LIPOPROTEIN METABOLISM IN THE HORSE

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ABSTRACT

Hyperlipaemia is a disease of ponies in which massive hypertriglyceridaemia is associated with fatty infiltration of body tissues and organ failure. The aim of the work described here was to identify the causal defect in lipoprotein metabolism.

Plasma lipid transport in healthy horses and ponies was first characterised. The high density lipoproteins (HDL) predominated and were homogeneous with respect to particle size and density. Three subfractions were found within the low density lipoproteins (LDL), and although the very low density lipoproteins (VLDL) were heterogeneous in size, discrete subpopulations were not identified. Two apolipoprotein (apo) B-100 like proteins were present in VLDL and LDL, and VLDL contained a third shorter species that appeared of hepatic origin. Lipoprotein lipase (LPL) and hepatic lipase (HL) were isolated and used to develop a selective assay for their measurement in post-heparin plasma. The activity of lecithin:cholesterol acyl transferase was dependent upon apoA-I and determined the mass of cholesteryl esters in HDL. Equine plasma lacked cholesteryl ester transfer activity because of a deficiency of the protein rather the masking of its function by an inhibitory factor. The plasma concentrations of triglyceride and VLDL were higher in Shetland ponies than in Thoroughbred horses. Moderate hypertriglyceridaemia was prevalent in Shetland pony mares during the last trimester of pregnancy and was associated with changes in VLDL composition similar to those found in ponies with hyperlipaemia.

Although adult ponies showed no evidence of post-prandial lipaemia in response to feeding or an oral fat tolerance test, chylomicrons were found in the plasma of suckling foals where triglyceride and VLDL concentrations, and the activities of LPL and LCAT were higher than in adults. The plasma concentrations of LDL were also higher in the foals, and these lipoproteins were enriched with free cholesterol and phospholipid, and contained albumin and apoA-I. In the mares, lactation was associated with increased LPL activity.

The plasma concentrations of triglyceride and VLDL were increased by an average of 165 and 20 fold, respectively, in ponies with hyperlipaemia. This was due to the emergence of larger, more buoyant particles that were enriched in triglyceride and free cholesterol and depleted of cholesteryl esters and protein, specifically of apoB-100. The plasma concentrations of free fatty acids (FFA) and the activities of LPL and HL were increased. These data suggested that hepatic overproduction of VLDL, rather than compromised catabolism, was responsible for the condition. This was confirmed by kinetic analysis of plasma FFA and VLDL-triglyceride (VLDL-TG) metabolism in two ponies with hyperlipaemia, where the hepatic synthesis of VLDL-TG was 30 times higher than that in four healthy control ponies. This was driven by an increased flux of FFA from plasma into the liver. The residence time and fractional catabolic rate of VLDL was unaltered. Recycling of FFA into the plasma precursor pool from VLDL-TG lipolysis became a significant component of the hepatic input.

This thesis concludes that lipid lowering agents that reduce VLDL synthesis by reducing FFA flux to the liver should prove effective in treating equine hyperlipaemia and that such agents might be prescribed to those ponies identified at risk from hyperlipaemia, for example those mares in the last trimester of pregnancy.

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ACKNOWLEDGEMENTS

The completion of the work described here owes much to the contribution of others.

Professor Max Murray and Dr. Sandy Love were instrumental in stimulating my initial interest in this project and I am appreciative of their continued support. The project became a reality when Professor James Shepherd committed his expertise and facilities, and his enthusiasm did much to confirm the potential of my ideas. The progress that I have since made has largely been due to the guidance of Dr. Chris Packard, whose inquiring mind has asked much of the horse and myself.

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I must also thank Philip Stewart for his help in preparing apolipoproteins A-I, A-II and C by high performance liquid chromatography, and acknowledge Dr. Helen Laird, of the Department of Veterinary Pathology, who performed the electron microscopy for me. I am also grateful to Muriel Caslake, Dorothy Bedford and Dr. Bruce Griffin for sharing with me their expertise and giving their advice so freely.

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Finally, the timely completion of this thesis owes as much to the inspiration of my supervisors, Professors Murray and Shepherd, as it does to my own perspiration. I only hope that the product of my labours meets the high standards that they set me.

AUTHOR'S DECLARATION

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged

T.D.G. Watson, November 1991.

DEDICATION

I dedicate this thesis to my wife, Carmel,

for without her strong support

and many sacrifices

little would have been possible.

CHAPTER I

INTRODUCTION

This thesis concerns the structure and function of the equine plasma lipoproteins and focuses on the disruption in their metabolism that leads to the hypertriglyceridaemic crisis in ponies called hyperlipaemia. In this introduction, the clinical features of this condition are described and followed by an account of lipoprotein metabolism, largely based on the human system, which shows how defects in the production and clearance of the triglyceride rich particles give rise to hypertriglyceridaemia. The information that is available on lipoprotein metabolism in the horse, in both healthy animals and ponies with hyperlipaemia, is then reviewed and the aims of the present studies defined.

1. EQUINE HYPERLIPAEMIA

1.1. The First Report

A disturbance in the lipid metabolism of ponies marked by gross lipaemia was first described in 1969 by Schotman and Wagenaar who called the condition hyperlipaemia. Over the seven year period to 1966 they saw a total of 138 cases at the State University of Utrecht, the majority were Shetland ponies and many were in foal, or had recently foaled or aborted. The disease thus appeared more common in the spring months coinciding with the seasonal pattern of horse breeding. The affected animals appeared dull and produced either no faeces or had severe diarrhoea, which was related to the fact that many (61%) had concurrent disorders of the gastro-intestinal tract such as "worms, sand in the intestines and enteritis". A further 22% of cases were suffering from afflictions that did not concern the gastrointestinal tract, chiefly upper respiratory tract infections. The hyperlipaemia was of unknown origin in 17% of the cases. The mortality rate was high, in the region of 65%, and fatty degeneration of the liver and other parenchymous organs was obvious at necropsy.

Schotman and Wagenaar (1969) recounted how in severe cases the blood took on a bluish colour and suggested that the lipaemia could be demonstrated by pouring blood onto a dark surface, such as the floor, where a thin blue surface could be seen within a few seconds. They reported that total blood lipids were increased from 130-190 mg/dl in healthy ponies to 1000-7000 mg/dl in hyperlipaemic ponies, due to elevated levels of total and free cholesterol, phospholipids, fatty acid esters and free fatty acids (FFA), and the ß-lipoprotein fraction. Blood glucose concentrations were

reduced in some cases and there was often evidence of hepatic damage as the serum activities of alkaline phosphatase, and sorbitol and lactate dehydrogenase were increased.

Anorexia was a consistent feature of the case histories and this prompted the authors to speculate that the hyperlipaemia was caused by failure of the animals to eat, often because of some underlying illness. This was supported by showing a four fold increase in total blood lipids in two healthy ponies that were fasted for seven days. They concluded that body fat was mobilised to satisfy energy demands and that this resulted in fatty infiltration of the liver. The prognosis for individual cases appeared to be determined by the nature of the primary illness and was inversely related to their total blood lipids. In addition to correcting any concurrent disease, the authors advocated that calories be provided with intravenous glucose and soaked grass meal administered through a nasogastric tube, and suggested using prednisolone to stimulate the appetite and heparin to help clear the plasma of neutral lipids. Their final comment was that "the treatment of hyperlipaemic ponies requires much effort and is often disappointing".

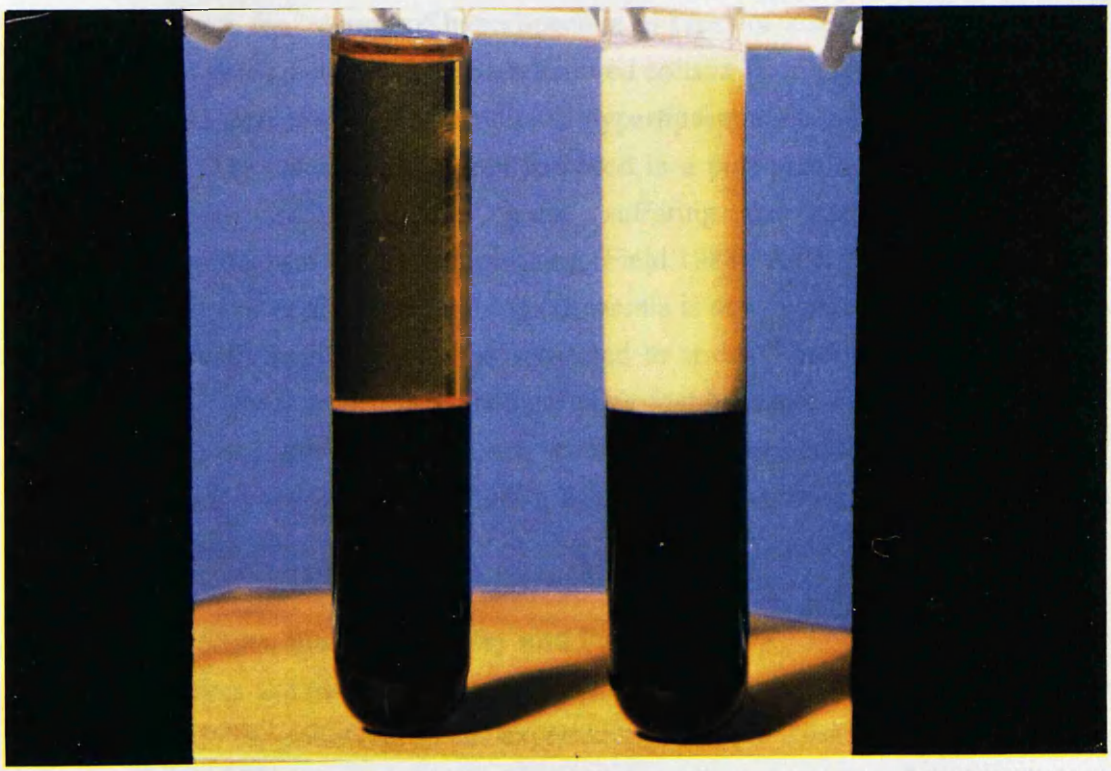


Figure 1. Lipaemia: the appearance of a centrifuged blood sample from a pony with hyperlipaemia (right) compared with that from a healthy pony (left).

1.2. Epidemiology

Equine hyperlipaemia has since been described in most parts of the world, including Denmark (Eriksen and Simesen 1970), Australia (Gay, Sullivan, Wilkinson, McLean and Blood 1978), North America (Naylor, Kronfeld and Acland 1980) and South Africa (Gilbert 1986). Meaningful data on the epidemiology of the condition came from a prospective survey of members of the Australian Pony Stud Book that ran for 12 months and covered a total population of 592 ponies (Jeffcott and Field 1985a). Thirty cases (5%) of hyperlipaemia were reported, 27 (90%) of which were Shetland ponies which comprised only 25% of the total population. Mares were also over-represented, 27 (90%) of the cases were females and of these, 80% were lactating and 15% were pregnant. The physical condition of the affected ponies was reported as good to overweight in 90% of the animals and 50% of the cases had been subjected to some form of stress, principally transportation or food deprivation, in the four weeks prior to the onset of disease. The average course of the condition was seven days and the total mortality rate was 57%. Although the study did not report details of any underlying or concurrent disease, the results confirmed the predisposition of the Shetland pony and the eminence of obesity, stress, pregnancy and lactation as risk factors for hyperlipaemia.

