution 1.320g/ml. 8 mls of the adjusted infranatant was overlayered with the salt solution of density 1.063g/ml and centrifuged for 24h, 40,000 r.p.m., 20°C. The top 2ml fraction was gently aspirated and the corresponding portion of tube washed twice with 500ul of the 1.063g/ml solution.

1.2.4.6.3 ISOLATION OF HDL

The LDL infranatant was mixed thoroughly and it's solvent density raised to 1.220g/ml by the addition of the salt solution with density 1.479g/ml. 8ml of the adjusted LDL infranatant was overlayed with the 1.220g/ml solution and centrifuged for 24h also at 40,000 r.p.m., 20°C. The HDL concentrate was removed in a 2ml fraction using the same procedure as that used for the isolation of VLDL and LDL. The top of the tube was washed twice with 500ul of the 1.220g/ml solution.

1.2.4.7 AGAROSE GEL ELECTROPHORESIS

Up to 5ul of plasma, d1.225g/ml lipoprotein concentrate or concentrated lipoprotein fractions were separated by electrophoresis on 1mm agarose plates (Corning-agarose gel electrophoresis system) in 0.05M Na-barbitone buffer pH8.6 (0.035% EDTA) at 90 volts for 35min. Plates were removed and dried thoroughly at 60°C. Lipoproteins were fixed in 2% acetic acid for 30min and the plates redried at 60°C. Lipoprotein bands were stained in a filtered 2% (w/v) sudan black solution (methanol:water (1:1)) for 2-3h. Destaining was achieved by briefly washing with a 50% ethanol solution.

1.2.5 EXTRACTION AND ANALYSIS OF LIPID COMPONENTS FROM PLASMA AND LIPOPROTEIN FRACTIONS

1.2.5.1 EXTRACTION

Lipids were extracted according to a modified method of Folch et al.

(1957);

To 1 ml of sample 15ml of chloroform:methanol (2:1 v/v) was added and mixed thoroughly. The solution was allowed to stand for 15min. One quarter of the total volume (4ml) of 0.03M HCl was added, mixed thoroughly and allowed to settle. The solutions were centrifuged at 15,000 r.p.m. in a Beckman JA 20 fixed angle rotor for 15min. The upper layer was removed by aspiration and one quarter of the volume of the lower phase (2.75ml) of water-methanol (1:1) added and the washing procedure repeated twice. The bottom phase contained the purified lipid. All lipid extracts were stored in chromic acid washed sealed glass ampoules at -15°C in the dark, under high purity nitrogen.

Prior to lipid extraction, 125ul of a 0.1uCi/ml glycerol tri(1-^{14}C) oleate solution was added per 1ml of plasma, or lipoprotein concentrate. Corrections for the loss of the lipid components were made after determining the residual activitiy remaining in the lipid extracts, on the assumption that the efficiency of the extraction procedure for each of the different lipid components was uniform.

1.2.5.2 TRIACYLGLYCERIDE DETERMINATION

TAG was measured according to a modified method of Carlson and Wadstrom (1959);

To 1ml of lipid extract approximately 50mg of activated 'zeolite mix' was added to remove phospholipids (Fletcher 1968). The zeolite mix contained by weight 80% zeolite (hydrated alkali-aluminium silicate $Na_2O.Al_2O_3(SiO_2)x.(H_2O)y$, 8% Fullers earth (Al-Mg-SiO_2), 8% $Ca(OH)_2$ and 4% $CuSO_4.5H_2O$, and was activated by heating at 110°C for 1h. The samples

were mixed occassionally over a 1h period, after which they were centrifuged at 3,000 r.p.m for 5min. An 800ul aliquot was taken for TAG determination. The solvents containing the lipid extract were evaporated under high purity nitrogen. 750ul of alcoholic KOH (90% ethanol v/v) was added. The glass tubes were capped and saponified for 30min in a 60°C water bath. After cooling to room temperature, 750ul of 0.7M H_2SO_4 followed by 4.0ml of diethyl ether were added. This was thoroughly mixed and the phases allowed to separate. The top ether layer was aspirated, and any remaining film of ether evaporated with a gentle stream of high purity nitrogen. Duplicate 300ul aliquots were taken from the bottom phase. To each sample 100ul of 0.02M $NaIO_4$ was added and mixed thoroughly. After exactly 15min 100ul of 0.2M $NaAsO_2$ was added to remove excess periodate. After mixing, the initially colourless solution goes yellow, then colourless again. After a 10min interval 3.0ml of freshly prepared chromotropic acid (15mM solution in 22M H_2SO_4) was added and mixed thoroughly. Each tube was capped and colour developed in a 100°C water bath for 30min.

The optical density of each sample at 547nm was determined and the concentration of TAG calculated by reference to standards (0-500nm).

1.2.5.3 PHOSPHOLIPID DETERMINATION

Phospholipid concentration was determined according to a modified method of Fiske and Subbarow (Bottcher et al 1961);
Reagents: Ammonium molybdate 8.6%(w/v) in water was mixed 1:1 (v/v) with 28% (v/v) H_2SO_4. The reducing agent was made up of 2.5g $NaHSO_4$, 0.5g $Na_2(SO_4)$ and 0.042g 1-amino-2napthol-4sulphonic acid in 250ml of water.

The solution was allowed to stand in the dark for several hours, after which it was filtered into a dark bottle. This was stable for 1 month when refrigerated.

A 500ul sample of lipid extract was evaporated under a stream of high purity nitrogen. 200ul of perchloric acid was added, the tubes capped and placed in a sand bath maintained at 180-200°C and digested for a minimum period of 40min. After cooling to room temperature, 1.2ml of ammonium molybdate and 1.2ml of the reducing agent were added. The solution was mixed thoroughly, capped and heated in a boiling water bath for 10min.

After cooling, absorbance was measured at 830nm. The samples could be diluted with water without loss of proportionality.

1.2.5.4 CHOLESTEROL AND CHOLESTEROL ESTER DETERMINATION

Plasma and lipoprotein cholesterol and cholesterol-esters were determined by gas chromatography. 100ul of internal standard (stigmasterol 1mg/ml in chloroform) was placed in 10ml Kimble tubes and the solvent evaporated under a stream of high purity nitrogen. To each tube, 100ul of plasma or 200ul of lipoprotein lipid extract was added and in the latter case, the solvent evaporated. The samples were hydrolysed in 2.1ml of 2% ethanolic KOH (95% ethanol v/v) at 60°C for 30min. After cooling, 2ml hexane and 1ml water were added and the tubes shaken vigourously for 1min. The phases were allowed to separate, after which an aliquot of the hexane phase was taken. This was evaporated with nitrogen and 50ul of both chloroform and trifluoroacetic anhydride were added. The tubes were capped and heated at 50°C for 20min. The reagents

were evaporated and the residue redissolved in 100ul of chloroform. Total cholesterol was determined on a Hewlett-Packard HP5710 gas chromatograph equipped with a 2M X 2mm glass column, packed with 1% OV-101 on Gas Chrom Q. The injector and detector were at 250°C and the oven at 220°C isothermal with nitrogen gas carrier at 30ml/min. HP3388 integrator operated in an internal standard mode.

To calculate the free and esterified component of the total cholesterol, cholesterol esters were determined by repeating the above procedure with the omittion of the hydrolysis procedure. Free cholesterol was then calculated by difference of the total cholesterol (free plus esterified) and the esterified component.

1.2.6 LIPOPROTEIN PROTEIN DETERMINATION

Lipoprotein protein was determined according to the modified Lowry method (Hartree 1972).

Reagents: solution A contained 2g potassium-sodium tartate and 100g $Na_2(CO_3)$ sodium carbonate made up in 1L of 0.5M NaOH.

Solution B; 2g potassium-sodium tartarate, 1g $CuSO_4.5H_2O$ in 100ml 0.1M NaOH.

Solution C; 6.6% Folin-Ciocalteau phenol reagent.

To 250ul of sample 225ul of solution A was added, mixed thoroughly and heated at 50°C for 10min. After cooling to room temperature 25ul of solution B was added and allowed to stand at room temperature for at least 10min with occassional mixing. 750ul of solution C was then added and mixed immediately. After colour development at 50°C for 10min, absorbance was determined at 650nm. Standards ranging 0-50ug of bovine

serum albumin were included in each assay. Contaminating lipids which may have interfered with the absorbance were removed by washing the colour developed solution twice with hexane and chloroform. Standards were also washed with the appropriate solvent.

1.2.7 NON-ESTERIFIED FATTY ACID DETERMINATION

For each 400ul of plasma 50ul of internal standard (heptadecanoic acid (17:0) 500mg/L in hexane) and 4ml of Dole reagent (2-propanol:heptane:1M sulphuric acid (400:100:10 (v:v:v:))) were placed in Kimble tubes and mixed thoroughly. 5ml of heptane and 2ml of water were added and the tubes shaken for a further 1min. The phases were allowed to separate and the upper heptane layer transferred to another Kimble tube. The solvent was evaporated with a stream of high purity nitrogen and the fatty acid residue methylated for 5min with excess ethereal diazomethane (in ether). The solvent was evaporated and the crude fatty acid methyl esters dissolved in 200ul of hexane. Each sample was applied to a 10mm X 6mm biosil column (activated at 60°C) and washed with 2ml of hexane. The fatty acid methyl esters were eluted with 2ml of 10% ether in hexane. The solvent was evaporated and the residue dissolved in 20ul of chloroform for injection into the gas chromatograph. The sample was chromatographed on a 50M SCOT capillary column of FFAP at 220°C isothermal, helium carrier at 4ml/min with injector and detector at 250°C. Quantitation was by a HP3388 computing integrator using the internal standard method.

1.2.8 TRANSMISSION ELECTRON MICROSCOPY OF OVINE LIPOPROTEINS

Lipoprotein fractions eluted by HPLC were negatively stained with sodium phosphotungstic acid, according to Forte and Nichols (1972). Negatively stained samples were viewed on 200 mesh copper grids coated with Formvar and supported with carbon, using a J.E.O.L. Jem 100CX transmission electron microscope.

1.2.9 MATERIALS AND REAGENTS

Alloxan monohydrate was purchased from Koch-Light Ltd. England. Glucose Oxidase and Peroxidase was purchased from Sigma Chemicals Australia. Glycerol tri(1-^{14}C)oleate (56mCi/mmol) was purchased from Amersham Australia Pty. Ltd. All chemicals and reagents were analytical reagent grade or the best commercially available grade. H.P.L.C. protein molecular weight markers were purchased from Pharmacia Pty. Ltd., Uppsala, Sweden.

Heparin and Li.EDTA tubes were purchased from Surgical and Medical Supplies, Australia. Nitrocellulose, ultraclear and polyallomer ultracentrifuge tubes (14mm X 89mm) were purchased from Beckman Instruments Australia. Agarose gel electrophoresis kit was purchased from Corning I.C.I. Australia Pty. Ltd.. Bio Gel A-5m (200-400 mesh) was purchased from Bio Rad Australia Pty. Ltd.. Toyo Soda G3000-SW and G5000-PW columns were purchased from Beckman Australia Ltd.. ETP-Kortek HPLC system was purchased from ETP-Kortek Australia Pty. Ltd.. Diaflo XM50 Ultrafiltraton Membranes were purchased from Amicon Australia Ltd.

All glassware was chromic acid washed. All solvents were glass redistilled and stored under high purity nitrogen in the dark. Only glass double distilled water was used.

1.3 RESULTS

1.3.1 SHEEP PLASMA

Plasma from healthy fed sheep is clear and slighly pink in colour. Induction of alloxan diabetes elevated plasma lipids, which was visually evident by loss of translucence and the development of a white coloured plasma best described as 'milky' (figure 1.2). When plasma isolated from severely diabetic sheep (blood glucose concentration greater then 10mM) was cooled to below 4°C, the coagulation of fat into small globules could be seen.

The mean plasma density of sheep plasma at 20°C was 1.307g/ml. Alloxan diabetes or fasting for 72h did not alter the plasma density.

1.3.2 TIME COURSE STUDIES

The time required to isolate ovine plasma lipoproteins by ultracentrifugation at a solvent density of 1.225g/ml was determined by prestaining plasma lipoproteins with sudan black and monitoring their rate of migration to the top of the tubes spectrophotometrically. Figure 1.3 represents the absorbance profile of sudan black stained sheep lipoproteins spun for 18h, 24h and 40h respectively. Tubes spun for a period less than 40h exhibited tailing in their absorbance profile, meaning that the complete recovery of the sheep plasma lipoproteins could only be achieved after a 40h ultracentrifugation period, under the prescribed experimental conditions. In contrast, lipoproteins from cattle plasma could be recovered in 24h (Mamo and

Figure 1.2

Figure 1.2 represents plasma from three fed and three diabetic wethers. Tubes containing the pink transluscent samples are plasma from normal fed animals (F1, F2, F3). The hyperlipidaemia associated with severely diabetic animals was reflected in clouded plasma (D1, D2, D3). (The tubes were being overlayed with a buffer, in preparation for lipoprotein isolation by ultracentrifugation.)

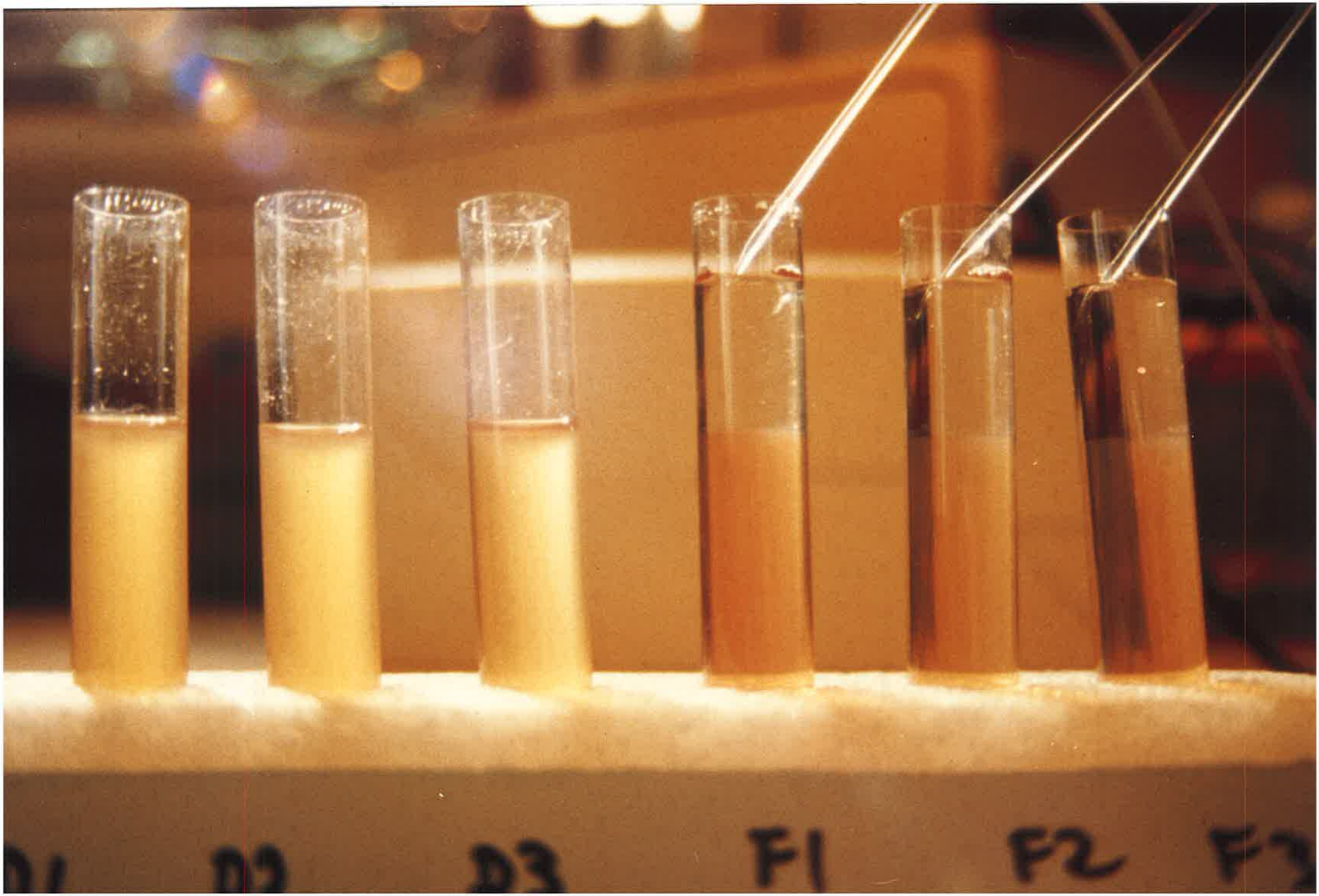
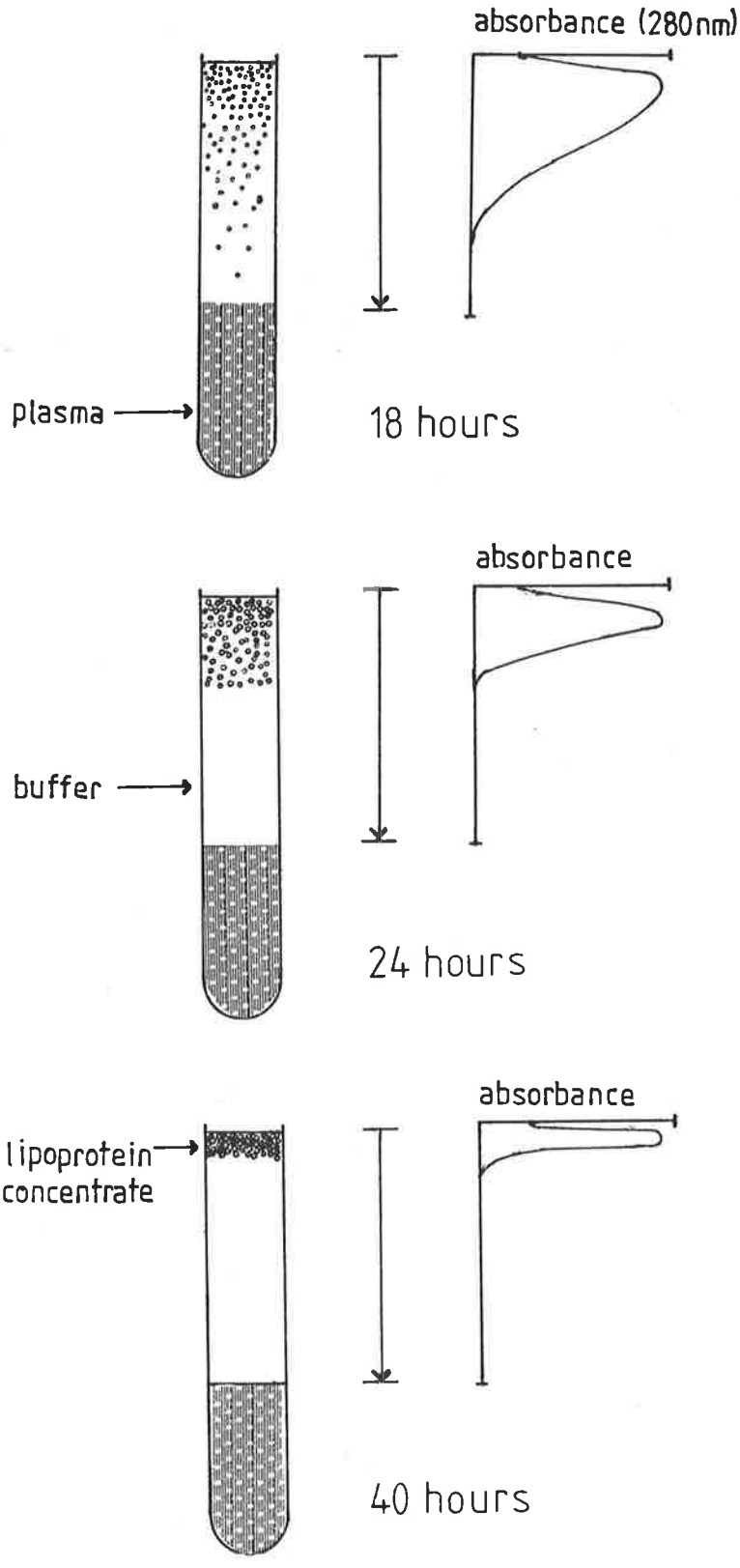

D3
F1
F2

Figure 1.3

Figure 1.3 represents the rate of migration of total sheep plasma lipoproteins when centrifuged under the conditions described in the text (1.2.4.1). Complete recovery of the lipoproteins could only be achieved after a 40h ultracentrifugation period.

fig. 1.3


absorbance (280nm)
plasma
18 hours
absorbance
buffer
24 hours
absorbance
lipoprotein concentrate
40 hours

Fishlock, unpublished observations).

1.3.3 SHEEP PLASMA LIPOPROTEIN CONCENTRATION

Sheep plasma lipoproteins isolated by ultracentrifugation at a solvent density of 1.225g/ml were dialysed, dried and weighed. The mean total plasma lipoprotein concentration for three fed and three diabetic sheep were 3.1mg/ml and 6.3mg/ml respectively.

1.3.4 AGAROSE GEL CHROMATOGRAPHY

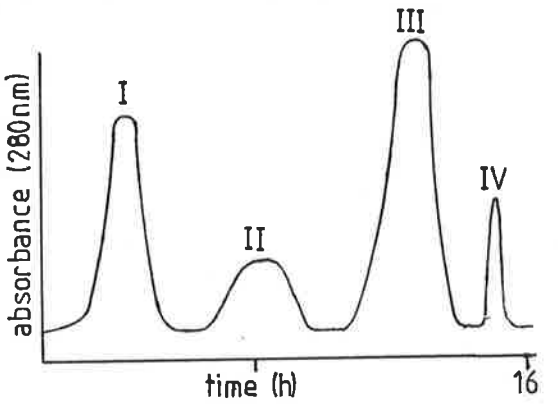
1.3.4.1 HUMAN PLASMA LIPOPROTEINS

Concentrated lipoprotein fractions isolated by ultracentrifugation at density 1.225g/ml were separated on the basis of size, through agarose gel columns. The typical elution profile of human plasma lipoproteins isolated from fasted subjects is shown in figure 1.4. Four lipoprotein classes were obtained, the largest lipoproteins being eluted first. Thus, peaks 1, 2, 3 and 4 were expected to contain VLDL, LDL, HDL and VHDL respectively. VHDL was not detected in all plasma samples. In addition, this component of the plasma lipoproteins could only be detected when Ellman's reagent and phenylmethylsulphonyl flouride were omitted from the isolation procedure, as these reagents were found to elute in the same region. The absorbance of each lipoprotein fraction (monitored at 280nm) does not represent an accurate reflection of their concentration as VLDL, LDL, HDL and VHDL have an increasingly greater protein content respectively. Nevertheless, qualitatively, HDL and VLDL

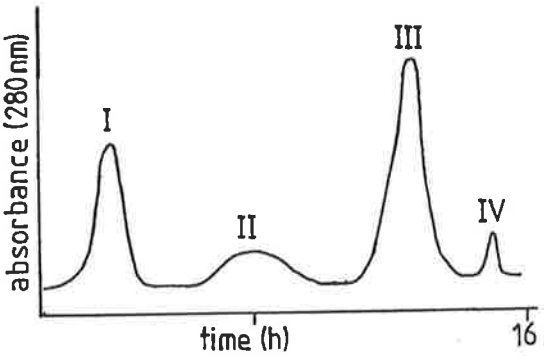
Figure 1.4

Figure 1.4 shows the representative elution profile of plasma lipoproteins from fasted humans, fed sheep, fasted sheep and diabetic sheep respectively, through agarose gel (5M) sizeing columns, as described in section 1.2.4.4. Peaks I, II, III and IV are very low density, low density, high density and very high density lipoproteins repectively.

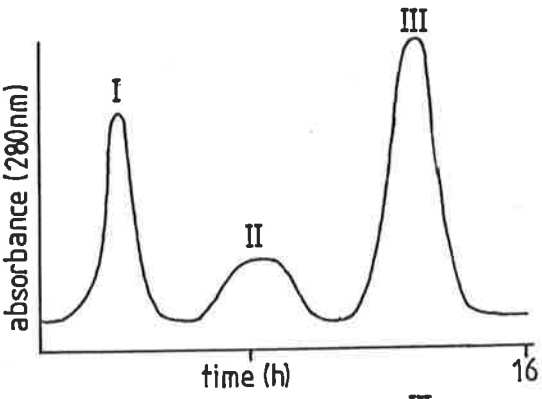
fig. 1.4



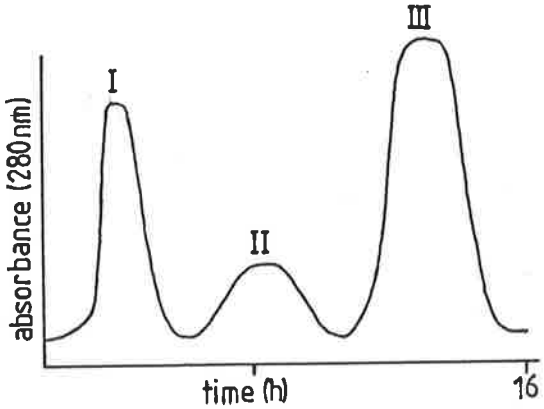
HUMAN
(fasted)



SHEEP
(fed)



SHEEP
(fasted)



SHEEP
(diabetic)

appeared to be the major plasma lipoprotein components in fasted human plasma. Peak II (LDL) may be more heterogeneous than the other lipoprotein fractions as it's absorbance profile was much broader. The total elution time for fasted human plasma lipoproteins was approximately 16h.

1.3.4.2 SHEEP PLASMA LIPOPROTEINS

Agarose gel filtration of fed sheep d1.225g/ml lipoprotein concentrate gave an elution profile not unlike that seen for humans (figure 1.4). The most striking feature was the significantly lower total lipoprotein absorbance profile (per unit of plasma) than that observed for humans. In addition VLDL and LDL did not appear to be as quantitatively significant as that for human plasma. HDL was the predominant lipoprotein. The VHDL fraction was not observed in all fed sheep. Similar elution of the d1.225g/ml lipoprotein concentrate from fasted sheep or alloxan diabetic wethers (figure 1.4) had a similar profile with the exception that no VHDL was present in either treatment. Each of the other fractions, and in particular HDL, appeared to be elevated in metabolically stressed sheep.

1.3.5 AGAROSE GEL ELECTROPHORESIS OF THE AGAROSE CHROMATOGRAPHY LIPOPROTEIN FRACTIONS

1.3.5.1 HUMAN FRACTIONS

Each of the tubes containing individual lipoprotein peaks were

pooled and concentrated using pressure dialysis. Samples of the concentrated lipoproteins were then separated by electrophoresis on agarose gels (figure 1.5). Fractions 1, 2 and 3 had migrations of pre-beta, beta and alpha respectively. This corresponds with VLDL, LDL, and HDL. It is apparent from the gels that each of the fractions were homogeneously distinct from the other lipoprotein classes. The VLDL fraction featured tailing towards the beta region. Similarly, LDL had a degree of streaking towards the pre-beta region. The HDL fraction had two distinct bands which overlapped. The slower and less abundant component had an electrophoretic mobility similar to that of the LDL fraction.

1.3.5.2 SHEEP FRACTIONS

Agarose gel electrophoretograms of plasma, d1.225g/ml lipoprotein concentrate and the respective lipoprotein fractions from fed, fasted and diabetic sheep are shown in figures 1.6a-1.6c respectively. Each of the bands corresponded to those observed in the equivalent human fractions. There were, however, some slight differences in electrophoretic mobility. The sheep VLDL fraction did not seem to be as homogeneous as that observed in human plasma, as indicated by the greater degree of tailing. Sheep LDL had a slightly greater mobility than that observed with the human LDL. Conversely, the major sheep HDL component had an electrophoretic mobility slightly less than the equivalent human HDL fraction.

The VLDL fraction from fed, fasted and diabetic sheep differed. Fasted sheep VLDL migrated more slowly than those either from diabetic

Figure 1.5

Figure 1.5 represents the agarose gel electrophoretic migration of human plasma and lipoprotein peaks I, II and III, derived from eluting human plasma lipoproteins through agarose (5M) sizeing columns.

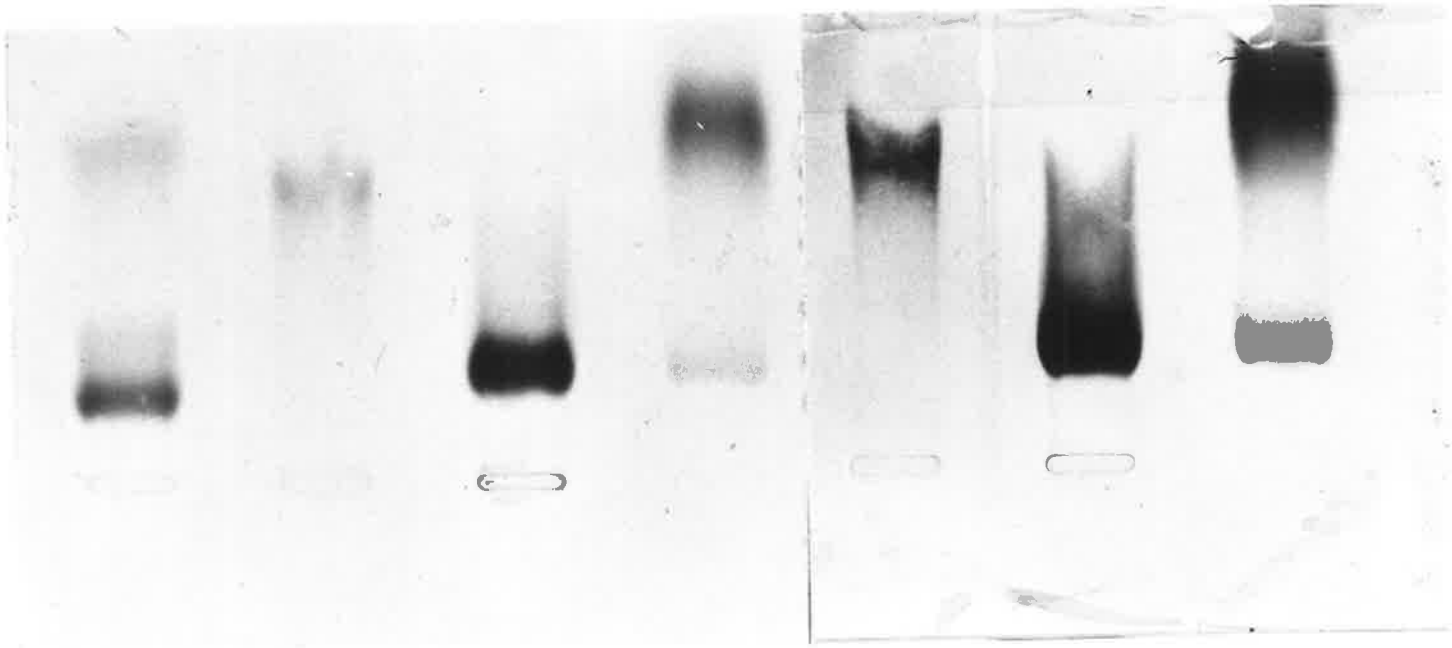
Lane 1 - plasma

Lanes 2 and 5 - agarose column peak I with pre-beta mobility (very low density lipoproteins)

Lanes 3 and 6 - agarose column peak II with beta mobility (low density lipoproteins)

Lanes 4 and 7 - agarose column peak III with beta-alpha mobility (high density lipoproteins)

Fig. 1.5



Lane 1 2 3 4 5 6 7

or fed sheep. The VLDL component from diabetic sheep appeared to consist of two overlapping components, both in the pre-beta region. The VHDL fraction observed only in fed sheep had two distinct components. The slower band migrated in the beta region, the faster band had an electrophoretic migration slightly greater than the HDL component, in the alpha region.

1.3.6 HIGH PERFORMANCE GEL FILTRATION

Up to 250ug of ovine lipoproteins could be separated into the major lipoprotein classes (VLDL, LDL, and HDL) by HPLC within 35min (at a flow rate of 1ml/min). A representative elution profile is shown in figure 1.7. It was immediately obvious that the spectrophotometric response was not proportional to that observed with the agarose gel eluted lipoproteins. VLDL and to a lesser degree LDL, gave a reduced response, whilst HDL gave an elevated response. VHDL was not detected by HPLC, a factor again complicated by the absorbance of the preservative reagents, in the region where VHDL would presumably be expected to elute. VLDL eluted at 23ml, LDL at 27.5ml and HDL at 32.5ml.

In order to ensure the HPLC peaks were in fact VLDL, LDL, and HDL, aliquots of sheep VLDL, LDL, and HDL fractions eluted by agarose 5M gel columns were applied to the HPLC. Each of the respective lipoprotein fractions gave rise to peaks which corresponded exactly with those derived from HPLC of d1.225g/ml lipoprotein concentrate. HPLC of the agarose 5M lipoprotein fractions indicated that each fraction was completely homogeneous from the others. An approximation of the molecular weight of each of the lipoproteins was achieved by

Figure 1.6

Figure 1.6 shows agarose gel electrophoretic migration of the lipoprotein fractions from (a) fed, (b) fasted and (c) diabetic sheep separated by agarose (5M) sizeing columns.

Lanes numbered 1 - sheep plasma

Lanes numbered 2 - density equal to 1.225g/ml ultracentrifuge lipoprotein concentrate.