There are few reports of hyperlipaemia in types of horses other than ponies. Naylor *et al.* (1980) included three Standardbred colts, a Thoroughbred colt and mare, and an Arab in their study, all of which had hyperlipaemia secondary to severe multi-organ failure. The condition has been recorded in a post-parturient Paso Fino mare (Murray 1985) and a Quarter horse suffering the combined effects of hyperadrenocorticalism and acorn poisoning (Field 1988). A donkey was in the series reported by Naylor *et al.* (1980) and hyperlipaemia is now recognised as a significant cause of mortality in obese donkeys subjected to stress (Fowler 1989). Congenital hyperlipaemia was described in a Shetland pony foal born to a hyperlipaemic mother (Gilbert 1986) and apart from the three severely compromised foals of 1, 3.5 and 7 months reported by Naylor *et al.* (1980), hyperlipaemia appears rare in animals less than two years of age.

1.3. Clinical Signs, Blood Chemistry and Pathology

The clinical signs and pathology associated with this condition were best described by Gay *et al.* (1978) following their experiences with 15 cases of hyperlipaemia. Dullness, depression and lethargy were the earliest features. As the disease progressed, the animals became reluctant to move and showed incoordination, weakness, and muscular fasciculations after minor exercise. Mild abdominal pain was present in some cases and usually lasted for one to two days and was followed by

diarrhoea with foetid faeces that persisted until death. Five ponies developed ventral subcutaneous oedema. Inappetance progressed to anorexia, and depression to signs of severe nervous dysfunction as the animals became recumbent with paddling convulsions, champing, and nystagmus, and mania as an occasional agonal event. The clinical course varied from six to ten days, and 12 of the 15 ponies died despite intensive therapy. In Jeffcott and Field's survey (1985a), failure to drink, loss of body condition and halitosis were recorded as less frequent and more variable signs.

Eriksen and Simesen (1970) showed that the major changes in blood lipid content related to triglycerides which were 38.1 $\pm$1.9 (mean $\pm$sd) mmol/l in four hyperlipaemic ponies compared with 0.09 $\pm$0.05 mmol/l in eight healthy ponies. Plasma concentrations of cholesterol and phospholipids were increased by an average of three and four fold, respectively. Similar changes were noted by Naylor *et al.* (1980), who emphasised that elevated plasma FFA concentrations might be significant in the aetiology of the disease.

In addition to confirming the evidence of hepatic pathology presented by Schotman and Wagenaar (1969), Eriksen and Simesen (1970) reported that blood urea and ketone concentrations were normal, that hypoglycaemia was present in some but not all cases, and that total clotting time was increased and bromosulphothalein clearance prolonged. Naylor *et al.* (1980) confirmed that ketonaemia was not a feature of the disease and showed that bilirubin levels were not elevated, consistent with the absence of icterus as a clinical sign. Both Gay *et al.* (1978) and Naylor *et al.* (1980) suggested that elevated blood urea and creatinine concentrations, and metabolic acidosis develop terminally.

At necropsy, Gay *et al.* (1978) found widespread fatty infiltration of body organs marked by pallor, swelling and a greasy texture. These changes were most severe in the liver and kidneys, to the extent that the liver had ruptured in two cases, and less so in skeletal muscle, adrenal cortex and myocardium. Focal haemorrhages were sometimes scattered throughout the carcase, there was extensive infarction of the left ventricular myocardium in one case, two animals had renal infarcts and another had thrombosis of the portal vein. Pulmonary oedema was a consistent finding and considered an agonal event. Histopathology confirmed the severity of the lipid infiltration and revealed extensive venous thrombosis in the lung, kidney and brain, as well as the heart, liver, adrenals and subcutis. In the kidneys, there was evidence of a proliferative glomerulitis superimposed upon the fatty change. Other findings included hyaline degeneration of skeletal muscle, nutritional atrophy of the exocrine pancreas and depletion of lymphoid follicles in the spleen and lymph nodes. The clinical signs were attributed to developing hepatic failure and encephalopathy, with renal failure and acidosis as terminal events. The vascular pathology was

peculiar to this report, but prompted the authors to speculate that the widespread vascular thrombosis might be initiated by the hyperlipidaemia and fat embolism.

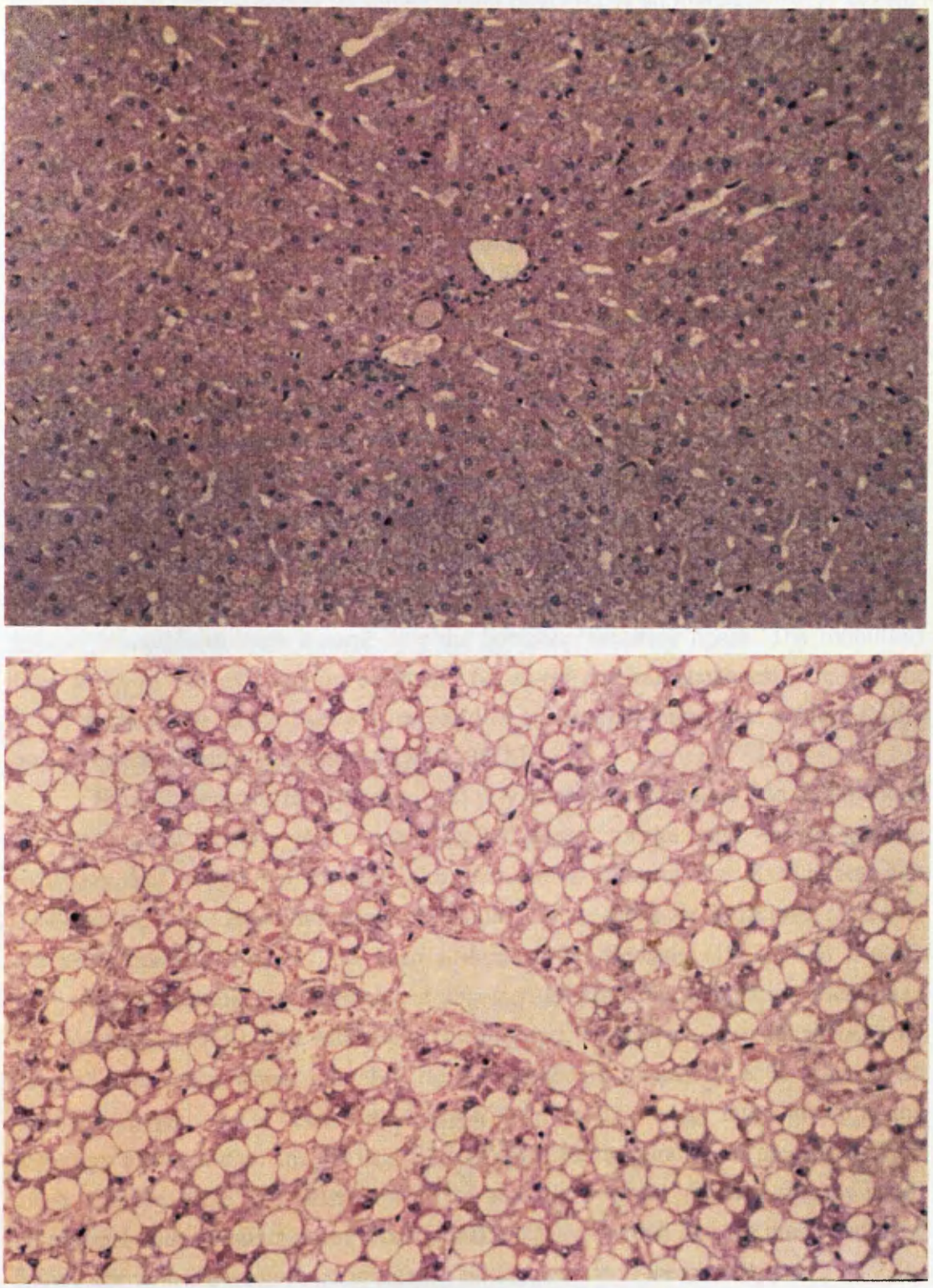


Figure 2. Fatty infiltration: histological sections of liver from a healthy pony (top) and a pony with hyperlipaemia (bottom) (hematoxylin and eosin, x400).

1.4. Current Concepts of Pathogenesis and Therapy

A number of studies have shown that plasma triglyceride levels rise when food is withheld from healthy ponies for three to eight days (Baetz and Pearson 1972; Morris, Zilversmit and Hintz 1972; Bauer 1983) and that this is preceded by increases in FFA concentrations (Naylor *et al.* 1980; Rose and Sampson 1982). These data have strengthened Schotman and Wagenaar's (1969) original belief that hyperlipaemia results from the mobilisation of body lipids in response to an energy imbalance. Such a deficit may arise due to the increased metabolic demands of pregnancy and lactation, simple undernutrition, and anorexia secondary to stress or underlying disease (Jeffcott and Field 1985b). However, because the disease is most commonly associated with states, such as obesity, stress, pregnancy and lactation, that in other species are associated with insulin insensitivity, attention has focused on what role insulin resistance might play in its aetiology. To this end, Jeffcott, Field, McLean and O'Dea (1986) demonstrated that ponies are glucose intolerant and insulin insensitive compared with larger horses, an insensitivity accentuated by obesity, and Fowden, Comline and Silver (1984) showed that insulin resistance is a natural feature of the equine pregnancy. It is therefore believed that once adipose lipolysis is initiated, the mobilisation of fatty acids proceeds in an exaggerated and unregulated fashion as insulin is unable to exert control over the hormone sensitive lipase. The mobilised fatty acids, as well as providing substrates for gluco- and ketoneogenesis, are thought to be re-esterified in the liver and secreted in to the circulation thus leading to the hypertriglyceridaemia (Jeffcott and Field 1985b).

Present therapies for hyperlipaemia rely on the identification and treatment of any underlying disease, the maintenance of energy intake with enteral glucose solutions and supportive therapy with intravenous electrolytes, B vitamins and anabolic steroids. A combination of insulin and glucose or galactose aimed at curtailing adipose lipolysis has been advocated by Wensing (1972), and heparin suggested to promote the clearance of triglycerides from the circulation (Schotman and Wagenaar 1969). The efficacy of insulin and heparin has questioned been by clinical experience where mortality rates remained in excess of 65% (Gay *et al.* 1978), and their use is controversial given the role for insulin insensitivity in the aetiology of the disease and the fact that impaired clotting function is present in many cases. Clearly, there is therefore a need for an effective lipid lowering therapy based on rational understanding of the biochemical mechanisms that link the mobilisation of fatty acids with the massive accumulation of triglyceride in the circulation.

2. LIPOPROTEIN STRUCTURE AND FUNCTION

The plasma lipoproteins have evolved to overcome the difficulties presented in distributing aqueous-insoluble lipids from their sites of assimilation and synthesis to the tissues of utilisation, storage and excretion. These particles contain the intensely hydrophobic neutral lipids (cholesteryl esters and triglyceride) in a spherical core that is protected from the hostile environment of plasma by a polar coat of free cholesterol, phospholipids and specialised proteins called the apolipoproteins (apo) (Fig. 3). A number of discrete populations of lipoproteins are identifiable in plasma on the basis of their size, hydrated density, and lipid and apolipoprotein compositions (Tables 1 and 2). While each class has evolved separate and diverse roles in the transport of lipids, they interact intimately with one another and, in health at least, are capable of providing a coordinate response to the demands placed upon them. The strong association of certain classes with the occurrence of coronary artery disease (CAD) in man and some animal species has been a major stimulus for intense interest in the biology of these particles. The structure, function and metabolism of the major lipoprotein classes are reviewed in the succeeding sections.