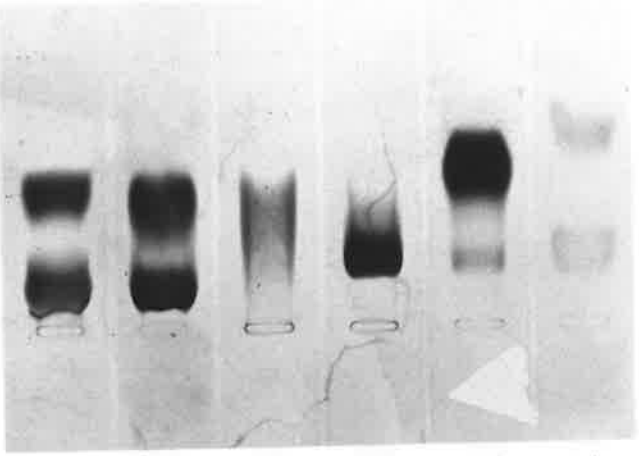
Lanes numbered 3 - agarose peak I (very low density lipoproteins)

Lanes numbered 4 - agarose peak II (low density lipoproteins)

Lanes numbered 5 - agarose peak III (high density lipoproteins)

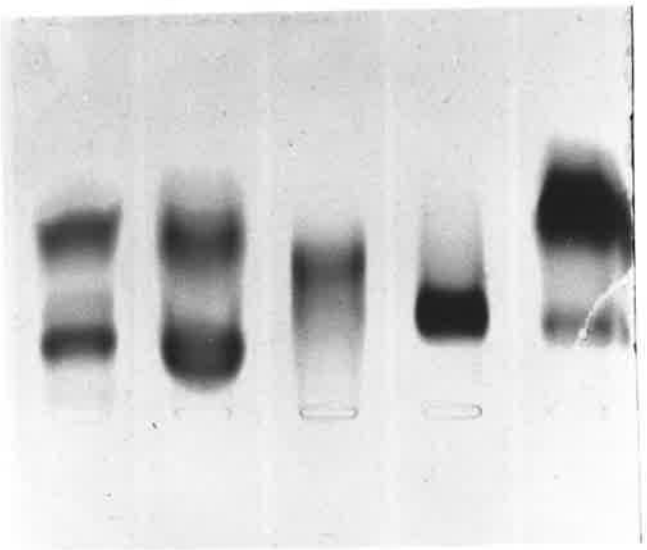
Lane numbered 6 - agarose peak IV (very high density lipoproteins)

Fig. 1.6



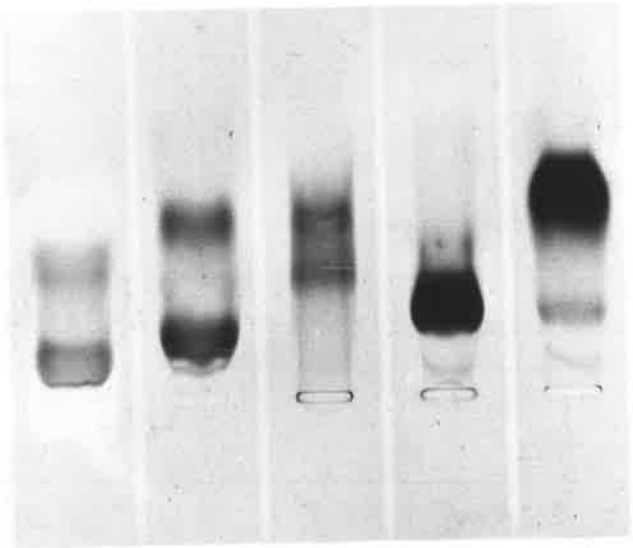
(a)

Lane 1 2 3 4 5 6



(b)

Lane 1 2 3 4 5



(c)

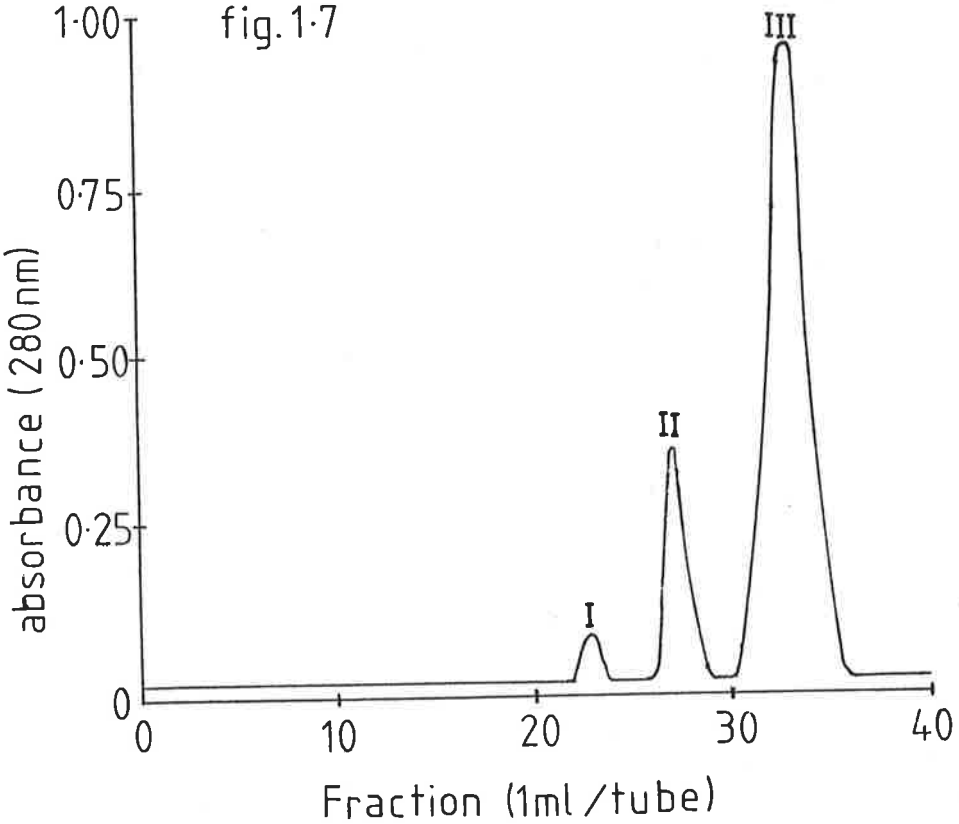
Lane 1 2 3 4 5

Figure 1.7

A representative elution profile of sheep plasma lipoproteins by high performance liquid chromatography is shown in figure 1.7. Peak 1 (Rv=23.0ml) is the very large very low density lipoproteins. Peak II (Rv=27.5ml) and Peak III (Rv=32.5ml) are the low density and high density lipoproteins respectively.

A representative elution profile of fed sheep

fig. 1·7


1·00
0·75
0·50
0·25
0
absorbance (280nm)
I
II
III
0
10
20
30
40
Fraction (1ml/tube)

interpolating the retention volumes of the lipoproteins, with the retention volumes of proteins with known molecular weights. Ovine VLDL had an estimated molecular weight in the order of 1,750,000 daltons. LDL and HDL eluted at volumes which correspond to molecular weights of approximately 800,000 and 350,000 daltons respectively.

1.3.7 SHEEP LIPOPROTEINS ISOLATED BY SERIAL ULTRACENTRIFUGATION

Serial centrifugation was also examined as a means of isolating ovine VLDL, LDL, and HDL.

The lipoprotein fractions isolated by this technique were eluted by HPLC and their electrophoretic mobility determined on agarose gels so as to verify their identity. Fractions 1 (fraction1< 1.006g/ml), 2 (1.006g/ml<fraction 2<1.063g/ml) and 3 (1.063g/ml<fraction 3<1.210g/ml) when eluted by HPLC gave rise to peaks with retention volumes of 23ml, 27.5ml and 32.5ml respectively. These corresponded with the elution of VLDL, LDL, and HDL as earlier determined.

HPLC of the serially isolated fractions indicated that there was little cross-contamination between groups. The VLDL fraction from diabetic sheep showed less than 5% LDL and HDL components (determined on an area percent absorbance of the total). Fraction 2 (LDL) from either fed or diabetic sheep had on average, less than 4% VLDL contamination and the HDL fraction from either fed or diabetic sheep, had a combined VLDL plus LDL content of approximately 3%.

Fractions 1, 2 and 3 yielded an expected electrophoretic mobility pattern of pre-beta, beta and alpha respectively. However, the migration of lipoproteins isolated from normal and alloxan diabetic animals by

serial centrifugation differed (figure 1.8a-1.8c). In all instances, VLDL, LDL and HDL from diabetic animals migrated further towards the anode end than the equivalent fraction from fed animals. This was not previously observed with the electrophoretic pattern of the lipoproteins isolated by gel filtration (figures 1.6a-1.6c). Other differences in the nature of migration of the sheep lipoprotein fractions isolated by the two techniques were observed. The serial centrifugation HDL fraction was considerably less homogeneous than the gel chromatographed fraction as indicated by the more diffuse banding. The migration of the slow and fast components of HDL from fed sheep were reduced in the serially isolated lipoproteins, as compared to the gel chromatographed fractions, though this was only observed in the slower component of HDL from diabetic sheep.

1.3.8 CHEMICAL CHARACTERIZATIONS OF SHEEP LIPOPROTEINS

1.3.8.1 FED SHEEP

The major chemical components of each of the classes of lipoproteins, namely VLDL, LDL and HDL for fed sheep is given in table 1.2. The VLDL were TAG rich with 51% of the molecular complex being made up of this lipid. The LDL were principally composed of cholesterol ester whilst HDL were found to be essentially of phospholipid composition. Phospholipids were found to be the next major component of VLDL and LDL respectively. Cholesterol, in all fractions was the smallest lipid component, approximating 5% of total lipoprotein lipids.

Figure 1.8

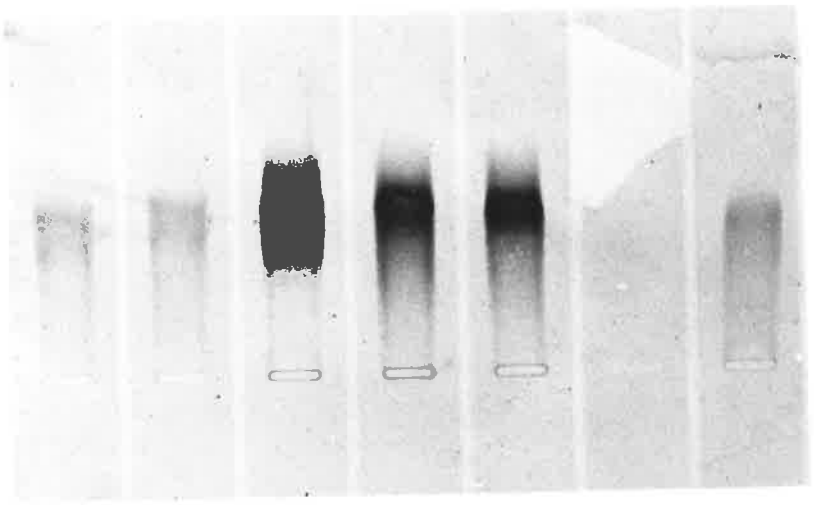
Figure 1.8 shows the electrophoretic migration of (a) very low density lipoproteins, (b) low density lipoproteins and (c) high density lipoproteins isolated by serial ultracentrifugation from fed and diabetic sheep.

(a) Lanes 1, 2 and 6 represent very low density lipoproteins from fed sheep. Lanes 3, 4 and 5 represent very low density lipoproteins from diabetic sheep.

(b) Lanes 1, 2 and 3 represent low density lipoproteins from fed sheep. Lanes 4, 5 and 6 represent low density lipoproteins from diabetic sheep.

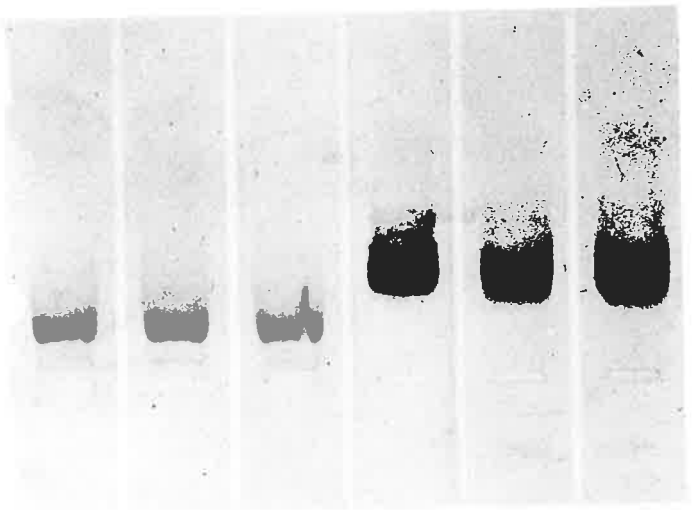
(c) Lanes 1,2 and 3 represent high density lipoproteins from fed sheep. Lanes 4, 5 and 6 represent high density lipoproteins from diabetic sheep.

Fig. 1.8



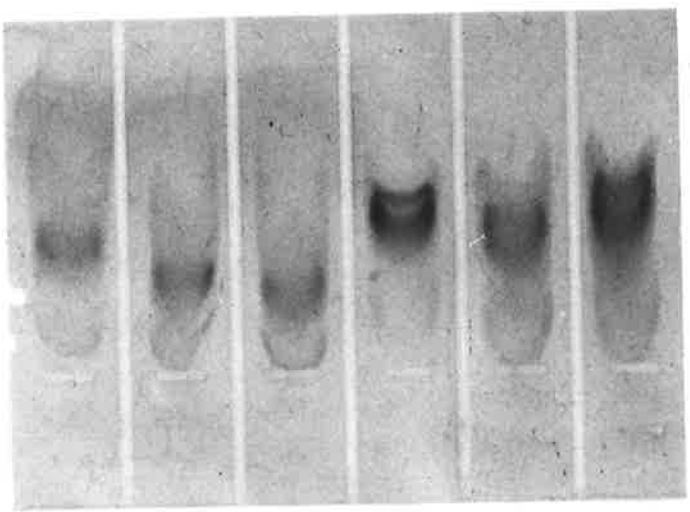
(a)

Lane 1 2 3 4 5 6



(b)

Lane 1 2 3 4 5 6



(c)

Lane 1 2 3 4 5 6

Table 1.2

Table 1.2 lists the major chemical components of each of the lipoprotein classes from fed and diabetic sheep. Very low density lipoproteins were rich in triacylglyceride, low density lipoproteins were principally composed of cholesterol ester and high density lipoproteins which were essentially of phospholipid composition. The lipid and protein content of the lipoproteins differed between treatments.

The results are for 3-6 animals in each treatment

LIPOPROTEIN AND SOURCE	TRIACYLGLYCERIDE ug/ml plasma	ug%	CHOLESTEROL-ESTER ug/ml plasma	ug%	CHOLESTEROL ug/ml plamsa	ug%	PHOSPHOLIPID ug/ml plasma	ug%	PROTEIN ug/ml plasma	ug%
VLDL-fed	176	51	57	17	13	4	83	24	21	6
VLDL-diabetic	2602	58	184	4	241	5	791	18	673	15
LDL-fed	139	15	465	50	59	6	221	24	73	8
LDL-diabetic	164	8	1246	62	116	6	415	21	138	7
HDL-fed	362	19	347	18	64	3	775	39	420	21
HDL-diabetic	322	10	681	21	126	4	1356	41	826	25

Table 1.2

1.3.8.2 DIABETIC SHEEP

The chemical profile of the lipoproteins from alloxan diabetic sheep differed considerably with respect to that from their fed counterparts (table 1.2). VLDL of diabetic sheep exhibited a greater TAG content and protein content, though a cholesterol ester content only 25% that of VLDL from fed animals. Conversely, the diabetic LDL fraction had a 50% lower TAG component and an elevated cholesterol ester component when compared to LDL from fed sheep. The protein content remained unchanged. Similarly HDL from diabetic sheep had a TAG component only half as much of that observed in the same fraction from normal animals. Cholesterol esters were elevated to a lesser degree. The protein component in the HDL fraction from diabetic animals (25%) may also have been slightly elevated with respect to the equivalent fraction from fed sheep (21%).

1.3.9 PLASMA LIPID PROFILE AND THE ROLE OF LIPOPROTEINS IN PLASMA LIPID TRANSPORT

1.3.9.1 FED SHEEP

Table 1.3 summarizes the plasma lipid profile and role of each of the lipoprotein classes as mediators of plama lipids in fed and diabetic sheep. In the former, phospholipids were the major plasma lipid (40%). TAG and cholesterol esters made up 20% and 35% of the plasma lipids respectively, with cholesterol the other major component (8%).

VLDL, LDL, and HDL were responsible for the transport of 11%, 34%

Table 1.3

Table 1.3 summarizes the plasma lipid profile and role of each of the major lipoproteins in transporting circulatory lipids in fed and diabetic sheep.

The results are for 3-6 animals in each treatment. The molecular weights used in the conversions are TAG,850;phospholipids,750;cholesterol ester,651; cholesterol,421.

	FED							DIABETIC						
	VLDL		LDL		HDL		Total	VLDL		LDL		HDL		Total
LIPID COMPONENT	nmol/ml plasma	nmol%	nmol/ml plasma	nmol%	nmol/ml plasma	nmol%	nmol/ml	nmol/ml plasma	nmol%	nmol/ml plasma	nmol%	nmol/ml plasma	nmol%	nmol/ml
Triacylglyceride	207	26	164	21	426	53	797	3063	84	193	5	379	11	3635
Cholesterol-ester	87	6	714	53	533	40	1334	282	9	1914	59	1046	32	3242
Cholesterol	30	9	140	42	153	46	323	573	50	277	24	299	26	1149
Phospholipid	111	8	295	20	1033	72	1439	1054	31	554	16	1807	53	3415
Total	435		1313		2145		3893	4972		2983		3531		10877

Table 1.3

and 56% of total plasma lipids respectively in fed sheep. As a result, HDL also mediated the greater proportion of each of the lipid components. 53% of plasma TAG was in the HDL fraction, though VLDL was also a significant contributor with 26% of total TAG.

1.3.9.2 DIABETIC SHEEP

Alloxan diabetic wethers with a blood glucose concentration greater than 10mM were found to be most severely hyperlipidaemic (table 1.3). There was a 137% increase in phospholipids, 356% increase in TAG, 146% increase in cholesterol esters and 256% increase in cholesterol. This was reflected in elevated plasma concentrations of HDL (65%), LDL (124%) and of most significance VLDL which was 12 fold higher than that observed in fed sheep.

The VLDL fraction in the alloxan diabetic wether became the predominant lipoprotein, mediating 50% of all plasma lipids and 89% of total plasma TAG. HDL was still responsible for 34% of plasma lipids and LDL 16%.

Recovery of each of the plasma lipid components, (as determined by the summation of the respective lipoprotein lipid components) was closely monitored. Recoveries for phopholipids, TAG, cholesterol esters and cholesterol were 92%, 93%, 84% and 93% respectively. These results are the mean of recoveries for three normal and three alloxan diabetic sheep.

1.3.10 TRANSMISSION ELECTRON MICROSCOPY OF SHEEP LIPOPROTEINS

Aliquots of sheep VLDL, LDL and HDL from both fed and diabetic animals isolated by H.P.L.C. were viewed by transmission electron microscopy. A minimum of one hundred particles in each fraction were approximated for their respective diameter based on an internal marker of known diameter (3nm), within the viewing chamber. Each particle was grouped within a 5nm classification for HDL and a 10nm group for LDL and VLDL. Figure 1.9 shows the frequency of particle size in each of the lipoprotein fractions from fed and diabetic animals. Figures 1.10a-1.10e are representative micrographs of each of the major ovine lipoprotein fractions. VLDL appeared as grey electron-transparent particles. Under high magnification the finer structural details could be resolved. Qualitatively in terms of size VLDL was the same in both fed and diabetic animals (10-30nm), though quantitatively there was a greater frequency of smaller particles in the latter. Similarly, this trend was observed in the LDL component of fed and diabetic sheep plasma (10-30nm) and was particularly notable in the HDL fraction of fed (5-20nm) and diabetic (5nm) animals. The LDL particles from fed animals appeared as smooth sigmoidal particles, whereas the equivalent fraction from diabetic sheep exhibited a rough surface, though these particles were generally spherical. HDL from both fed and diabetic animals were the most homogeneous in terms of shape and appearance. Particles were spherical, but due to their small size, differences in the nature of the particles apart from size, was difficult to determine.

Figure 1.9

Figure 1.9 shows the size frequency of each of the major sheep plasma lipoprotein fractions between fed and diabetic animals, determined by transmission electron microscopy.

fig. 1.9

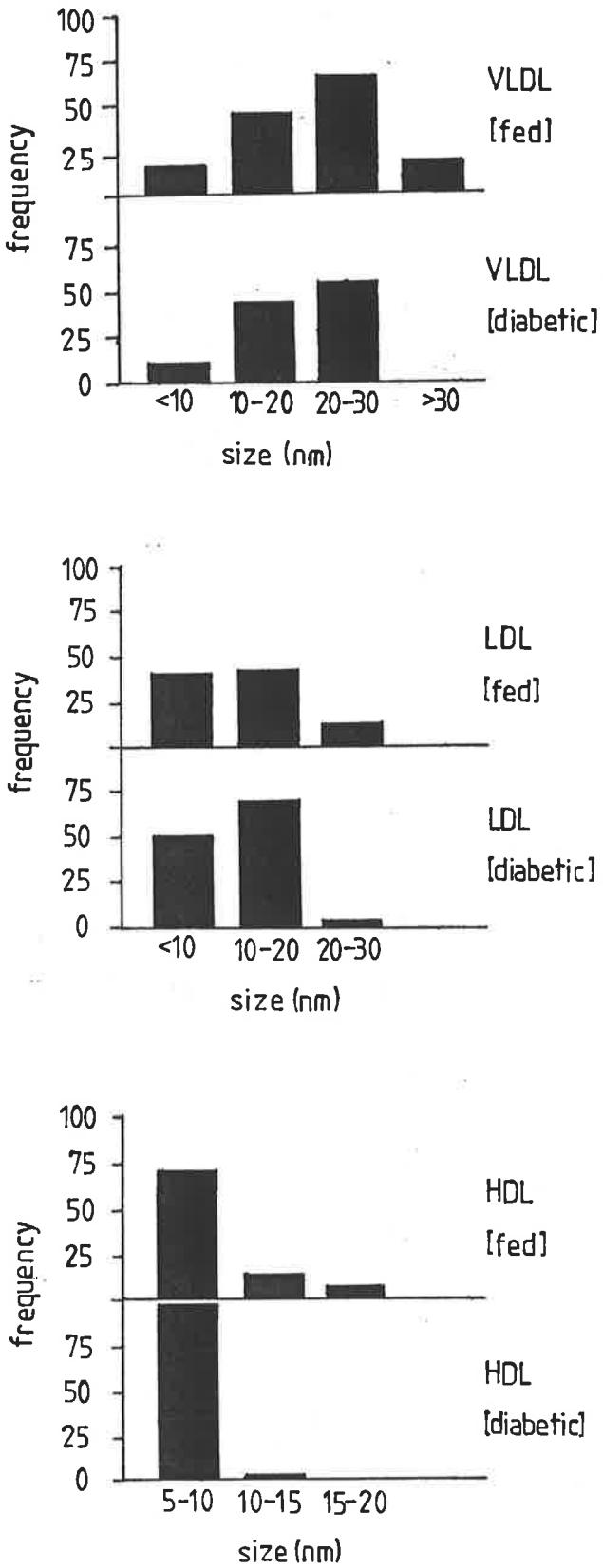

100
75
50
25
frequency
VLDL
[fed]
75
50
25
0
VLDL
[diabetic]
<10
10-20
20-30
>30
size (nm)
100
75
50
25
frequency
LDL
[fed]
75
50
25
0
LDL
[diabetic]
<10
10-20
20-30
size (nm)
100
75
50
25
frequency
HDL
[fed]
75
50
25
0
HDL
[diabetic]
5-10
10-15
15-20
size (nm)

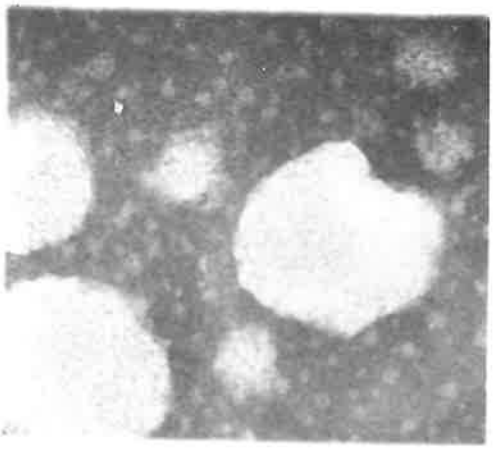
Figure 1.10a

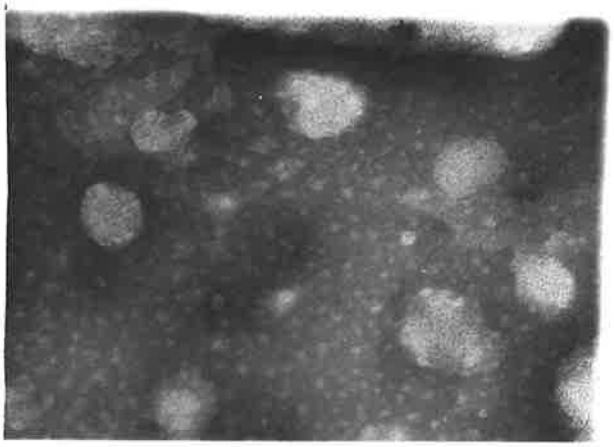
Figure 1.10a represents negatively stained very low density lipoproteins from fed sheep. Particles were approximately 25-30nm.

top right - approximate magnification 160,000

top left - approximate magnification 250,000

bottom figure - approximate magnification 10 X 250,000





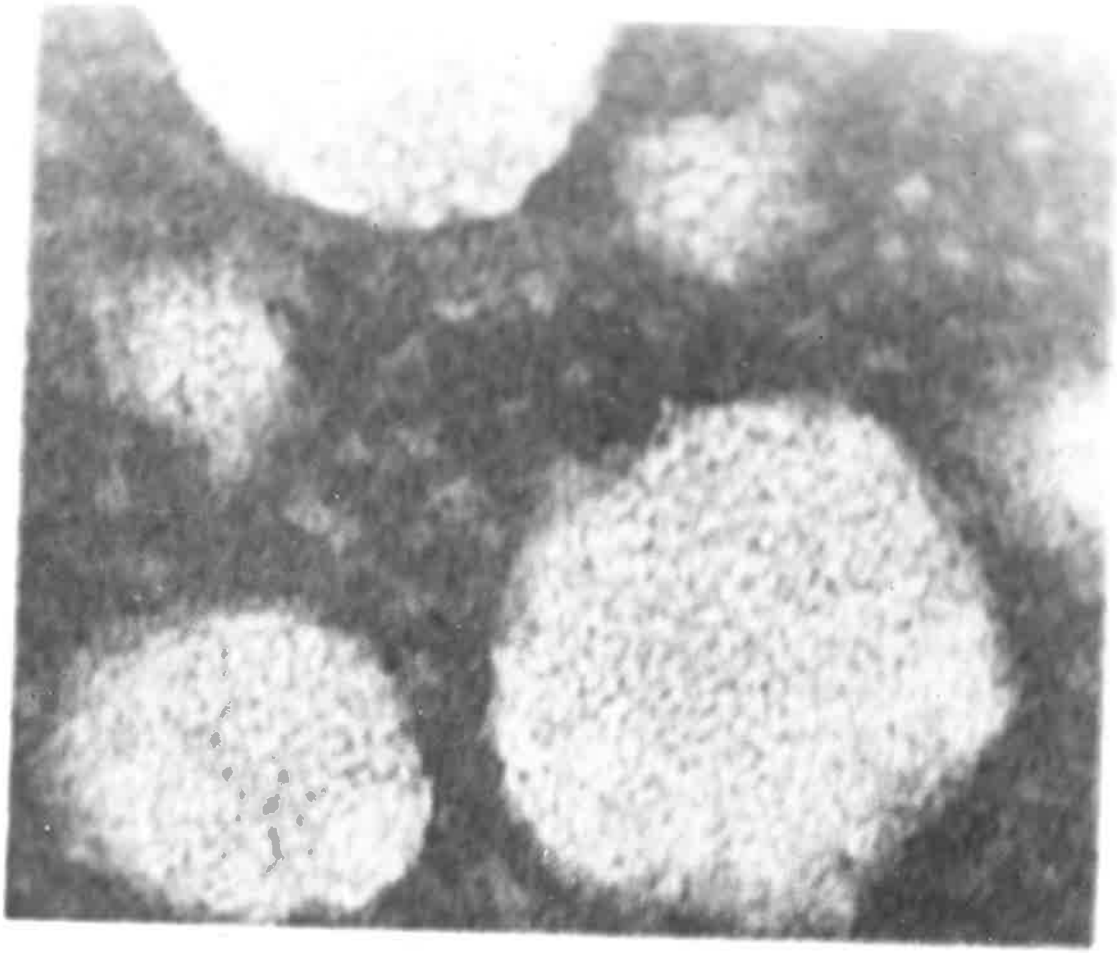


Figure 1.10b

Figure 1.10b represents negatively stained very low density lipoprotein particles from diabetic sheep at 5 X 66,000 times magnification. The majority of these particles were approximately 10nm.

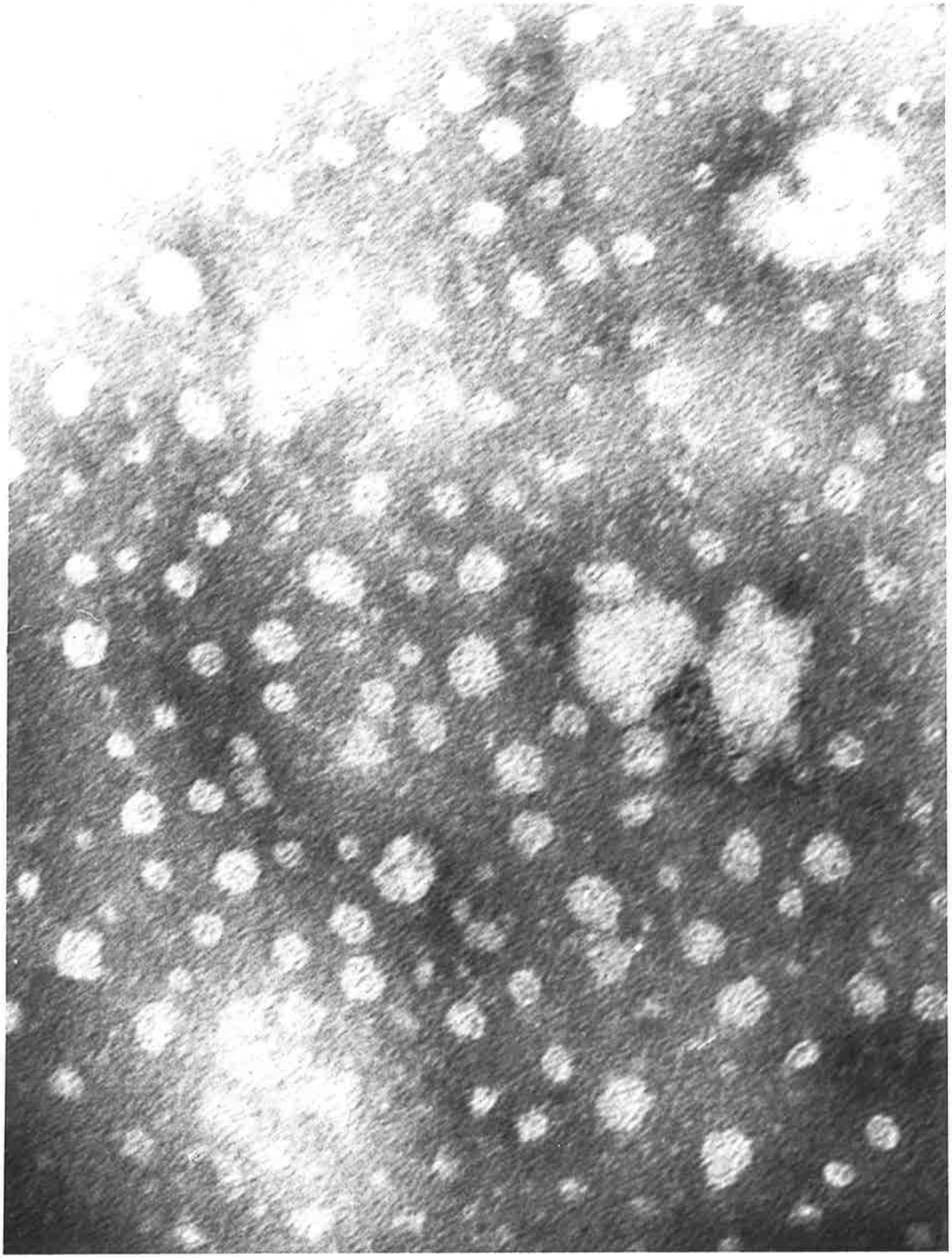
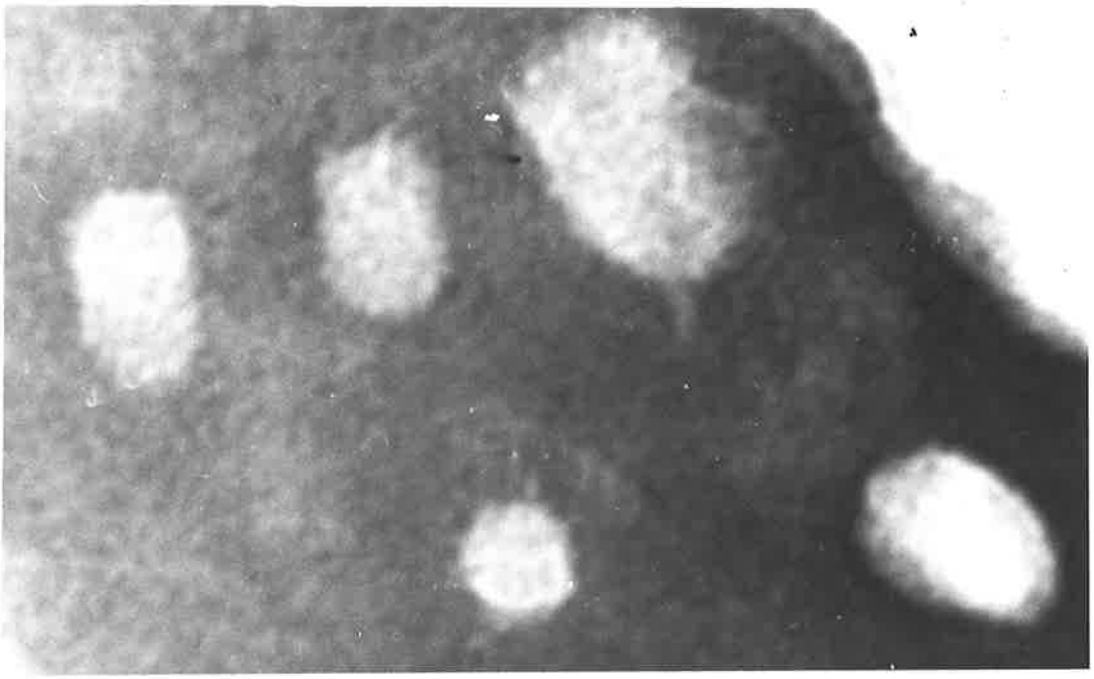


Figure 1.10c

Figure 1.10c shows the low density lipoproteins from both fed (top figure) and diabetic sheep (bottom figure). These particles from fed animals were approximately 10-20nm in size and symmetrical in shape. The low density lipoproteins from diabetic sheep had an approximate diameter of 10nm, but in contrast, their surface seemed less ordered.

top figure - approximate magnification 10 X 250,000.

bottom figure - approximate magnification 2 X 160,000



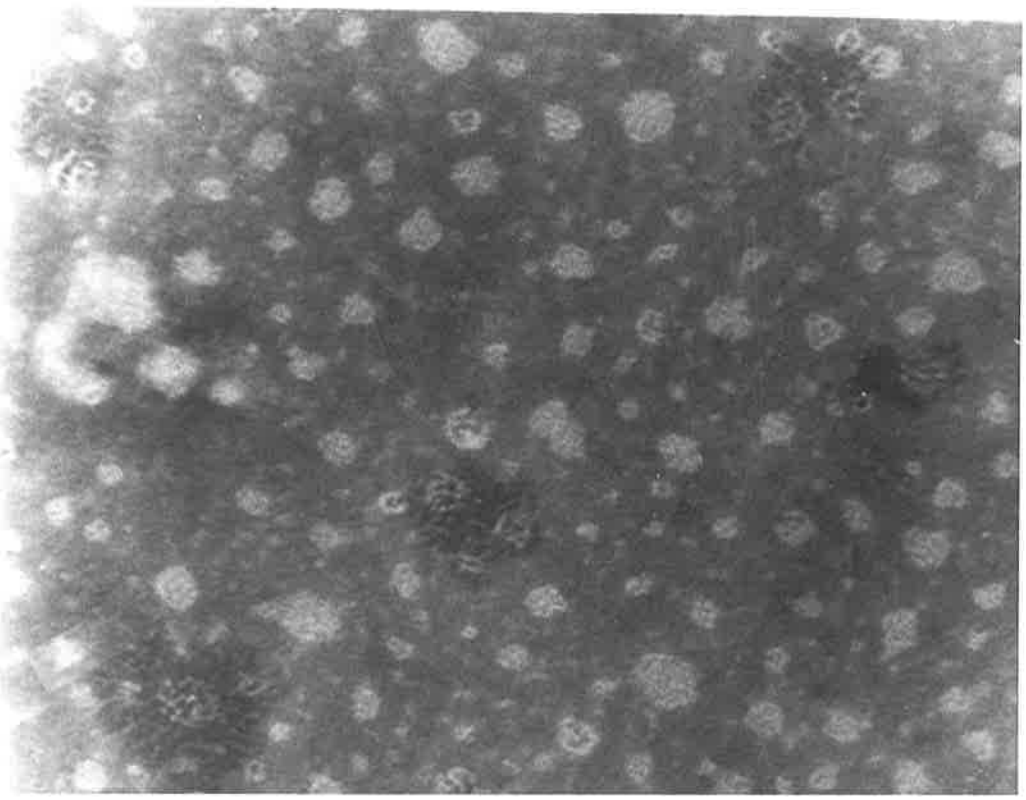


Figure 1.10d

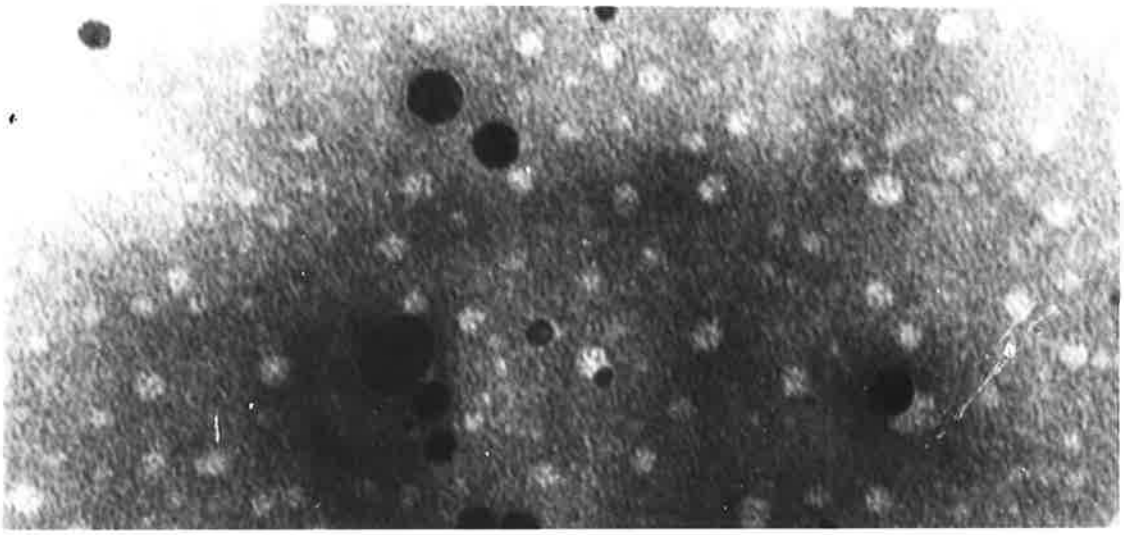
Figure 1.10d represents negatively stained high density lipoproteins from fed sheep at differing magnification. These particles were very small with an approximate diameter of 5-10nm.

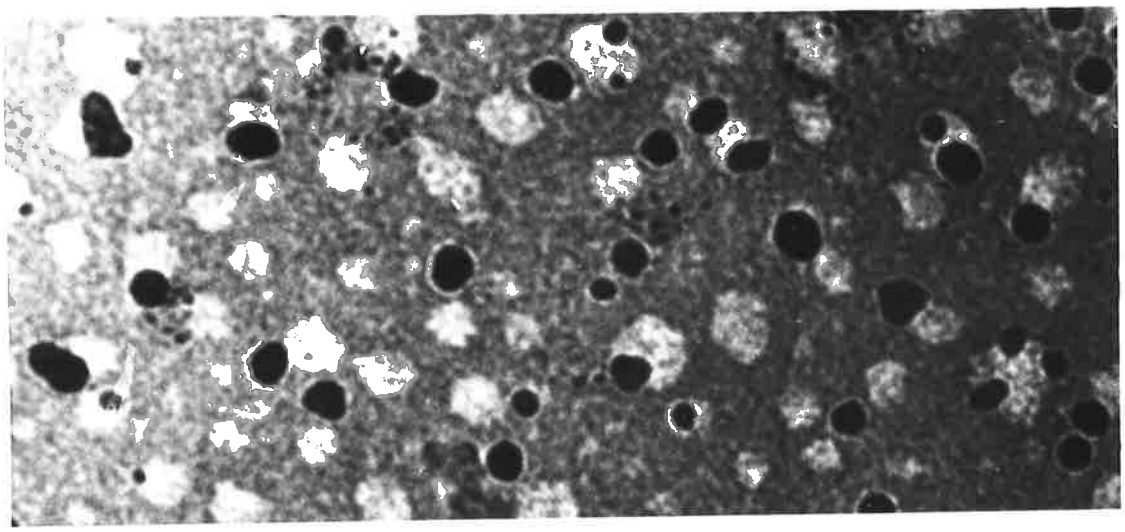
top figure - approximate magnification 4 X 160,000

middle figure - approximate magnification 10 X 100,000

bottom figure - approximate magnification 5 X 250,000

(black spots are phosphotungstic acid precipitate)





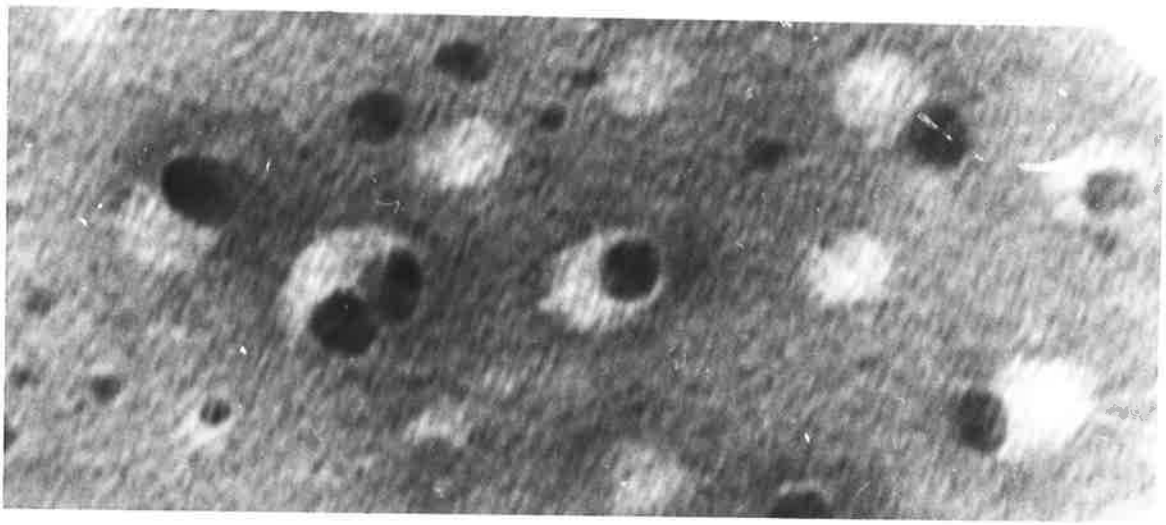


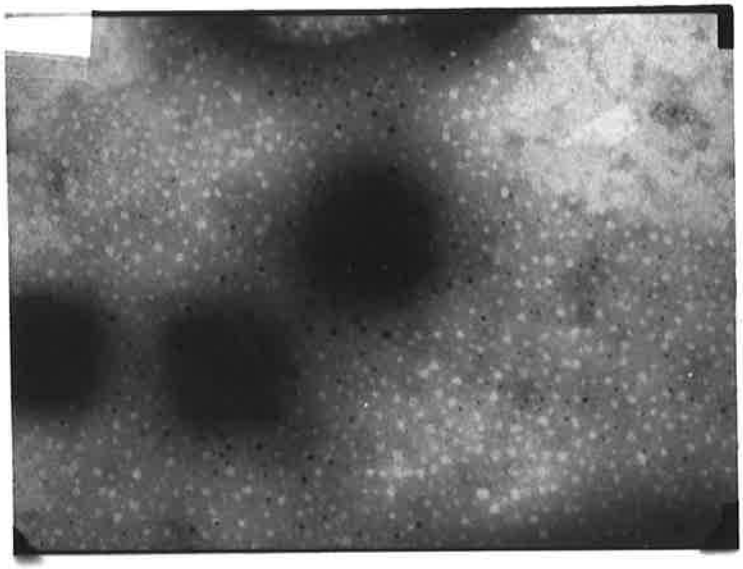
Figure 1.10e

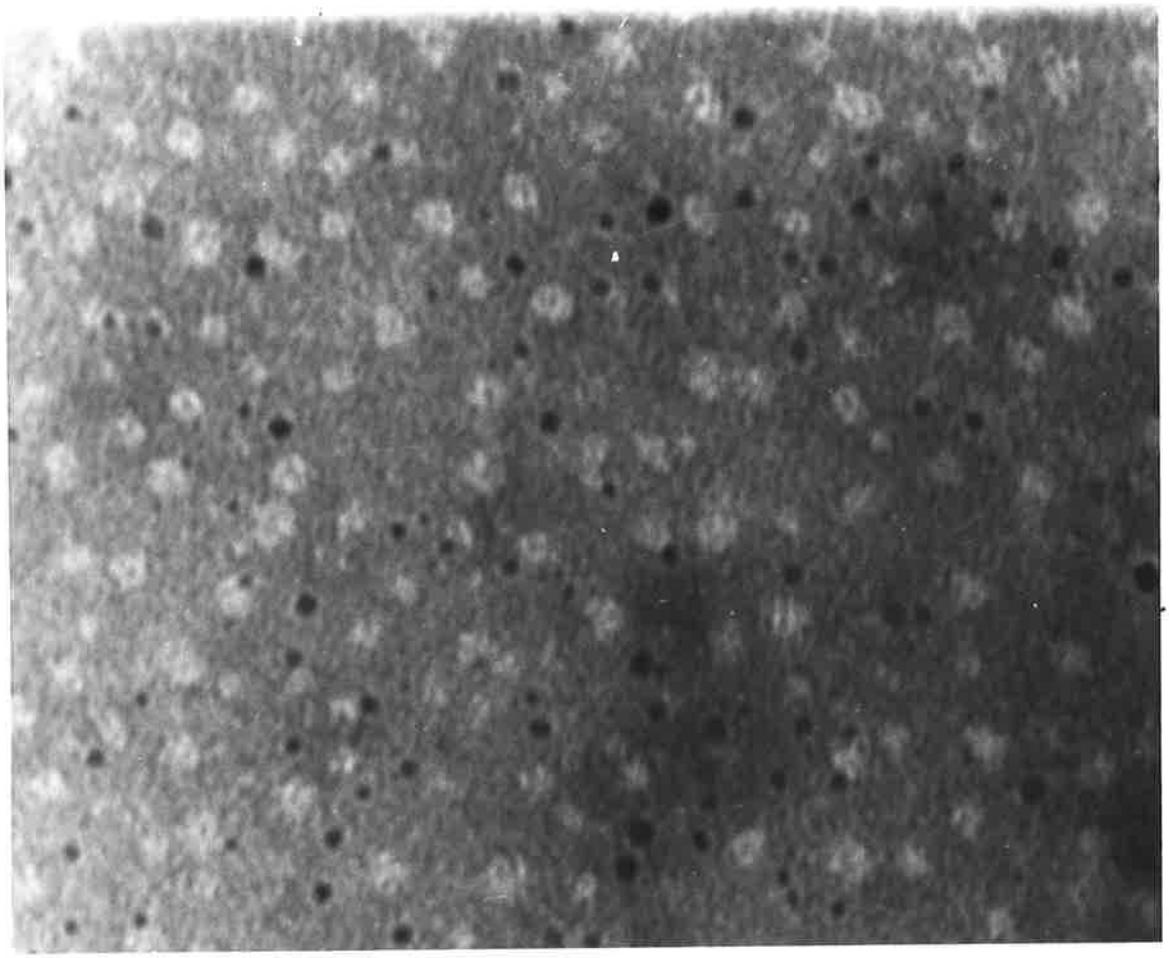
Figure 1.10e shows negatively stained high density lipoproteins from diabetic sheep. Particles were very symmetrical with an approximate diameter of only 5nm.

top figure - approximate magnification 130,000

bottom figure - approximate magnification 5 X 130,000

(black spots are phosphotungstic acid precipitate)





1.4 DISCUSSION

The total lipid composition of sheep plasma has been studied often (Garton and Duncan 1964, Leat 1967, Nelson 1969), but the contribution of the plasma lipoproteins to circulatory fats has received little attention. All published data, either on whole serum, plasma, or isolated lipoproteins, agree that the total lipid is very low when compared to monogastric omnivores such as the rat or human. Nelson (1973) has reported that low levels of circulatory TAG are reflected in the virtual absence of VLDL, with less than 0.2% of plasma lipids in this fraction. Similarly, Leat et al. (1976) attributed the contribution of VLDL mediating plasma lipids as being less than 5%. However, sheep metabolically stressed as a result of fasting or diabetes are not unlike the monogastric species in that they exhibit accute hyperlipidaemia. We have previously shown that the elevation of circulatory TAG was in part attributable to an increased hepatic release of this lipid (Mamo et al. 1983). Nonetheless TAG rapidly accumulate in the liver of these animals, suggesting that synthesis far outweighs secretion. In view of this, the first part of this study had several objectives. The precise nature of sheep plasma lipoproteins remained unclear and so initially, this thesis was aimed at determining whether the parameters of size, electrophoretic mobility and density by which human plasma lipoproteins are most commonly isolated, can be applied to sheep. Having established the appropriate methodologies, quantitative and qualitative analysis of these particles in fed animals and their role in the hypertriacylglyceridemia in diabetic sheep was determined.

Lipoproteins are prone to rapid physiochemical degradation and as

such require expeditious isolation, analysis and storage under strictly controlled conditions. Degeneration or modification of their composition and structure, results through the activity of several endogenous plasma enzymes. The best characterized of these are lecithin cholesterol acyl transferase (LCAT) and lipoprotein lipase. To minimize the effects of these enzymes, Ellman's reagent to inhibit LCAT, and phenylmethyl-sulphonyl fluoride, to inhibit proteolytic enzymes were added. Azide and thimerosol were also included as bacteriocides, the latter having the additional merit of inhibiting lipoprotein lipase (Lee 1976).

It has been known for many years that lipoproteins are susceptible to oxidative degradation (Ray et al. 1954), catalyzed by heavy metals (Schuh et al. 1978). Oxidation was minimized by the addition of disodium ethylenediamine tetraacetate to sequester heavy metal catalysts. In addition, isolated lipoprotein fractions were maintained at low temperatures in chromic acid washed glassware, under high purity nitrogen and in the dark. Analysis of lipoproteins for which precautions for their preservation have not been strictly adhered to, may be considered as a futile exercise.

Many of the physical principles by which lipoproteins are isolated may also bring about significant changes to their structure and composition. For example, the quantitative loss of HDL apoprotein subunits by prolonged ultracentrifugation has been well documented (Scanu and Granda 1966), but little is known of the effects of high pressure filtration on the lipoprotein molecules, when chromatographed by HPLC. Thus, the process of isolating plasma lipoproteins is dependent on the nature of an intended study. The techniques used, are dictated by the balance of quantitative and qualitative recovery of the particles.

Preliminary investigations in this laboratory in isolating bovine lipoproteins at a solvent density of 1.225g/ml, showed that a 24h ultracentrifugation time was sufficient to isolate all of the plasma lipoproteins. This was a substantial decrease compared with the 40h required to float the plasma lipoproteins from rabbit, rhesus monkey or humans under the same conditions (Rudel et al. 1974). In order to ascertain the minimum period of ultracentrifugation required to achieve full recovery of ovine plasma lipoproteins, the rate of migration of sudan black stained lipoproteins was monitored at selected time intervals. The results showed that to reduce ultracentrifugation time of the lipoproteins, could only be achieved at the expense of recovery.

Elution of the sheep 'd1.225 lipoprotein concentrate' through agarose (5M) sizeing columns gave rise to a profile, which qualitatively was similar to that observed for humans. The generally smaller absorbance profile of the ovine lipoproteins (per unit plasma), suggested that this species had lower levels of total particles, which was expected, in view of their low plasma lipid concentration. The mean concentration of plasma lipoproteins in adult human males is approximately 8.5mg/ml (Hatch and Lees 1968), somewhat higher than the ovine plasma concentration of 3.1mg/ml obtained in this study for fed sheep. The sheep 'd1.225 lipoprotein' elution profile also suggested that although HDL was the main ovine plasma lipoprotein, VLDL was also a major component of this spectrum (peak I, figure 1.4), particularly if one considers that due to the low protein content of these particles, absorbance at 280nm is low. The elution profile of the total sheep plasma lipoproteins from fasted and diabetic animals suggested that all of the major lipoprotein fractions had been elevated with respect to

their normal fed counterparts, although it would appear that HDL were the principal element of this increase.

Analysis of sheep plasma by agarose gel electrophoresis (figure 1.6) showed only two bands corresponding to beta-protein (LDL) and alpha-protein (HDL) stained for lipid. The pre-beta band, which was a characteristic of human plasma, was not detected in adult sheep plasma, due principally to the overlap of the beta and alpha components. The electrophoretic mobility of the individual sheep lipoprotein fractions (namely VLDL, LDL and HDL) has not been previously reported. However, electrophoretograms of peaks 1, 2 and 3 from agarose gel filtration and similarly that of fractions 1, 2 and 3 from serial ultracentrifugation yielded bands which stained for lipid in the pre-beta (VLDL), beta (LDL) and alpha (HDL) regions respectively. Their migration clearly indicated that VLDL, as well as the other major classes of lipoproteins, were indeed present in sheep plasma, and therefore in terms of mobility, like that found in human plasma.

The tailing of the VLDL fractions towards the beta region and the streaking of the LDL fraction towards the pre-beta region, observed in both human and ovine fractions (figures 1.5 and 1.6), reflects the association of the two lipoprotein classes. LDL is formed by the process of VLDL catabolism by lipoprotein lipase. Thus at any one time, plasma will contain a heterogeneous mixture of partially metabolized VLDL (or intermediate density lipoprotein). Ovine LDL had a slightly faster migration than human LDL, suggesting that sheep have a greater amount of this lipoprotein of smaller size. Like humans, sheep HDL had two distinct bands. The slower migrating fraction is HDL1, a subclass of the HDL fraction, which is arguably a low density lipoprotein, though by

tradition is described as an HDL. The difference in electrophoretic mobility of the faster HDL component between the two species, reiterates the heterogeneity usually attributed to this lipoprotein class.

The electrophoretic patterns of ovine lipoproteins isolated by either gel chromatography or serial ultracentrifugation, yielded lipoprotein fractions which differed slightly in their rate or nature of migration. These differences may simply be a reflection of the mode of plasma lipoprotein isolation, or in the stressed sheep, a response to elevated levels of plasma lipoproteins, or modification of the lipoprotein particles. Nonetheless, from the reduced mobility of VLDL from fasted sheep and the two overlapping components observed in diabetic VLDL isolated by gel filtration (figure 1.6), it appears that there may be an accumulation of a less dense LDL fraction, namely intermediate density lipoprotein. This would suggest that there could exist a defect in the catabolic processes of VLDL metabolism in fasted and alloxan diabetic sheep.

The migration of both components of HDL from fed sheep, was reduced in the serially isolated lipoproteins, as compared to the gel chromatographed fractions (figures 1.6 and 1.8). This was only observed in the slower component of HDL from diabetic sheep (figure 1.8c). In addition, the banding of the serially isolated HDL lipoproteins were diffuse in comparison with the chromatographed HDL lipoproteins. It is difficult to interpret these differences, however, the HDL fractions from serially ultracentrifuged plasma may have undergone changes in their physical characteristics, due to the prolonged ultracentrifugation time required to isolate VLDL, LDL and finally HDL.

A very high density lipoprotein fraction was found only on

occassions in fed wethers and ewes and was absent in the plasma of fasted or alloxan diabetic sheep. As a result, for the purpose of this study it was not considered a major ovine lipoprotein and thus not investigated further. Gel electrophoresis of VHDL yielded two distinct bands (figure 1.6a), the slower corresponding to beta-protein and the faster a little greater than the electrophoretic mobility of ovine HDL.

Agarose gel electrophoresis of diabetic sheep lipoproteins isolated by serial centrifugation migrated further towards the anode compared to native VLDL, LDL and HDL. This is a characteristic of proteins which have been glucosylated, in this instance as a result of the hyperglycaemia associated with alloxan diabetes in these animals.

HPLC has only recently been utilized as a tool for separating the major classes of lipoproteins. It holds particular promise in vastly reducing the time usually required to achieve separation and hence possibly particle degradation. The major ovine lipoproteins in the 'd1.225 lipoprotein concentrate' were successfully separated by HPLC within 35min, as compared to 16-18h by agarose gel filtration and 48-72h by serial centrifugation.

HPLC analysis of the lipoprotein fractions isolated by agarose gel filtration or serial ultracentrifugation detected a small degree (less than 5%) of cross-contamination in fractions 1, 2 and 3 from the latter. As with the lipoproteins isolated by agarose chromatography, HPLC lipoprotein fractions were homogeneously distinct, with no overlap of components. Another advantage of HPLC was the capacity to load, separate and detect small quantities of lipoproteins, which should prove particularly useful to the study of ruminant lipoproteins whose plasma lipoprotein content is low. However, the response of the HPLC detector

was not proportional to the concentration of plasma lipoproteins and in particular, due to the extremely low protein content of VLDL, the absorbance of this fraction was poor. In the advent of improved spectrophotometers, or by prestaining lipoproteins so that detection is not protein dependent (Busbee et al. 1981), this aspect should be overcome. In addition, it was extremely difficult to obtain enough individual lipoprotein material, without pooling equivalent fractions from several elutions, to either chemically characterize or make subject to electrophoresis, and thus its current application is somewhat limited. Recently, HPLC columns with the ability to quantitatively determine all major lipid components (when used in conjunction with light scattering detectors), have become commercially available.

An approximate molecular weight of each of the major ovine lipoproteins was determined by referring the HPLC elution volume of each of the lipoproteins, to the elution volume of proteins with known molecular weights. Sheep VLDL, LDL and HDL had molecular weights comparitive to that for the equivalent fractions in man, that is approximately 2 million, 800,000 thousand and 350,000 thousand daltons respectively.

Both gel filtration and serial ultracentrifugation yielded homogeneously distinct fractions of each of the major classes of ovine lipoproteins, with little or no overlap of components, as demonstrated by the respective electrophoretigrams and HPLC elution profiles. However, gel chromatographed lipoprotein fractions were very dilute and required concentrating prior to further analyses. Freeze drying, reverse dialysis and pressure filtration were investigated as means of concentrating lipoprotein samples. Although qualitative recovery

appeared unaffected, substantial losses of lipoprotein material was experienced in all instances. Serial centrifugation resulted in classes of lipoproteins in a concentrated form relative to plasma. Thus it was considered that ovine lipoproteins isolated by this means would be best characterized for their chemical components, as the need for concentrating the lipoproteins is removed and the sensitivity of detecting the lipid and protein components is increased.

The concentration of the plasma lipids in fed sheep in the present investigation were similar to those previously reported for sheep (Nelson 1973, Leat et al. 1976) (and during the course of this study Noble et al. 1984). Thus phospholipids were the principal component (43%) and were accompanied by smaller quantities of TAG (24%), cholesterol-esters (22%) and cholesterol (10%). However, in contrast with the previous investigations which reported that sheep plasma VLDL was virtually absent, a significantly greater proportion of total plasma lipids were mediated by VLDL, that is, 11% in fed Merino wethers. This agrees with recent work by Noble and Shand (1983) who reported that in pregnant ewes, VLDL accounted for 12% of total plasma lipids. In both of the earlier studies it is difficult to interpret the qualitative nature or 'purity' of the lipoprotein fractions, which could account for their very low values. In terms of plasma TAG, VLDL-TAG accounted for 26% of the total. It appears therefore that whilst the plasma VLDL concentration in fed sheep is still relatively small, the role of VLDL in mediating plasma lipids in previous investigations, has been greatly underestimated. The low circulatory levels of VLDL relative to monogastric omnivores may be due to rapid metabolism of these particles

by extrahepatic tissues coupled with low rates of hepatic release (Mamo et al. 1983), or conversely, may simply be a reflection of the nature of their diet.

A high proportion of the plasma lipids (53%) were associated with the HDL fraction, and although this is not unique to ruminants, it is in marked contrast to many other species, including man, where the LDL fractions account for a high proportion of total circulatory lipid (Eisenberg and Levy 1975).

The chemical composition of the lipoproteins isolated from fed sheep were similar to those reported for human lipoproteins. The VLDL were rich in TAG (50%), cholesterol esters were the major lipid component of LDL (50%), and HDL, the smallest of the lipoproteins, had a high phospholipid content (40%).

Alloxan diabetic sheep were found to be accutely hyperlipidaemic with an elevated plasma lipid concentration of greater than 300% (table 1.3). There was a two fold increase in plasma phospholipid and esterified cholesterol concentration, a 250% increase in circulatory cholesterol and a 350% increase in plasma TAG. Similarly, all lipoprotein fractions were increased, but none more so than VLDL which was elevated twelve fold. A good estimate of the total lipoprotein content of sheep plasma can be obtained by multiplying the total phospholipid concentration by (100-17)/n (VHDL phospholipid = 17% of total phospholipid; n = mean percentage of phospholipid in plasma lipoproteins). Thus, the calculated values of 2.9mg/ml and 5.7mg/ml lipoprotein for fed and diabetic sheep respectively, agrees well with that calculated by weighing total lipoprotein isolated by centrifugation at a solvent density of 1.225g/ml.

The chemical constituents of the lipoproteins from diabetic animals also differed from their normal fed counterparts (table 1.2). VLDL from diabetic animals contained a slightly greater percentage of TAG, significantly less cholesterol esters, a smaller percentage of phospholipids and a substantial elevation in protein. This suggests that the VLDL molecules from these animals, may in fact be smaller than those isolated from their fed counterparts, which was supported by the greater frequency of larger particles observed by transmission electron microscopy in the latter. This was most surprising, as in view of the elevated hepatic lipoprotein synthesis and secretion, one would have expected the VLDL particles to accommodate more lipid per unit particle, rather than less. The higher TAG content of VLDL from diabetic animals probably only reflected the lower cholesterol ester content of these particles. Alternatively, this may be a result of an increased packaging process in VLDL synthesis, or may reflect a defective plasma catabolism. In addition, plasma TAG may transfer between lipoproteins mediated by the enzyme plasma cholesterol ester (TAG) transfer protein (Rajaram and Barter 1980) and though the presence of this enzyme in sheep plasma has not been shown, it may be that its activity has increased in favour of this process. In contrast to VLDL, LDL from normal fed animals had a two fold greater TAG component, and relatively lower cholesterol-ester with respect to the same fraction from diabetic animals. It is difficult to determine if there has been any change in the size of the particles, because although in the diabetic animals more LDL were determined as being in the intermediate size of 10-20nm, fewer particles exceeding this were observed. Similarly, HDL-TAG from diabetic animals was only half of that observed in the equivalent fraction from fed sheep.

Cholesterol-ester and the lipoprotein surface components phospholipids and protein appeared to be elevated. Electron microscopy of HDL from diabetic sheep suggested that these particles on average, were smaller than those from fed animals. The smaller percentage TAG component of both diabetic LDL and HDL with respect to the same fractions from fed animals, suggests that metabolism of the TAG of these particles is not depressed, but rather, may be enhanced.

The sheep liver obviously has a substantial capacity to synthesize and secrete VLDL, and indeed, this laboratory has previously shown that severely diabetic sheep have elevated rates of hepatic TAG release, seven days after alloxan induction (Mamo et al. 1983). However, if synthesis of this lipid outweighs rates of release, or if the diabetic sheep is unable to maintain this elevated rate of synthesis and secretion, hepatic accumulation will result. Hepatic output may be limited by the rate of lipoprotein-apoprotein components, or simply be due to a finite capacity of the plasma to transport lipids. However, the greater protein content observed in diabetic sheep VLDL would suggest that apoprotein synthesis is not limiting.

The hypertriacylglyceridaemia associated with metabolically stressed sheep cannot be solely attributed to an increased hepatic output of this lipid. Plasma accumulation of lipid will only result if clearance is outweighed by rates of release. In view of this, the LDL fraction in diabetic sheep (which represents the end product of VLDL metabolism by endothelial lipases), was only increased 89% whereas VLDL concentration was elevated by 1200%, suggesting that the catabolism of these particles has decreased in these animals.

Impaired catabolism of VLDL by lipoprotein lipase may be due to

reduced production and/or activity of the enzyme (chapter two) or a physical or chemical modification of the substrate which could prevent binding and subsequent hydrolysis of the TAG. The nature of the impairment in the latter may be related to quantitative changes in the apolipoprotein composition. Bar-On et al. (1976), reported that VLDL of diabetic rats showed differences in their apoprotein C composition (which activate/inhibit lipoprotein lipase) compared to VLDL of non-diabetic animals. More recently, VLDL of diabetic origin were shown to be deficient in apoprotein E (Bar-On et al. 1984). This apoprotein is thought to play a role in the recognition of the VLDL particles or their remnants by peripheral tissues and liver (Innerarity and Mahley 1978, Shelburne et al. 1980 and Windler et al. 1980). In the latter study, VLDL isolated from the plasma of diabetic rats and reinjected into normal recipients had a significantly higher half life than the corresponding VLDL of non-diabetic rats.

VLDL catabolism may also be impaired as a result of structural alterations in the protein moiety brought about by increased glucosylation of the lipoproteins in diabetes (Gonen et al. 1981, Schleicher et al. 1981 and Witzum et al. 1982). Curtiss and Witzum (1985) have demonstrated the non-enzymatic post-translational glucosylation of the free amine of lysine residues of plasma lipoproteins. They found that the majority of glucosylated proteins in the lipoprotein fraction of density less than 1.225g/ml in hyperglycaemic subjects was in the TAG rich lipoproteins. In diabetic subjects, apoproteins AI, AII, B, CI and E were all glucosylated. A number of studies (Gonen et al. 1981, Sasaki and Cottam 1982a,b and Witzum et al. 1982) have shown that extensive glucosylation of LDL

apoprotein B (greater than 40% of lysine residues) totally abolishes the ability of LDL to be recognized by the LDL receptor.

There is evidence that the lysine residues of the various apoproteins are required for various functional activities, including receptor recognition (Weisgraber et al. 1978 and Mahley et al. 1979), enzyme interaction and activation (Musliner et al. 1979 and Vainio et al. 1983), lipid binding (Sparrow and Gotto 1982) and the regulation of cellular proliferation (Noel et al. 1981). Thus glucosylation of the various apoproteins could have a profound influence on the function of that apoprotein.

Yamamoto et al. (1986) reported a significant reduction in the rate of binding and degradation of glucosylated VLDL in human skin fibroblasts compared to native VLDL. They suggested that glucosylation of apoprotein E results in the impairment of the receptor binding capacity. This study also showed that glucosylated VLDL on agarose gel electrophoresis migrated further towards the anode, compared to native VLDL. Similarly, agarose gel electrophoresis of sheep lipoprotein fractions from diabetic animals appeared to be glucosylated (figures 1.8a-1.8c).

Finally, elevated plasma VLDL levels may also be due to defective hepatic VLDL synthesis in the first instance. Berry et al. (1981) showed that severe insulin deficiency increased hexosamine incorporation into VLDL in the perfused liver system.

The first part of this study has shown that normal fed sheep possess a lipoprotein compliment similar to that seen for other species, in that all of the major lipoprotein fractions, namely VLDL, LDL and HDL

are present. Previous investigations in this laboratory have also shown that sheep respond to metabolic stress (as a result of diabetes), by increasing the synthesis and release of lipoproteins. This has been extended here, in that the nature of the hyperlipidaemia associated with this increase has been determined and is reflected principally in the VLDL-TAG lipoprotein fraction. The plasma compartment has a finite capacity to transport TAG (and indeed all lipids) which is regulated not only by secretion of these particles, but also rates of clearance. The catabolism of VLDL is generally attributed to two enzymes, lipoprotein lipase and hepatic lipase and it may be that the activity of one or both of these enzymes has diminished in diabetic sheep. In view of this, the second part of this thesis was aimed at examining the catabolism of VLDL-TAG by these enzymes in fed, diabetic and fasted sheep.

CHAPTER 2

2.1.1 INTRODUCTION

In chapter one it was shown that the metabolic stress of diabetes produced a substantial rise in sheep plasma lipids. This increase was not uniform in all lipoproteins, but rather, there was a disproportionate elevation in VLDL-TAG, which is a reflection of the increased secretion of these particles from the liver (Mamo et al. 1983). However, the steady state concentration of plasma TAG, is also critically regulated by the lipolytic rate of the tissues which utilize TAG-fatty acids and therefore, this process has important implications, in terms of the hypertriacylglyceridaemia and hepatic accumulation of this lipid seen in these animals. In man and other monogastric species thus far studied, there are essentially two enzymes involved in the catabolism of circulating VLDL-TAG, namely lipoprotein lipase (LPL) and hepatic lipase (HL). LPL has been isolated in sheep and its biochemical characteristics determined. In summary, it was found to be not unlike that reported for other species (Clegg 1981b and Vernon 1981). However, there have only been few communications of investigations concerned with activity of this enzyme in sheep under stressed conditions, namely pregnancy and lactation (Vernon et al. 1980, 1981 and Smith and Walsh 1984). Ovine HL has not been previously reported and so the role of both enzymes in the metabolism of VLDL-TAG in metabolically stressed sheep are at present unknown.

The second part of this project had several objectives, which could be divided into essentially biochemical and animal production related

aspects of TAG metabolism. Initially, the first part of this study was aimed at establishing the presence of HL in sheep and thereafter, to determine changes in the rates of TAG hydrolase activity of both LPL and HL in fed, fasted and diabetic animals and correlate these with differences in the plasma lipoprotein lipid profile. During the course of these investigations, it became clear that such activities were regulated by both steroidal and genetic factors and so the second part of the results presented in this chapter are concerned with lipase activities in rams, castrates, ewes and genetically 'lean' and 'obese' sheep. The latter part of this study is discussed in view of local animal husbandary practices.