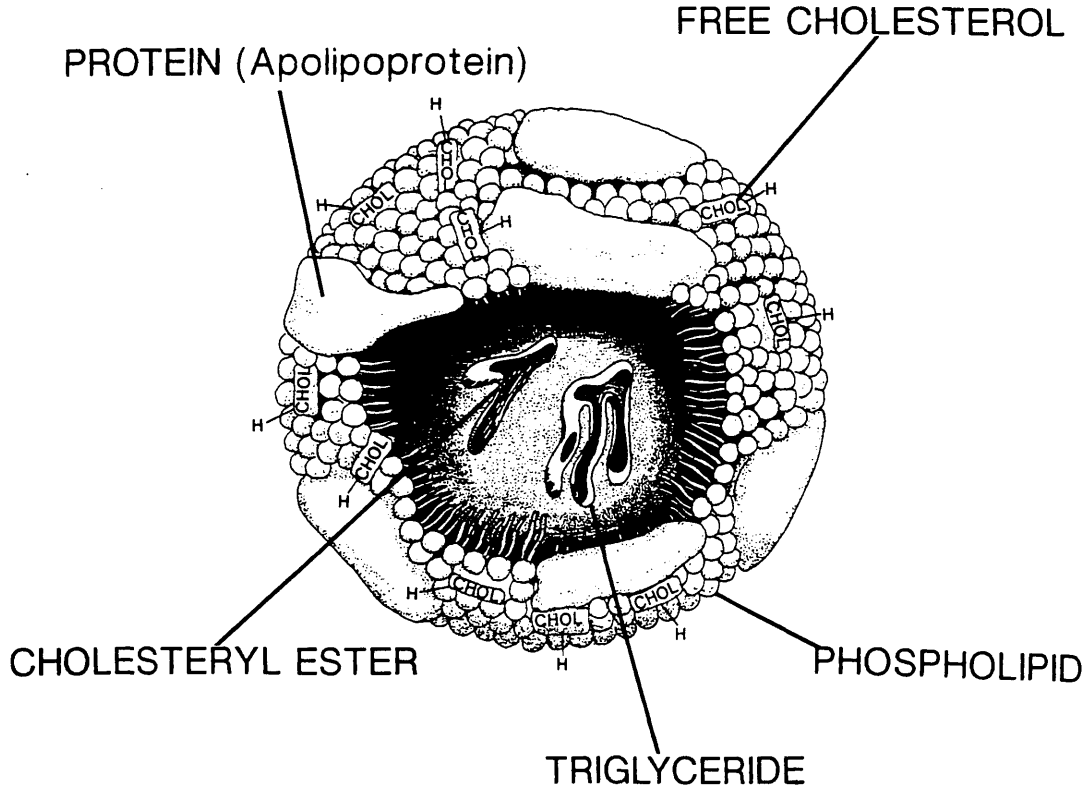


Figure 3. Schematic representation of a lipoprotein particle.

Lipoprotein	Electrophoretic Mobility	Diameter nm	Weight k Da	Density g/ml
Chylomicrons	Origin	75-1200	>400,000	<0.93
Very Low Density	pre-ß	30-80	10-80,000	0.93-1.006
Intermediate Density	slow pre-ß	25-35	5-10,000	1.006-1.019
Low Density	ß	18-25	2,300	1.019-1.063
High Density; HDL_2	α	9-12	360	1.063-1.125
HDL_3	α	5-9	175	1.125-1.210

Table 1. The physical properties of the human plasma lipoprotein classes.

	Percentage Particle Mass				
Lipoprotein	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
Chylomicrons	2	90	1	5	2
VLDL	13	54	7	16	10
IDL	34	20	9	20	17
LDL	41	4	11	21	23
HDL_2	13	5	5	35	42
HDL_3	15	3	3	23	56

Table 2. Typical composition of the human plasma lipoproteins.

2.1. Chylomicrons

The human diet is rich in fat, each day we consume approximately 100g of triglycerides, 2.2g of phospholipids and 1g of cholesterol and these are distributed to their target organs by chylomicrons, the largest and most buoyant of the lipoprotein classes. The ingested lipids are hydrolysed in the upper small intestine and enter adjacent mucosal cells by passive diffusion. Triglyceride, phospholipid and cholesteryl esters are re-synthesised within the smooth endoplasmic reticulum (SER) and are complexed with apolipoproteins B-48, A-I, A-II and A-IV near to the junction with the rough endoplasmic reticulum (RER). The particles are then transported to the Golgi apparatus, from where secretory vesicles migrate towards and fuse with the lateral plasma membrane and discharge their contents into the intercellular space (Sabesin and Frase 1977). From there, the chylomicrons enter the intestinal lacteals passing through the lymphatic system so that one hour after fat consumption they enter the general circulation.

The transfer of the chylomicron lipids to tissues is accomplished in two stages. First, the core triglyceride is hydrolysed by the enzyme lipoprotein lipase (LPL) releasing fatty acids that are taken up by adipose tissue and skeletal muscle for energy production or storage. The activity of this enzyme requires the presence of apoC-II on the chylomicron surface and this is acquired from HDL, along with apoC-III and apoE, as the particles enter the circulation. As triglyceride is progressively removed, the core of the chylomicron shrinks leaving an excess of surface material (phospholipid, free cholesterol, apoA-I and apoA-II) that is transferred to HDL (Tall, Green, Glickman and Riley 1979). Once approximately 75% of the triglyceride has been removed, apoC-II is also lost from the particle and lipolysis stops. The remnant particles formed by this process are cleared by a hepatic receptor that recognises apoE so that cholesterol of dietary origin reaches the liver where it is used for bile salt synthesis, stored, or secreted in VLDL (Rubinsztein, Cohen, Berger, van der Westhuyzen, Coetzee and Gevers 1990). The identity of this chylomicron remnant receptor had remained elusive until the recent description of an LDL receptor related protein (LRP) which satisfies the functional criteria applied to the remnant receptor (reviewed by Brown, Herz, Kowal and Goldstein 1991).

The assimilation and clearance of dietary fat is an efficient process that returns plasma triglycerides to fasting levels within 6 to 8 hours of finishing a meal. In response to the digestion and absorption of fat, the synthesis of apoB (Davidson, Magun, Brasitus and Glickman 1987), apoA-I (Go, Schonfield, Pfleger, Cole, Sussman and Alpers 1988), and apoA-IV (Black, Rohwer-Nutter and Davidson 1990) are increased. In fact, synthesis of apoB is an absolute requirement for chylomicron assembly as shown by the absence of these particles in patients who inherit the

condition abetalipoproteinaemia and are deficient in this protein (Bouma, Beucler, Pessah, Heinzmann, Lusis, Naim, Ducastelle, Leluyer, Schmitz, Infante and Aggerbeck 1990). The clearance of chylomicrons is influenced by the activity of LPL (Patsch, Prasad, Gotto and Patsch 1987), the availability and function of apoC-II and apoC-III, and there is recent evidence that the uptake of chylomicron remnants is dependent on the activity of hepatic lipase (Sultan, Lagrange, Jansen and Griglio 1990), and the quantity (Annuzzi, Holmquist, and Carlson 1989) and phenotype of apoE (Weintraub, Eisenberg and Breslow 1987).

2.2. Very Low Density Lipoproteins

The very low density lipoproteins (VLDL) are composed predominantly of triglyceride, which in contrast to the chylomicrons, is of endogenous origin. These particles are the precursors of LDL and so serve as vehicles for the distribution of both triglyceride and cholesterol from the liver to peripheral tissues. Nascent VLDL particles are continuously secreted by the liver and to a minor extent by the intestine (Green and Glickman 1981), and are stabilised by a single molecule of apoB-100 (Elovson, Chatterton, Bell, Schumaker, Reuben, Puppione, Reeve and Young 1988). The assembly and secretion of hepatic VLDL has recently been the subject of an extensive review by Gibbons (1990). Following synthesis in the RER, apoB is complexed with cholesteryl esters and transferred to a transitional area at the junction with the SER. There triglycerides, synthesised in the SER, are added and the immature particles transferred to the Golgi apparatus where the surface lipids are added. The nascent particles are held in secretory storage vesicles that bud off from the Golgi in to the space of Disse where the majority of the other apolipoproteins found on VLDL, namely apoE and apoC, are acquired.

Once in the circulation, VLDL enter the LPL lipolytic cascade and are stripped of their core triglyceride in a stepwise manner identical to that described for chylomicrons. This gives rise to smaller particles that are further processed to IDL and LDL by hepatic lipase (HL), or cleared from the circulation following recognition of their apoE component by the LDL receptor (Brown *et al.* 1991). There is presently debate as to what role the LRP might play in the clearance of these remnants (Eisenberg 1990). Considerable heterogeneity exists within the VLDL class and is evident when particles are separated according to size (Sata, Havel and Jones 1972) or hydrated density (Patsch, Patsch, Kostner, Sailer and Braunsteiner 1978). Indeed, as particle size decreases and density increases along the VLDL spectrum, the contribution of triglyceride and non-apoB proteins declines as the particles become enriched in cholesteryl esters and apoB (Kuchinskiene and Carlson 1982). It appears that the smaller particles continue through the lipolytic cascade to LDL, while the

remnants of larger VLDL are cleared directly from the circulation (Packard, Munro, Lorimer, Gotto and Shepherd 1984).

The hepatic synthesis of triglyceride and apoB, and the secretion of VLDL, *in vitro* at least, appear sensitive to nutritional and metabolic changes. Triglyceride synthesis is enhanced in fed compared with fasted animals (Wilcox and Heimberg 1987), in animals fed carbohydrate precursors of triglyceride (Yamamoto, Yamamoto, Tunaka and Ontko 1987), in hyperphagic rats (Vance and Russell 1990), and is related to the supply of FFA (Dashti and Wolfbauer 1987). The neutral lipid composition of VLDL is largely directed by the lipid content of the hepatocyte so that the cholesterol content of VLDL is increased by feeding cholesterol, in exchange for a reduction in VLDL triglyceride content (Davis, McNeal and Moses 1982). Hepatic VLDL secretion is enhanced by glucose and inhibited by insulin (Durrington, Newton, Weinstein and Steinberg 1982). This latter effect appears due to decreased secretion rather than synthesis of VLDL and may result in the accumulation of triglyceride within the liver (Patsch, Franz and Schonfeld 1983). Glucagon appears to inhibit triglyceride secretion while the thyroid hormones and oestrogens have the reverse effect (Haagsman and Van Golde 1984).

As for chylomicrons, the catabolism of VLDL is largely dependent upon the activity of LPL. In addition, evidence has recently emerged from human (Eisenberg, Friedman and Vogel 1988) and animal (Mahley, Weisgraber, Hussain, Greenman, Fisher, Vogel and Gorecki 1989) studies to suggest that the removal of VLDL remnants may also be saturable and limited by the quantity and quality of apoE in plasma and VLDL.

2.3. Intermediate Density Lipoproteins

Removal of VLDL core triglyceride causes the particle to shrink in size and become more dense. These intermediate density lipoproteins (IDL) are enriched in cholesteryl esters and protein relative to their precursors. The loss of surface components during lipolysis means that apoB comprises the majority of their protein moiety. The plasma concentrations of IDL are normally low, reflecting their rapid conversion to LDL by hepatic lipase and their direct removal from the circulation by cell surface receptors (Demant, Shepherd and Packard 1988).

2.4. Low Density Lipoproteins

The completion of IDL hydrolysis and continued loss of surface components produces a particle extremely rich in cholesteryl esters and which contains very few apolipoproteins other than a single molecule of apoB. These low density lipoproteins (LDL) are the major human cholesterol transporter and as such are the *agents*

provocateurs of atherosclerosis. This is evidenced by the strong correlation between the plasma concentration of LDL and the incidence of CAD observed in large scale epidemiological studies such as that of Framingham (Kannel, Castelli, Gordon and McNamara 1971), and by the reduction in CAD that can be achieved by reducing LDL levels (The Lipid Research Clinics Coronary Primary Prevention Trial Results 1984).