2.1.2 LIPOPROTEIN LIPASE AND HEPATIC LIPASE

LPL and HL are important regulators of plasma lipoprotein concentrations and therefore are implicated in related disease states in man such as atherogenesis. As such, both enzymes have enjoyed extensive investigation in monogastric animals. Some recent reviews of LPL and HL, which emphasize various aspects of their synthesis, activity, mode of regulation and metabolic significance, are listed (Robinson 1970, Scow et al. 1976, Smith et al. 1978, Tan 1978, Augustin and Greten 1979, Nilsson-Ehle et al. 1980, Kinnunen et al. 1983 and Breckenridge 1985). This overview briefly describes the major characteristics attributed to these enzymes, with particular emphasis on the catabolic processes of VLDL-TAG within the plasma compartment.

2.1.2.1 LIPOPROTEIN LIPASE

LPL is bound to the capillary endothelium (Pedersen et al. 1983) of those cells which utilize plasma TAG fatty acids for oxidation such as heart, lung and skeletal muscle (Twu et al. 1976 and Gal et al. 1982), or resynthesis of TAG for storage such as adipose tissue or mammary gland (Jansen et al. 1979, Clegg 1981a). In addition LPL is also a component of milk (Egelrud and Olivecrona 1972) and its presence in macrophages has also been demonstrated (Kinnunen 1981).

LPL, which was referred to as clearing factor lipase in older literature has been isolated and purified from a number of tissues and species, and is thought to have an approximate molecular weight of 34,000-73,000 (Smith et al. 1978 and Quinn et al. 1983). This enzyme is essentially a TAG hydrolase, showing highest rates of activity towards TAG in large lipoproteins (Fielding and Higgins 1974), with preference for the sn-1-position of the TAG moiety (Morley and Kuksis 1972 and Nilsson-Ehle et al. 1974). To a lesser degree, LPL also exhibits hydrolase activity towards diacylglycerides, monoacylglycerides and phospholipids (Quinn et al. 1983 and Kinnunen et al. 1983).

Different forms of LPL exist, which would seem to be a reflection of their site of isolation. For example, a high molecular weight LPL (69,250) appears to correspond to a low affinity enzyme from adipose tissue (Km=0.70mM TAG in rats) and a low molecular weight form (37,000), to a cardiac high affinity enzyme (Km=0.07mM TAG) (Fielding et al. 1974, 1977 and Fielding 1976). There are, however, immunological similarities between LPL from different tissues and species (Miller and Gotto 1982).

LPL requires the presence of apoprotein CII for expression of

hydrolase activity. This protein is a normal constituent of the TAG rich lipoprotein fractions. The precise mechanism of apoprotein CII activation remains to be defined, though recently Kinnunen et al. (1983) proposed a mechanism for this activation, namely, apoprotein CII accepts the fatty acyl groups from an enzyme intermediate, and transfers these to albumin. Maximum activation is achieved in a 1:1 apoprotein molar ratio with the enzyme (Chung and Scanu 1977 and Fielding 1978).

2.1.2.2 HEPATIC LIPASE

Hepatic lipase (HL) is similar to LPL, in that the enzyme is bound to the capillary endothelium by electrostatic interaction with mucopolysaccharides (Cheng et al. 1981). As the name suggests, the liver is thought to be the major source of this enzyme, although it is also found on the plasma membranes of steroidogenic organs which utilize lipoprotein cholesterol (Jansen et al. 1980a and Jansen and De Greef 1981). There are however, difficulties in determining the contribution of these other tissues (Boberg et al. 1964, La Rosa et al. 1972, Assmann et al. 1973 and Krauss et al. 1974). In the liver, the enzyme is thought to be synthesized by the parenchymal cells (Jansen et al. 1979) and after secretion, binds to the hepatic endothelial cells that possess receptors for this lipase. The enzyme has also been located in coated pits on the cell surface (Kinnunen and Virtanen 1980).

HL has been isolated and purified and is reported to have an apparent molecular weight of 53,000 (Kuusi et al. 1974 and Jensen and Bensadoun 1981). HL has also been reported to be immunologically distinct from LPL (Huttunen et al. 1975, Twu et al. 1984), although like

LPL, HL is a serine-histidine hydrolase (Kinnunen et al. 1983). HL does not require any known cofactor for activity, although apoprotein AII enhances it's lipolytic action (Jahn et al. 1983).

2.1.3 ROLE OF LIPOPROTEIN LIPASE AND HEPATIC LIPASE IN THE METABOLISM OF VLDL-TAG

The currently perceived physiological role of LPL and HL in the catabolism of VLDL-TAG is depicted in figure (2.1). Upon entry into the plasma, VLDL is converted to the mature particle by the acquisition of apoproteins from the large pool of circulating HDL. HDL are considered to act as a plasma reservoir for apoprotein CII (and other apoproteins), which transfers to newly secreted VLDL and chylomicron particles, but which are returned to HDL during lipolysis of the core TAG. (Apoprotein regulation of LPL and HL activity is discussed in chapter three). The TAG rich particles having attained a full compliment of apoproteins, bind with LPL at the plasma membrane of the various tissues containing this enzyme, whereby apoprotein stimulated activation results. The TAG core is progressively hydrolysed, resulting in the formation of smaller intermediate (IDL) particles and eventually LDL particles. During this process, apoproteins are lost (principally CII and CIII) or transferred to smaller HDL2 particles (Patsch et al. 1978, Eisenberg et al. 1979 and Tam et al. 1981). LPL will at different sites sequentially hydrolyze up to 70% of VLDL-TAG. The rate-limiting step in the removal of circulating plasma VLDL and chylomicron TAG, has been demonstrated to be the hydrolysis of this lipid by this enzyme (Garfinkel et al. 1967, Huttunen et al. 1976, Kompiang et al. 1976 and Bensadoun and Kompiang 1979).

Figure 2.1

Figure 2.1 depicts the plasma triacylglyceride catabolism of very low density lipoproteins by lipoprotein lipase and hepatic lipase. Triacylglyceride rich very low density lipoproteins attach to the endothelial surfaces containing lipoprotein lipase, whereby apoprotein CII stimulated lipolysis results. The particles are progressively hydrolysed to smaller intermediate density lipoproteins, which in turn, may be further hydrolysed via hepatic lipase. Loss of apoproteins during this process transfer to other plasma lipoproteins, principally the high density lipoproteins.

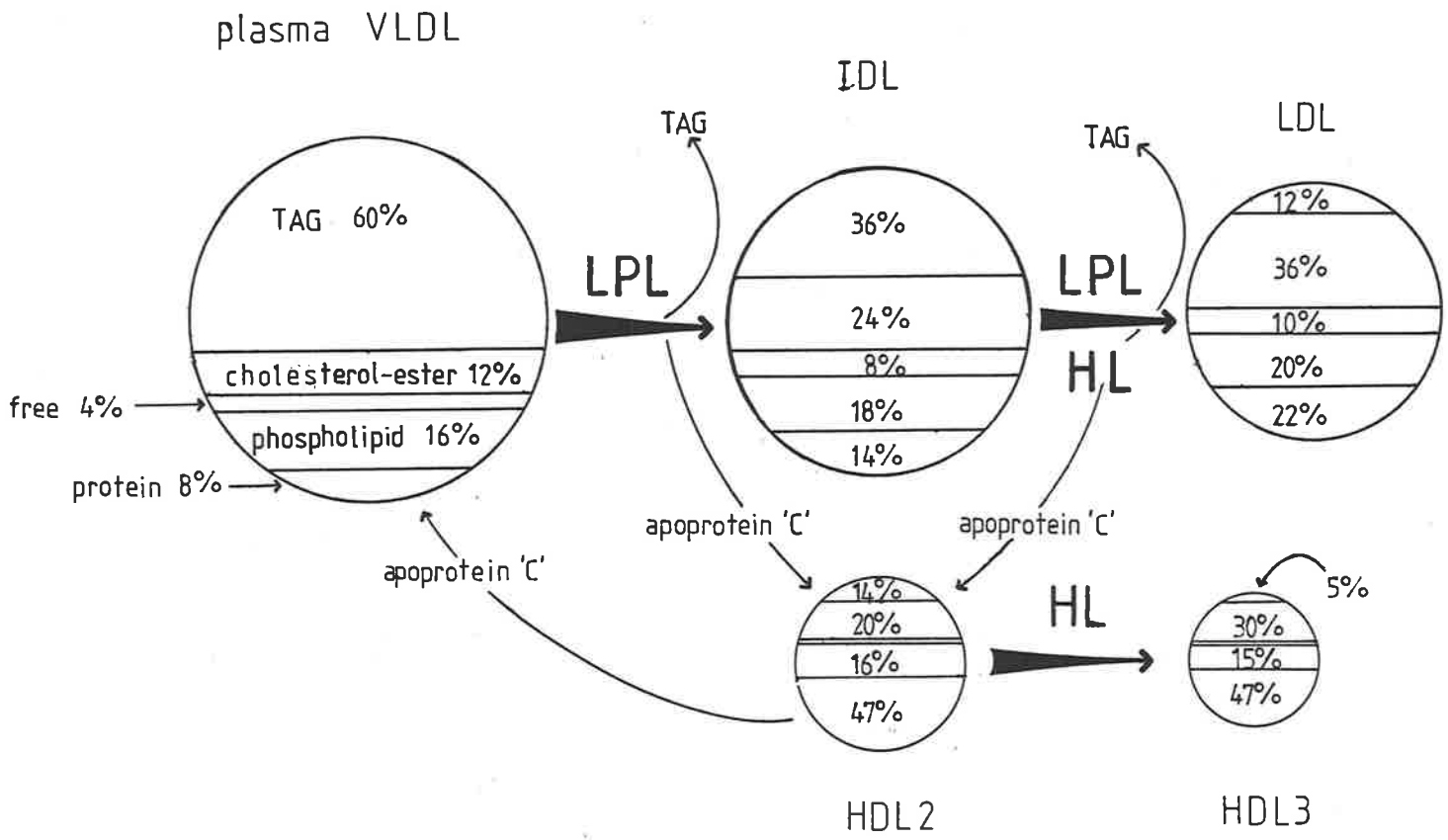

plasma VLDL
IDL
LDL
TAG
TAG
TAG 60%
LPL
LPL
36%
12%
36%
24%
10%
8%
HL
20%
18%
22%
14%
cholesterol-ester 12%
free 4%
phospholipid 16%
protein 8%
apoprotein 'C'
apoprotein 'C'
apoprotein 'C'
14%
20%
HL
5%
30%
16%
15%
47%
47%
HDL2
HDL3

fig. 2.1

Although HL exhibits a potent TAG and phospholipase activity of which the former is highest towards IDL, LDL and HDL (Musliner et al. 1979a), the precise physiological role of HL, is at present, unknown. The enzyme has been implicated in the clearance of remnant lipoproteins (LDL) and HDL by the liver, as administration of anti-HL antibodies results in a marked accumulation of cholesterol and phospholipid in LDL and HDL (Kuusi et al. 1979b). That is, HL is now believed to catalyse the further hydrolysis of IDL-TAG to produce LDL and by its combined TAG and phospholipase activities to convert HDL2 into HDL3 (Kuusi et al. 1979b, Jansen et al. 1980b, Reardon et al. 1982). A deficiency of this enzyme in humans leads to an accumulation of IDL, an LDL enhanced in TAG and a pronounced elevation in HDL2 (Breckenridge et al. 1982). HL can hydrolyze tri-, di- and monoacylglycerides and pholpholipids (La Rosa et al. 1972, Assman et al. 1973, Waite and Sisson 1973, 1974, Jansen and Hulsmann 1974, 1975 and Ehnholm et al. 1975b) and it is considered by many, that this enzyme has a distinct role in the metabolism of VLDL-TAG (Grosser et al. 1981, Murase and Itakura 1981 and Goldberg et al. 1982), whilst others believe this is not the case (Tikkanen et al. 1985 and Miller and Gotto 1982).

2.1.4 POSTHEPARIN PLASMA LIPOPROTEIN LIPASE AND HEPATIC LIPASE

Plasma under normal circumstances contains little or no TAG hydrolase activity of any kind. However, both LPL and HL are readily released from their respective tissue plasma membranes into the circulatory system, by intravenous administration of heparin. The interaction of this glycosaminoglycan with the enzymes, has suggested

that the binding of the enzymes to the endothelial cells may be due to the presence of this type of compound on the cell membrane (Dolphin 1985). Postheparin plasma lipase activities have received much investigation, as this provides a simple 'in-vivo-in-vitro' method of assaying TAG hydrolase. Both LPL and HL have been purified and characterized (Baginsky and Brown 1977 and Clegg 1979), which has enabled postheparin plasma lipolytic activity to be readily resolved into either of these components. This is very important, as their activities invariably do not change in parallel in different metabolic and pathological conditions (Krauss et al. 1974, Ehnholm et al. 1975a, Greten et al. 1976, 1977, Klose et al. 1977, Mordasine et al. 1977 and Nikkila et al. 1977). When measuring postheparin plasma LPL and HL activity, it is now usual to determine total hydrolase activity, then to inhibit either LPL or HL, and measure the activity of the remaining enzyme. The activity of the second enzyme is then determined by difference. Most frequently, either sodium chloride or protamine sulphate are used to inhibit LPL. These compounds quantitatively reverse the apoprotein CII dependent activation of LPL, but they do not inhibit any apoprotein CII independent activity, nor do they dissociate the enzyme-substrate complexes (Fielding and Fielding 1976). The NaCl effect is an anionic dependent action (Fielding and Fielding 1976). In addition, apoprotein CII is omitted from the assay. Alternatively, specific antibodies, or sodium dodecyl sulphate to inhibit HL can be used to resolve the two enzymes in postheparin plasma (Krauss et al. 1974, Huttunen et al. 1975 and Greten et al. 1976).

2.1.5 REGULATION OF LIPOPROTEIN LIPASE AND HEPATIC LIPASE

Investigations of LPL have shown that activity correlates with its site of isolation to the metabolic and nutritional state of the animal (Bezman et al. 1962, Garfinkel et al. 1967, Austin and Nestel 1968 and Cryer et al. 1976). This provides a means for distributing the TAG fatty acids to different tissues or organs, according to their metabolic requirements. It is not surprising therefore, that LPL in heart and LPL from adipose tissue are reciprocally related, depending on food intake (Cryer et al. 1976). This, in addition to the differences in apparent Km, would enable preferential saturation of the heart enzyme when animals are on a plane of nutrition too low to support fat depostion (Breckenridge 1985). Similarly, LPL activity in the mammary gland is increased during lactation (Scow et al. 1976 and Vernon and Flint 1982). The hormonal and nutritional mechanisms which differentially regulate activity in vivo, are however, poorly understood (Patten 1970, Faergeman and Havel 1975, Spooner et al. 1979, Ashby and Robinson 1980, Bordeaux et al. 1980 and Pedersen et al. 1981). Insulin and glucocorticoids have been shown to stimulate adipose tissue LPL activity in both man and animals by increasing the synthesis and secretion of the enzyme in vivo (Garfinkel et al. 1976, Vydelingum et al. 1983 and Speake et al. 1985).

HL has not been as widely investigated as LPL, presumably because its precise physiological significance has not been resolved. Nevertheless, as this enzyme is often associated with steroidogenic organs, it is not surprising that the respective hormones contribute to the regulation of this enzyme's activity. Administration of oestrogens or androgens have been reported to decrease and increase, respectively,

the activity of postheparin plasma HL (Ehnholm et al. 1975a and Applebaum et al. 1977).

2.2 METHODS AND MATERIALS

2.2.1 ANIMALS

Adult Merino wethers (35-60kg) and rams (60-85kg) were used. Each sheep was housed individually and maintained on a diet of lucerne chaff and pellets, with water available ad-libitum. Fasting animals were studied after 72h of food deprivation with water available ad-libitum. Alloxan diabetes was induced by an intravenous injection of sterile alloxan (50mg/kg) into the jugular vein one week prior to experimentation. A diabetic condition was confirmed by blood glucose concentration greater than 10mM. Wistar rats were weight and age matched and maintained on laboratory chow with water available ad libitum. Fasted rats were without food for 16h.

2.2.2 ACETONE POWDER PREPARATIONS OF LIVER AND ADIPOSE TISSUE

For tissue sampling and powder preparations, animals were slaughtered at approximately 0900h. Portions of liver and adipose tissue (omental and perirenal) were immediately frozen in liquid nitrogen and crushed. To 10g of tissue, 20ml of acetone was added and blended on ice for 1min using a Polytron no. PCU2. The resulting solution was homogenized in a glass-teflon Potter-Elvehjem grinder for 1min. Four such samples were pooled and delipidated by suction washing with 400ml acetone and 200ml diethyl ether, on Whatman number 42 filters. The resulting powders were dried under vacuum at room temperature. Powders were stored at -15°C.

2.2.3 ADIPOSE LIPOPROTEIN LIPASE AND HEPATIC LIPASE ACETONE POWDER ENZYME PREPARATIONS

3g of the respective acetone powder preparation was blended on ice with 60ml 5mM NH_4OH-NH_4Cl pH7.5 for 1min. The solution was stirred continuously for 3h at 4°C, after which samples were centrifuged at 15,000 r.p.m. for 15min. The resulting supernatant was collected and used as the source of TAG hydrolase immediately.

2.2.4 SHEEP AND RAT POSTHEPARIN PLASMA

Sheep post heparin blood samples for the TAG hydrolase (LPL and HL) assay were drawn from the jugular vein and transferred to heparinized tubes, 15min after injection of heparin (100U/kg) into the jugular vein. Rat postheparin blood samples were collected from ether anaesthetized rats through the abdominal aorta, 4min after heparin administration (50U/250g) through the common iliac vein. Plasma was separated by centrifuging at 4°C at 3000 r.p.m. for 13min and stored on ice. The postheparin plasma LPL and HL assay was done immediately.

2.2.5 LIPOPROTEIN LIPASE AND HEPATIC LIPASE ASSAY

LPL and HL TAG hydrolase activity were measured according to a modified method of Nakai et al. (1979). Substrate was made up of the following components per millilitre of assay mixture; 10umol of Glycerol tri(1-^{14}C) oleate (45000 d.p.m.), 60mg of bovine serum albumin (purified and lyophilized), 30mg gum arabic, 25umol ammonium sulphate, 40ug

phosphatidyl choline and 50ul rat or sheep serum (LPL assay only). (^{14}C)-Triolein and phosphatidyl choline were added to a 25% gum arabic solution and sonicated four times for 2min at 1min intervals, in a cold water bath, at a setting of 100 watts (Labsonic 1510, 9.5mm probe). Albumin and ammonium sulphate were added to the emulsion and the pH adjusted to 8.5 (unless stated otherwise) by the addition of KOH.

For the adipose LPL and liver HL enzyme preparations, the reaction mixture contained 500ul enzyme preparation and 500ul of TAG substrate. For total lipase (LPL plus HL) in postheparin plasma, the assay contained either 100ul of postheparin plasma and 400ul of 0.15M NaCl, or 200ul of plasma and 300ul of 0.15M NaCl and 500ul of substrate. The plasma HL assay contained the same components except that the 0.15M NaCl was replaced with 2M NaCl or with 0.15M NaCl containing 2mg/ml protamine sulphate. In addition the HL assay did not contain serum. LPL activity was determined by subtracting the activity in an assay containing 2M NaCl (HL) from the activity in an equivalent assay containing 0.15M NaCl (LPL plus HL). All assays were run at 37°C in a shaking water bath for 0-60min. Each assay was terminated and free fatty acids extracted according to a modified Dole procedure as described by Kaplan (1970). Activity was counted in a Packard liquid scintillation counter (Tri Carb 460CD), with inbuilt corrections for quench and efficiency and conversion of all activity to d.p.m..

2.2.6 HEPARIN-SEPHAROSE AFFINITY CHROMATOGRAPHY OF SHEEP LIVER TISSUE ENZYME HOMOGENATES AND POSTHEPARIN PLASMA

For affinity chromatography of the liver tissue enzyme homogenate,

1-3ml of extract was loaded on heparin-sepharose CL6B columns (30cm X 1cm), pre-equilibrated with 0.15M NaCl-barbitone buffer solution (5mM, pH 7.5). The columns were sequentially eluted with 50ml of 0.15M, 25ml of 0.45M, 50ml of 0.72M and 50 ml of 1.5M NaCl buffer solutions. A maximum of 5ml of sheep postheparin plasma was similarly eluted through the columns at any one time.

2.2.7 ISOLATION OF VLDL FROM FED AND DIABETIC SHEEP

VLDL from fed and diabetic animals was isolated by centrifuging sheep plasma at a solvent density of 1.0063g/ml as described in section 1.2.4.6.1. Three animals were used for each treatment and the samples pooled. The concentration of VLDL-TAG in fed and diabetic fractions was adjusted to 3mM with a NaCl diluent such that the final concentration of this salt was 0.15M.

2.2.8 HYDROLYSIS OF VLDL-TAG FROM FED AND DIABETIC SHEEP IN POSTHEPARIN PLASMA FROM FED SHEEP

For total rates of VLDL hydrolysis (LPL plus HL) in postheparin plasma, the assay contained 300ul of postheparin plasma, 25ul rat serum (heat inactivated at 60°C for 10min), 75ul 0.15M NaCl and 300ul of TAG adjusted VLDL. The postheparin plasma HL assay contained the same components except that the 0.15M NaCl was replaced with 100ul of 4M NaCl and the assay did not contain rat serum. All assays were run at 37°C in a shaking water bath for 0, 20 or 40min. Each assay was terminated and free fatty acids extracted as described in section 2.2.5. NEFA

released as a result of lipase hydrolysis were calculated by determining total fatty acids at 20min and 40min and subtracting from this the initial concentration of unesterified fatty acids at zero time. NEFA were determined as described in section 1.2.7. VLDL hydrolysis as a result of LPL activity was determined by subtracting the activity in an assay containing 4M NaCl (HL) from the activity in an equivalent assay containing 0.15M NaCl (LPL plus HL).

2.2.9 BLOOD GLUCOSE, TRIACYLGLYCEROL AND NON-ESTERIFIED FATTY ACIDS

Blood glucose, plasma TAG and NEFA were determined as described in sections 1.2.2, 1.2.5.2 and 1.2.7 respectively. Proteins were determined using a modified Biuret method (Itzhaki and Gill 1964).

Statistical evaluation was by one way analysis of variance.

2.2.10 MATERIALS AND REAGENTS

Chemicals: Glycerol (1-^{14}C) trioleate (56mCi/mmol) was purchased from Amersham Australia Pty. Ltd.. Alloxan monohydrate was purchased from Koch-Light Ltd., England. Bovine serum albumin (fraction V, 99% pure) and L-alpha-phosphatidyl choline (type1-EH) were purchased from Sigma chemical company. Sodium heparin (170U/mg) and insulin (Isophane) were purchased from Commonwealth Serum Laboratories Australia. Ready Solv EP scintillation fluor was purchased from Beckman Instruments Inc., Australia. Heparin-sepharose CL6B was purchased from Pharmacia Pty. Ltd. Uppsala, Sweden.

2.3 RESULTS

2.3.1 CHARACTERIZATION OF ACETONE POWDER ENZYME HOMOGENATES

The alkaline endothelial lipases LPL and HL, can be measured either in homogenates from their respective tissues, or in postheparin plasma. However, whilst LPL has been studied in sheep, HL in this species has not been previously reported. So to establish the identity of this enzyme and its potential contribution to ovine heparin releasable plasma TAG hydrolase activity, acetone powder homogenates of sheep liver were prepared and assayed for activity. Furthermore, so as to validate the methods described herein and determine if conformity exists with other species, equivalent extracts from sheep adipose and rat adipose and liver tissue were also assayed for TAG hydrolase activity.

2.3.1.1 SHEEP AND RAT LIVER EXTRACTS

Buffered extracts of sheep liver acetone powder preparations exhibited a capacity to hydrolyze TAG. This activity was similar to the HL activity of equivalent rat liver extracts, in that optimal activity was observed at pH 7 for sheep and pH 8 for rats (figure 2.2). 75% of the TAG hydrolyzing capacity of the sheep preparation was retained at NaCl concentrations of up to 1.5M, though only 30% of lipolysis was observed with the rat hepatic fraction at this level (figure 2.3). Addition of heparin up to 10Iunits/ml inhibited activity by approximately 25% (figure 2.4). TAG hydrolase activity of the sheep liver extract was linear with increasing triolein substrate up to 10mM

Figure 2.2

Figure 2.2 shows the effect of pH on triacylglyceride hepatic lipase activity in sheep (o———o) and rat (+ ——— +) acetone powder liver homogenates. Bars represent the standard deviation of the mean.
At least 3 animals per treatment were used

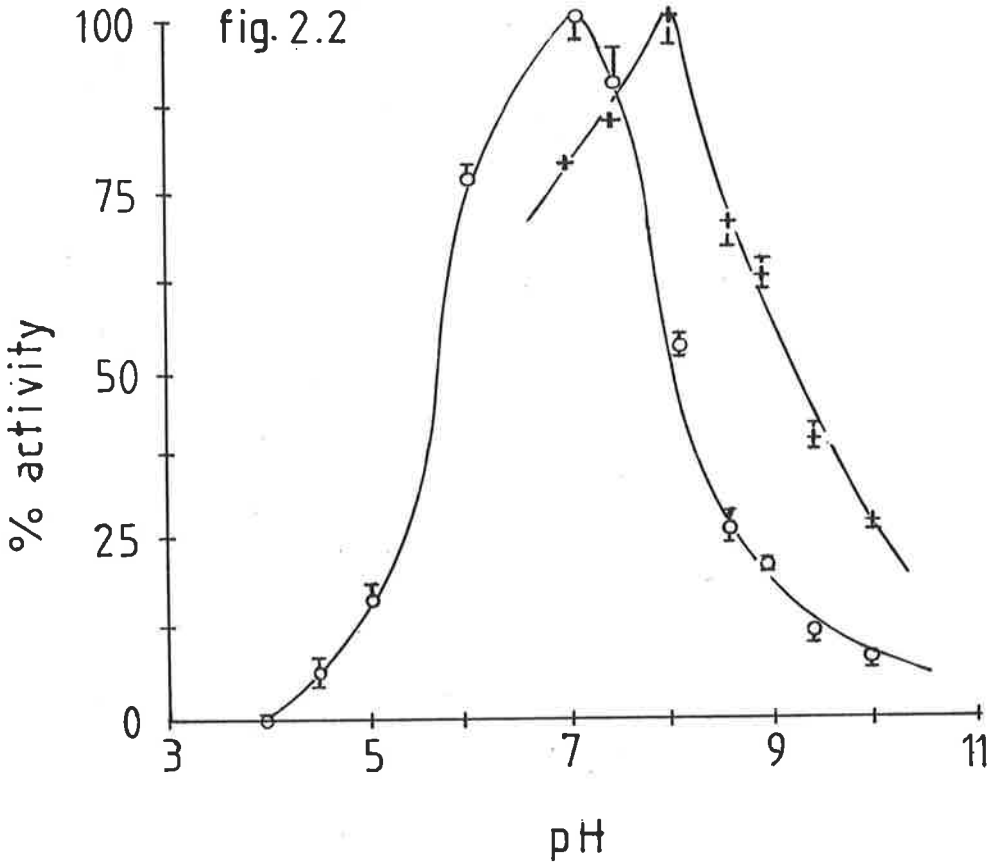


fig. 2.2
100
75
50
25
0
% activity
3
5
7
9
11
pH

Figure 2.3

Figure 2.3 shows the effect of increasing sodium chloride concentration on triacylglyceride hepatic lipase activity in sheep (o——o) and rat (+——+) acetone powder liver homogenates. Bars represent the standard deviation of the mean for three experiments.

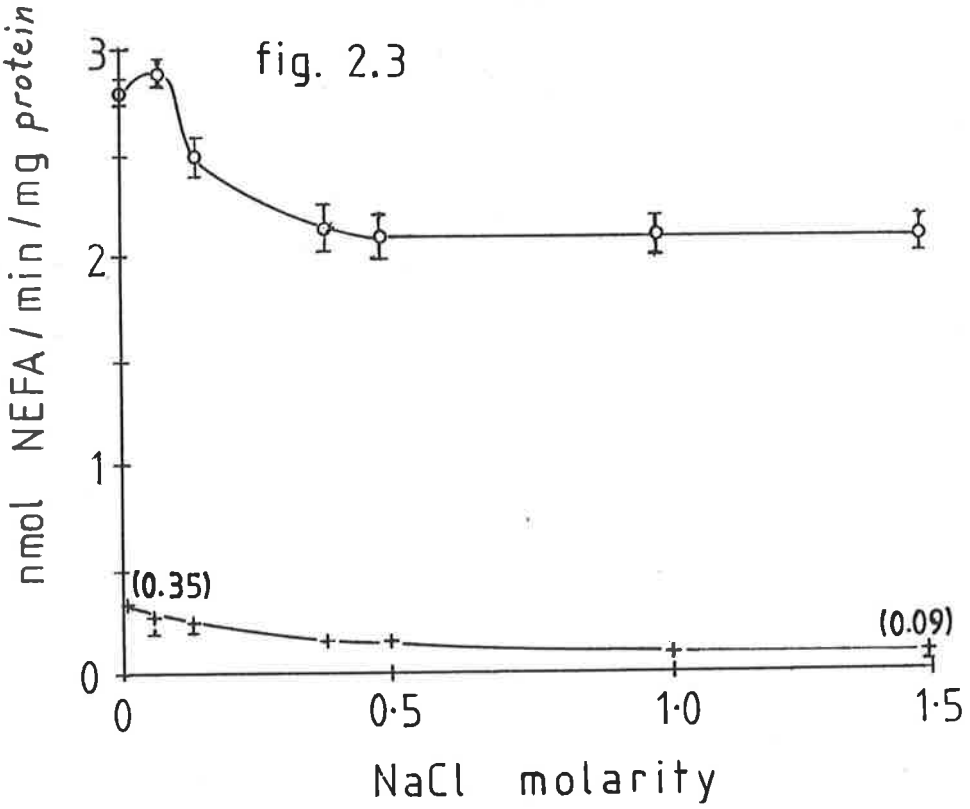
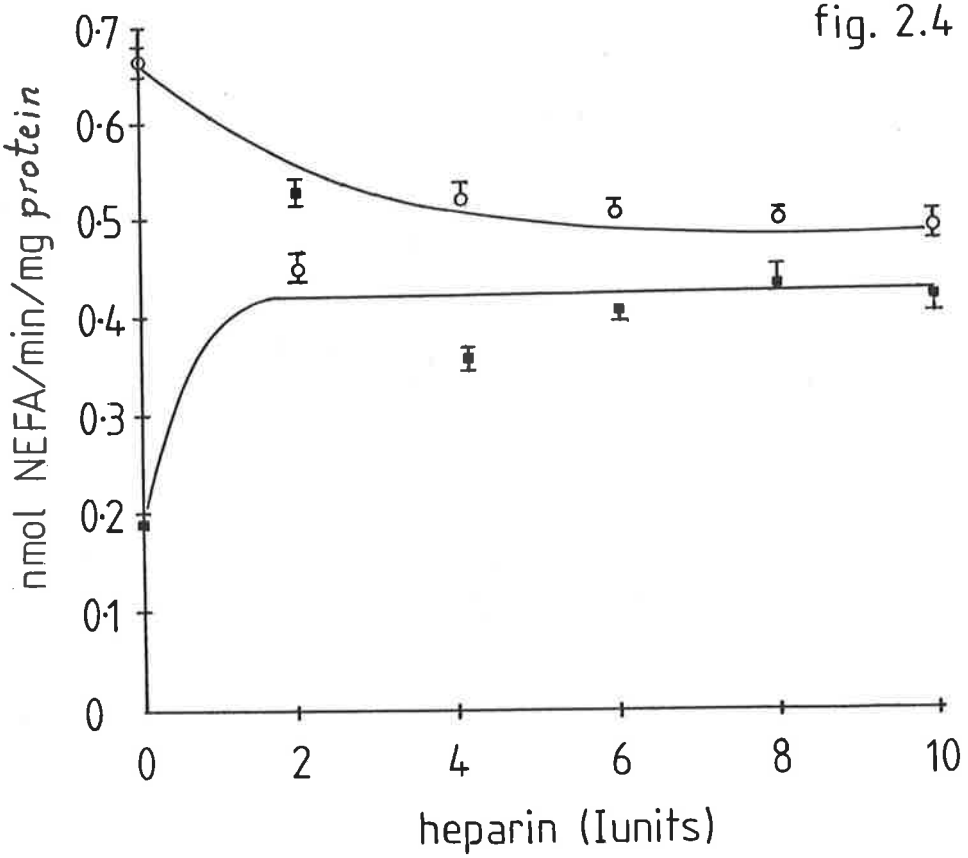


fig. 2.3
nmol NEFA / min / mg protein
3
2
1
0
(0.35)
(0.09)
0
0·5
1·0
1·5
NaCl molarity

Figure 2.4

Figure 2.4 shows the effect of increasing heparin per millilitre of the enzyme and substrate mixture, on sheep triacylglyceride hepatic lipase (o——o) and sheep adipose lipoprotein lipase (■———■), from the respective acetone powder homogenates. Bars represent standard deviation of the mean for three experiments.

fig. 2.4


0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
nmol NEFA/min/mg protein
0
2
4
6
8
10
heparin (Iunits)

(figure 2.5). Activity, however, diminished rapidly with time, having a biological half life of 30min (figure 2.6). There was a small decline in activity with increasing levels of serum (figure 2.7). Rates of TAG hydrolysis were significantly higher in sheep fractions, (approximately 2.8nmol NEFA released/min/mg protein) than that of rat fractions (approximately 0.5nmol/min/mg protein). Gradient NaCl elution of the sheep liver enzyme preparation through heparin-sepharose affinity columns, resulted in a shouldered peak in the 0.72M NaCl-barbitone fraction (figure 2.8). TAG hydrolase activity was not however confined to these peaks. 54% of total activity was not bound to the columns, but rather was eluted in the 0.15M NaCl to 0.45M NaCl wash fractions.