With kinetic analysis of apoB metabolism it has become clear that LDL particles do not originate exclusively from VLDL precursors (Demant *et al.* 1988) and that as many as 50% may be secreted directly into the circulation (Soutar, Myant and Thompson 1977; Goldberg, Le, Ginsberg, Paterniti and Brown 1983). In support of this, lipoproteins with the characteristics of LDL are secreted by hepatocytes *in vitro* (Dashti and Wolfbauer 1987). Advancements in understanding the origins of LDL have been accompanied by the realisation that this lipoprotein class is composed of a number of discrete subpopulations that are heterogeneous with respect to size, density and lipid composition (Shen, Krauss, Lindgren and Forte 1981). These subspecies differ in their kinetic behaviour (Marzetta, Foster and Brunzell 1989) and may possess varying potentials for promoting atherosclerosis, as a preponderance of the smaller more dense particles appears to be associated with CAD (Griffin, Caslake, Yip, Tait, Packard and Shepherd 1990).

The majority of LDL (80-90%) is cleared from the circulation by a glycoprotein cell surface receptor, the LDL receptor, that recognises and binds apoB-100 (Brown and Goldstein 1984). Much of this clearance occurs in the liver which contains 50-70% of the body's receptor complement, although high concentrations of receptor activity are found on the adrenals and ovaries reflecting their high cholesterol requirement for hormone synthesis (Fong, Bonney, Kosek and Cooper 1989). The expression of LDL receptor activity is closely regulated by the level of cholesterol within the cell so that when the supply of cholesterol exceeds requirements the transcription of the gene is suppressed and *vice versa* (Brown and Goldstein 1975). This has provided the basis for the cholesterol lowering drugs that upregulate receptor activity and so reduce plasma LDL levels, by inhibiting cholesterol biosynthesis or blocking the enterohepatic circulation of bile acids (Shepherd and Packard 1986). Defective expression of the receptor is the basis for the genetic disorders found in man (Familial hypercholesterolaemia) and the rabbit (Watanabe heritable hyperlipidaemia) which results in massive plasma LDL concentrations and premature atherosclerosis (Goldstein and Brown 1989).

The remainder of LDL clearance occurs by receptor independent pathways that appears in a number of tissues as a function of the ambient plasma LDL concentration (Spady, Meddings and Dietschy 1986). Further to this, macrophages

express high affinity binding sites for chemically modified LDL residues which avidly scavenge such particles in an unregulated fashion initiating and aggravating atherosclerosis (Mahley and Innerarity 1983).

2.5. High Density Lipoproteins

The high density lipoproteins are the smallest and most dense of the major classes and, in terms particle numbers, are the most abundant lipoprotein in human plasma. In contrast with the other classes, the HDL have protein as their major constituent and the majority of particle mass (80%) is attributable to shell rather than core components (Patsch and Gotto 1987). The major proteins in HDL are apoA-I and A-II, which together account for 75-85% of the protein mass, with minor contributions from apoA-IV, apoC and apoE. Interest in the metabolism of HDL stems from the inverse relationship that exists between plasma HDL concentrations and the incidence of CAD, suggesting a cardioprotective function for these lipoproteins (Miller and Miller 1975).

It has long been recognised that the human HDL class is composed of a number of thermodynamically stable subspecies that have diverse metabolic functions (reviewed by Eisenberg 1984). This heterogeneity is obvious when HDL is subjected to zonal ultracentrifugation (Patsch, Schonfeld, Gotto and Patsch 1980) and non-denaturing polyacrylamide gel electrophoresis (Blanche, Gong, Forte and Nichols 1981) when two major subclasses, HDL_2 and HDL_3, are identified. To complicate matters, the HDL class can be alternatively divided, by immunological means, into particles that contain apoA-I and apoA-II in a 2:1 molar ratio (LpA-I/A-II), and particles with apoA-I but no apoA-II (LpA-I) (Cheung and Albers 1982). It is apparent that the LpA-I particles exist at both extremes of the HDL spectrum with the bulk in HDL_2, whereas the LpA-I/A-II particles are largely distributed within the HDL_3 compartment (Atmeh, Shepherd and Packard 1983).

Mature HDL evolve from disc-shaped bilayers of phospholipids, free cholesterol and protein that are secreted directly into the circulation by the liver and intestine (Musliner, Michelfender and Krauss 1988). The liver and intestine appear to share equally in this, the hepatic product being rich in apoA-I and apoE, whereas the nascent intestinal HDL contains only apoA-I (Wu and Windmueller 1979). Other sources of HDL precursors include the surface remnants of triglyceride rich lipoproteins undergoing lipolysis and apolipoprotein/phospholipid/free cholesterol complexes that form spontaneously in the circulation following the loss of individual surface components from other lipoproteins and from cell membranes (Patsch and Gotto 1987). The transformation of the discoidal particles deficient in neutral lipid is mediated by the enzyme lecithin:cholesterol acyl transferase (LCAT) which esterifies

free cholesterol present on the surface of HDL. These esters, because of their hydrophobicity, move into the core of the particle thus creating a concentration gradient down which more free cholesterol can move into HDL from cell membranes and other lipoproteins so that the particle expands and evolves into mature HDL_3.

Transfer of cholesteryl esters from HDL to the lower density lipoproteins in exchange for triglyceride, which is mediated by the cholesteryl ester transfer protein (CETP), then helps maintain this gradient and provides a route by which cholesterol of peripheral origin can be returned to the liver (via the LDL receptor) for excretion or redistribution. Because of the larger molecular volume of triglyceride, this exchange process results in a further increase in particle size and decline in density so that the particles enter the HDL_2 spectrum. These lipoproteins are then susceptible to hydrolysis by HL which reduces the core volume and returns them to the HDL_3 range (Patsch, Prasad, Gotto and Bengtsson-Olivecrona 1984). Thus the circulating HDL are subject to extensive remodelling with the enzymes LCAT, CETP, HL and LPL all working together to coordinate the redistribution of HDL components (Newnham and Barter 1990) with the balance of this cycle shifted towards larger HDL in the post-prandial period and back towards smaller HDL during fasting (Tall 1990).

The exact fate of HDL has been harder to follow than that of the apoB-containing particles as all the lipid and protein components enter and leave independently and, in contrast, to the other lipoproteins, HDL has a significant extravascular distribution (Shepherd and Packard 1984). However, the majority of HDL appears to be cleared by the liver, with the adrenals and ovaries also showing strong uptake activity (Stein, Dabach, Hollander, Halperin and Stein 1983). The mechanism of this uptake is unclear. The presence of apoE on a small minority of HDL particles will direct them to the LDL receptors for direct hepatic clearance (Willingham 1989). Since the discovery of the LDL receptor, the search has been on for a similar mechanism to explain HDL uptake, and two candidate proteins have recently been described that bind both apoA-I and apoA-II (Tozuka and Fidge 1989).

2.6. Miscellaneous Lipoproteins

Lipoprotein (a) (Lp(a)) is a LDL particle with an additional surface apolipoprotein (apo(a)) linked to its apoB molecule by a disulphide bond (reviewed by MBewu and Durrington 1990). These particles are distinct from LDL on the basis of a different electrophoretic mobility (pre-ß), larger size and higher hydrated density (Table 3). Interest in this unusual lipoprotein stems from its association at high concentrations with both the incidence of myocardial infarction (Rhoads, Dahlen, Berg, Morton and Dannenberg 1986) and severity of CAD (Dahlen, Guyton, Attar, Farmer, Kautz and Gotto 1986). The plasma concentrations of Lp(a) are not distributed normally in the

population, but are skewed in a positive direction with the majority of the population clustering at the lower end of the concentration range. This appears to result from genetic polymorphism at the apo(a) locus causing variable expression of several isoforms of the protein that differ in the number of pretzel-like domains called kringles (Utermann 1989).

Little is known of the origins, function and fate of Lp(a). The cDNA and protein sequences of apo(a) show a high degree of homology with those of plasminogen (McLean, Tomlinson, Kuang, Eaton, Chen, Fless, Scanu and Lawn 1987) and the genes that code for both proteins are close together on the long arm of chromosome 6 (Utermann 1989). The major site of apo(a) synthesis appears the liver as demonstrated by the high quantities of mRNA in HepG2 cells (McLean *et al.* 1987) and the change in Lp(a) phenotype that is seen in patients after liver transplantation (Kraft, Menzel, Hopplicher, Vogel and Utermann 1989). However, Lp(a) does not have a VLDL precursor and it is unclear whether the particles are secreted ready assembled by the liver or LDL and apo(a) combine in the circulation. The site of Lp(a) clearance is also unknown. While Lp(a) concentrations are elevated in familial hypercholesterolaemia, suggesting a role for the LDL receptor (Utermann, Hoppichler, Dieplinger, Seed, Thompson and Boerwinkle 1989), stimulation of receptor activity in heterozygotes for this condition fails to reduce Lp(a) concentrations (Kostner, Gavish, Leopold, Bolzano, Weintraub and Breslow 1989).

	Diameter	Density	% Particle Mass				
Lipoprotein	nm	g/ml	CE	TG	FC	PL	Protein
Lp(a)	25	<1.090	36	3	9	34	18
Lp-X	35	1.019-1.063	5	3	25	61	6
ß-VLDL	30	<1.006	25	35	6	24	12
HDL_1	15	<1.125	20	1	10	34	35

Table 3. Average composition of the unusual human lipoproteins (CE, cholesteryl esters; TG, triglyceride; FC, free cholesterol; PL, phospholipid).

The mechanisms by which Lp(a) causes and aggravates atherosclerosis are speculative. Because of its homology with plasminogen, it has been suggested that apo(a) competes for the plasminogen binding sites present on endothelial cells reducing the formation of thrombolytic factors and setting up a precoagulant state that increases the risk of myocardial infarction (Scott 1989). Lp(a) also binds to fibrin, and while this may represent a route by which cholesterol is delivered to recently injured tissues to aid repair, it may also contribute to the cholesterol load of a compromised atherosclerotic artery (Brown and Goldstein 1987).

Lipoprotein X (LpX) is found in the plasma of patients with extra- or intrahepatic cholestasis (Seidel, Buff, Fauser and Bleyl 1976). These particles float in the LDL density range, but are composed almost entirely of phospholipids and free cholesterol and are devoid of apoB, having albumin, apoA-I, apoC and apoE as their proteins (Table 3). These lipoproteins are believed to originate from the regurgitation of bile salts in to the circulation, although somewhat similar particles are also found in patients with familial LCAT deficiency due to disturbed cholesterol esterification (Riesen and Kloer 1989).

In patients homozygous for the defective isoform of apoE (E2), the removal of chylomicron and VLDL remnants is impaired causing these cholesteryl ester enriched intermediates to accumulate in the circulation. These particles reside in the VLDL density range but migrate in the ß-position on electrophoresis and are hence called **ß-VLDL** (Table 3). Similar lipoproteins are found in dogs when fed high fat/high cholesterol diets and are avidly taken up by macrophages in a receptor mediated process that leads to cholesteryl ester deposition (Goldstein, Ho, Brown, Innerarity and Mahley 1980)

Feeding cholesterol to a number of species, including man, pigs and dogs, results in the appearance in plasma of large HDL particles that are enriched in cholesteryl esters and apoE and referred to as **apoE rich HDL**, alternatively $\mathbf{HDL_c}$ or $\mathbf{HDL_1}$ (Mahley and Innerarity 1983). These lipoproteins are present in small amounts in plasma from normolipidaemic humans (Weisgraber and Mahley 1978). The enrichment of this fraction with apoE appears functionally beneficial in directing the particles to the liver for the reverse transport of cholesterol (Koo, Innerarity and Mahley 1985).

3. THE APOLIPOPROTEINS

It is clear from the preceding discussion that the apolipoproteins serve a number of functions vital to the transport of lipids, the foremost of which is their ability to bind and emulsify aqueous insoluble lipids into lipoprotein packages. For this they are well

adapted as much of their secondary structure consists of repeating amphipathic helical regions of 22 amino acids, in which the charged residues are localised to the polar surface of the helix extending into the aqueous phase and thereby contributing to the solubility by hydrogen bonding with water molecules (Edelstein, Kézdy, Scanu and Shen 1979). The opposite face of these regions contains mostly hydrophobic amino acids that are intimate with the fatty acyl chains of the surface phospholipids and to a lesser extent with the neutral lipids in the particle core. The apolipoproteins have subsequently evolved, by acting as enzyme cofactors (apoA-I, apoC-II) and receptor ligands (apoB, apoE), central roles in the direction and regulation of lipoprotein metabolism.

3.1. Apolipoprotein B

Two distinct species of apoB are found in human plasma (Kane, Hardman and Paulus 1980). The first, apoB-100, is one of the largest monomeric proteins known (4,563 amino acids, approximately 549k Da), it is the structural protein of VLDL, IDL and LDL, and is synthesised in the liver and to a small extent in the small intestine. The second, designated apoB-48 on the basis that its apparent molecular weight (264k Da) is 48% of that of apo-100, is synthesised only in the small intestine where it is central to the production of chylomicrons.

It was initially thought that the two proteins were the product of separate genes or that apoB-48 arose from posttranslational cleavage of apoB-100. However, it has been shown that both the cDNA and protein sequences of apoB-48 (2,152 amino acids) are colinear with the amino-terminal half of apoB-100 and that the two proteins are the product of a single gene (Chen, Habib, Yang, Gu, Lee, Weng, Silberman, Cai, Deslypere, Rosseneu, Gotto, Li and Chan 1987). It is now clear that apoB-48 is synthesised following editing of the apoB message so that a stop translation codon (UAA) is found at position 2153 rather than the codon (CAA) that codes for glutamine in apoB-100 (Scott, Wallis, Davies, Wynne, Powell and Driscoll 1989). This organ specific editing appears to be catalysed by an enzyme that converts cytosine to uracil at a single position in the mRNA molecule (Brown and Goldstein 1987). Both proteins are synthesised in the liver of the rat (Wu and Windmueller 1981) and this has helped provide evidence that the editing mechanism is subject to metabolic (Coleman, Haynes, Sand and Davis 1988; Davidson, Carlos, Sherman and Hay 1990) and developmental (Jiao, Moberly and Schonfeld 1990) regulation.

The human apoB gene resides on the short arm of chromosome 2 and spans 43 kb. The structure of its product, apoB-100, has largely been deduced from amino acid sequence analysis which predicts frequent alternation of hydrophilic and hydrophobic sequences (Rosseneu and Labeur 1990). Much of the secondary structure (33%) of

apoB is in the form of ß-sheet (Goormaghtigh, De Meutter, Vanloo, Brasseur, Rosseneu and Ruysschaert 1989) which would explain the relative insolubility of delipidated apoB compared with the other apolipoproteins where the alpha helix predominates. This also suggests that apoB interacts more intimately with the hydrophobic core of the lipoprotein than the other proteins (Yang, Kim, Weng, Lee, Yang and Gotto 1990). Using the HepG2 cell culture system, it has become apparent that apoB secretion is increased by cholesterol and fatty acids and reduced by insulin due to changes at a co- or posttranslational, rather than a transcriptional, level (reviewed by Scott 1990). Apolipoprotein B-100 is subject to posttranslational modification including glycosylation (Yang, Gu, Weng, Kim, Chen, Pownall, Sharp, Liu, Li, Gotto and Chan 1989), acylation (Hoeg, Meng, Ronan, Demosky, Fairwell and Brewer 1988) and phosphorylation (Sparks, Sparks, Roncone and Amatruda 1988), the latter two of which may be involved in lipid binding and intracellular transport during lipoprotein assembly.

The precise location on the molecule of the receptor binding domain remains unclear, but it is in the region of residues 3330-3800 that is rich in basic amino acids (Milne, Theolis, Maurice, Pease, Weech, Rossart, Fruchart, Scott and Marcel 1989). This region is missing from apoB-48, explaining why the smaller protein does not interact with the LDL receptor. Recently, a condition named familial defective apoB-100 (FDB) has been described in which a point mutation at amino acid 3500 abolishes the receptor binding of LDL (by approximately 70%in in heterozygotes) and produces a hyperlipidaemia identical to familial hypercholesterolaemia (Innerarity, Mahley, Weisgraber, Bersot, Krauss, Vega, Grundy, Friedl, Davignon and McCarthy 1990).

3.2. Apolipoprotein E

Apolipoprotein E is the most cosmopolitan of the apolipoproteins exchanging freely between the lower density lipoproteins and HDL. The human apoE gene is located on chromosome 19 where it is closely linked to the genes for apoC-I and apoC-II and more distantly to the gene for the LDL receptor (Lusis, Heinzmann, Sparkes, Scott, Knott, Geller, Sparkes and Mohandas 1986). Messenger RNA is found in human liver cells and monocyte macrophages and translates a mature peptide of 299 amino acids (molecular weight 34.2k Da) after co-translational cleavage of an 18 amino acid signal peptide (Zannis, McPherson, Goldberger, Karathanasis and Breslow 1984). The protein forms a four-helix bundle with the LDL receptor binding domain at the NH_2-terminal of helix four, which is unusually rich in basic residues. The binding of apoE to the surface of lipoproteins is mediated by the COOH-terminal region (Wilson, Wardell, Weisgraber, Mahley and Agard 1991).

In man, three major isoforms of apoE, which are the products of genetic polymorphism at the apoE locus, are identifiable by isoelectric focusing (Menzel and Utermann 1986). These isoforms differ by amino acid substitution at one or both of two sites (residues 112 and 158) and are coded for by distinct alleles; E4 has arginine, and E2 cysteine at both sites, whereas E3 has cysteine at 112 and arginine at 158. These determine six genotypes that have a significant impact on lipid and lipoprotein concentrations, the most striking of which is the hyperlipoproteinaemia associated with the E2/2 phenotype where the remnants of triglyceride rich lipoproteins accumulate because apoE2 is an ineffective ligand for receptor binding (Davignon, Gregg and Sing 1988). Further evidence of the essential role that apoE plays in the catabolism of these remnants is found in patients with familial apoE deficiency (Schaefer, Gregg, Ghiselli, Forte, Ordovas, Zech and Brewer 1986).

3.3. Apolipoprotein A

Apolipoprotein A-I is the major protein of HDL and is found in small quantities on chylomicrons and VLDL. Mature apoA-I (molecular weight 28k Da) is the prototypic amphipathic apolipoprotein and is the activator of LCAT. Human apoA-I is synthesised in the liver and intestine as a 267 amino acid preproprotein, which undergoes co-translational cleavage to produce a proprotein of 249 residues that is converted to mature apoA-I in lymph and plasma by the loss of a hexapeptide (Bojanovski, Gregg, Ghiselli, Schaefer, Light and Brewer 1985). A number of genetic variants of apoA-I, resulting from single amino acid substitutions, have been identified in man, some of which may influence plasma apoA-I and HDL concentrations and the activity of LCAT (Ordovas 1990).

The human apoA-I gene is located on the long arm of chromosome 11, alongside the gene for apoA-IV, with the gene for apoC-III on the opposite strand of that chromosome. These three genes, and those that code for apoA-II, apoC-II and apoE, all share a similar structural organisation with four exons and three introns suggesting that they have evolved from a common ancestor through partial or complete gene duplications (Breslow 1987).

Apolipoprotein A-II is a minor component of HDL. It is synthesised primarily in the liver (Eggerman, Hoeg, Meng, Tombragel, Bojanovski and Brewer 1991) and exists in the circulation as a dimer made up of two identical 77 residue polypeptide chains (each 8.5k Da) linked by a disulphide bond between the cysteines at residue 6 (Brewer, Lux, Ronan and John 1972). The metabolic functions of apoA-II are unclear; there is *in vitro* evidence that it can modulate the activity of LCAT (Nishida, Nakanishi, Yen, Arai, Yen and Nishida 1986), and that it may play a role in the activation of HL (Jahn, Osbourne, Schaefer and Brewer 1983).

Apolipoprotein A-IV is a component of newly secreted chylomicrons and in the circulation is found only in small amounts in HDL with the majority (>80%) present in the lipoprotein free fraction following ultracentrifugation (Bisgaier, Sachdev, Megna and Glickman 1985). The function of this protein is also unclear, although it is a potent activator of LCAT *in vitro* (Steinmetz and Utermann 1985) and may be required for the efficient transfer of apoC-II from HDL to triglyceride rich lipoproteins in preparation for hydrolysis (Goldberg, Scheraldi, Yacoub, Saxena and Bisgaier 1990). The plasma concentrations of apoA-IV increase after fat feeding (Bisgaier *et al.* 1985) prompting speculation that the protein is involved in chylomicron production, recently corroborated by the mild fat malabsorption seen in a kindred deficient in apoA-IV (Ordovas, Cassidy, Civeira, Bisgaier and Schaefer 1989).

The human apoA-IV gene is located with those for apoA-I and apoC-III on chromosome 11; its mRNA is most abundant in the small intestine with less than 2% of the total message being of hepatic origin (Elshourbagy, Walker, Boguski, Gordon and Taylor 1986). Polymorphism of human apoA-IV has been described, but has apparently little effect on plasma lipoprotein metabolism (de Kniff, Rosseneu, Beisiegel, de Keersgieter, Frants and Havekes 1988). Proteins with the features of apoA-IV are detectable in the rat, where there is significant hepatic synthesis, (Swaney, Reese and Eder 1977) and the dog (Weisgraber, Bersot and Mahley 1978).

3.4. Apolipoprotein C

Apolipoproteins C-I, C-II and C-III are to varying extents present on chylomicrons, VLDL and HDL, and are synthesised predominantly in the liver with a minor contribution from the small intestine. Although they are collectively referred to as apoC, they are products of separate genes and have metabolically diverse functions.

There is evidence that there are two copies of the **apoC-I** gene, both located on chromosome 19; the first is adjacent to the apoE gene and the second is 3' to this apoE/apoC-I gene complex (Li, Tanimura, Luo, Datta and Chan 1988). Whether both genes are transcriptionally active is at present unclear as only a single protein of 6.5k Da is found in plasma (Breslow 1987). The metabolic function of apoC-I has not been defined, although it is a weak activator of LCAT *in vitro* (Soutar, Garner, Baker, Sparrow, Jackson, Gotto and Smith 1975).

In contrast, the essential role that **apoC-II** plays in the activation of LPL is well established and shown most strikingly by the severe hypertriglyceridaemia that is found in patients with familial apoC-II deficiency. The apoC-II gene is located between the genes for apoE and apoC-I on chromosome 19 and codes for a mature protein of 79 amino acids (9k Da) (Fojo, Taam, Fairwell, Ronan, Bishop, Meng,

Hoeg, Sprecher and Brewer 1986). The LPL activation site is in residues 77-79 and residues 44-55 possess the ability to bind to phospholipids (Breslow 1987).

Apolipoprotein C-III is the most abundant of the C proteins; it is a glycoprotein that contains none, one or two molecules of sialic acid so that three isomers identifiable by isoelectric focusing (Breslow 1987). ApoC-III has been shown to inhibit the activity of LPL in a non-competitive fashion both *in vitro* (Brown and Baginsky 1972) and *in vivo* (Wang, McConathy, Kloer and Alaupovic 1985) and may also conceal the apoE on triglyceride lipoproteins from cell surface receptors so that lipolysis is completed prior to remnant removal. The apoC-III gene is located between those for apoA-I and apoA-IV on chromosome 11 and codes for a mature protein of 79 amino acids (9k Da) (Sharpe, Sidoli, Shelly, Lucerno, Shoulders and Baralle 1984). Genetic variation within this gene complex has recently been associated with hyperlipidaemia (Shoulders, Ball and Baralle 1989).