2.3.1.2 SHEEP AND RAT ADIPOSE EXTRACTS

Figures 2.9 and 2.10 show the effects of NaCl and pH on the TAG hydrolase activity of sheep and rat adipose tissue. Activity was progressively depressed with increasing concentration of NaCl and in fact at 1.5M NaCl was completely inhibited. Maximum activity was observed at pH 7 and pH 8.5 for sheep and rat extracts respectively (though activity was present over a wide pH range (6-10)). Addition of heparin at 2Iu/tube increased ovine TAG hydrolase activity by 100%, but no further increases were observed with further additions (figure 2.4). Activity was linear with substrate concentration up to 10mM triolein (figure 2.5) and time up to 60 min (figure 2.6). Serum only marginally stimulated LPL activity in these extracts (figure 2.7). TAG hydrolase activity per unit protein was roughly equivalent between sheep and rat adipose fractions, that is, approximately 2nmol NEFA released/min/mg

Figure 2.5

Figure 2.5 shows the effect of increasing concentrations of triolein substrate on the sheep triacylglyceride hepatic lipase (o———o) and sheep adipose lipoprotein lipase (▪——▪), from the respective acetone powder homogenates. Bars represent the standard deviation of the mean for three experiments.

fig. 2.5

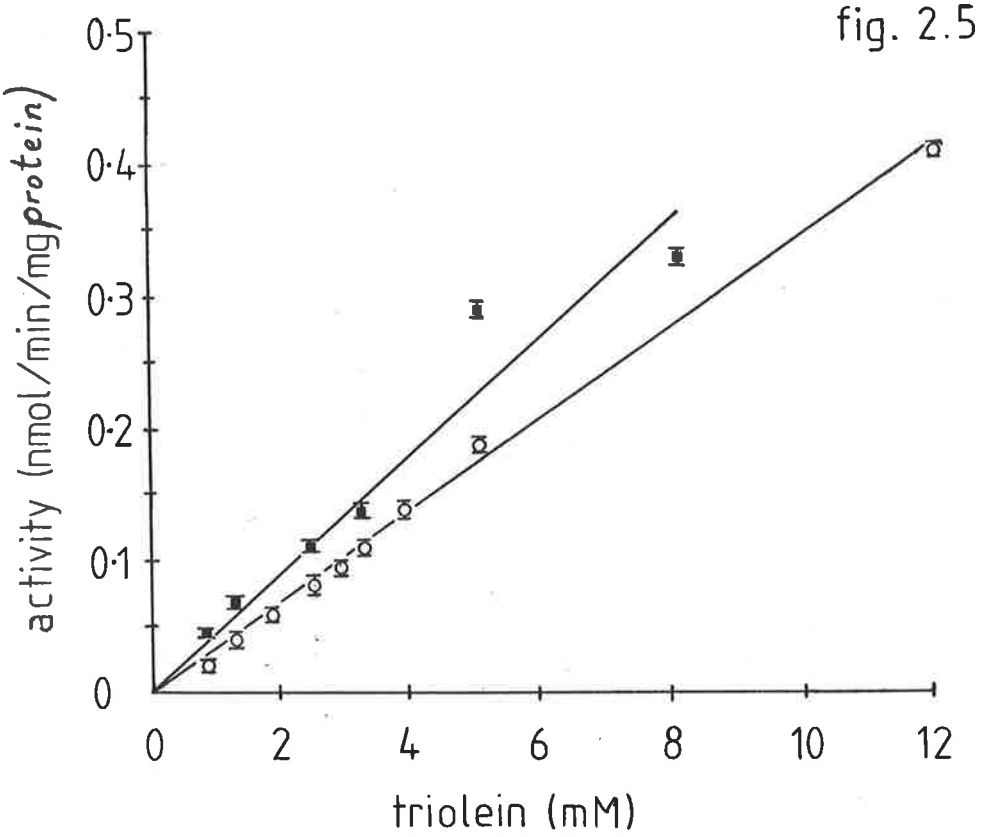

0·5
0·4
0·3
0·2
0·1
0
activity (nmol/min/mg protein)
0
2
4
6
8
10
12
triolein (mM)

Figure 2.6

Figure 2.6 shows the effect of time on sheep triacylglyceride hepatic lipase (o——o) and sheep adipose lipoprotein lipase (▪——▪), from the respective acetone powder homogenates. Bars represent the standard deviation of the mean for three experiments.

fig. 2.6

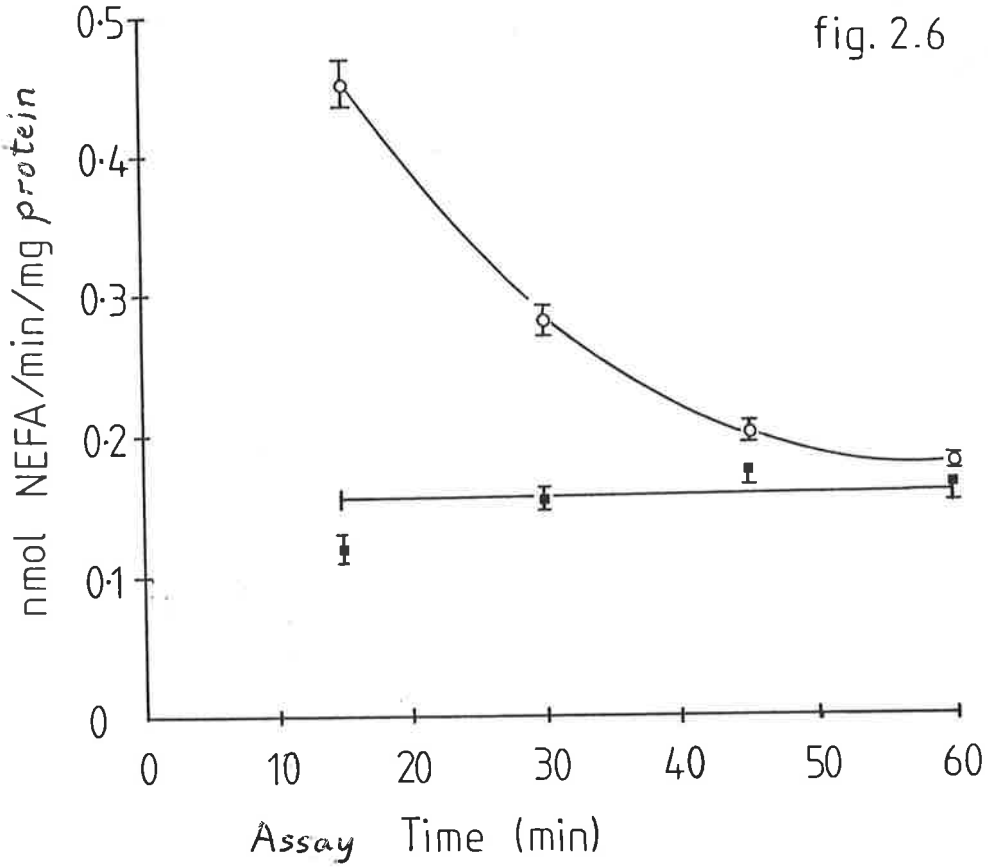

0·5
0·4
0·3
0·2
0·1
0
nmol NEFA/min/mg protein
0
10
20
30
40
50
60
Assay Time (min)

Figure 2.7

Figure 2.7 shows the effect of increasing levels of heat inactivated exogenous sheep or rat serum (as a source of apoprotein CII) to sheep triacylglyceride hepatic lipase (o——— o) and sheep adipose lipoprotein lipase (▪——▪), from the respective acetone powder homogenates. Bars represent the standard deviation of the mean for three experiments.

fig. 2.7

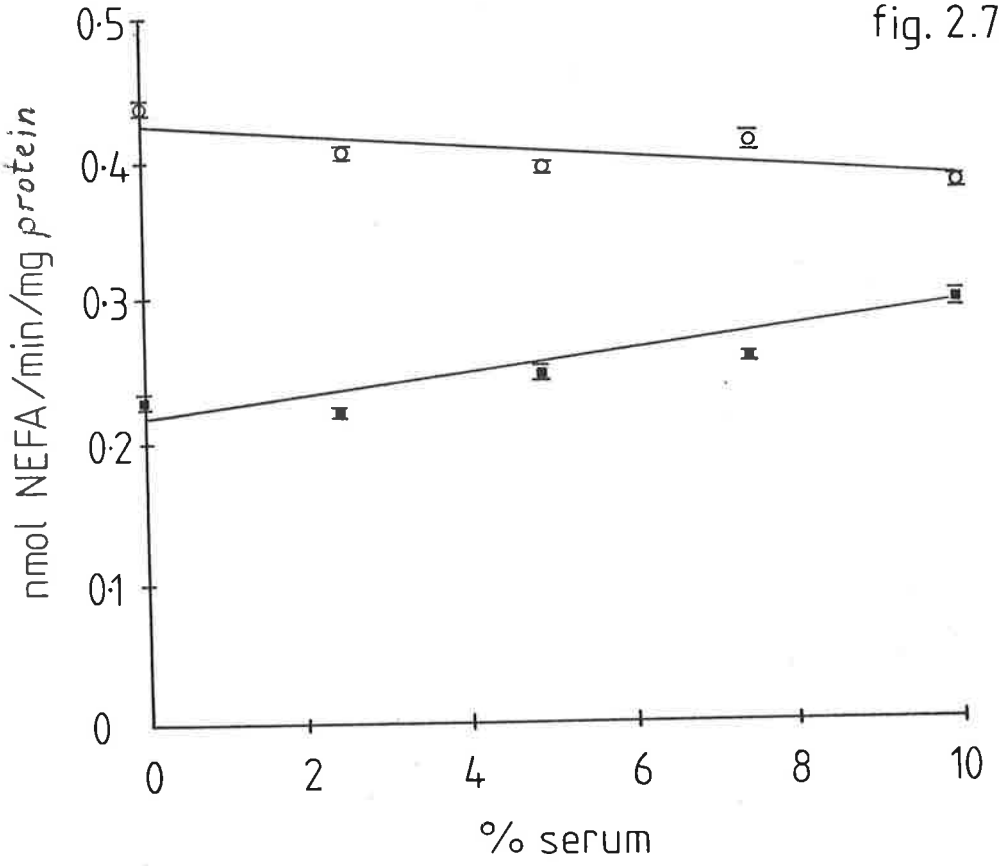


Figure 2.8

Figure 2.8 shows the sodium chloride gradient elution of sheep acetone powder hepatic homogenates through heparin-sepharose affinity columns (————). The corresponding triacylglyceride hepatic lipase activity is shown (×—×—×).

fig. 2.8

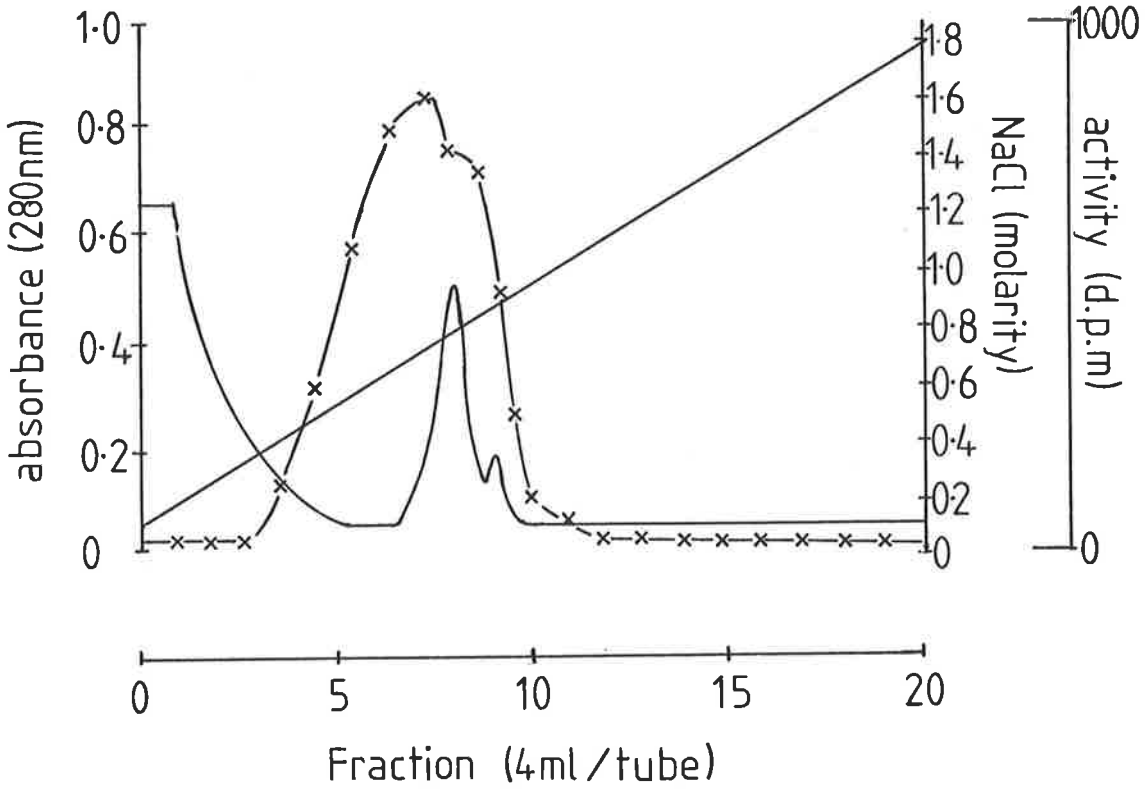

absorbance (280nm)
1·0
0·8
0·6
0·4
0·2
0
NaCl (molarity)
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
activity (d.p.m)
1000
0
0
5
10
15
20
Fraction (4ml / tube)

Figure 2.9

Figure 2.9 shows the effect of increasing sodium chloride concentration on adipose lipoprotein lipase in sheep (▪——▪) and rat (▫——▫) acetone powder homogenates. Bars represent the standard deviation of the mean for three experiments in replicate.

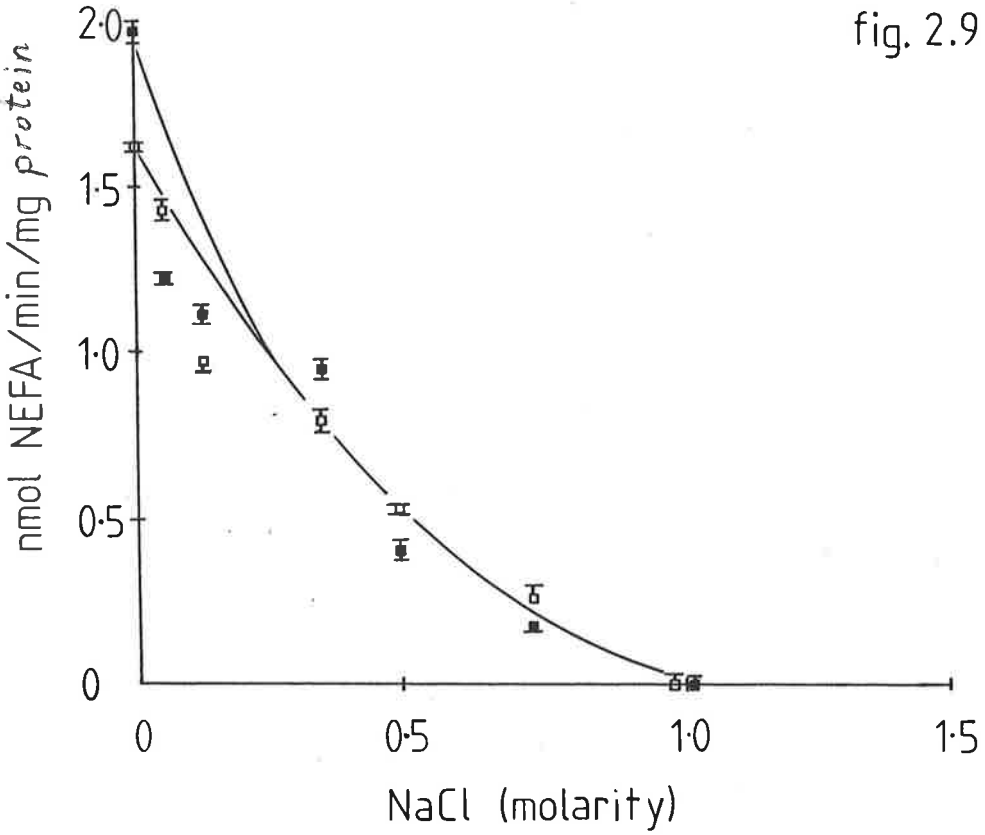
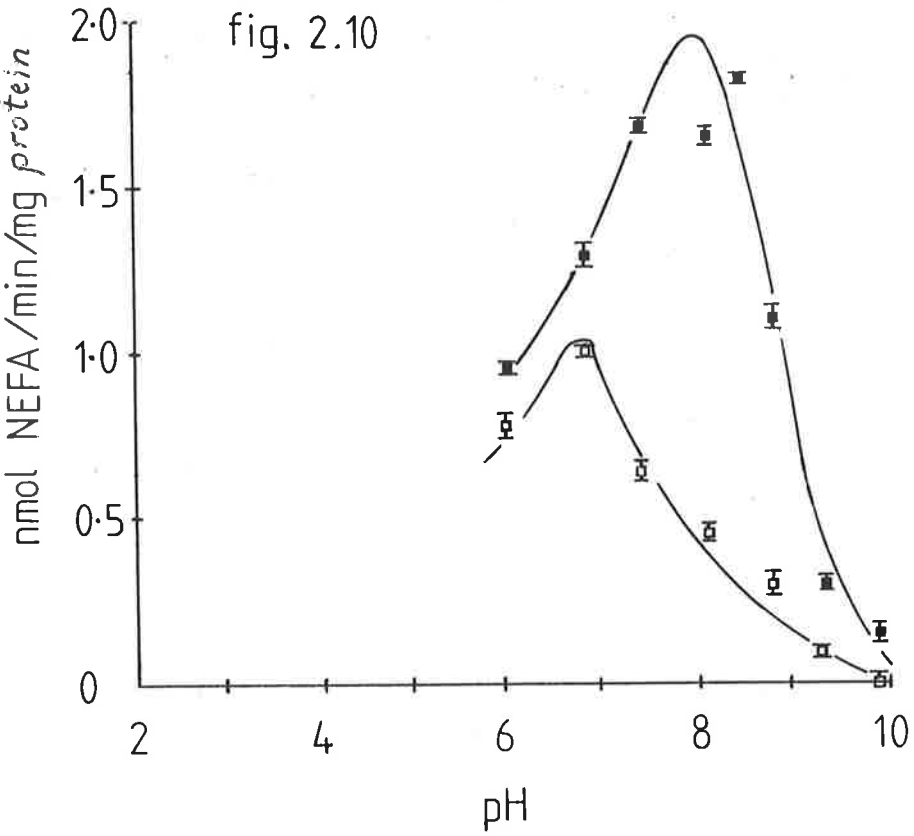


fig. 2.9

Figure 2.10

Figure 2.10 shows the effect of pH on adipose lipoprotein lipase in sheep (▪——▪) and rat (▫——▫) acetone powder homogenates. Bars represent the standard deviation of the mean for three experiments in replicate.

fig. 2.10



protein (at optimum pH).

2.3.2 POSTHEPARIN PLASMA LIPASE ACTIVITY

2.3.2.1 RAT POSTHEPARIN PLASMA

Prior to determining the TAG lipase activity in sheep postheparin plasma, the activities of LPL and HL were determined in rats as described in sections 2.2.4 and 2.2.5. The method used initially was that described by Nakai et al. (1979). Table 2.1 shows that in rats fasted overnight, total TAG hydrolase activity was approximately 14.6umol NEFA/ml plasma/h, which was divided equally between LPL and HL. However, preliminary investigations in this laboratory suggested that extraction of the unesterified fatty acids was inadequate due to volume fluctuations in the aqueous/solvent phases. Having increased the specific activity of the triolein substrate and utilized the modified Dole extraction procedure (Kaplan 1970), higher rates of both LPL and HL in rat postheparin plasma were obtained (table 2.1). Blanks with a known concentration of unesterified fatty acids were extracted under the same conditions to ascertain that this process was complete. Furthermore, radioactivity in aliquots of the 'Dole' extract were determined before and after elution of unesterified fatty acids through Biosil columns, to determine whether or not tri-di-or mono-acylglycerides were similarly extracted. The results showed that the mean total hydrolase activity measured was nearly threefold greater than that previously determined. The proportions of the two enzymes remained essentially unchanged, in that LPL made up approximately 60% of the total activity.

Table 2.1

Table 2.1 lists rat (fasted) postheparin plasma total lipase, lipoprotein lipase and hepatic lipase determined by the method described by Nakai T., Yamada S., Tamai T., Kobayashi T., Hayashi T. and Takeda R. (1979) Metabolism 28, 30-40;and the modified Dole extraction procedure as described in the text.

(n) ± x.x=number of animals ± standard deviation of the mean

	TOTAL LIPASE	LIPOPROTEIN LIPASE	HEPATIC LIPASE
	(umoles of non-esterified fatty acids released / ml. plasma / h)		
Method 1 *	14.6 (3) ± 1.5	7.4 (3) ± 0.9	7.2 (3) ± 0.8
Method 2 **	50.1 (6) ± 9.8	30.1 (6) ± 7.1	20.0 (6) ± 7.2

Table 2.1

* based on extraction procedure described by Nakai et al. (1979)

** based on modified Dole extraction procedure as described in text

2.3.2.2 SHEEP POSTHEPARIN PLASMA

Intravenous administration of heparin to sheep resulted in a rapid rise of TAG lipase activity which was maximal 15min after injection (at 100U/kg). Approximately 70% of this activity was inhibitable by 1M NaCl or protamine sulphate, with no further change up to 3M NaCl (figure 2.11). The NaCl resistant component of total TAG hydrolase activity diminished with time (figure 2.12). Heparin-sepharose affinity chromatography resulted in the elution of two peaks (figure 2.13). The first in the 0.72M NaCl-barbitone fraction was considered to be 'salt resistant' HL. The second peak in the 1.5M NaCl-buffer fraction is LPL. Both LPL and HL in sheep postheparin plasma had an alkaline pH optimum of 8 and 9 respectively (figure 2.14).

Having characterized both components of plasma TAG hydrolase activity, namely LPL and HL, the effects of fasting and diabetes were determined.

2.3.2.3 POSTHEPARIN PLASMA LIPOPROTEIN LIPASE AND HEPATIC LIPASE IN FED, FASTED AND DIABETIC SHEEP

Total plasma TAG hydrolase, LPL and HL activities are shown in table (2.2a). In fed wethers total plasma TAG hydrolase activity was significantly higher than lipolytic rates found in fasted (81%) and diabetic wethers (178%). In normal fed wethers, LPL activity represented approximately 70% of the total and in fasting wethers, this proportionality was maintained, as an equivalent reduction in both LPL and HL was observed. However, in the diabetic animals, despite a

Figure 2.11

Figure 2.11 shows the effect of increasing concentration of sodium chloride on sheep postheparin plasma triacylglycerol lipase activity. Bars represent the standard deviation of the mean for three experiments in replicate.

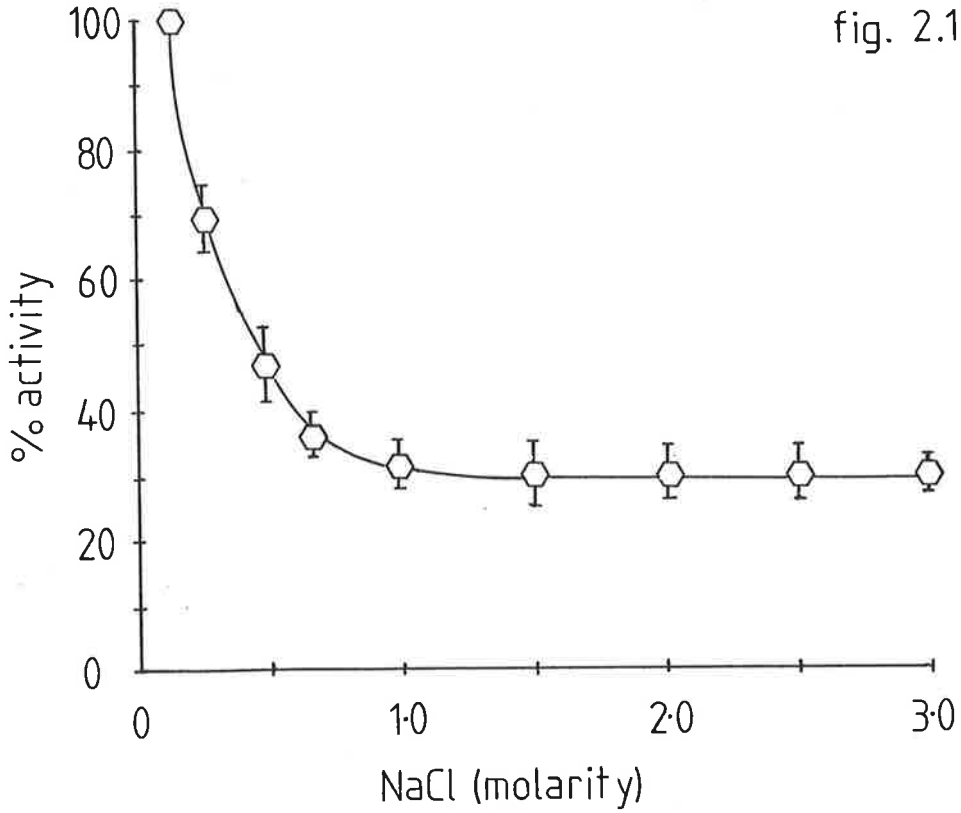


fig. 2.11

Figure 2.12

Figure 2.12 shows the effect of time on sheep postheparin plasma lipoprotein lipase (●——●) and hepatic lipase (×——×) determined by (1) protamine sulphate inhibition of lipoprotein lipase or, (2) sodium chloride inhibition of lipoprotein lipase. Bars represent the standard deviation of the mean for three experiments.

fig. 2.12

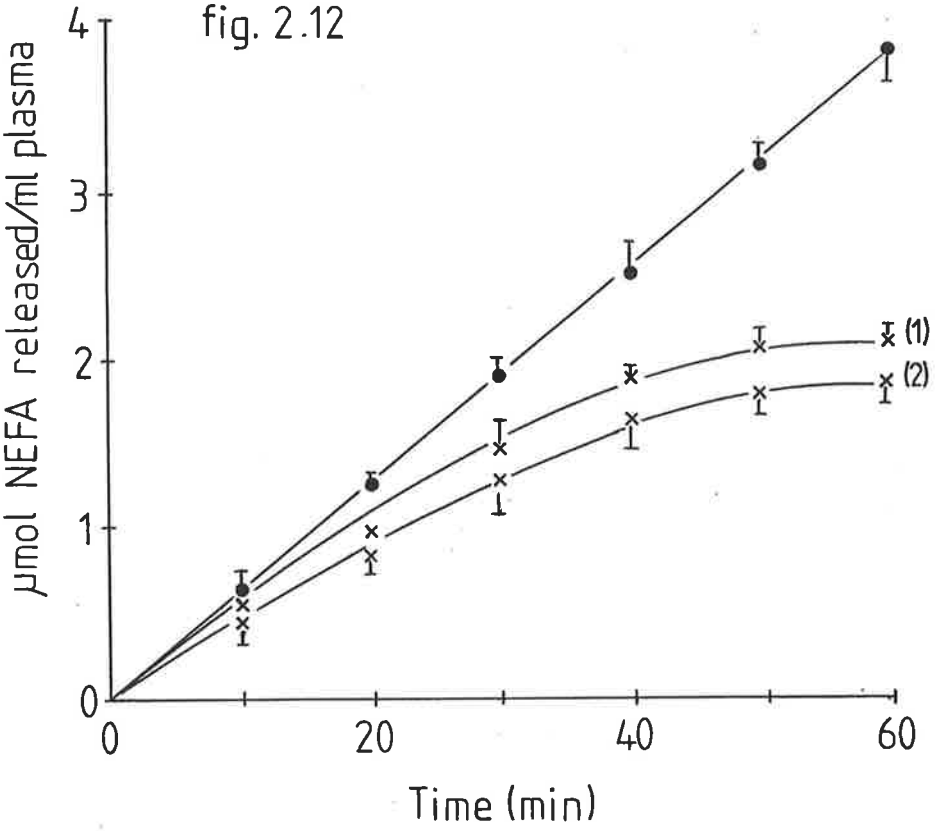


Figure 2.13

Figure 2.13 represents the sodium chloride gradient elution profile of sheep postheparin plasma through heparin-sepharose affinity columns. The first peak in the 0.72M NaCl fraction was identified as hepatic lipase. The peak eluting in the 1.5M NaCl region is lipoprotein lipase.

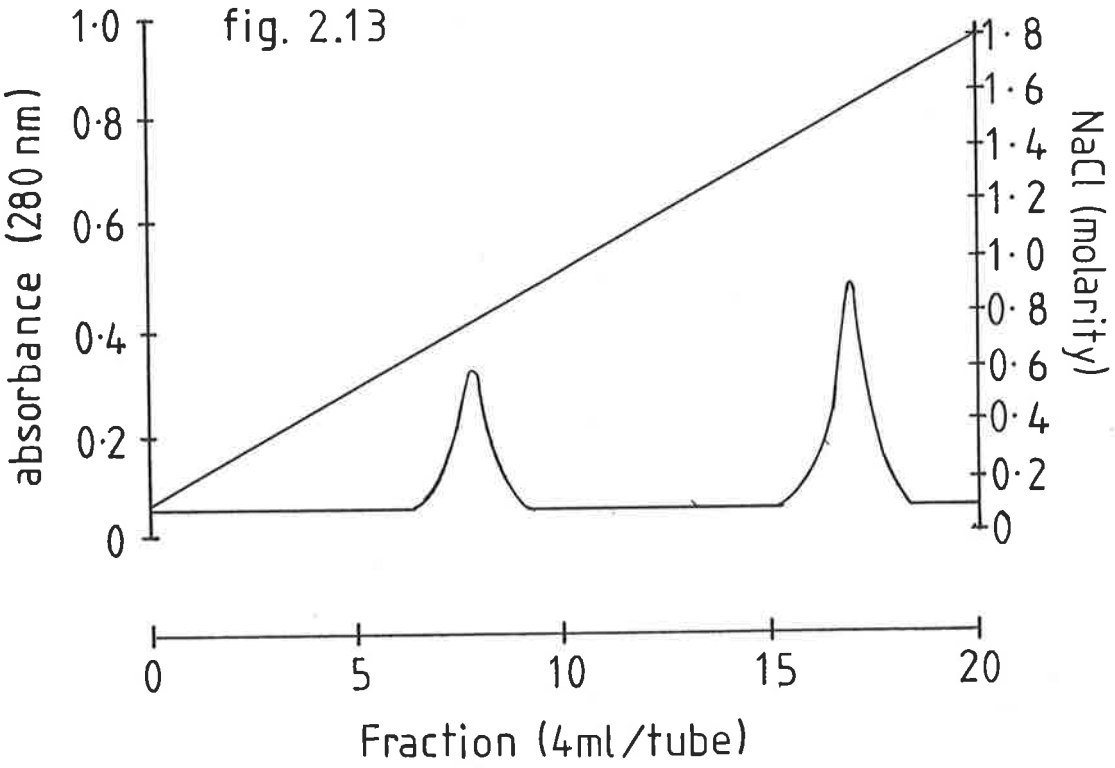


fig. 2.13
1·0
0·8
0·6
0·4
0·2
0
absorbance (280 nm)
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
NaCl (molarity)
0
5
10
15
20
Fraction (4ml/tube)

Figure 2.14

Figure 2.14 shows the effect of pH on sheep postheparin plasma lipoprotein lipase (●——●) and hepatic lipase (x——x). Bars represent the standard deviation of the mean. for three experiments.

fig. 2.14

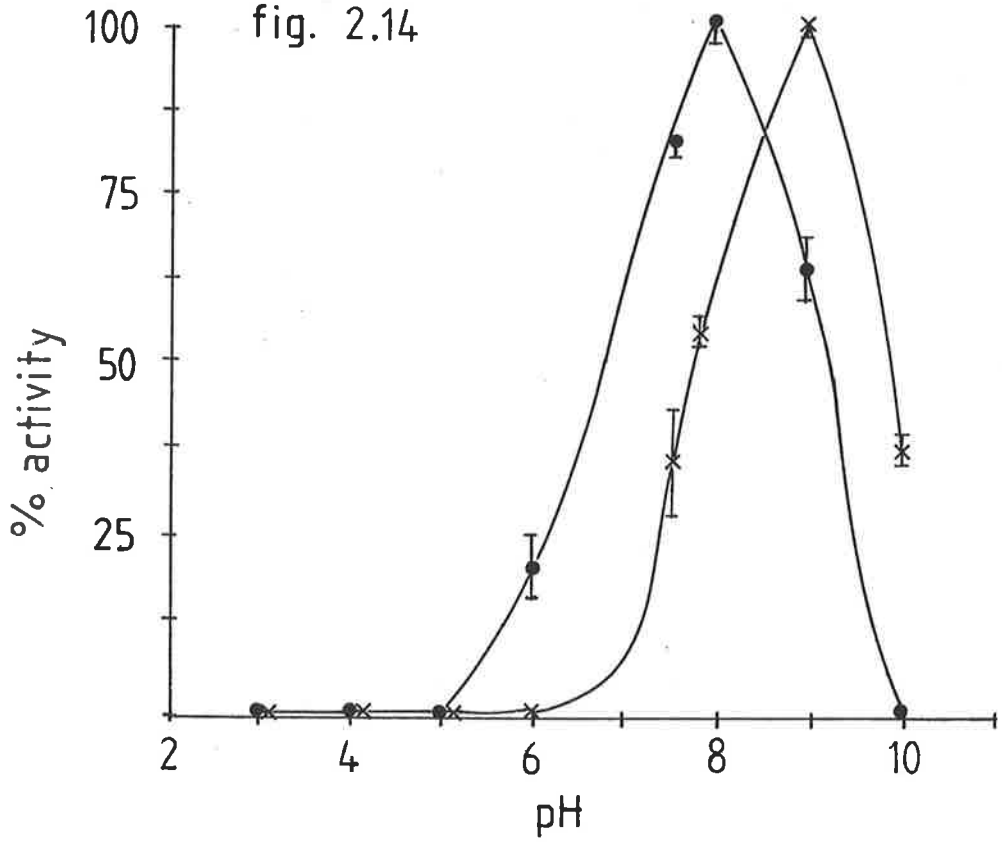


Table 2.2

Table 2.2 (a) lists sheep postheparin plasma total lipase, lipoprotein lipase and hepatic lipase activities in fed wethers, fasted wethers, diabetic wethers and (b) fed ewes and rams.

(n) ± x.xx=number of animals ± standard deviation of the mean.

Table 2.2

TREATMENT	TOTAL LIPASE	LIPOPROTEIN LIPASE	HEPATIC LIPASE
	(umoles of non-esterified fatty acids released / ml. plasma / h)		
FED WETHERS	7.8 (10) ± 1.69	5.7 (10) ± 1.83	2.1 (10) ± 0.76
FASTED WETHERS	4.3 (4) ± 1.49 (a)	3.0 (4) ± 1.70 (a)	1.3 (4) ± 0.66 (a)
DIABETIC WETHERS	2.8 (9) ± 0.67 (a,b)	0.5 (9) ± 0.50 (a,b)	2.5 (4) ± 0.84 (a,b)
FED EWES	8.5 (5) ± 3.42 (d)	6.0 (5) ± 1.98	2.6 (5) ± 2.32
FED RAMS	4.6 (5) ± 0.70 (d,f)	3.2 (5) ± 1.00 (c,e)	1.4 (5) ± 0.60 (g)

[a] [b]

(a) significant against fed at p> 0.5%
(b) " " fasted at p> 0.5%
(c) " " fed at p> 1.0%
(d) " " fed at p> 2.5%
(e) " " ewes at p> 2.5%
(f) " " ewes at p> 5.0%
(g) " " fed at p> 7.5%

significant increase in HL activity (20%), total activity was depressed to 36% of that seen in the normal fed state. This was due to the decline in LPL activity, which was only 0.5umol NEFA released/ml plasma/h, or 20% of the total. The changes in heparin releasable TAG hydrolase activity correlated with fluctuations in blood lipids. In a typical alloxan diabetic wether, total activity was reduced from 4.0umol NEFA/ml/h to 2.4umol/ml/h and plasma NEFA and TAG increased from 77umol/ml for the former, and 262umol/ml for the latter, to 1,258umol/ml and 2,531umol/ml respectively. Administration of exogenous insulin restored activity to 7.6umol NEFA/ml/h, and reduced plasma NEFA to 126umol/ml and TAG to 300umol/ml. Subsequent withdrawal of the insulin showed hydrolase activity was again reduced to 4.1umol NEFA/ml/h, and concentrations of plasma NEFA and TAG elevated to 685umol/ml and 1962umol/ml respectively.