4. THE ENZYMES OF LIPOPROTEIN METABOLISM

4.1. Lipoprotein Lipase

Lipoprotein lipase plays a key role in the metabolism of triglyceride rich lipoproteins of endogenous and exogenous origin and therefore in the generation of chylomicron and VLDL remnants and IDL. The human enzyme is coded for by a 30 kb gene containing 10 exons (Deeb and Peng 1989), that has been mapped to the p22 region of chromosome 8 (Sparkes, Zollman, Klisak, Kirchgessner, Komaromy, Mohandas, Schotz and Lusis 1987), and which predicts a 448 amino acid protein of molecular weight 50,314 Da (Wion, Kirchgessner, Lusis, Schotz and Lawn 1987). Comparison of the LPL cDNA sequences from man (Wion *et al.* 1987), the mouse (Kirchgessner, Svenson, Lusis and Schotz 1987), cattle (Senda, Oka, Brown, Quasba and Furichi 1987) and the guinea pig (Enerbäck, Semb, Bengtsson-Olivecrona, Carlsson, Hermansson, Olivecrona and Bjursell 1987) reveals that the enzyme is extraordinarily conserved amongst species. Further, it is apparent that LPL is a member of a family of proteins that includes hepatic lipase and pancreatic lipase, which are believed to originate from a single ancestral gene (Kirchgessner, Chuant, Heinzmann, Etienne, Guilhot, Ameis, Pilon, D'Auriol, Anadalibi, Schotz, Galibert and Lusis 1989).

While LPL mRNA can be identified in a number of body tissues, including adipose, skeletal and cardiac muscle, lactating mammary gland, brain, adrenal, lung, ovary and kidney (Goldberg, Soprano, Wyatt, Vanni, Kirchgessner and Schotz 1989), it is in the heart and adipose tissue that the signal is strongest and the enzyme activity metabolically most significant (Semenkovich, Chen, Wims, Luo, Li and Chan 1989). Following synthesis and glycosylation in the endoplasmic reticulum, the protein is

transported to the Golgi apparatus where the N-linked oligosaccharide chains are processed allowing the enzyme to fold, adopt a dimeric conformation and acquire hydrolytic activity (Vannier and Ailhaud 1989). Only approximately 30% of newly synthesised LPL is released, the remainder being degraded in an intracellular compartment that might represent a reservoir of secretory capacity (Vannier, Amri, Etienne, Négrel and Ailhaud 1985). The mature enzyme attaches to the vascular endothelium via the glycan chains of heparan sulphate proteoglycans and high affinity binding sites on the luminal surface of these cells direct the enzyme to its site of functional activity (Blanchette-Mackie, Masuno, Dwyer, Olivecrona and Scow 1989). This association is not permanent and the enzyme appears to move along the vascular tree eventually reaching tissues remote from those of LPL synthesis, including the liver where the enzyme is taken up and degraded (Olivecrona and Bengtsson-Olivecrona 1987).

In addition to the heparin binding domain, the enzyme has a site that anchors the substrate particle (lipid interface recognition site), a site for its cofactor apoC-II and a catalytic site that hydrolyses tri- and monoglycerides (Brunzell 1989). There is evidence that LPL possesses phospholipase activity *in vitro* (Scow and Egelrud 1986) and that the enzyme might be important in the transfer of vitamin E from chylomicrons to peripheral tissues (Traber, Olivecrona and Kayden 1985). The mechanism by which apoC-II activates LPL is unclear; the protein is not required for substrate binding but may serve as a recognition signal for the lipase (Olivecrona and Bengtsson-Olivecrona 1987). Enzyme activity is also modulated by apoC-III, which appears to inhibit apoC-II activation in a non-competitive fashion (Wang *et al.* 1985), and by apoA-IV which may play a role in releasing apoC-II from HDL for the hydrolysis of nascent particles (Goldberg *et al.* 1990). Recent evidence suggests that apoE may inhibit the activity of LPL and direct remnants of triglyceride rich lipoproteins for removal (McConathy and Wang 1989).

The regulation of LPL activity *in vivo* appears complex. Feeding and fasting have profound and opposite effects; adipose LPL activity increases post-prandially and returns to normal on fasting, while muscle LPL decreases on feeding and increases during fasting (Olivecrona and Bengtsson-Olivecrona 1990). This coordinate response seems a rational mechanism by which lipids of dietary origin can be stored in plenty without deleterious effects on skeletal and cardiac function, and are then redistributed to these organs when required during times of fasting. This appears to be controlled posttranslationally as adipose and cardiac LPL mRNA levels and rates of LPL synthesis do not correlate with changes in LPL activity on fasting (Doolittle, Ben-Zeev, Elovson, Martin and Kirchgessner 1990). Rather, these authors suggest that LPL activity is modified by diverting newly synthesised enzyme towards

or away from the intracellular degradation compartment to alter the amount of enzyme secreted. The major signal responsible for this regulation appears to come from insulin, although glucagon, glucocorticoids, thyroxine and the catecholamines may also play roles (Kissebah and Schectman 1987). Elevated insulin levels in obesity and hyperinsulinaemia are associated with increased adipose LPL activity (Pykälistö, Smith and Brunzell 1975) and insulin has been shown to increase human adipose LPL activity (Sadur and Eckel 1982) by increasing both the synthesis (Ong, Kirchgessner, Schotz and Kern 1988) and secretion (Chan, Lisanti, Rodriguez-Boulan and Saltiel 1988) of LPL from adipocytes. In contrast, insulin seems to decrease LPL activity in skeletal muscle (Kiens, Lithel, Mikines and Richter 1989).

Tumour necrosis factor is a powerful inhibitor of adipose LPL synthesis and activity *in vitro* (Fried and Zechner 1989) and may play an important role in the genesis of the hypertriglyceridaemia that accompanies infection and malignancy. Mammary gland LPL activity increases greatly at the onset of lactation in humans (Neville, Waxman, Jensen and Eckel 1991) and the cow (Iverius and Östlund-Linqvist 1976). This is largely accomplished by prolactin stimulation of mammary tissue and is accompanied by a decrease in adipose LPL activity that serves to channel plasma triglycerides for milk production (Scow and Chernick 1987). Recently, additional roles have been ascribed for LPL in the provision of fatty acids to neurones (Ben-Zeev, Doolittle, Singh, Chang and Schotz 1990) and in generating fatty acids to participate in lung alveolar defences (Coonrod, Karathanasis and Lin 1989).

4.2. Hepatic Lipase

Hepatic lipase, also known as the salt resistant lipase or hepatic triglyceride lipase, is an endothelial bound glycoprotein that shares with LPL the ability to bind to lipid surfaces, a common catalytic site that hydrolyses tri- and monoglycerides, and a heparin binding site. In contrast to LPL, HL does not require a serum cofactor and has significant phospholipase activity (Ehnholm, Shaw, Greten and Brown 1975). The functional significance of HL has only become clear within the last decade as lipoprotein metabolism has been studied in animals following blockade of the enzyme (Grossner, Schreckner and Greten 1981; Goldberg, Le, Paterniti, Ginsberg, Lindgren and Brown 1982) and in human patients with a familial deficiency of the enzyme. The enzyme is essential for the final stages of VLDL and IDL catabolism and the conversion of IDL to LDL, for the interconversion of HDL subfractions (HDL_2 to HDL_3) and for the remodelling of large, buoyant LDL to smaller and denser particles. In addition, there is recent evidence to suggest a role in the uptake of chylomicron remnants both *in vitro* (Sultan, Lagrange, Le Liepvre, and Griglio 1989) and *in vivo* (Sultan *et al.* 1990).

The human HL gene has been mapped to the q21 region of chromosome 15 (Sparkes *et al.* 1987); it is 35 kb in length and translates a 477 amino acid protein of 53k Da (Cal, Wong, Chen and Chan 1989). Evidence from the rat indicates that although HL activity is found in hepatic, adrenal and ovarian tissue (Doolittle, Wong, Davis and Schotz 1987), HL synthesis is confined to the liver (Semenkovic *et al.* 1989) and that the enzyme in extrahepatic tissues results from transport along the vascular bed and the presence of specialised binding sites in these tissues. In common with LPL, oligosaccharide processing is essential for the secretion of active HL (Verhoeven and Jansen 1990) and the native enzyme appears to exist as a complex of four subunits (Twu, Garfinkel and Schotz 1984).

In contrast to LPL, little is known of the regulation of HL activity. There is evidence that apoC-III may inhibit triglyceride but not monoglyceride or phospholipid hydrolysis *in vitro* (Twu *et al.* 1984) and that the recognition of surface lipid by the enzyme is sensitive to the apolipoprotein configuration of the substrate particle (Taskinen and Kuusi 1987). The modulation of HL by sex hormones is well established. Oestrogen supplementation decreases HL activity in post-menopausal women (Applebaum-Bowden, McLean, Steinmetz, Fontana, Matthys, Warnick, Cheung, Albers and Hazzard 1989) and hepatic mRNA levels rise in ovarectomized rats and decrease when oestrogens are supplemented (Staels, Jansen, van Tol, Stahnke, Will, Verhoeven and Auwerx 1990). Conversely, androgen derived progestins appear to increase HL activity (Tikkanen, Nikkilä, Kuusi and Sipinen 1982).

4.3. Lecithin:Cholesterol Acyl Transferase

When plasma is incubated at 37°C, a net esterification of cholesterol occurs as fatty acids are transferred from the 2-position of lecithin (phosphatidylcholine) to the 3-hydroxy group of cholesterol forming cholesteryl esters and lysolecithin (Glomset 1968). This reaction is catalysed by lecithin:cholesterol acyl transferase (LCAT) and is the major source of cholesteryl esters in human plasma (Barter, Hopkins and Rajaram 1987). Following synthesis in the liver (McLean, Wion, Drayna, Fielding and Lawn 1986), LCAT is released into the circulation where it complexes with the lipids present on the surface of HDL and thus becomes intimate with its substrates and obligate cofactor apoA-I. The conversion of free cholesterol to cholesteryl esters is central to the maturation of HDL from their precursors, and is essential for maintaining the concentration gradient down which excess free cholesterol can move from tissue membranes into the plasma compartment in the first step of reverse cholesterol transport (Reichl and Miller 1989). Under experimental conditions, up to 50% of the cholesteryl esters synthesised by LCAT are derived from cholesterol of

cell membrane origin (Fielding, Davison, Karasek and Fielding 1982), the remainder coming from free cholesterol secreted in nascent HDL particles and from the transfer of surface remnants from lower density lipoproteins (Francone, Fielding and Fielding 1990).

The physiological significance of LCAT activity is confirmed by studies of cholesterol metabolism in patients with the rare condition of familial LCAT deficiency. Here, plasma cholesterol esterification is minimal, the lipoproteins are almost entirely devoid of cholesteryl esters (Glomset, Norum and King 1970), and the HDL particles remain small and discoidal in shape resembling nascent HDL of hepatic and intestinal origin (Chen, Applegate, King, Glomset, Norum and Gjone 1984). The patient's inability to generate a concentration gradient results in the accumulation of free cholesterol in cell membranes (Hovig and Gjone 1974) which is manifested by diffuse corneal opacities, increased red blood cell fragility leading to a normochromic anaemia, and proteinuria with late renal insufficiency (Gjone 1974).