2.3.2.4 POSTHEPARIN HYDROLYSIS OF VLDL-TAG FROM FED AND DIABETIC SHEEP

VLDL was isolated from fed and diabetic sheep and incubated with postheparin plasma from fed animals to determine whether the changes in postheparin plasma lipase activity may have been a result of physiochemical changes of these particles. Figure 2.15 shows rates of NEFA released with time. VLDL-TAG from diabetic animals was hydrolyzed at a rate 280% greater than that of VLDL-TAG from fed sheep. Table 2.3 lists the contribution of both HL and LPL to this activity. LPL which comprised 94% of the total lipolytic rate in VLDL substrate from fed sheep, was increased 2.6 fold with diabetic VLDL substrate. Similarly,

Figure 2.15

Figure 2.15 shows the rate of non-esterified fatty acids released from very low density lipoproteins from fed and diabetic sheep, when incubated with normal fed sheep postheparin plasma. Bars represent the standard deviation of the mean.
Three animals per treatment.

fig. 2.15

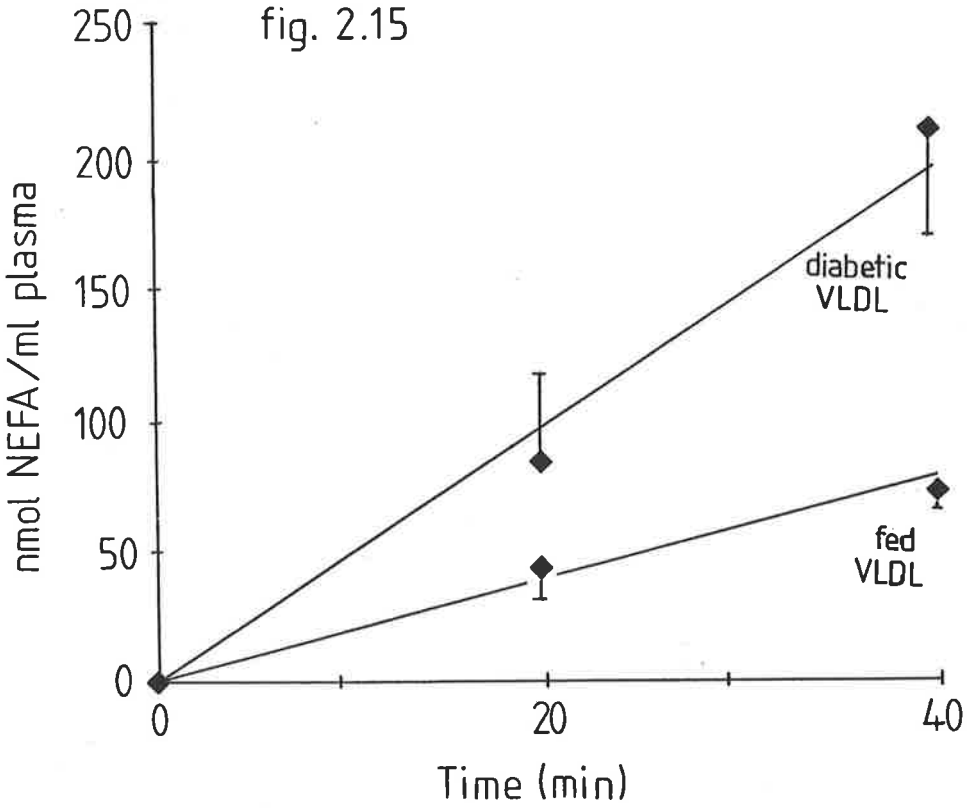

250
200
150
100
50
0
nmol NEFA/ml plasma
diabetic
VLDL
fed
VLDL
0
20
40
Time (min)

Table 2.3

Table 2.3 lists the total lipase, lipoprotein lipase and hepatic lipase activities of normal fed sheep postheparin plasma when incubated with very low density lipoproteins from fed and diabetic sheep.

(n) ± x=number of animals ± standard deviation of the mean.

SUBSTRATE	TOTAL LIPASE	LIPOPROTEIN LIPASE	HEPATIC LIPASE
	(nmol of non-esterified fatty acids released / ml plasma / h)		
Fed VLDL	298 (2) ± 118	282 (2) ± 118	16 (2) ± 7
Diabetic VLDL	845 (2) ± 207	747 (2) ± 277	99 (2) ± 69

Table 2.3

the HL component was also elevated in the latter, though substantially more so (6.2 fold higher). As such, HL comprised 12% of the total postheparin plasma activity, when incubated with VLDL from diabetic animals.

2.3.2.5 POSTHEPARIN PLASMA LIPASE ACTIVITIES IN RAMS, WETHERS AND EWES

Table 2.2b depicts differences in the TAG hydrolase activity of sheep postheparin plasma LPL and HL between ewes, wethers and rams. Ewes had a significantly greater total TAG hydrolyzing capacity (9%) and conversely, rams had a significantly lower total TAG hydrolyzing capacity (41%) than wethers. In ewes, the higher rates of lipolysis were attributable solely to the LPL component, which was 88% higher than that of rams. Similarly LPL in wethers was 78% higher than that of rams. HL was found to vary considerably in ewes and although activity of this enzyme was 46% higher than that of rams, this was not significant. However, HL in rams was significantly lower than fed wethers (33%).

2.3.2.6 POSTHEPARIN PLASMA LIPASE ACTIVITIES IN 'LEAN' AND 'OBESE' SHEEP

Heparin releasable plasma TAG hydrolase activities were determined in 'lean' (Merino) and 'obese' (Romney X Dorset X Merino) sheep, to ascertain whether different breeds of sheep, not subject to feed restriction, are genetically predisposed to lipase activities and as such, a potential degree of adiposity. Table 2.4 lists the total TAG hydrolase activity, LPL and HL of pre-ruminant lambs from both groups.

Table 2.4

Table 2.4 lists the postheparin plasma total lipase, lipoprotein lipase and hepatic lipase activities in genetically 'lean' and 'obese' preruminating and ruminating lambs. Hepatic triacylglyceride release (determined by Triton WR1339 method described in text) is shown for preruminating 'lean' and 'obese' lambs.

(n) ± x.xx=number of animals ± standard deviation of the mean.

TREATMENT	TOTAL LIPASE	LIPOPROTEIN LIPASE	HEPATIC LIPASE	PLASMA TAG CONCENTRATION	HEPATIC TAG SECRETION RATE
	(umoles of non-esterified fatty acids released / ml. plasma / h)			(nmol / ml)	(nmol / min / kg B.Wt)
PRERUMINATING FAT LAMBS	15.1 (4) ± 2.00	12.4 (4) ± 1.50	2.7 (4) ± 0.50	368 (6) ± 54.5	249 (6) ± 55.4
PRERUMINATING LEAN LAMBS	12.4 (6) ± 2.95	11.0 (6) ± 2.57	1.4 (6) ± 0.39 (a)	318 (6) ± 78.6	194 (6) ± 45.2
RUMINATING FAT LAMBS	11.8 (5) ± 1.39 (f)	9.2 (5) ± 1.86 (d)	2.6 (6) ± 0.24		
RUMINATING LEAN LAMBS	9.2 (5) ± 1.33 (b,g)	7.1 (5) ± 1.06 (b,e)	2.1 (5) ± 0.50 (c,h)		

(a) preruminating lean lambs significant against preruminating fat lambs at p> 1.0%
(b) ruminating " " " " ruminating fat lambs at p> 2.5%
(c) " " " " " ruminating fat lambs at p> 7.5%
(d) " fat " " " preruminating fat lambs at p> 1.0%
(e) " lean " " " " lean lambs at p> 2.5%
(f) " fat " " " " fat lambs at p> 2.5%
(g) " lean " " " " lean lambs at p> 7.5%
(h) " " " " " " lean lambs at p> 7.5%

Table 2.4

Total activity was 15umol NEFA released/ml plasma/h for the crossbreds, and although total activity was only 10umol NEFA/ml/h in the Merino lambs, this was not significantly different. Nonetheless, HL in the lean merino sheep was significantly lower than their crossbred counterparts (38%).

Table 2.4 also shows the postheparin plasma total TAG hydrolase activity, LPL and HL for both 'lean' and 'fat' animals after weaning and at a stage where weight gain was at its highest. Total activity was significantly different between these animals namely 11.70umol NEFA/ml plasma/h and 9.07umol NEFA/ml/h for 'fat' and 'lean' groups respectively. The lower rates of hydrolysis in the latter group were solely attributable to LPL which was 23% of that observed in the crossbreds. Mean HL activity in the 'lean' animals was 2.04umol NEFA/ml/h and significantly higher (25%) than that in the 'fat' animals (2.56umol NEFA/ml/h).

2.3.3.1 TRIACYLGLYCEROL SECRETION RATE IN PREWEANED 'LEAN' AND 'OBESE' LAMBS

Triton WR1339, was used to inhibit plasma clearance of TAG and so measure hepatic release of lipid in both 'lean' and 'obese' animals, to see if this could be correlated with the higher rates of TAG lipolysis observed in the latter group.

Mean plasma TAG concentrations were 318umol/ml plasma and 368umol/ml for 'lean' and 'fat' lambs respectively (table 2.4). There was considerable variation, and results were not significantly different. Similarly, hepatic secretion of TAG was 194nmol/min/kg body

weight and 249umol/min/kg body weight respectively and although in each animal secretion was constant, (r greater than 0.96 over 4.5 hours) there was considerable variation between animals.

2.3.3.2 TOXICITY OF TRITON WR1339

Immediately after the TAG secretion study, the animals were returned to open grazing within a 2 acre paddock. Approximately two weeks after Triton administration, a number of sheep had lost weight and general body condition. They developed black chapped patches on areas which were generally exposed, such as nose, lips and rump and eventually a number of sheep died. Autopsies on each of the deceased lambs revealed consumption of toxic material was not the cause of death (as suggested by the symptoms). There was, however, gross hepatic cellular damage, which could not be further characterized.

2.4 DISCUSSION

Ovine LPL has been isolated, characterized and shown to be similar to that reported in other species, in that it has an absolute requirement for apoprotein CII. The role of this enzyme in relation to the hypertriacylglyceridaemia and hepatic accumulation of this lipid exhibited in diabetic sheep, has not however, been previously reported. The activity of this enzyme in other species such as humans and rats made diabetic, has been widely investigated, but both of these differ from sheep, in that diabetes results in a reduced hepatic output of TAG. Similarly, ovine HL has not been previously reported and the role of this enzyme in the metabolism of VLDL-TAG also remains to be defined. Purification of this enzyme was beyond the scope of this study, though an investigation of the catabolism of sheep VLDL-TAG would have been incomplete without determining changes in it's activity. As such, this section of this study had several objectivies. The first was aimed at establishing if sheep liver possessed a TAG hydrolase activity which had characteristics resembling membrane bound HL as reported for other species. This was achieved by extracting TAG lipolytic enzymes from acetone powder preparations of sheep liver and comparing these with similar fractions from rat liver and published data on such fractions for the latter (Jansen and Hulsmann 1975 and Hulsmann et al. 1977). Furthermore, to establish conformity with other species, equivalent homogenates of sheep and rat adipose tissue exhibiting LPL activity were also characterized. Acetone powder homogenates have been a traditional means where such enzymes and the characteristics attributable to them, may be identified without the need for further purification. Such

fractions do however, represent a crude protein extract, which in addition to LPL and HL, may contain other proteolytic enzymes active under the described experimental conditions.

Acetone powder preparations of ovine liver contained TAG hydrolase activity which resembled the membrane bound HL reported in other species, in that activity was expressed under conditions of high salt concentration (75% retained). However, the sheep HL activity did not exhibit the higher alkaline optimum usually associated with this enzyme and as observed here for the equivalent fractions from rats. A number of liver lipases have been reported, including the membrane bound enzyme which is NaCl resistant and has an alkaline optimum, as well as a lysosomal lipase, which is inhibited by NaCl and has an acid optimal pH (Assman et al. 1973, Jenson et al. 1980 and Cordle et al. 1983). Although the presence of these activities have not been previously reported in sheep liver, it is most probable that these extracts contain both enzymes, thus accounting for the lower pH optimum and 25% reduction in activity at NaCl concentrations of 1.5M. Similarly, the reduction in rat liver TAG lipolysis observed under high NaCl concentrations, could also be attributable to the presence of these enzymes. The lower rates of TAG lipolysis per unit protein in rats, compared to sheep is of potential significance, in that this may suggest differences in the TAG hydrolysing capacity of HL between species. Activity was linear with triolein substrate concentrations of up to 10mM, suggesting that the sheep liver enzyme has a low affinity though high capacity for the substrate. Serum added to the assay had a depressing effect as reported for rat HL (La Rosa et al. 1972 and Kubo et al. 1980) and bovine HL (Cordle et al. 1983). Gradient NaCl elution of the sheep liver enzyme

preparations through heparin-sepharose affinity columns, gave rise to a shouldered peak in the 0.72M NaCl fraction. This is a key feature of HL and in terms of this study, was considered as sufficient confirmation of the presence of this enzyme in sheep liver. In addition, there was a TAG hydrolysing enzyme(s) which did not bind to the column indicating the presence of other lipases. HL enzyme activity after heparin-sepharose elution was unstable in that it diminished rapidly with time. This was also found to be the case with sheep liver extracts and the NaCl resistant component of postheparin plasma after 30min. Jensen and Bensadoun (1981) and Jensen et al. (1982) have previously reported that Triton X-100 is required to stabilize HL which was absent in the procedure described here. Nevertheless, as the postheparin plasma lipase assay was terminated at 30min, addition of Triton was not deemed necessary.

LPL in sheep has been characterized and as such, it was not intended here to replicate these findings. Rather, it was considered that identifying the characteristics attributable to this enzyme, would, by validating the methods, indirectly support the identification of HL in the sheep liver homogenates.

In a recent study by Tume et al. (1983), who looked at LPL in sheep and rat adipose tissue homogenates, it was reported that LPL in either aqueous or acetone powder extracts was inhibited by 0.6M NaCl. Similarly, in this study, lipase activity in defatted homogenates of rat or sheep adipose tissue was totally inhibited by NaCl. However, these authors also reported that LPL activity could only be detected when the tissues were maintained at 37°C, that activity diminished rapidly with

time, was dependent on the presence of serum (as a source of apoprotein CII), that 80% of activity was lost when heparin was excluded from the assay and that activity was optimal at pH8-9. Conversely in the experimental protocol described here, it was found that immediate freezing of the tissue in liquid nitrogen and extraction of LPL at 4°C for 3h, did not incur such a loss of activity. Heparin was found to stimulate LPL activity, though there was substantial activity in it's absence. The pH optimum reported here was similar, namely pH7.5-8.5. Tume et al. (1983) reported that sheep adipose LPL had a high affinity (Km=0.4mM triolein), wheras LPL in the homogenate described here was not saturated at 10mM. In addition, full activity was retained for up to 60min. The reasons for these differences are unclear. It was considered that a high affinity LPL in sheep adipose tissue would be unlikely, in view of the plasma concentration of TAG and particularly, in terms of the metabolic priority of this tissue, which would be of least importance. The animals used in the former study were crossbred (Border-Leicster X Merino) sheep which generally have a greater degree of adiposity. These animals also had a diet supplemented with pellets available ad libitum and the differences in enzyme activity may be attributable to these factors. There is little doubt that LPL from sheep also requires apoprotein CII for activity, though it was found here that 75% of lipolytic potential was retained in it's absence, perhaps due to endogenous amounts of this type of protein in the enzyme homogenates.

Having established the presence of a NaCl resistant lipase in sheep liver, postheparin plasma was examined for both LPL (NaCl inhibitable) and HL (NaCl resistant) components. Heparin administration and optimal

time of blood sampling after injection were established, such that maximal rates of total TAG hydrolase activity were attained. Similar such studies have been widely reported in other species and so postheparin plasma from rats were also assayed to validate the analytical procedures. The method used to measure postheparin LPL and HL activity was essentially that described by Nakai et al (1979). Replication of this method gave rise to rates of LPL and HL activity similar to those reported (table 2.1). However, preliminary investigations in this laboratory showed that the ratio of the volume of aqueous phase and solvent phase containing the fatty acids was found to be highly variable, which potentially could give rise to substantial errors. Subsequently, the specific activity of the substrate was increased (originally to allow for expected low hydrolase activities in sheep plasma) and a modified 'Dole' extraction procedure was used (Kaplan 1970). Replication was excellent and higher rates of both enzymes were calculated for rats (table 2.1). Extraction of fatty acids as blanks showed that this process resulted in full recovery of these, without contaminating mono-, di-, or triacylglycerols. The ratio of LPL/HL was in close agreement with what has previously been reported for rats (Krauss et al. 1973 and Elkeles et al. 1977). The specific activity of the substrate described here, was found to be the minimum required to achieve a measure of ovine postheparin lipase activity by this technique.

Gradient elution of sheep postheparin plasma gave rise to two peaks. The first in the 0.72M NaCl fraction corresponded to the 'salt' (NaCl) resistant HL of the liver enzyme extracts and HL of other species. The second peak in the 1.5M NaCl fraction was the salt

inhibitable LPL. Ideally, the effects of NaCl, substrate concentration and pH would have been best determined on the enzyme fractions and plasma eluted through the affinity columns, rather than the acetone powder homogenates, however, activity was found to be very low in the former. This was particularly true for LPL, after desalting through sephadex G25 columns. Concentration of the proteins was not successful, as no significant increase in specific activity could be achieved, due to loss or degradation of the enzyme. Purified lipoprotein lipase and hepatic lipase are characteristically unstable (Clegg 1979, Jensen and Bensadoun 1981 and Jensen et al. 1982). Nonetheless, it was considered that the part characterizational analysis of the acetone powder enzyme fractions, coupled with the affinity chromatography of these fractions and postheparin plasma, was sufficient for the purposes of this study, in establishing the identity of both LPL and HL in sheep postheparin plasma.

Total plasma TAG hydrolase activity was much lower in sheep than that for rats, suggesting that the low levels of circulating VLDL-TAG in the former was not due to high rates of hydrolase activity as proposed in the overview.

The contribution of LPL or HL to total activity also differed between these two species. LPL comprised approximately 75% and 60% of total activity in sheep and rats respectively. This suggests that LPL is the principle means by which sheep metabolize circulating TAG. Increasing dosages of intravenous heparin, or delaying time of blood sampling after injection in sheep, did not alter the ratio of LPL/HL.

In order to establish the role of LPL and HL in the hypertriacylglyceridaemia and hepatic accumulation of this lipid in metabolically stressed sheep, animals were deprived of food for 72h, or made diabetic by intravenous administration of alloxan. Fasting for three days resulted in a significant decrease (28%) in total activity, which was due to a 47% decline in LPL and a 38% decline in HL. Thus, in addition to an enhanced rate of release of VLDL-TAG by the liver in these animals, the plasma accumulation was also attributable to a decreased catabolism of these particles. The decline in LPL activity was probably a response to low plasma insulin levels (Steiner et al. 1975 and Bouchat et al. 1981). Tume et al. (1983) have during the course of this study also reported lowered LPL activity in powder preparations from fasted sheep adipose tissue. Although postheparin plasma LPL activity has been reported to increase in fasted rats (Nakai et al. 1979), it is generally considered that this enzyme decreases in activity, when the plane of nutrition is below that required to maintain homeostasis (Bezman et al. 1962, Garfinkel et al. 1967, Austin and Nestel 1968, Wing and Robinson 1968, Persson et al. 1970, Cryer et al. 1976, Taskinen and Nikkila 1979, Fried et al. 1983, Lasuncion and Herrera 1983 and Stam et al. 1984). LPL from different tissues are often reciprocal in their relationship to TAG lipolysis.

The lower rates of TAG hydrolysis in fasted sheep postheparin HL activity is similar to that reported for fasted rats (Jansen and Hulsmann 1974 and Nakai et al. 1979) or fasted cows (Liesman et al. 1984). As this enzyme is thought to be involved in the further hydrolysis of TAG in VLDL remnants, it was not considered unusual that the activity of this enzyme paralleled that of LPL.

Total TAG lipase was significantly reduced in diabetic wethers when compared to fed sheep (64%) or even fasted animals (35%). This was despite a significant increase in HL compared to fed wethers (16%) and a 1.9 fold increase compared to fasted animals. The reason for this was the very low LPL activity which was only 8% of that in fed sheep. (In some animals LPL was below the threshold of detection). In diabetic rats and man, insulin has been shown to be a critical regulator of LPL (Nikkila et al. 1977 and Stam et al. 1984) and the results here with insulin stabilized sheep clearly showed that this is also true for this species. The increase in HL in diabetic animals was however, extremely surprising. A reciprocal insulin regulation of HL was considered unlikely and in view of the lower HL activity seen in fasted sheep (which also have lower concentrations of plasma insulin) shown not to be so. This agrees with the recent report by Murase and Inoue (1985), who found that in perfused rat livers, HL was not an insulin dependent enzyme. Similar results have just been published in a study of diabetic dogs (Muller et al. 1985), whose postheparin HL activity was also elevated against normals. The relationship between HL activity, plasma insulin and TAG concentration remains unclear. In humans, plasma HL activity remains constant in either insulin dependent diabetes (Pfeifer et al 1983) or normotriacylglyceridaemic insulin dependent diabetics (Nikkila et al. 1977), though conversely, streptozotocin treated rats exhibit a reduced HL activity (Nakai et al. 1979 and Stam et al. 1984). It is difficult to perceive why HL increased in diabetic sheep. It may simply be a physiological response to the elevated plasma TAG levels, as a result of increased secretion and reduced LPL catabolism, or may involve a complex hormonal regulation. The synthesis and secretion of

this protein as a result of diabetes may also be increased, though such a mechanism is difficult to perceive.

VLDL from both fed and diabetic animals were incubated with postheparin plasma from fed sheep, to determine if changes in either or both the physical nature or chemical nature of these particles, may have been responsible for differences in rates of LPL and HL lipolysis observed between treatments. Higher rates of VLDL hydrolysis were obtained in particles isolated from diabetic animals as opposed to the same fraction from normals. This has not been previously reported and is in contrast to what is generally considered to occur in diabetic subjects. Radiolabelled VLDL and chylomicrons isolated from diabetic rats and reinjected into normal recipients have been shown to have a reduced rate of clearance (Bar-On et al. 1984 and Levy et al. 1985). These authors concluded that the longer plasma half life of VLDL particles may be related to qualitative changes in the apoprotein profile. However, this qualification may also be true for the higher rates of hydrolysis seen in this study. Radiolabelling lipoprotein particles and subsequently monitoring rates of clearance in vivo has a number of deficiencies which must be considered. The labelling process may modify the nature of the particle, such that in vivo lipolysis is altered. In addition, there may be a considerable degree of transfer of the radiolabelled proteins or lipid components between lipoprotein fractions, thus not portraying true rates of lipolysis. Similarly, although clearance may be impaired in diabetic subjects, this may reflect a reduction in the removal of lipoprotein remnants produced as a result of the lipolytic process, rather than reflect a reduction in the activities of LPL and HL. The HL (NaCl resistant) component of

postheparin plasma lipase activity was very low in the VLDL-TAG assay from fed sheep, though based on the postheparin rates of lipolysis of the triolein emulsion, this activity was expected to represent only 25% of total activity. Nonetheless, LPL and HL activities of fed sheep postheparin plasma were clearly stimulated when incubated with VLDL from diabetic sheep, and whilst LPL remained the major component of lipolytic rate, quantitatively, HL activity exhibited a greater increase.

Depressed LPL activity in diabetic sheep was probably caused by low levels of plasma insulin, which stimulates synthesis and secretion of LPL. In contrast, the increased rates of HL in these animals seemed to reflect physiochemical modifications of the lipoproteins. The higher protein content of VLDL from fed sheep may account for this increase, or perhaps qualitative changes in the apoprotein compliment. Alternatively, glucosylation of these particles may have altered lipase activities (as suggested in chapter one).

HL is thought to have a physiological role in regulating VLDL-TAG metabolism, as an impaired removal of these particles occurs after the administration of HL specific antibodies (Grosser et al. 1981, Murase and Itakura 1981 and Goldberg et al. 1982). HL also plays a role in the conversion of HDL2 type particles to the smaller HDL3 type particles (Van Tol et al. 1980 and Reardon et al 1982). Specifically, HL removes TAG and phospholipid from IDL, thereby increasing its density (Homma et al. 1985). A deficiency of this enzyme leads to an accumulation of IDL, an LDL enhanced in TAG and elevation in HDL2 type particles (Breckenridge et al. 1982). Furthermore, Breckenridge (1985) suggested that HL may also promote the formation of cholesterol-ester rich HDL particles. With these postulated roles for this enzyme, one can predict

a change in the lipoprotein spectrum in diabetic sheep as a result of an increase in HL activity. In chapter one it was observed that the VLDL of diabetic origin had less TAG and was richer in cholesterol esters with respect to the equivalent fraction from fed animals. Considering LPL was depressed in diabetic sheep, this suggests that HL was an important enzyme in the metabolism of TAG rich VLDL. Applebaum-Bowden et al. (1985) recently reported in a population study of men and women, that HL was directly correlated with VLDL-TAG concentration. Furthermore, as the VLDL fraction of diabetic sheep was richer in cholesterol-esters, HL may also be implicated in the formation of cholesterol-esters, possibly from VLDL surface cholesterol. The LDL from diabetic animals was enhanced in TAG and contained relatively less cholesterol esters, but this does not necessarily mean that HL does not hydrolyze VLDL(-IDL-LDL)-TAG, rather, this could reflect the virtual absence of LPL activity. In other words, the catabolism of VLDL to LDL in these animals may be solely attributable to HL action. Similarly, Nozaki et al. (1986) showed that HL could be inversely correlated with the cholesterol content of IDL, the phospholipid component of IDL and the enzyme activity was directly proportional to the ratio of HDL2/HDL3 cholesterol. The HDL fraction in diabetic animals had a lower TAG content and as a result, a greater protein per unit lipid ratio, even though these particles had a greater cholesterol ester compliment, in comparison to HDL from fed animals. It would appear therefore, that diabetic sheep had an HDL profile which resembled smaller HDL3 particles (as supported by the frequency of smaller HDL particles observed by transmission electron microscopy). The lipid complement of HDL from fed and diabetic animals also correlated with HL activity in a manner such

that this enzyme may be implicated in the hydrolysis of lipoprotein TAG and formation of lipoprotein cholesterol esters.

LPL and HL were determined in fed ewes, wethers and rams. This represents an examination of postheparin plasma lipase activity in animals actively producing oestrogens to animals producing androgenic hormones. Total lipase activity was highest in ewes (8.5umol NEFA/ml plasma/h) with wethers and rams exhibiting 91% and 54% of this activity respectively. This trend was seen in both the LPL and HL component of total lipase activity. HL activity varied considerably in ewes and as a result, was not significantly different from fed wethers or rams, even though it was on average 83% greater than that of rams. Nonetheless rams exhibited an HL activity which was significantly lower (33%) than that of wethers. It has been well established Christie(1979) that wethers have a body composition comparable to ewes, in that they have a greater degree adiposity. The similar rates of LPL observed in these animals, which was almost twofold higher than that of rams, showed that the process of castration and hence, removal of androgenic synthesis is the causative agent for the greater degree of fat deposition. Similarly, HL activity progressively decreased from ewes, to wethers and rams reflecting its association with the metabolism of IDL-TAG produced as a result of LPL lipolysis. Oestrogens and androgens have been reported to decrease and increase respectively, the activity of HL in postheparin plasma (Ehnholm et al. 1975a, Tikkanen et al. 1982, Augustin and Greten 1979 and Applebaum et al. 1977, 1985), whereas the results here indicate that the exact opposite occurs in sheep. The hormonal regulation of this enzyme has not been widely researched and it is apparent that the

oestrogenic/androgenic control of HL requires further investigation.

During the course of this study, access was gained to sheep designated as genetically 'lean' (Merino) and 'obese' (Merino X Romney X Dorset), providing a natural extension of examining a different aspect of sheep plasma TAG metabolism. Postheparin plasma TAG lipase activities were determined in preruminating and postweaned (ruminating) lambs, to see if these could be correlated with hepatic plus gut release of TAG and adiposity. In preruminating lambs, plasma TAG concentration was 318nmol/ml and 367nmol/ml for 'lean' and 'fat' animals respectively and TAG hepatic secretion was 194nmol/min/kg and 249nmol/min/kg respectively. These however, were not significantly different. Similarly, total lipase activity which was 33% higher in 'fat' type animals than in 'lean', could only be attributed to a 57% lower HL in the latter, as LPL in lean animals (which was 72% that of fat lambs) did not differ significantly. This was probably due to the considerable degree of variation within groups. Unfortunately, additional animals were not available at the time of writing and furthermore, the same animals could not be replicated, as Triton WR1339 has a long plasma half life in sheep (Mamo et al. 1983). Both LPL and HL did however, differ between treatments in ruminating postweaned animals. LPL was 30% higher and HL 25% higher in the 'fat' type lambs. The higher rates of postheparin plasma LPL activity seen in the crossbreds, reiterates the relationship of this enzyme with adiposity. Similarly, HL activity positively correlated with LPL activity.

Total postheparin lipase activity was lower in both 'fat' and 'lean' animals after weaning, probably due to the decline in TAG rich milk, the

main dietary component of preruminating lambs. The differences in TAG hydrolase activity, particularly LPL which was the principal component of this reduction, between 'lean' and 'fat' animals on an identical plane of nutrition, suggests that different breeds have a genetically predetermined potential degree of adiposity. In terms of producing leaner sheep in lieu of consumer health concerns, this avenue of research has received little attention.

The toxic symptoms exhibited in Triton WR1339 treated sheep has not been observed in other such studies in this laboratory and has not been previously documented (although cats rapidly exhibit annaphylaxis when given intravenous Triton (Dr. David Topping, personal communication)). However, in the earlier studies, all sheep were housed individually in sheltered pens, whereas the animals in this study were grazed openly. As the chapped black patches were observed only on exposed areas of skin and in addition, as Triton WR1339 is a surfactant which absorbs light strongly in the ultraviolet region, it was considered that the sheep may have suffered extensive sunburn as a result of the circulating Triton. The loss of weight and subsequent death would have resulted from an inability to eat, due to the extensive damage around the lip region.

It has been shown in chapter two that sheep liver possesses a TAG hydrolase comparable to the membrane bound endothelial HL reported for other species. The regulation of this enzyme and LPL, are however, inversely related, in that while LPL activity decreases as a result of diabetes, HL activity is increased. In addition, this investigation has shown that the VLDL particles undergo a physiochemical transformation which actually promotes the activities of these enzymes. It is most

probable that such differences are reflected in the protein compliment of these particles. Chapter three examines for the first time the apoprotein profile of sheep plasma lipoproteins and determines qualitative changes in the VLDL, LDL and HDL apoprotein compliment as a result of diabetes.

CHAPTER 3

3.1 INTRODUCTION

The hypertriacylglyceridaemia in metabolically stressed diabetic or fasted sheep, is a result of an increased rate of hepatic secretion of VLDL associated TAG and a decrease in the subsequent catabolism of these particles by the endothelial lipases. Associated with these differences were changes in the physiochemical composition of the lipoproteins, as demonstrated by differences in their rate of electrophoretic migration and protein/lipid ratio. Apoproteins are the means by which the metabolic processes of lipoprotein metabolism are directed and controlled and so the differences in secretion and catabolism of these particles are probably reflected in shifts in the apoprotein profile. At the start of this project, ovine apoproteins had not been previously reported, though recently, Forte et al. (1983) in a study of sheep lung lymph lipoproteins, described differences in the apoprotein profile of LDL and HDL isolated from the plasma and lung lymph. Isolation and quantification of all the sheep lipoprotein apoproteins was considered to be beyond the aims of this investigation. Rather, this section of this study was concerned with establishing the qualitative apoprotein profile of the major sheep lipoprotein fractions between fed and diabetic animals and to see if differences between treatments, correlated with differences in TAG-VLDL metabolism. Apoprotein B can be readily precipitated by established techniques and its concentration determined. This protein is essential for the release of the TAG rich VLDL and so its content in these particles from both fed and diabetic

animals was also determined.

Apoproteins have been widely investigated particularly in monogastric omnivores and many functions have been attributed to particular proteins. Thus, apoproteins are necessary for the secretion of TAG rich lipoproteins from both the liver and intestine, they mediate receptor binding to hepatic and extrahepatic tissues and regulate the enzymes which promote or inhibit release and uptake of their lipid components. The subsequent uptake of the remnant lipoprotein particles, which result from metabolism by peripheral tissues, is also dictated by the apoprotein compliment, through specific apo-lipoprotein receptors. In addition, apoproteins also play an important structural role. In conjunction with the polar head groups of the phospholipids and unesterified cholesterol, they form the hydrophyllic shell surface of the lipoprotein molecule (as discussed in Chapter one).

Studies of ruminant apoproteins, are however, relatively few and indeed the full compliment of ovine plasma apoproteins has not been previously reported. Limitations, defects or changes in the secretion of hepatic VLDL, lipolysis of VLDL-TAG and uptake of the remnant particles attributable to an abnormal apoprotein profile, can thus only be speculated upon, based on the wealth of information available for humans, rats and other monogastric animal species used principally as models of man. These extensive investigations of the structure and function of the different apoproteins are summarized in a number of reviews (Hatch and Lees 1968, Eisenberg and Levy 1975, Jackson et al. 1976, Morrisett et al. 1977, Osborne and Brewer 1977, Schaefer et al. 1978, Smith et al. 1978, Eisenberg 1979, Havel 1980, Scanu and Landsberger 1980, Brewer 1981, Miller and Gotto 1982, Kane 1983, Turpin

and De Gennes 1983, Mahley et al. 1984, Dolphin 1985 and Sparks and Sparks 1985). It is not intended here to provide an exhaustive and comprehensive treatise, but rather, to briefly summarize the major apoproteins and discuss their role in relation to lipoprotein metabolism, particularly TAG-rich VLDL.

3.1.1 HUMAN APOPROTEINS; STRUCTURE AND FUNCTION

Table (3.1) lists the major human apoproteins, their sites of biosynthesis, plasma lipoprotein distribution and ascribed function. The apoproteins are very different in both structure and function, with molecular weights some 60 fold different. Nevertheless, the apoproteins can generally be divided into those required for de novo synthesis of lipoprotein particles, enzyme activation and receptor binding.

Apoproteins AI and AII constitute greater than 90% of HDL protein. Of this 65% is AI and 25% AII. Human apoprotein AI is also present on chylomicrons, but is rarely found in significant amounts on their remnants, VLDL, IDL or LDL. Both apoproteins have highly ordered amphiphylic helical structures, which are thought to be of critical importance in the binding properties of all of the associated apoproteins, with the lipid components of the lipoprotein particle. (Jackson et al. 1975). Apoprotein AI is a single polypeptide of 243 amino acids and a molecular weight of 28,100, of which several isoforms are known to exist. It is synthesized in both the liver and intestine, though little is known of their relative contributions and factors which regulate them. Hepatic apoprotein AI is thought to enter the circulation in association with nascent HDL particles which have little or no core

Table 3.1

Table 3.1 lists the major human plasma apoproteins, their sites of biosynthesis, plasma apoprotein distribution and ascribed function.

Apoprotein	Estimated Mol. Wt.	No. amino acid residues	Major site of biosynthesis	Carbohydrates present?	Plasma distribution	Ascribed functions
AI	28,100	243	Intestine and liver	No	HDL3, chylomicron	LCAT activation; receptor binding
AII	17,000	77	Intestine and liver	No	HDL3, chylomicron	HL activator
AIII	22,700-32,500	?	?	Yes	HDL2, HDL3, VHDL	Unknown
AIV	46,000	371	Intestine	No	HDL, chylomicron, VHDL	LCAT activation
Bh*	549,000	?	Liver	Yes	VLDL, LDL	receptor binding; particle formation
Bl*	264,000	?	Intestine	Yes	chylomicron	particle formation
CI	6,605	57	Liver	No	chylomicron, VLDL, HDL	LCAT activator; LPL activator?
CII	8,840	79	Liver	No	chylomicron, VLDL, HDL	LPL activator
CIII	8,750	79	Liver	Yes	chylomicron, VLDL, HDL	LPL inhibition; inhibits hepatic clearance of particles
E	34,200	299	Liver	Yes	chylomicron, VLDL, LDL, HDL	receptor binding; LCAT activation

* Two species of apoprotein B have been identified in humans and rats (Sparks and Sparks (1985)).

Table 3.1

of cholesterol ester. As such, it is not surprising that apoprotein AI serves as a cofactor or activator of LCAT (Fielding et al. 1972a). This mechanism is not entirely understood, though Soutar et al. (1975) showed that the degree of activation was dependent on the fatty acid composition of the phospholipid substrate.

Apoprotein AII is a dimer (in man and chimpanzee) of 77 amino acids with a molecular weight of 17,000. Apolipoprotein AII has been reported to enhance the activity of HL (Jahn et al. 1983) and may thus promote further hydrolysis of IDL-TAG to form LDL particles. Apoprotein AII is an antagonist of apoprotein AI, in that it reduces the ability of this protein to activate LCAT (Fielding et al. 1972a).

Apoprotein AIV is a prominant component of newly secreted chylomicrons, although it is not found in significant amounts in the remnant particles, VLDL or LDL. There also appears to be interspecies variation, as unlike human HDL this apoprotein is a major constituent of rat HDL (Mahley et al 1984). Apoprotein AIV is synthesized almost exclusively by the liver and intestine. Its amino acid sequence of 371 residues, means this protein has an apparent molecular weight of 46,000. Like a number of the apoproteins, AIV is a glycoprotein containing carbohydrate prosthetic groups. Apoprotein AIV also activates LCAT, but its ability to do so is only 25% that of apoprotein AI (Albers et al. 1984).

Apoprotein AIII (which is also referred to as apoprotein D) has a molecular weight of approximately 22,700-32,500 daltons. It is a glycoprotein containing 18% carbohydrate (Kostner 1974, and Fielding et al. 1972a). The site of synthesis of apo AIII and its function are as yet unresolved.

Apoprotein E is the most widely distributed protein unit of lipoproteins being a constituent of chylomicrons and their remnants, VLDL, LDL, and HDL (Curry et al. 1976 , Kushwaha et al. 1977). Apoprotein E is composed of 299 amino acids, has a high arginine content (17%) and a molecular weight of 34,200 daltons. Many isoforms have been reported, though the molecular basis for the presence of these is unknown. Apoprotein E has been extensively studied and as a result, a number of functions have been ascribed to it. The major role of apoprotein E appears to be its involvement in receptor mediated binding and uptake of lipoproteins through either the apoprotein B,E (LDL) or apoprotein E receptors. It is therefore a critical regulator of cholesterol transport. Apoprotein E is also involved in the formation of cholesterol ester-rich particles, as this protein also activates LCAT (though its ability to do so is much less than apoprotein AI). Apoprotein E has been implicated in the processing of beta-VLDL, inhibition of mitogenic stimulation of lymphocytes and in the metabolic regulation of the central nervous system (Mahley et al 1984). The liver is thought to be the major site of apoprotein E synthesis, although recently, a number of peripheral tissues including the brain, adrenal, spleen, ovary, kidney and muscle have also been shown to possess the capacity to synthesize this protein. (Basu et al. 1981, Blue et al. 1983, Driscoll and Getz 1983, Boguski et al. 1984 and Reue et al. 1984).

Apoprotein B is an obligatory structural component of the TAG rich lipoproteins, as demonstrated by subjects with abetalipoproteinaemia (who cannot synthesize apoprotein B), which feature a plasma lipoprotein component devoid of VLDL or chylomicrons respectively (Gotto et al. 1971 and Malloy and Kane 1982). Unlike the other apoproteins, apoprotein B

does not transfer to other particles, but rather remains an integral part of the lipoprotein. Thus, apoprotein B is also a major component of the remnant chylomicron particles and the metabolic products of VLDL catabolism, namely IDL and subsequently LDL. In man, apoprotein B represents 10-20% of chylomicron protein, 40% of VLDL protein and 90% of LDL protein. Apoprotein B has proven immensely difficult to characterize due to its insolubility in aqueous buffers after lipid removal, sensitivity to oxidation and susceptability to cleavage by proteases. Nevertheless, in recent years progress has been made in its characterization. Kane et al. (1980, 1983) and Malloy et al. (1981) identified two distinct forms of apoprotein B, namely apoprotein B100 (or Bh) which is synthesized by the liver and so is the obligatory component of VLDL and apoprotein B48 (or Bl) which is synthesized by the intestine and is thus usually associated with chylomicrons. In the rat however, the liver synthesizes both apoproteins B100 and B48 and both forms of the apoprotein are associated with VLDL particles in this species (Sparks et al. 1981 and Bell-Quint et al. 1981). There have been many estimates of the molecular weight of apoprotein B, which reflect the inherent difficulties associated with its characterization. The general consensus of opinion now, is that apoprotein B100 has a molecular weight of 549,000 and apoprotein B48-264,000. Apoprotein B possesses some 4-8% carbohydrates (Sparks and Sparks 1985). Apoprotein B100 is also critical for the receptor mediated uptake of LDL through the apoprotein B,E receptor, which is the means by which the majority (50% in man) of LDL are catabolized (Brown and Goldstein 1983).

Other molecular weight variants of apoprotein B have been identified whose metabolic significance remain unclear. As such,

consideration of these proteins is beyond the scope of this review.

There are three apoproteins other than B in VLDL, namely the low molecular weight proteins designated as apoproteins CI, CII and CIII. They are secreted predominantly by the liver, although there is some intestinal contribution (Windmueller et al. 1973, Imaizumi et al. 1978 and Glickman et al. 1978). In man they make up some 5-40% of the total VLDL protein, however, they are also found in significant quantities in chylomicrons and HDL. The C apoproteins are generally associated with the catabolic processes of lipoprotein metabolism and hence, are inevitably involved with the activation or inhibition of enzymes. The C apoproteins are not directly involved with receptor mediated clearance though the presence of some of these proteins may inhibit binding of the lipoprotein particle. This class of apoproteins causes significant conformational change when binding to lipids and so they may also play an important role in the structure of the lipoprotein.

Apoprotein CI has a molecular weight of only 6605 with 57 amino acids. This protein activates LCAT though to a lesser extent than apoprotein AI (Soutar et al. 1975).

Apoprotein CII is a single polypeptide chain of 79 amino acids with a calculated molecular weight of 8,840. Like apoprotein CI this protein does not contain any carbohydrate. In VLDL, apoprotein CII is approximately 10% of the total protein and thus, is a major protein component of this particle. Apoprotein CII is an essential cofactor in activating LPL (La Rosa et al. 1970 and Havel et al. 1973).

Apoprotein CIII is the major apoprotein C component of VLDL and in fact may comprise upto 30% of the total protein in this fraction. Apoprotein CIII also makes up approximately 45% of the total protein in

chylomicrons and 3-10% in HDL (Brewer et al. 1974). Apoprotein CIII is a single polypeptide chain of 79 amino acid residues with a molecular weight of 8,750. There are three isoforms of the protein which differ in their sialic acid content. The precise metabolic role of apoprotein CIII and the significance of the sialic acid heterogeneity is unclear. Apoprotein CIII has a nonspecific inhibitory effect on LPL (Brown and Baginsky 1972 and Krauss et al. 1973b). Shelburne et al. (1980) and Windler et al. (1980) suggested that the presence of apoprotein CIII may modulate the uptake of TAG rich remnants by hepatic receptors. Apoprotein CIII has also been demonstrated to activate LCAT (Jonas et al. 1984).

In addition to the apoproteins already discussed, there are others of minor amounts, whose functions have not yet been elucidated.

3.1.2 METABOLISM OF TRIACYLGYCERIDE RICH-LIPOPROTEINS; ROLE OF APOPROTEINS

The apoprotein regulation of lipoprotein metabolism through various anabolic/catabolic processes requires a continual shift in the spectrum of the apoproteins. This is achieved in a directed cycling fashion, whereby the apoproteins exchange between newly secreted lipoproteins and existing plasma lipoproteins. The binding or release of specific apoproteins from the surface of the particles is modulated by three factors, the lipid binding properties of the particular apoprotein, the composition of the surface lipids of the lipoprotein and also the size of the particle. This relationship is bi-directional, as subsequently, the nature and ratio of the surface lipids and the core lipid components

are modulated by the activity of the apoprotein stimulated enzymes and proteins, which mediate the exchange of the lipid components.

Apoprotein B is essential for both the secretion of TAG-rich VLDL and for the uptake of the product of its metabolism, namely LDL, by the apoprotein B,E (LDL) receptor. It does not however, appear to be a major regulatory factor in the catabolism of these particles. The synthesis and secretion of VLDL has already been discussed in the general overview. Of primary interest now is whether the genetic, dietary and hormonal factors which affect hepatic TAG synthesis and release, mediate this through the quantity of VLDL particles (apoprotein B secretion) secreted.

The newly secreted VLDL are considered to be relatively inert, even though they contain a full compliment of the apoproteins usually associated with this particle (Marsh and Sparks 1979, 1982, Rash et al. 1981 and Bell-Quint and Forte 1981), probably because the presence of the C apoproteins are at a much lower proportion in nascent VLDL, than in plasma VLDL (Hamilton et al. 1976). The metabolic transformations surrounding nascent VLDL and the catabolic events which transpose these TAG rich particles to LDL are depicted in figure 3.1 . The newly secreted particles attain a further complement of C and E apoproteins, essentially from the large pool of plasma HDL. It should be realized that the apoprotein content of VLDL varies depending on the protein in question. Apoprotein B per VLDL particle remains constant (one apoprotein B molecule per lipoprotein particle) and does not differ significantly from that of an LDL particle (Eisenberg and Levy 1975), because this protein remains with the particle throughout its biological catabolic life. Howevever, in contrast, the concentration of apoproteins

Figure 3.1

Figure 3.1 is a diagramatic representation of the apoprotein regulated metabolic transformations surrounding nascent very low density lipoproteins and the catabolic events which transpose these triacylglyceride rich particles to low density lipoproteins, within the human plasma compartment.

 - apoprotein AI

 - apoprotein AIII

 - apoprotein B

 - apoprotein C

 - apoprotein E

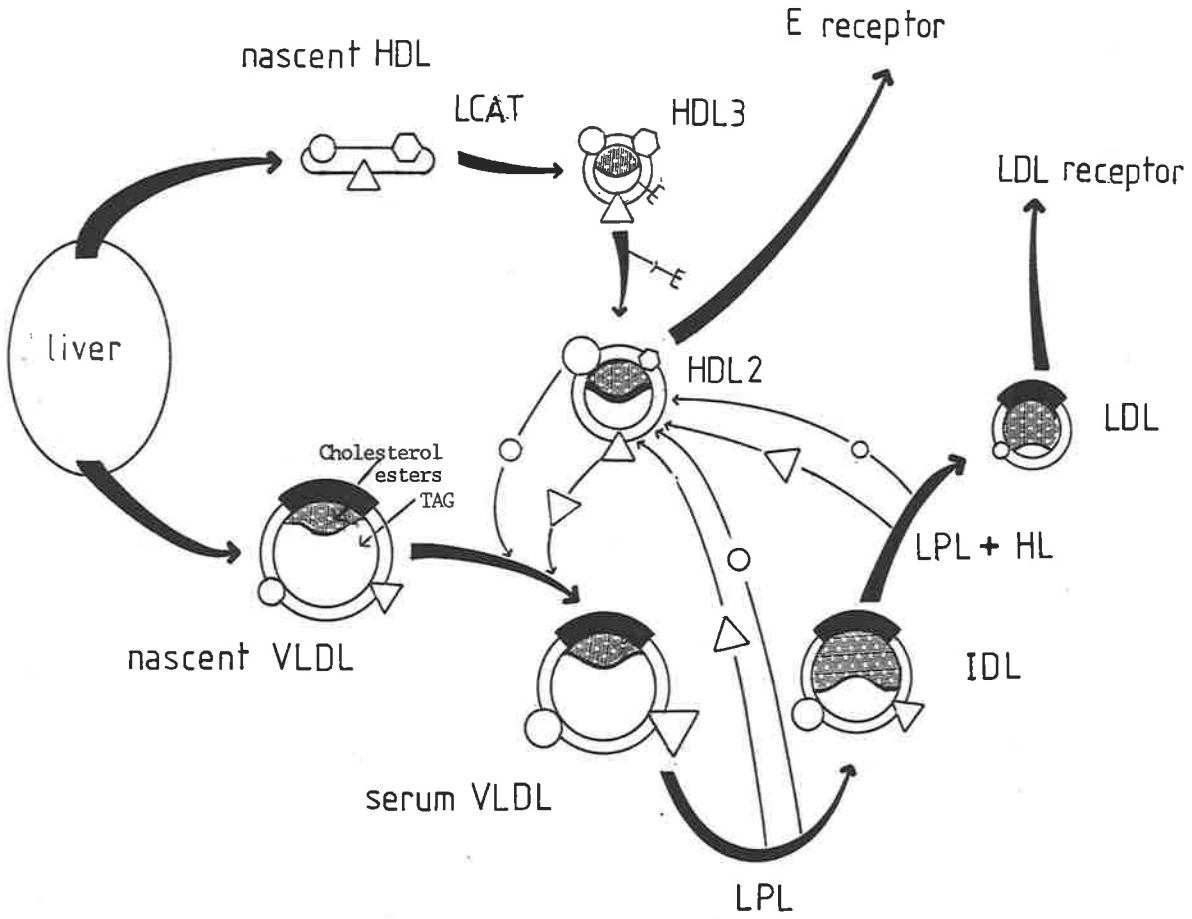

nascent HDL
LCAT
HDL3
E receptor
LDL receptor
liver
HDL2
LDL
Cholesterol
esters
TAG
LPL + HL
nascent VLDL
IDL
serum VLDL
LPL

fig. 3.1

CI, CII, CIII and E in plasma VLDL is dependent upon the density (or stage of catabolism) of the particle. That is, apoprotein C content increases whilst apoprotein E decreases relative to apoprotein B, as the TAG component is hydrolysed and the density of the particle is increased (Eisenberg 1979). Apoprotein CII is required as an obligatory cofactor for the LPL mediated hydrolysis of VLDL-TAG to diacylglycerides, monoacylglycerides and free fatty acids (Havel et al. 1970). An apoprotein CI stimulated LPL has been reported (Fielding and Fielding 1976), though generally this protein is thought to inhibit LPL (Bensadoun et al. 1974). Reaction of VLDL with LPL results in a marked reduction in particle size and depletion of core TAG. Surface components including cholesterol, apoproteins CII and CIII and some apoprotein E are lost as a result of this hydrolysis and are transferred to particles which isolate in the HDL2 range. The decline in apoprotein E content in VLDL is, however, slower than the decline in apoprotein C (Mjos et al. 1975). Apoprotein CIII also has a nonspecific inhibitory effect on LPL activity and it may be that the ratio of apoproteins CII/CIII, control lipolytic rate (Dolphin 1985). Apoprotein E has also been reported to inhibit LPL (Ganesan et al. 1976) but this could not be confirmed (Fielding and Fielding 1977). Apoprotein CIII, a major constituent of essentially unmetabolized VLDL inhibits the interaction of the lipoprotein particle with the apoprotein E receptor (Shelburne et al. 1980 and Windler et al. 1980), thus preventing premature removal of the TAG-rich particle. As apoprotein CIII is lost during VLDL catabolism to LDL, the inhibition of interaction of the particle with the LDL receptor is removed (Schonfeld et al. 1979). Although potentially apoprotein E could also interact with the apoprotein E receptor, in humans this does

not appear to be the case and factors governing this preferential uptake remain unresolved. It may be that the apoprotein B molecule interferes with the binding of the apoprotein E and its receptor. There have been numerous investigations into the apoprotein B,E receptor pathway, discussion of which is beyond the scope of this review.

3.2 METHODS AND MATERIALS

3.2.1 ANIMALS

Fed sheep and alloxan induced diabetic sheep were housed and maintained as already described (Section 1.2.1).

The major lipoprotein fractions were isolated by serial ultracentrifugation as described in section 1.2.4.6. The VLDL fraction was washed by recentrifuging the lipoprotein concentrate for 20h at 20°C with the 1.96M NaBr buffer of solvent density 1.0063g/ml (unless stated otherwise).

3.2.2 PROTEIN EXTRACTION

A modified procedure of the method described by Cham and Knowles (1976) designed for the delipidation of plasma lipoproteins from grossly hypertriacylglyceridaemic subjects was used.

To 200ul of lipoprotein fraction in Eppendorf tubes 800ul of a mixture of n-butanol/diethyl ether (15:85 v/v) was added. The tubes were mixed thoroughly by rotating end over end for 45-60min at room temperature. The samples were centrifuged for 5min and the organic supernatant discarded. The process was repeated by the addition of another 800ul of solvent. After aspirating the solvent phase, the lower aqueous phase was kept under a gentle stream of high purity nitrogen for 5min to remove all traces of solvent.

3.2.3 APOPROTEIN B AND SOLUBLE APOPROTEIN DETERMINATION

Total protein content of each fraction was determined by the modified Lowry method described in section 1.2.6. Selective precipitation of apoprotein B was achieved by addition of iso-propanol, as described by Holmquist and Carlson (1977). Total soluble apoproteins (A,C, and E) were then determined and the apoprotein B content calculated by subtracting the soluble proteins from the total proteins. Standard protein solutions of bovine serum albumin also contained 10% isopropanol for the soluble protein assay.

3.2.4 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the apoproteins was run on 180mm X 200mm X 0.7mm or 180mm X 200mm X 1.5mm gels, with a 4% to 30% acrylamide gradient containing 2.4% crosslinker (bis-acrylamide), made up in a modified Laemmli discontinuous buffer (0.375M Tris-HCl, pH 8.8, 0.1% SDS). Plates were cast using a Pharmacia GSC-2 gel casting apparatus. Each gel was overlayed with 2-3cm of stacking gel (4% acrylamide, 2.7% crosslinker, 0.125M Tris HCl, pH 6.8, 0.1% SDS). Acrylamide gels were polymerised with 10% ammonium persulphate and N,N,N',N'-Tetramethylethylenediamine (TEMED).

To each aliquot of protein extract an equivalent amount of Laemmli sample buffer, containing 0.01M Tris-HCl, pH 8.0, 0.001 EDTA, 1% SDS and 5% mercaptoethanol was added, thoroughly mixed and heated at 100°C for 5-10min. Glycerol (50% solution) was added to increase the density of

the protein solution and bromophenol blue (0.1%) was also added to monitor the rate of electrophoretic migration.

If the protein concentration of lipoprotein fractions was low (less than 5ug/50ul), samples were concentrated under vaccum (100Kpa) in a Savant Speedvac centrifuge (approximately 1500 r.p.m.) at 30°C until a suitable concentration was achieved.

5-20ug of lipoprotein protein was added for each lane of the gel. Each lane containing protein was separated by a lane containing buffer only. Each gel had a complete compliment of both high and low molecular weight markers (section 3.2.5).

The SDS-acrylamide gradient gels were subject to electrophoresis in 0.05M Tris-HCl, 0.348M glycine, pH 8.3, 0.1% SDS, in a Pharmacia GE 2/4LS water cooled, circulating tank. Constant voltage was maintained at 100V until each sample had migrated through the stacking gel and into the separating gel. Voltage was then increased (150V) and maintained for a total of 1500Vh. The gels were carefully removed, fixed and stained in 0.1% Coomassie Blue R-250 made up in methanol:acetic acid:water (250:100:650) for a minimum of 6h. Destaining took approximately 17h with 3 X 1L changes of methanol:acetic acid:water (250:100:650). Gels were dried in a Bio Rad dual temperature slab gel dryer (model SE1125B) at 60°C for 2-3h.

Nth order exponential regression was applied to the molecular weight protein standards of each gel. Regression was better than $r^2 = 0.92$. The Rf of each sample band was then interpolated according to the standards, a molecular weight determined, and a tentative identity assigned.

3.2.5 MATERIALS AND REAGENTS

Acrylamide and bis acrylamide (electrophoresis grade) and TEMED (99% pure) were purchased from Sigma Chemical Company. Low and high molecular weight markers were purchased from Bio-Rad Australia. The low molecular weight group contained lysozyme (14,400) soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200) and phosphorylase B (92,500). The high molecular weight markers were bovine serum albumin, phosphorylase B, beta galactosidase (116,250) and myosin 200,000. The gel casting apparatus, and electrophoretic tank were purchased from Pharmacis Pty. Ltd., Uppsala, Sweden.

3.3 RESULTS

3.3.1 APOPROTEIN PROFILE OF FED AND DIABETIC SHEEP

A number of gradient gels which differed in their thickness, running time, amount of total protein and source of apoprotein were run, the results of which are tabulated in table 3.2. Apoprotein components of particular lipoprotein fractions were not always detected in all acrylamide gels, principally because either the lipoprotein concentration was very low, or the respective band was overlapped with adjacent more abundant proteins of similar molecular weight. Nonetheless, a qualitative apoprotein profile of each of the major ovine lipoprotein classes has been given. The apolipoprotein distribution of sheep plasma VLDL, LDL and HDL on SDS-PAGE (4-30%) is shown in figures 3.2 and 3.3. The qualitative nature of the lipoprotein apoproteins contained proteins with molecular weights similar to apoprotein C's (less than 11,000 daltons), apoprotein AII (15,500 daltons), apoprotein AIII (21,500 daltons), apoprotein AI (25,500 daltons), apoprotein E (33,500 daltons) apoprotein AIV (47,500 daltons) and apoprotein B (greater than 65,000 daltons) (table 3.2). The methods described did not permit absolute identification of the apoproteins due to the unavailability of suitable antisera. However, the respective apoproteins which are common to rat VLDL, namely the B, C and E apoproteins, had similar rates of migration and molecular weights within the given ranges used to identify the ovine compliment (table 3.2, figures 3.2 and 3.3).

The major apoprotein components of VLDL on this basis from fed sheep were apoprotein B (molecular weight bands 60,000-465,000; these

Table 3.2

Table 3.2 lists the apoprotein compliment of each of the major sheep plasma lipoproteins, in both fed and diabetic animals. The identity of the apoproteins were based on their molecular weight determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and the apoprotein profile of rat plasma very low density lipoproteins.

			VLDL		LDL		HDL	
total number of gels;			Fed (17)	Diabetic (17)	Fed (6)	Diabetic (6)	Fed (5)	Diabetic (6)
Estimated molecular weight (daltons).	Ascribed apoprotein	Estimated mol. wt. of rat apoproteins	number of gels detected		number of gels detected		number of gels detected	
65,000-475,000	B	-	17	17	6	6	1*	2*
39,800-53,000	AIV	45,000-50,100	6	6	3	4	4	5
31,000-36,000	E	28,000-33,400	11	11	3	3	4	5
23,000-28,000	AI	27,500	4	10	5	3	4	4
21,000-22,000	AIII	-	-	-	-	3	4	4
11,700-16,500	AII	14,000-19,500	2	9	1	-	4	4
6,600-10,000	C	11,000	2	6	-	-	-	-

* these bands were not replicable

Table 3.2

Figure 3.2

The apoprotein composition of ovine lipoproteins given by sodium dodecyl sulphate polyacrylamide gel electrophoresis is shown in figure 3.2. Coomassie blue stained gradient gels as indicated are;

(a) lane 1 - low molecular weight markers
lane 2 - high molecular weight markers
lane 3 - fed sheep VLDL
lane 4 - diabetic sheep VLDL
lane 5 - fed sheep LDL
lane 6 - fed sheep HDL

(b) lane 1 - high molecular weight markers
lane 2 - fed sheep VLDL
lane 3 - diabetic sheep VLDL
lane 4 - diabetic sheep LDL
lane 5 - diabetic sheep HDL

(c) lane 1 - low molecular weight markers
lanes 2 and 3 - rat VLDL
lane 4 - high molecular weight markers

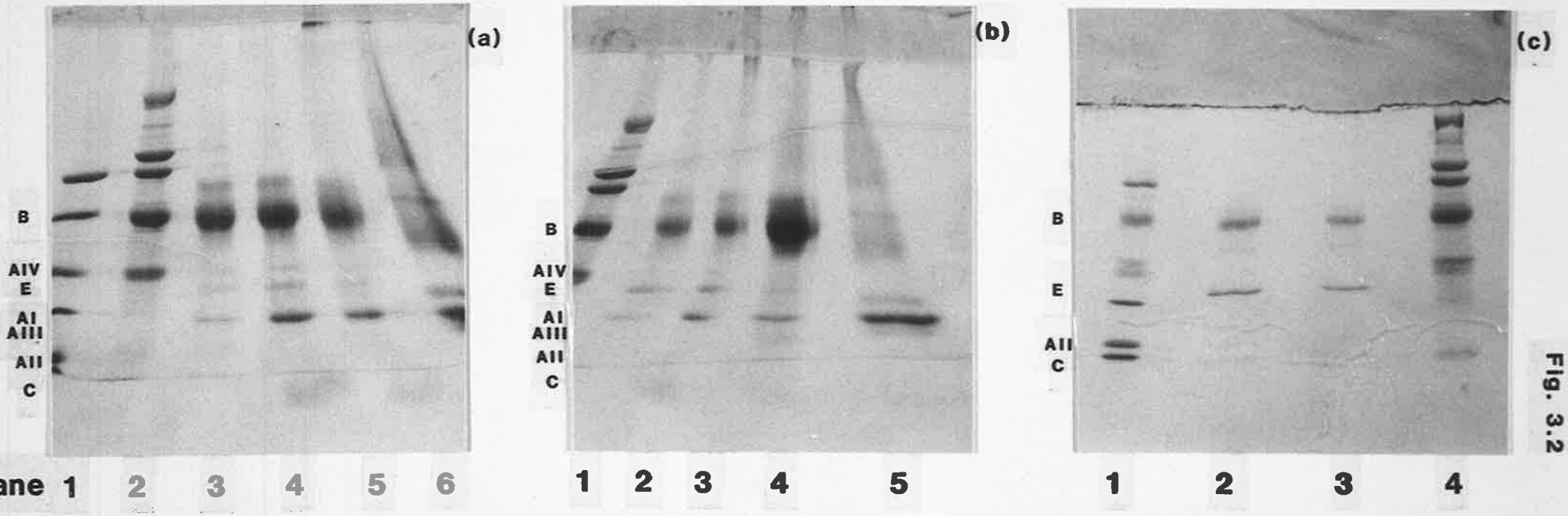

(a)
B
AIV
E
AI
AIII
AII
C
Lane 1 2 3 4 5 6
(b)
B
AIV
E
AI
AIII
AII
C
1 2 3 4 5
(c)
B
E
AII
C
1 2 3 4

Fig. 3.2

Figure 3.3

The apoprotein composition of ovine lipoproteins given by sodium dodecyl sulphate polyacrylamide gel electrophoresis is shown in figure 3.3. Coomassie blue stained gradient gels as indicated are;

(a) lane 1 - fed sheep VLDL

lane 2 - rat VLDL

lane 3 - low molecular weight markers

(b) lanes 1 and 5 - low molecular weight markers

lanes 2 and 6 - high molecular weight markers

lane 3 - fed sheep HDL

lane 4 - diabetic sheep HDL

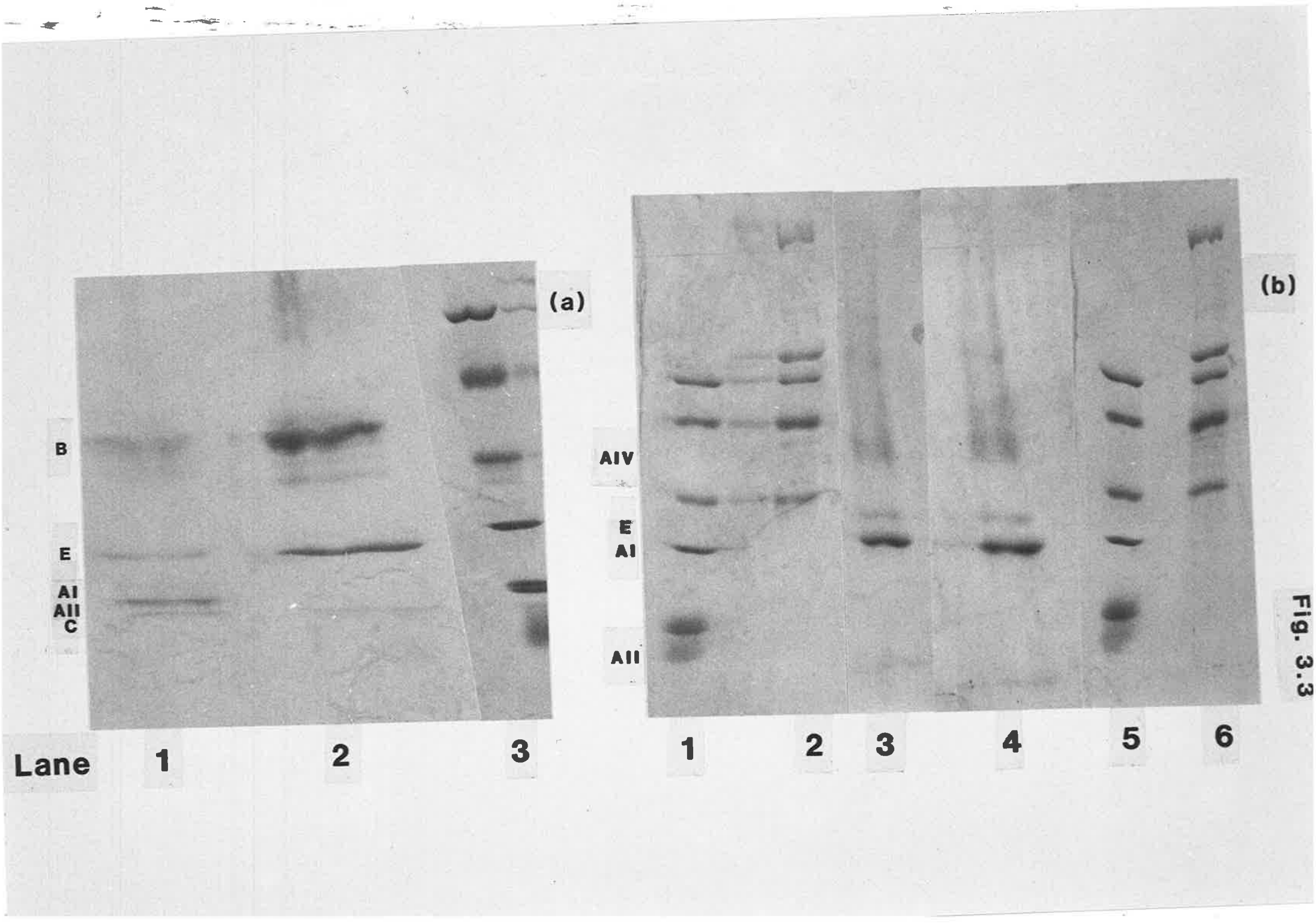


Fig. 3.3

were proteolytic products of this protein), apoproteins AI, AII and AIV, the C group apoproteins and E. The rate of migration of apoprotein AII did not differ markedly from the C group proteins and though on the basis of molecular weight (11,700-16,500 daltons)this protein has been considered as apoprotein AII, it may in fact have reflected a C apoprotein. Visual examination suggested that after apoprotein B, in order of decreasing amounts, apoprotein AI predominated, followed by apoprotein E, apoprotein AII and of lesser significance, apoproteins AIV and C. There were no qualitative differences in the apoprotein profile of VLDL from fed or diabetic origin.

The apoprotein LDL profile from fed sheep was predominantly apoprotein B. However, other apoproteins which featured prominantly (in decreasing significance) were apoproteins AI, E and AIV. Apoprotein AII was apparent in only one rather heavily loaded gel. The apoprotein LDL profile from diabetic sheep was different from that of their fed counterparts in that apoproteins AII and AIV were not detected in any of the gels. Conversely, in diabetic LDL, an additional band near the apoprotein AI region and of approximate molecular weight 22,000, but nonetheless distinct from apoprotein AI was visualized in a number of gels. This was considered to be the 'thin-line peptide' apoprotein AIII. There were no bands in LDL from either fed or diabetic animals, which stained for proteins in the apoprotein C region.

The apoprotein HDL compliment from either fed or diabetic animals was not different between these two groups. Apoprotein AI was the major HDL component with AII also staining in most gels. Bands in the apoprotein C region were not detected. In all apoprotein HDL gels, there was a protein staining in the 31,000-36,000 dalton range. Although this

was regarded as apoprotein E, this protein migrated between the apoprotein AI and apoprotein E (23,000-36,000 daltons) of VLDL and LDL. This protein may be a reflection of an isoform of apoprotein E, or alternatively, may represent another protein such as apoprotein F whose molecular weight is estimated at 30,000 daltons. Apoprotein AIV was also present in ovine HDL. However, its molecular weight under these conditions was approximately 53,000 daltons, somewhat higher than the molecular weight of 44,000 daltons attributable to this apoprotein in the sheep VLDL and fed LDL fraction. On several occasions high molecular weight proteins (greater than 80,200) were found in the sheep HDL fractions. These however, were not replicable.

3.3.2 APOPROTEIN B CONTENT OF SHEEP LIPOPROTEINS

Table 3.3 lists the total apoprotein content of VLDL in fed and diabetic sheep. There was an increase of 358% total protein in diabetic sheep, however, the proportion of apoprotein B versus the total soluble apoproteins (A, C, and E) remained constant. Similar analysis of the pooled LDL fraction from three fed animals and three diabetic sheep showed that apoprotein B made up 73% and 93% of the total protein content respectively. Analysis of apoprotein HDL from either fed or diabetic animals, showed that the total protein was soluble in isopropanol and therefore, that no apoprotein B was present.

3.3.3 EFFECT OF ULTRACENTRIFUGATION ON APOPROTEIN RECOVERY

Because the three diabetic sheep used in this experiment were

Table 3.3

Table 3.3 lists the total apoprotein and percentage apoprotein B content of each of the major sheep plasma lipoproteins from both fed and diabetic animals.

(n) ± x.x=number of animals ± the standard deviation of the mean

LIPOPROTEIN FRACTION	Total plasma apoprotein ug/ml	% apoprotein B	mg apoprotein B per 100mg plasma lipoprotein
Fed VLDL	84(3) ± 42	49(3) ± 1%	1.6(3) ± 0.9
Diabetic VLDL	301(3) ± 80	50(3) ± 2%	7.5(3) ± 1.7
Fed LDL *	73	73%	5.6
Diabetic LDL *	138	93%	6.2
Fed HDL *	420	0%	-
Diabetic HDL	826	0%	-

* These results are based on the pooled lipoprotein fraction from three sheep.