The presence of LCAT activity in equine serum is well documented, first by Yamamoto, Tunaka and Sugano (1979a), who showed that the enzyme exhibited some specificity to form the linoleate ester in the cholesterol ester product, and in common with human LCAT was completely blocked by DTNB. Although the enzyme was active in all lipoprotein fractions isolated by precipitation or ultracentrifugation, HDL appeared the preferred substrate accounting for 53-80% of the decrease in free cholesterol (Yamamoto, Tunaka and Sugano 1979b). This, together with the significant activity that they found in the density >1.225 g/ml fraction, suggested that the equine enzyme is also associated with HDL particles and dissociates from this complex during ultracentrifugation. This group recently purified the enzyme free of apolipoproteins, it had an apparent molecular weight on polyacrylamide gel electrophoresis of 64k Da and was resistant to reduction by 2-mercaptoethanol suggesting that it was constructed of a single polypeptide chain (Yamamoto, Yamamoto, Tunaka and Sugano 1987). Chen and Albers (1983) found that LCAT in equine plasma was activated by apoA-I isolated from man, horses, sheep, goats and rabbits, and that equine apoA-I was an effective activator of LCAT in plasma from these species.

4.4. The Plasma Lipid Transfer Proteins

In man and some animal species, the exchange and net transfer of lipids between lipoprotein particles is facilitated by two specialist lipid transfer proteins (LTP) (Tall 1986). The first of these, the cholesteryl ester transfer protein (CETP) also known as LTP-I, promotes the redistribution of cholesteryl esters from HDL to the lower density lipoproteins (VLDL and LDL) in exchange for triglycerides (Chajek and

Fielding 1978). This 74k Da protein (Jarnagin, Kohr and Fielding 1987) is synthesised by the liver, small intestine, adrenals and spleen in man (Drayna, Jarnagin, McLean, Henzel, Kohr, and Fielding 1987) and chiefly in the liver in the rabbit (Nagashima, McLean and Lawn 1988). In common with LCAT, CETP appears to be complexed to HDL by electrostatic attractions that are be separated by ultracentrifugation at density >1.215 g/ml (Marcel, McPherson, Hogue, Czarnecka, Zawadzki, Weech, Whitlock, Tall and Milne 1990). Evidence suggests that CETP acts as a carrier between lipoproteins with single binding sites for cholesteryl ester, triglyceride and phosphatidylcholine (Swenson, Brocia and Tall 1988) and that the kinetics of transfer are favoured by the formation of a ternary collision complex comprising CETP and the donor and acceptor particles (Ihm, Quinn, Busch, Chataing and Harmony 1982). While CETP has some capacity to exchange phospholipids *in vitro* (Tall, Abreu and Shuman 1983), the majority of human phospholipid transfer is mediated by a second protein (LTP-II) that has no cholesteryl ester or triglyceride transfer capacity, but which may enhance cholesteryl ester transfer mediated by LTP-I (Tollefson, Ravnik and Albers 1988).

The transfer of LCAT derived cholesteryl esters to the apoB containing lipoproteins provides a route by which cholesterol of peripheral origin may be returned to the liver in the second step of reverse cholesterol transport. To help this, there is evidence that CETP may directly influence cholesteryl ester efflux from peripheral cells into the vascular compartment (Morton 1988). As the LDL receptor is not confined to the liver, the benefits of this process must be reappraised in the light of the atherogenic potential of cholesteryl ester rich lower density lipoproteins (Griffin *et al.* 1990). Because CETP plays a prominent role in the remodelling of lipoproteins in the vascular compartment, particularly in the interconversion between HDL subclasses (Lagrost, Gambert, Dangremont, Athias and Lallemant 1990) and the genesis of LDL heterogeneity (Gambert, Bouzerand-Gambert, Athias, Farnier and Lallemant 1990), the activity of this protein may directly influence the atherogenicity of an individual's lipoprotein phenotype and their risk of CAD.

The physiological impact of CETP has only been recently confirmed by studies of lipoprotein metabolism in humans with an inherited deficiency of the protein (Brown, Inazu, Hesler, Agellon, Mann, Whitlock, Marcel, Milne, Koizumi, Mabuchi, Takeda and Tall 1989). These patients have markedly increased HDL concentrations, enlarged HDL particles that are enriched with cholesteryl esters and depleted of triglyceride, and a reduced cholesteryl ester content of VLDL and LDL (Koizumi, Mabuchi, Yoshimura, Michishita, Takeda, Itoh, Sakai, Sakai, Ueda and Takeda 1985). These findings are supported by examination of animal species with little or no CETP activity (Ha and Barter 1982), by studies in which antibodies have

been used to inhibit rabbit transfer protein activity *in vivo* (Whitlock, Swenson, Ramakrishnan, Leonard, Marcel, Milne and Tall 1989; Abbey and Calvert 1989), and where CETP has been injected into a deficient species (Ha, Chang and Barter 1985; Groener, van Gent and van Tol 1989). These all show that in the absence of CETP, HDL particles expand and become enriched in cholesteryl esters (through the agency of LCAT) and apoE so that they resemble HDL_1. When CETP is added or its blockade removed, these particles are lost as the HDL fraction is depleted of cholesteryl esters and enriched in triglyceride, and the cholesteryl ester content of the lower density lipoproteins increases.

The species distribution of CETP has been well documented by Ha and Barter (1982) who divided animals into groups of high (possum, rabbit and trout), intermediate (man, wallaby guinea pig, chicken, turkey, toad, lizard and snake) and low (cow, sheep, pig, dog and rat) activity. More recent studies have shown that low CETP activity might be the result of an inhibitor to transfer activity which is expressed variably amongst species, as transfer activity in plasma from rats and pigs has been unmasked by separation from the inhibitor on phenyl sepharose (Tollefson, Liu and Albers 1988). An inhibitor to CETP has been isolated from human plasma (Son and Zilversmit 1984) and recently characterised (Nishide, Tollefson and Albers 1989).

5. THE METABOLIC BASIS OF HYPERTRIGLYCERIDAEMIA

The prevalence of hyperlipidaemia in the human populations of developed countries led Fredrickson and colleagues (1967) to define five types of hyperlipoproteinaemia based on changes in the concentration and composition of the plasma lipoproteins. This phenotypic classification has, however, been made redundant by improvements in understanding the nature of the genetic, molecular and metabolic defects that disturb lipoprotein metabolism. The origins of increased plasma triglyceride concentrations in man are considered here as the model mechanisms on which investigations of the aetiology of equine hyperlipaemia were based.

Hypertriglyceridaemia represents the accumulation of triglyceride rich lipoproteins in the circulation and because both chylomicrons and VLDL are large particles, they scatter light when present in high concentrations and impart upon plasma the turbidity or lactescence that is recognised as lipaemia. In addition, the buoyancy of chylomicrons causes them to float to the top of plasma when left to stand at 4°C to form a creamy layer, the "creaming in the cold test" (Brunzell and Bierman 1982).

5.1. Familial Deficiencies of Lipoprotein Lipase and Apolipoprotein C-II, Familial Inhibitor of Lipoprotein Lipase

Havel and Gordon (1960) first described an inherited deficiency of LPL as the cause of defective chylomicron clearance in three siblings with massive hypertriglyceridaemia. This is a rare condition, occurring in approximately one person in a million, which presents in childhood with recurrent pancreatitis accompanied by hepatosplenomegaly, eruptive xanthomatosis and lipaemia retinalis (Brunzell 1989). A presumptive diagnosis can be made on the basis of a marked reduction in triglycerides after a week on a severely fat restricted diet and confirmed by the measurement of negligible LPL activity in post-heparin plasma or adipose tissue biopsies.

This condition appears to be inherited in an autosomal recessive manner and a number of point mutations in the LPL gene have been described that lead to the synthesis of a truncated inactive protein (Emi, Hata, Robertson, Iverius, Hegele and Lalouel 1990), and defects in the lipid interface recognition site (Henderson, Ma, Hassan, Monsalve, Marais, Winkler, Gubernator, Peterson, Brunzell and Hayden 1991), the heparin binding site (Beg, Meng, Skarlatos, Previato, Brunzell, Brewer and Fojo 1990) and the catalytic site of the enzyme (Monsalve, Henderson, Roederer, Julien, Deeb, Kastelein, Peritz, Devlin, Bruin, Murthy, Cagne, Davignon, Lupien, Brunzell and Hayden 1990). A more extensive internal gene duplication (Devlin, Deeb, Brunzell, and Hayden 1990) and major rearrangement of the gene have been described (Langlois, Deeb, Brunzell, Kastelein and Hayden 1989). The treatment of familial LPL deficiency rests on reducing chylomicron synthesis by limiting dietary fat intake and providing medium chain triglycerides, which do not contribute to chylomicron formation, as energy supplements.

Although LPL deficiency was long held synonymous with Fredrickson's type I hyperlipoproteinaemia, two other genetic accidents have subsequently been recorded as causes of this lipid phenotype. In the first of these, LPL activity is normal *in vitro* but the patient's plasma is deficient in apoC-II and therefore unable to activate the enzyme *in vivo* (Breckenridge, Little, Steiner, Chow and Poapst 1978). **Familial deficiency of apoC-II** has now been recognised in 11 families world-wide and appears to be inherited in an autosomal recessive fashion (Brunzell 1989). This condition tends to be milder and later in onset than LPL deficiency and infusion of plasma from healthy donors, or biologically active apoC-II, corrects the hypertriglyceridaemia and may offer some therapeutic potential (Baggio, Manzato, Gabelli, Fellin, Martini, Enzi, Verlato, Baiocchi, Sprecher, Kashyap, Brewer and Crepaldi 1986). Recently an inactive variant of apoC-II has been described in a

kindred with familial apoC-II deficiency (Sprecher, Taam, Gregg, Fojo, Wilson, Kashyap and Brewer 1988). More recently, type I hyperlipoproteinaemia was described in a mother and son who had an **inhibitor of LPL** in their plasma, which appeared to be inherited in an autosomal dominant fashion (Brunzell, Miller, Alaupovic, St. Hilaire, Wang, Sarson, Bloom and Lewis 1983).

5.2. Other Inherited Forms of Hypertriglyceridaemia

Familial hypertriglyceridaemia (endogenous hypertriglyceridaemia, type IV hyperlipoproteinaemia) is a heterogeneous disorder in which increased production of VLDL saturates the removal system and causes moderate hypertriglyceridaemia (Brunzell, Albers, Chait, Grundy, Groszek and McDonald 1983). Some patients also have an underlying defect in LPL activity, a subset of which may be heterozygotes for LPL deficiency (Babirak, Iverius, Fujimoto and Brunzell 1989; Wilson, Emi, Iverius, Hata, Wu, Hillas, Williams and Lalouel 1990). In these cases, reduced transfer of core and surface remnants would explain the low concentrations of LDL and HDL that accompany the hypertriglyceridaemia (Packard, Shepherd, Joerns, Gotto and Taunton 1980)

Familial combined hyperlipidaemia defines a condition characterised by moderate elevations of plasma cholesterol and/or triglyceride and the presence of hyperlipidaemia in one or more family members (Brunzell *et al.* 1983; Austin, Brunzell, Fitch and Krauss 1990). Kinetic studies of apoB show that VLDL is overproduced and that increased mass transfer to LDL accounts for the elevated plasma and LDL cholesterol concentrations (Chait, Foster, Albers, Failor and Brunzell 1986). Overproduction of VLDL is also thought to be the mechanism behind **hyperapobetalipoproteinaemia**, a condition where plasma and LDL cholesterol concentrations appear normal but apoB levels are elevated because of a preponderance of small dense LDL (Teng, Sniderman, Soutar and Thompson 1986).