Table 3.3

grossly hypertriacylglyceridaemic, recentrifugation (20h) of the plasma of these animals in the less than 1.0063g/ml solvent density range was required to totally recover this fraction. An average of 26% of VLDL was not recovered on the first centrifugation (20h) in these animals. Total plasma VLDL from fed sheep was recovered with only one period of centrifugation.

To investigate the effects of prolonged ultracentrifugation on the apoprotein content of these particles, the buffer overlay of density 1.0063 used to 'wash' the VLDL fraction was also assayed for total protein, soluble protein and apoprotein B. Table 3.4 shows that substantial losses of apoprotein resulted. The ratio of apoprotein B versus total (or soluble) apoproteins was equivalent to that of the intact particles, namely a 49% apoprotein B content. This being the case, analysis of VLDL protein before and after 'washing', indicated that in fed and diabetic sheep an 82% and 62% loss of apoprotein components occured as a result of this procedure respectively.

Table 3.4

Table 3.4 lists the recovery of very low density lipoprotein apoproteins based on the protein content of the 1.006g/ml buffer overlay used to 'wash' the lipoprotein fraction.

LIPOPROTEIN FRACTION	Buffer total protein ug/ml plasma	% recovery	Buffer soluble protein ug/ml plasma	% recovery	Buffer apoprotein B ug/ml plasma	% recovery	% apoprotein B
Fed VLDL	473(3) ± 167	18(3) ± 1%	244(3) ± 82	18(3) ± 1%	226(3) ± 84	18(3) ± 1%	49(3) ± 1%
Diabetic VLDL	792(3) ± 205	38(3) ± 2%	401(3) ±101	36(3) ± 2%	391(3) ± 111	41(3) ± 1%	49(3) ± 1%

Table 3.4

3.4 DISCUSSION

This study represents the first examination of the apoprotein profile of all sheep plasma lipoproteins. Identity has been based on rates of migration, molecular weights and homogeneity with the documented apoprotein compliment of rat VLDL using SDS-PAGE. Confirmation of these by other means, such as antibodies raised against specific apoproteins, could not be done in this laboratory at the time of writing. Nevertheless, bearing in mind that this report represents preliminary investigations, the sheep apoprotein profile which has been presented will be discussed, in view of the role these proteins have in lipoprotein TAG metabolism within the plasma compartment.

The qualitative profile, in terms of the total spectrum of plasma apoproteins, shows that sheep are similar to other species such as the rat or humans, in that each of the major known apoproteins, namely the A, B, C and E group of proteins, were present. The distribution of these between VLDL, LDL and HDL, did however, reveal some unusual features in comparison to that for man.

Sheep VLDL contained proteins which in terms of molecular weight, corresponded with apoproteins AI, AII, AIV, B, C and E. In man the B and C proteins are the major components with AI, AII and AIV present only in trace amounts. Unfortunately, a gel density scanning device was not at the disposal of this laboratory and so quantitation of these was not possible. Nevertheless, visual examination of the acrylamide gels, clearly showed that apoprotein AI was the major component of sheep VLDL after apoprotein B. Though of less significance, apoproteins E, AII, AIV and C were also detected on a number of occassions. The protein

recognised as apoprotein AII had a migration not greatly different from the C group apoproteins and though its calculated molecular weight was equivalent to that usually associated with apoprotein AII, this band may in fact represent a C apoprotein. The A group of proteins are together responsible for the activation of LCAT, stimulation of hepatic lipase and receptor binding and so the results presented here suggest that the liver could play a major role in the catabolism of TAG rich VLDL particles. In chapter two, it was seen that HL formed an integral part of the membrane bound TAG lipase potential associated with plasma and furthermore, that this activity was significantly increased in diabetic wethers. It may be that an increase in these components, or perhaps a change in the ratio of particular apoproteins in diabetic VLDL promoted this activity. The higher rates of VLDL-TAG HL hydrolysis in particles from diabetic animals also suggest that this may be an apoprotein stimulated effect (perhaps increased apoprotein AII). Streptozotocin induced diabetic rats, unlike their fed counterparts, also have an apoprotein AIV component in their VLDL fraction, (Bar-On et al. 1976) though these rats are associated with a lowered HL activity (Nakai et al. 1979).

Another observation of potential interest in the sheep VLDL fraction was that the C group apoproteins did not appear to be of major significance, as this class of apoproteins was detected in relatively few gels (20% total VLDL gels). If however, the protein designated as apoprotein AII is in fact a C apoprotein, then their contribution to the total protein spectrum will of course be much greater. Assuming that this is the case, the results presented here suggest that the C apoproteins have molecular weights considerably higher than that

reported in other species. A low apoprotein C content in sheep VLDL could explain the relatively low postheparin plasma lipase activities, (in comparison to rats or man) observed in these animals under fed conditions. Alternatively, the apoprotein CII/CIII (CI:CII:CIII?) ratio may be such that activity is not optimized. Low levels of apoprotein CIII in sheep VLDL, would also support an enhanced hepatic contribution to the metabolism of these particles, as the inhibitory affect of this protein on the apoprotein B,E receptor would be reduced. The 'C' apoproteins were more readily detected in diabetic VLDL, suggesting that the low LPL activity in fed sheep was not a result of deficient apoprotein C in these particles. In fact, the higher rates of diabetic sheep VLDL-TAG LPL-lipolysis, which was nearly three fold higher than the same fraction from fed animals, could reflect an improved apoprotein C (perhaps greater CII) compliment. Alternatively, the lipoproteins of diabetic sheep were physiochemically different from those derived from normals, probably a result of glucosylation, as suggested by the greater rates of electrophoretic migration. Curtiss and Witzum (1985) have shown that in hyperglycaemic diabetic subjects, apolipoproteins AI, AII, B, CI and E of the TAG rich lipoprotein fraction were glucosylated and it is possible that the higher rates of diabetic VLDL lipolysis may reflect an enhanced capacity for the apoproteins to stimulate the lipase enzymes. However, the physiological significance of apoprotein glucosylation will only be known, when it can be demonstrated that the apoprotein structural changes that accompany glucosylation, also lead to functional changes.

Apoprotein E, was not surprisingly, a readily detected protein in sheep VLDL. It's presence signifies that VLDL is metabolized by the

apoprotein B,E (E?) receptor. It would be interesting to determine the apoprotein CIII/E ratio in VLDL from both fed and diabetic animals in view of the role of these proteins in regulating hepatic clearance of these particles.

Apoprotein B was the major component of both fed and diabetic sheep VLDL, comprising 49% of the total protein compliment in each group. In chapter one, it was shown that the VLDL particles actually contained more protein per unit lipid (table 1.2). The ratio of apoprotein B to total protein remained constant between fed and diabetic animals, and so it could be calculated that there was approximately 1.6mg of apoprotein B per 100mg VLDL in fed animals, which is similar to that found in man (1-5mg/100mg VLDL (Sparks and Sparks 1985)). In diabetic sheep, there was nearly a 5 fold increase of this protein per unit VLDL, that is 7.5mg per 100mg VLDL. The secretion of these particles in metabolically stressed sheep was therefore not reduced or impaired by the rate of synthesis of this obligatory protein.

It was hoped that the electrophoretic pattern of apoprotein B would give an estimation of its molecular weight. However, in consequence, it was considered that inclusion of Ellman's reagent in the centrifugation procedure and extraction of lipid from VLDL with a butanol based solvent mixture, may potentially promote cleavage of this protein into smaller high molecular subfractions (Professor Julian Marsh personal communication). These replicable protein subunits (greater than 65,000) were at first thought to be albumin binding to the lipoproteins, however, the absence of such bands in the HDL fraction, which on the basis of the methods used here to isolate the lipoprotein fractions, would contain the most albumin, coupled with the absence of precipitable

protein by isopropanol in this fraction, indicated that these bands were not albumin. If apoprotein B did not undergo any oxidation or cleavage, then the approximated molecular weight of this protein in sheep VLDL and LDL would be 66,000-80,000, similar to that derived by Olofsson et. al. (1980) who used a 5% SDS-PAGE system coupled with urea gel filtration.

The apoprotein LDL profile of sheep was again different from that usually associated with man. In addition to proteins staining in the apoprotein B and E region, there were others which on molecular weight correlated with apoproteins AI, AII and AIV. Apoproteins AI and AIV are minor components of human LDL and apoprotein AII is not usually associated with this fraction. The protein staining in the AII region was only detected on one occassion, suggesting that this is only a minor component of sheep LDL. Alternatively, if this was a C apoprotein, then this may simply reflect intermediate particles between the transition of VLDL to LDL. Forte et al. (1983) did not detect any 'A' apoproteins in ovine plasma LDL, though conversely, they did report that apoprotein AI constituted 68% of lung lymph LDL. Similarly, in the results presented here, this protein appeared to be the major component in sheep plasma LDL after apoprotein B. The apoprotein AI and AIV component of these particles is readily associated with their high cholesterol ester content (48% in fed sheep and 60% in diabetic animals), both proteins being activators of LCAT. LDL from diabetic sheep were shown to have a higher content of cholesterol-esters and less TAG than this fraction from fed animals. This may reflect an increased apoprotein AI and AIV stimulated LCAT activity and the increased rate of hepatic lipase in diabetic sheep. Apoprotein AII which stimulates HL was not detected in any of the gels for diabetic sheep and so if higher rates of hepatic

lipase are responsible for lower levels of LDL-TAG, then this would not be an effect of this protein in this fraction. Conversely, this could be a result of an increased apoprotein AII promoted activity of this enzyme, in the precursors of LDL, that is, VLDL. In addition, the diabetic LDL fraction featured a protein closely associated with the protein referred to as apoprotein AI, considered to be the thin line peptide apoprotein AIII. This was not observed in the VLDL fraction of these animals and so it can be concluded that this protein was acquired during or after metabolism of the TAG component. The function of this protein is unclear, however, it may be that it too has the capacity to stimulate HL and perhaps LCAT. It is difficult to perceive why this protein was not detected in any of the gels containing fed sheep LDL, but it may be that this emphasises the different catabolic pathways of VLDL between different treatments. The absence of the 'C' apoproteins in ovine LDL was probably a reflection of the same process which occurs in man, namely the hydrolysis of TAG by LPL results in a decreased density of these particles and subsequent loss of the C group apoproteins.

Apoprotein B was the major protein (72%) of LDL from fed sheep, however, it was not as significant as that usually attributable to man (90-95%). This again supports the possibility that the A group apoproteins may be of greater significance in the former species. Conversely, as these results are the expression of pooled plasma from three sheep, they may not portray an accurate measure of apoprotein B in this fraction. Nonetheless, these same sheep having been made diabetic, exhibited a much greater apoprotein B profile (92%), or conversely, a lower soluble protein content. The total protein per unit lipid of LDL from fed and diabetic sheep did not differ significantly (8% and 7%

respectively). However, as their respective metabolic precursors had less protein and more lipid for fed VLDL, or conversely more protein and less lipid for diabetic VLDL, it can be inferred that the metabolism of the core lipid components was greater in fed animals, than in the diabetic ones. In view of the depressed LPL activity observed in the latter, this was not surprising. The greater soluble protein content of fed LDL, suggests that during the catabolism of VLDL, less of these proteins are lost, than during the same process with VLDL of diabetic origin.

The apoprotein HDL profile of man is associated with a high apoprotein AI and AII content. This too, was true for ovine HDL. There was, in addition, a protein staining at 53,000 referred to as apoprotein AIV. This was considerably higher than the approximated molecular weight of 46,000 derived for this protein in the VLDL and LDL fractions and may reflect an isoform of apoprotein AIV. Alternatively this protein may be another apoprotein such as H with a reported molecular weight of 54,000 (Polz et al. 1981), but whose function is unknown. Similarly, the protein desiganted as HDL apoprotein E migrated at a slightly slower rate (36,000 daltons) than that of the same protein in VLDL and LDL (31,000-33,000 daltons). Many isoforms of this protein have been reported, of which this difference in migration may be a reflection. Alternatively, this may be apoprotein F whose function is also unknown (Olofsson et al. 1978). Should this in fact be so, then ovine HDL would appear to have no apoprotein E.

The C group apoproteins could not be detected in the HDL from either fed or diabetic origins based on the molecular weight groupings used in this study. In view of the low levels of these proteins in VLDL,

it may be that their concentration was too low to detect under the conditions described here. Forte et al. (1983) reported that sheep plasma HDL contained a major protein of molecular weight 28,000 daltons, which was considered to be apoprotein AI. The remaining 12-16% of total protein stained in the 8,000 and 12,000 dalton range (possibly C apoproteins). Not unlike the study described here their methods did not allow them to determine if one of these small molecular weight proteins was monomeric apoprotein AII. Therefore, the protein described here as apoprotein AII in this study, may in fact represent a C group protein (as already discussed). If the sheep plasma HDL apoprotein C concentration is relatively small, transfer of these proteins to nascent VLDL may also be limited, which could in part contribute to the relatively low rates of LPL activity observed in fed sheep. The nascent VLDL, could however, acquire C apoproteins from the process of lipolysis of plasma VLDL, by LPL and HL.

In humans, nascent discoidal HDL particles are rapidly transposed into the small, spherical particles known as HDL3. This class of lipoproteins is rich in apoproteins AI and AII and in lesser amounts has a compliment of apoprotein AIII and the C apoproteins, but has no apoprotein E, presumably to avoid premature hepatic removal by the apoprotein E receptor. The HDL3 particles acquire additional cholesterol esters, via the action of LCAT on the surface cholesterol components, and become enlarged to HDL2 type particles, which are also rich in apoprotein AI, and in lesser amounts, apoproteins C and E. They do not have any apoprotein AIII. The loss of this protein with the transformation of HDL3 to HDL2, accompanied by an increased content of cholesterol esters, again suggests that this protein may be associated

with the core lipid components. Lipoprotein analysis in this study does not distinguish between HDL2 and HDL3, however, it was observed in chapter 1 (table 1.2) that the HDL from diabetic animals contained relatively more cholesterol esters (20% fed:23% diabetic) and less TAG (20%fed:11%diabetic). This may be a reflection of differences in apoprotein stimulated lipase and LCAT activities, in which AIII may be involved.

In view of the apoprotein profile which has been described in this study, a proposed pathway for the metabolism of the TAG-rich VLDL particles in both fed and diabetic animals is presented in figure 3.4. Plasma VLDL particles secreted by the liver vary in their composition between treatments. Fed components contain less protein per unit lipid. These particles are metabolised by apoprotein CII stimulated LPL and apoprotein AII stimulated HL, though the contribution of these two enzymes is significantly different between treatments. C apoproteins could, during this process be transferred to newly secreted VLDL. Other proteins such as A and E may also be transferred between intermediate density particles and other plasma lipoproteins. Through some unknown mechanism, LDL in diabetic animals acquire apoprotein AIII, presumably from the HDL particles. This difference in treatments may be a result of physiochemical modifications which occur as a consequence of diabetes, or simply reflect different catabolic processes between groups. The acquisition of apoprotein AIII could then mediate further hydrolysis of the TAG component, by the endothelial lipases and promote esterification of surface cholesterol. The reduction in density of the particle mediates further losses of apoproteins C and eventually AIII, which are transferred to nascent VLDL and HDL particles respectively. The

Figure 3.4

Figure 3.4 is a schematic diagram representing the apoprotein regulated catabolism of very low density lipoproteins in normal and alloxan diabetic sheep, within the plasma compartment, based on the qualitative apoprotein profile presented in this study.

 - apoprotein AI

 - apoprotein AII

 - apoprotein AIII

 - apoprotein AIV

 - apoprotein B

 - apoprotein C

 - apoprotein E

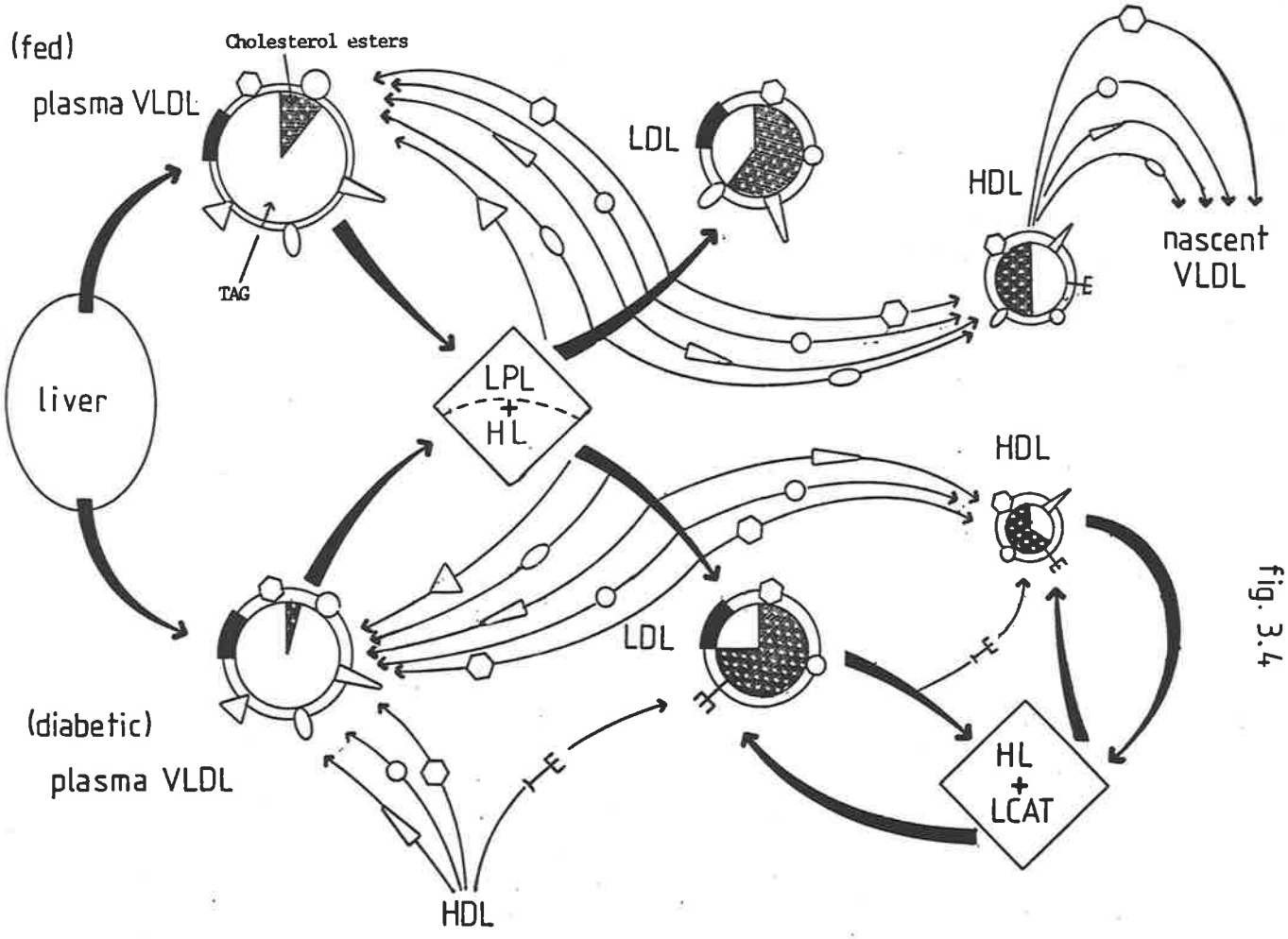

(fed)
plasma VLDL
Cholesterol esters
TAG
liver
LPL
+
HL
LDL
HDL
nascent
VLDL
E
HDL
LDL
E
HL
+
LCAT
(diabetic)
plasma VLDL
HDL

fig. 3.4

resulting LDL particles in the diabetic sheep will thus contain a greater percentage of cholesterol esters and lower amounts of TAG, than the LDL components from fed animals (as was observed). The presence of apoprotein AIII on HDL, results in part hydrolysis of its TAG core and esterification of surface cholesterol, whereby its subsequent reduction in density (diabetic HDL have a higher protein per unit lipid content- table 1.2) causes a similar loss of apoprotein AIII, back to VLDL, which are acquiring this protein as a result of lipolysis.

Total protein and apoprotein B was determined in the buffer fraction used to 'wash' isolated VLDL, because there remains a considerable amount of disagreement in the literature as to the benefits of this procedure for removing contaminating plasma proteins, versus the potential loss or degradation of apoproteins from the lipoprotein particles. The results presented in this chapter showed that recentrifugation of sheep VLDL resulted in a redistribution of protein, such that only 18% and 38% of total protein for fed and diabetic sheep respectively, was recovered with the 'intact' lipoprotein fraction at the top of the ultracentrifuge tubes. The ratio of soluble proteins versus apoprotein B remained constant (51%:49%) indicating that there was no contamination of plasma proteins, or the unlikely possibility, that an equivalent contamination of soluble versus precipitable proteins occurred. The reason why diabetic VLDL underwent a smaller degree of breakdown may be due to a protective factor of having a greater concentration of lipids. It was concluded that the 'washing' procedure for ovine VLDL was unnecessary, causing excessive loss and therefore subsequent underestimation of the apoproteins.

4 GENERAL DISCUSSION

Hepatic accumulation of triacylglyceride in sheep has been associated with a number of naturally occuring or clinically induced pathological conditions. This was thought to be due to an inability to maintain or increase hepatic release of this lipid in response to an increased hepatic uptake of unesterified fatty acids. However, recently we have reported that unlike other species such as the rat (Otway and Robinson 1967 and Agius et al. 1981), Rhesus monkey (Fiser et al. 1974) and rabbit (Topping and Turner 1976), the secretion of hepatic triacylglyceride is elevated in fasted and diabetic animals (Mamo et al. 1983). One of the first main findings of the work presented in this thesis was the determination of the nature of the lipoproteins mediating this increased secretion. It was found that under normal fed conditions, sheep have low levels of circulating plasma triacylglyceride, reflected in low levels of plasma very low density lipoproteins. In stressed animals, as a result of diabetes, this fraction was the major lipoprotein, transporting not only the majority of triacylglyceride, but infact all plasma lipids. In addition, the very low density lipoprotein fraction contained less lipid per unit protein, suggesting that limitations in the synthesis of this fraction was not the causative agent for hepatic accumulation. Very low density lipoproteins are the largest of the liver lipoproteins and it may be that if the hepatic sinusoids are surrounded by a fenestrated membrane, secretion could be retarded. The much smaller low and high density lipoproteins would theoretically be free to pass through the lamina, thus accounting for the lack of accumulation of other lipid components in the liver. If secretion was impeded by this membrane, the increased

hepatic release of very low density lipoproteins, might reflect a 'pressure' induced effect of increased hepatic synthesis of these particles.

Diabetic and fasted sheep were also grossly hypertriacylglyceridaemic and it was considered that whilst this reflected increased hepatic secretion of this lipid, plasma accumulation would only occur if clearance was not similarly increased, or impaired. Because of this, the hyperlipidaemia in these animals may at some point reach a maximum, such that hepatic release of further lipid is not possible and accumulation results. The second part of this study was thus aimed at determining rates of plasma triacylglyceride lipolysis. Triacylglyceride hydrolysis in postheparin plasma was shown to be dependent on lipase activities which resembled lipoprotein lipase and hepatic lipase from sheep adipose tissue and sheep liver respectively. Clearance of plasma triacylglyceride was infered in both fasted and diabetic animals. In the former, this was due to decreased activity of both lipoprotein lipase and hepatic lipase, whereas in the diabetic sheep, despite a significant increase in hepatic lipase activity, lipolysis was decreased due to the lipoprotein lipase component which was severely depressed. The increase in hepatic lipase activity in diabetic sheep appeared to reflect physiochemical changes in the very low density lipoproteins, as indicated by the greater rate of unesterified fatty acids released, when this lipoprotein fraction from diabetic animals as opposed to that from fed sheep, was incubated with postheparin plasma from fed animals. Lipoprotein lipase activity was also stimulated by modifications in the very low density lipoproteins, but an increase in activity was not realised in diabetic sheep, due to low plasma levels of insulin, which

when administered exogenously, completely restored lipoprotein lipase activity.

The secretion and subsequent catabolism of very low density lipoprotein triacylglyceride is controlled by the apoprotein compliment of the particle and so the higher rates of very low density lipoprotein lipolysis in particles isolated from diabetic animals may have reflected an improved apoprotein compliment. Similarly, deficiencies in the quantity or quality of apoprotein B, which is essential for the secretion of very low density lipoproteins, may in part, have contributed to hepatic triacylglyceride accumulation. The apoprotein profile of the sheep plasma lipoproteins presented in this study, was based principally on molecular weight as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and the apoprotein profile of rat very low density lipoproteins. It was unfortunate that the recent commercial availability of antisera to some of these proteins could not be used to confirm identity, as the antibodies were raised in sheep in the first instance. Nevertheless, on the basis of the ascribed apoprotein profile of the sheep plasma lipoproteins presented in this study, a postulated apoprotein regulated control of very low density lipoprotein triacylglyceride lipolysis was made. Three main points were raised. Firstly, the apoprotein 'A' compliment of the very low density lipoproteins and low density lipoproteins suggest that in sheep, hepatic lipase may play a major role in the catabolism of nascent very low density lipoproteins. Furthermore, greater hydrolase activity of lipoprotein lipase and hepatic lipase, stimulated by very low density lipoprotein particles isolated from diabetic sheep, may have been due to a greater apoprotein 'C' and 'A' compliment. Secondly, apoprotein AIII

correlated with particles which contained a smaller component of triacylglyceride and a greater fraction of cholesterol esters, suggesting that this protein may stimulate the endothelial lipases and lecithin cholesterol acyl transferase. The third main observation regarding the ovine apoproteins was that very low density lipoproteins from diabetic animals actually contained more apoprotein B than particles from normal fed sheep. This suggested that synthesis of this protein was not limiting hepatic secretion of these particles and therefore, was not a causative agent for hepatic accumulation of triacylglyceride. However, whether the nature of this protein remained the same, cannot be determined until this protein can be isolated intact.

The main objectives of this project revolved around lipoprotein triacylglyceride metabolism in normal and stressed sheep. However, during the course of this study, it was found that postheparin plasma lipase activities differed according to gender. An examination of differences in plasma lipolytic activities between sexes was a logical extension of the overall aims of this study and so lipoprotein lipase and hepatic lipase were determined in rams, castrates and ewes. The results reflected differences in activities due to the loss of androgen synthesis as a result of castration and converesly, the effects of oestrogen production. The oestrogenic/androgenic control mechanisms of very low density lipoprotein triacylglyceride kinetics in other species, is well defined for lipoprotein lipase, but in the case of hepatic lipase, remains a somewhat contentious issue. Like other species, lipoprotein lipase was progressively higher in rams, wethers and ewes respectively, which is reflected in a greater degree of

adiposity in the latter. Similarly, hepatic lipase activity paralleled that of lipoprotein lipase, indicating that the loss of androgen synthesis, or alternatively, the synthesis of oestrogens promotes activity of this enzyme which is in contrast to that reported for rats. It is apparent, therefore, that the steroidal regulation of hepatic lipase requires further investigation. In view of the widespread Australian farm practice of castration, the potential of producing a slower growing animal with a greater body fat content means that this process is undesirable.

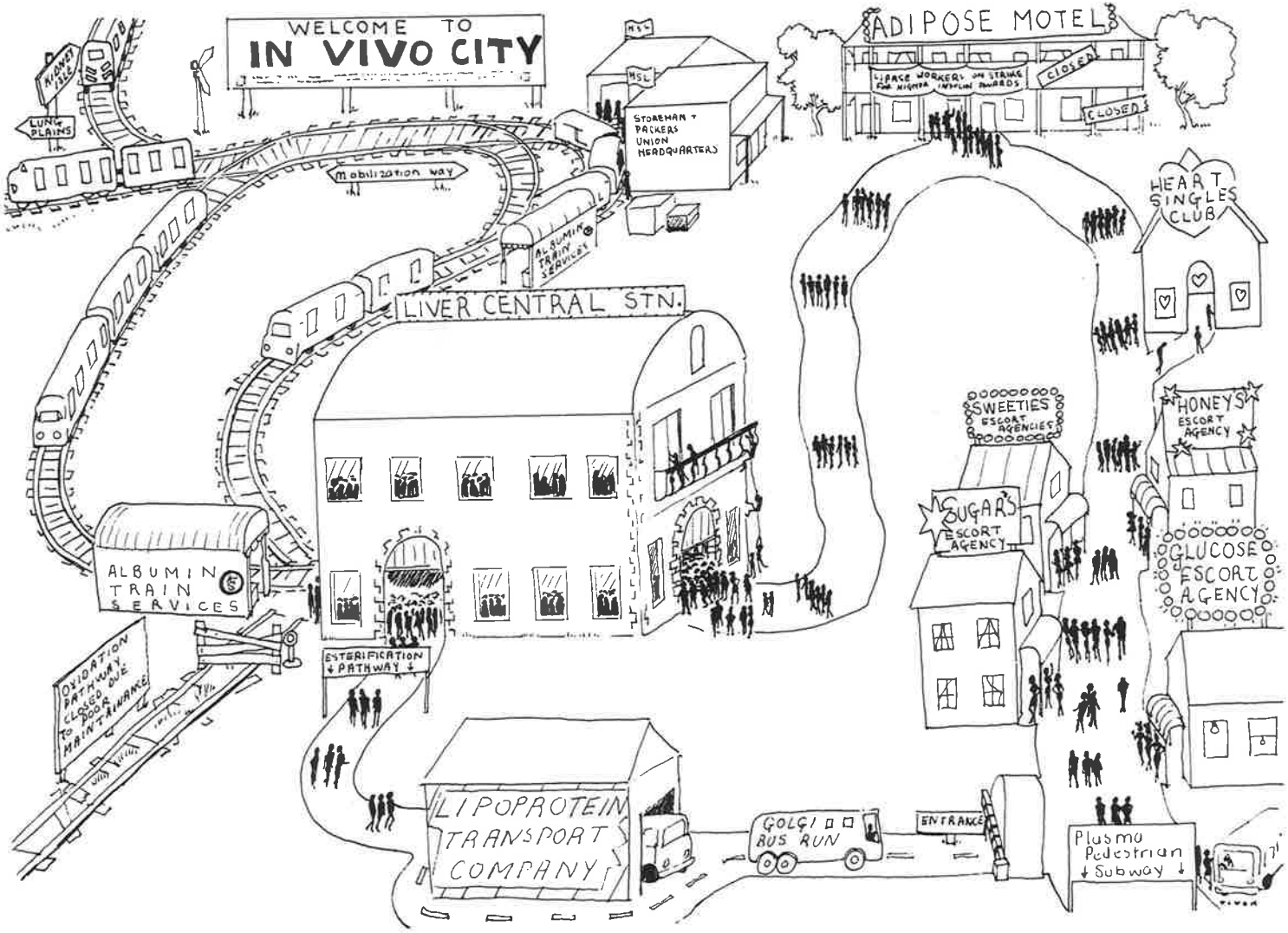
I was fortunate in having gained access to sheep designated as genetically 'lean' and 'obese', which again provided a further extension of lipoprotein triacylglyceride lipolysis and its relationship with fat deposition. The results showed that if nutrition is not a limiting factor, potential adiposity is in part, predetermined by differing rates of lipoprotein lipase. Further investigations may in future provide a rapid means of selecting animals which are generally leaner.

Figure 4 summarises the current concepts of difficulties associated with plasma lipoprotein triacylglyceride metabolism in metabolically stressed sheep, based on the results from this project:.

Metabolically stressed sheep obviously possess a tremendous capacity to synthesize and secrete triacylglyceride-rich very low density lipoproteins, in response to a large influx of unesterified fatty acids. It is difficult to conclude however, if this release is impaired by a basal lamina surrounding the sinusoid, or by a saturable capacity of the plasma compartment to accomodate lipids. In diabetic animals, the quality of these very large, very low density lipoproteins, in terms of their suitability as substrates

Figure 4

Figure 4 is a diagramatic representation of the problems associated with plasma very low density lipoprotein triacylglyceride metabolism in metabolically stressed sheep.


WELCOME TO
IN VIVO CITY
KIDNEY ISLE
LUNG PLAINS
Mobilization Way
HSL
STOREMAN + PACKERS UNION HEADQUARTERS
ADIPOSE MOTEL
LIPASE WORKERS ON STRIKE FOR HIGHER WAGES
CLOSED
CLOSED
HEART SINGLES CLUB
ALBUMIN TRAIN SERVICES
LIVER CENTRAL STN.
SWEETIES ESCORT AGENCIES
HONEYS ESCORT AGENCY
SUGAR'S ESCORT AGENCY
GLUCOSE ESCORT AGENCY
ALBUMIN TRAIN SERVICES
OXIDATION PATHWAY CLOSED TO BOOST MAINTENANCE DUE
ESTERIFICATION PATHWAY
LIPOPROTEIN TRANSPORT COMPANY
GOLGI BUS RUN
ENTRANCE
Plasma Pedestrian Subway

for lipoprotein lipase and hepatic lipase, is improved in comparison to these particles from fed sheep, as they are able to stimulate the activity of both endothelial lipases. This may be due to an improved protein lipid ratio, an improved apoprotein compliment, or simply a response to glucosylation of these particles. Although hepatic lipase is elevated in diabetic animals in response to these 'improvements', this is adverse to the needs of the liver in diabetic sheep. The triacylglyceride fatty acids are by this means returning to the liver, which is already trying to remove them by increasing very low density lipoprotein triacylglyceride secretion. Other extrahepatic tissues possessing lipoprotein lipase are unable to utilize the plasma triacylglycerides, even though the nature of the particles means that lipolysis by this route is stimulated, because of low levels of plasma insulin concentration, which directly determines the synthesis and secretion of this enzyme.

The objectives of this study have been achieved, but like many scientific investigations, much more work is needed to answer a number of questions. Although sheep are able to increase rates of hepatic very low density lipoprotein triacylglyceride release, accumulation of this lipid in the liver continues. The possibility that a basal lamina surrounds the hepatic sinusoid needs to be clarified, as it may be that this membrane inhibits the passage of the very large triacylglyceride rich very low density lipoproteins. In addition, the sheep liver lipase described in the defatted liver extracts, needs to be purified and it's biochemical properties characterised, to determine if this is infact the

same as hepatic lipase in other species. Promotion of plasma lipase activity by very low density lipoproteins from diabetic animals contradicts current conceptions as to problems associated with the clearance of plasma very low density lipoproteins in human diabetic subjects. Because very low density lipoprotein lipolysis and subsequent formation of low density lipoprotein is directly associated with atherogenesis (which is the major complication and cause of death in human diabetes), the implications of 'improving' a lipoprotein particle in terms of its substrate potential are very important. The ovine apoprotein investigation reported here, is in a sense preliminary. Much more work is required to purify and identify these components, so that the processes of sheep very low density lipoprotein triacylglyceride metabolism can be elucidated. Results presented in this study raised a number of additional aspects of sheep, or rather ruminant triacylgyceride metabolism. Steroidal regulation of plasma lipases remains to be defined and in terms of body composition and current animal production practices, genetic regulation of lipase activity has received little attention.

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