Plasma triglyceride and cholesterol concentrations are elevated in **familial dysbetalipoproteinaemia** (type III hyperlipoproteinaemia, broad/floating ß disease, remnant removal disease) where there is defective clearance of chylomicron and VLDL remnants due to the apoE2 isoform. The condition is marked by the presence of ß-VLDL, characteristic striate palmar and tuberose xanthomata, and premature CAD (Durrington 1989a). The expression of this hyperlipidaemia is not, however, a strict consequence of the E2/2 phenotype and in some patients is dependant on secondary factors such as obesity, diabetes mellitus and hypothyroidism. A similar lipid phenotype is seen in patients with the rare condition of familial hepatic lipase deficiency (Breckenridge, Little, Alaupovic, Wang, Kuksis, Kakis, Lindgren and Gardiner 1983)

The treatment for each of these conditions involves restriction of fat intake, in particular of saturated fatty acids, supplemented by lipid lowering drugs that act to reduce VLDL secretion. Such agents include nicotinic acid and its analogues (Fattore and Sirtori 1991), and the fibrate derived group of drugs, some of which also increase LPL activity (Gaw and Shepherd 1991).

5.3. Secondary or Acquired Causes of Hypertriglyceridaemia

A number of conditions have secondary effects on the metabolism of chylomicrons and VLDL and give rise to hypertriglyceridaemia, either alone or by superimposing upon the more subtle familial defects in the metabolism of triglyceride rich lipoproteins outlined above.

The most common of these is **diabetes mellitus**, where hypertriglyceridaemia in concert with low levels of HDL, hypertension and hyperinsulinaemia, presents a major increase in CAD risk. In patients with both uncontrolled insulin dependent (IDDM) and non-insulin dependent (NIDDM) diabetes mellitus, there is increased synthesis of VLDL that is triglyceride enriched with an increased triglyceride/apoB ratio (Howard 1987). This probably relates to the poor regulation of adipose tissue lipolysis in these people resulting in increased flux of FFA to the liver rather than any direct effect of insulin, in deficiency or excess, on hepatic triglyceride synthesis (Brunzell, Chait and Bierman 1985). Decreased fractional clearance of VLDL and chylomicrons may also contribute to the hypertriglyceridaemia as the permissive effects of insulin on the expression of LPL activity are lost (Abbate and Brunzell 1990). Plasma triglyceride concentrations are normalised with good glycaemic control in patients with IDDM but not in NIDDM. This latter fact may be related to the prevalence and effects of chronic hyperinsulinaemia, obesity, high alcohol intake, concurrent renal disease and drug therapy (diuretics and ß blockers) in people with NIDDM (Durrington 1989b).

Obesity, and in particular abdominal rather than gluteal-femoral fat, is associated with insulin resistance, glucose intolerance, hypertension and hypertriglyceridaemia and as such represents a major CAD risk factor (Kissebah and Peiris 1989). The hypertriglyceridaemia appears to result from a greater rate of fatty acid turnover in abdominal adipose tissue, leading to high portal FFA concentrations and increased triglyceride synthesis and VLDL secretion (Després 1991).

Mild hypertriglyceridaemia is occasionally observed with overt **hypothyroidism**, although increases in plasma and LDL cholesterol are more usual because of reduced receptor clearance of LDL (Abrams and Grundy 1981). In cases where triglyceride levels are elevated, the clearance of triglyceride rich lipoproteins is

reduced (Abrams, Grundy and Ginsberg 1981) because of a decrease in post-heparin plasma LPL activity (Valdemarsson, Hansson, Hedner and Nilsson-Ehle 1983).

Chronic excessive **alcohol** consumption results in hypertriglyceridaemia by increasing adipose lipolysis and reducing hepatic oxidation and gluconeogenesis so that the proportion of FFA channelled into triglyceride synthesis is increased (Taskinen, Nikkilä, Välimäki, Sane, Kuusi, Kësaniemi and Ylikahri 1987). However, some consolation comes from the evidence that moderate alcohol consumption increases LPL activity and may therefore buffer mild increases in VLDL production, that is until the lipolytic capacity is exceeded (Schneider, Liesenfeld, Mardsini, Schubotz, Zofel, Vandre-Plozzitzka and Kaffarnik 1985).

Although hyperlipidaemia is commonly seen in people with the **nephrotic syndrome,** the precise nature of the disturbances in lipoprotein metabolism are unclear (Keane and Kasiske 1990). Approximately 69% of cases have hypertriglyceridaemia, alone or in combination with hypercholesterolaemia, which is entirely due to increased VLDL, and not chylomicron, concentrations (Joven, Villabona, Vilella, Masana, Albertí and Vallés 1990). Increased secretion and reduced fractional catabolism of VLDL has recently been reported in these patients (Warwick, Packard, Demant, Bedford, Boulton-Jones and Shepherd 1991).

Other secondary causes of mild to moderate hypertriglyceridaemia include therapy with ß blockers, diuretics, oestrogen supplements, and corticosteroid excess due to Cushing's syndrome or exogenous glucocorticoid administration (Durrington 1989b).

6. LIPOPROTEIN METABOLISM IN THE HORSE

Awareness of lipoprotein metabolism in animals has been stimulated in recent years as certain species have proved useful as experimental models of hyperlipidaemia (Reue, Warden and Lusis 1990) and atherosclerosis (Armstrong and Heistad 1990). The horse was instrumental in providing Macheboeuf (1929) with the earliest source of a soluble lipid-protein complex. Since then, relatively little research effort has been devoted to understanding how the horse accomplishes plasma lipid transport, such that in Chapman's comprehensive review of animal lipoproteins (1980), the horse occupied half a paragraph while insects, fish, reptiles and birds commanded 27 pages between them. The information that is available concerning the structure and function of the equine plasma lipoproteins, in both healthy animals and in ponies with hyperlipaemia, is reviewed in the next two sections.

6.1. The Equine Plasma Lipoproteins

The earliest quantitative data on the blood lipids of the horse were published as recently as 1960 by Forenbacher, Keler-Bacoka and Pucar (1960) who measured the concentrations of cholesterol, phospholipids and total lipids in serum from 61 horses. The mean cholesterol was 98.8 mg/dl (2.56 mmol/l) with a range of 66 to 218 mg/dl (1.71-5.65 mmol/l), and was higher in geldings (104.4 mg/dl) and stallions (113 mg/dl) than mares (89 mg/dl), and also in eight animals being fed a mix of brewers' grain and sunflower seed cake (152.4 mg/dl) compared with 53 horses on the common diet of hay and maize (88 mg/dl). The mean phospholipid concentrations were also higher in the stallions (129.5 mg/dl) and geldings (134.3 mg/dl) than the mares (106.4 mg/dl). The total lipids were unaffected by sex or diet, and none of the three parameters measured were influenced by age. Fifteen years later, Robie, Janson, Smith and O'Connor (1975a) found that serum phospholipid and cholesterol concentrations were higher in Morgan than Thoroughbred horses and Shetland ponies, and that the ponies had higher FFA and lower glucose concentrations than the two breeds of horse. Triglyceride concentrations appeared to decline in the two horse breeds during the cold months (December to March), but remained unchanged in the Shetland ponies whose serum FFA concentrations rose during this period.

Forenbacher *et al.* (1960) also used paper strip electrophoresis to show that equine serum consisted predominantly of α-lipoproteins, with minor contributions from ß-lipoproteins and neutral fats [*sic*] that stayed at the origin. Campbell (1963) subsequently found only two areas of staining on paper electrophoresis of serum from 10 horses that corresponded to the α- and ß-lipoproteins of human serum. These accounted for 71.9% and 28.1% of total lipoprotein lipid, respectively. Robie, Smith and O'Connor (1975) found a third peak in a post-α position using slab polyacrylamide gel electrophoresis. This band represented a constant 26% of the lipoprotein spectrum in both the Morgan and the Thoroughbred horses, whereas the relative percentage of the α-lipoproteins was higher and that of the ß-lipoproteins lower in the Thoroughbred than in the Morgan horses. Later, van Dijk and Wensing (1989) showed, by using a combination of ultracentrifugation and precipitation, that plasma VLDL levels were higher in three ponies than four horses of undefined breeding.

In the third of their series of papers, Robie, Janson, Smith and O'Connor (1975b) isolated lipoproteins by preparative ultracentrifugation at densities of <1.006 (VLDL), <1.063 (LDL) and >1.063 g/ml (HDL). There were no significant differences in the lipid composition of each fraction between the two breeds, 76% of the total lipid was in HDL, 17% in LDL and 7% in VLDL. The VLDL class was typically triglyceride rich, and the LDL and HDL were reported as consisting

predominantly of FFA and phospholipid, respectively. Most of the total serum phospholipid and cholesterol was found in the HDL and the triglyceride was divided between VLDL and HDL. Electrophoresis of these fractions showed that the post-α band previously reported was a component of the density >1.063 g/ml fraction, and that the density <1.063 g/ml fraction migrated in the ß-position. The authors noted that there was a minor ß-component in the HDL fraction and commented that the VLDL fraction did not migrate in the electrophoresis systems used, yet a pre-ß band is obvious on the gel scans presented in the paper. Leat, Northrop, Buttress and Jones (1979) subsequently reported a prominent pre-ß band in two horses by electrophoresis on cellulose acetate. In addition, these authors used analytical ultracentrifugation to quantify the relative amounts of HDL (81.1%) and LDL (18.9%) and showed that the LDL was of complex composition consisting of two peaks.

More recent studies have employed density gradient ultracentrifugation. In the first of these, Terpstra, Sanchez-Muniz, West and Woodward (1982) pre-stained the serum with Sudan Black to visualise the lipoproteins after ultracentrifugation and found, in the two horses examined, that the LDL was divided into two subfractions while the HDL presented a single band. The fractions corresponding to human VLDL, LDL and HDL were collected from the two equine samples by tube slicing and their cholesterol concentrations (mmol/l serum) measured as 0.04 and 0.09 for VLDL, 0.70 and 0.92 for LDL, and 1.98 and 1.81 for HDL.

In the second study, Hollanders, Mougin, N'Diaye, Hentz, Aude and Girard (1986) unloaded their gradients through a fractionator and monitored the protein content at OD_{280nm}. They found LDL in the single horse examined within the density limits of 1.022 to 1.068 g/ml, with a peak at 1.035, and commented that this class was polydisperse. The HDL peak was found between 1.092 and 1.095 g/ml and was almost perfectly symmetrical with no evidence of heterogeneity. The lipoprotein concentrations of VLDL, LDL and HDL were 33.3, 47.7 and 222.1 mg/dl serum, respectively. The HDL fractions were delipidated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which revealed that apoA-I was the major protein of this lipoprotein, and identified apoA-II as a dimer, and a single apoC band with a molecular weight of 12.5k Da. The molecular weights of apoA-I and apoA-II were not reported, but a 45k Da protein assumed to be apoA-IV was found in the density >1.16 g/ml fraction.

In the third and most comprehensive study of this type, Le Goff, Nouvelot, Fresnel, and Silberzahn (1987) employed the lipid staining method used by Terpstra *et al.* (1982) to isolate lipoproteins from six Thoroughbred horses. Four major colour bands were found (I, II, III, IV) that corresponded in electrophoretic mobility and chemical composition to human VLDL (I), LDL (II and III) and HDL (IV). The

VLDL fraction contributed 5% of the total lipoprotein mass and was very similar to that of human VLDL in lipid composition, but was composed of somewhat smaller particles (mean diameter 22.3nm) when measured by electron microscopy. The LDL class represented 19% of the lipoprotein mass and was almost equally divided between two subfractions (LDL_1) and (LDL_2) found in the density ranges 1.028-1.044 and 1.044- 1.066 g/ml, respectively. These two fractions were distinguishable by the higher protein and lower cholesteryl ester content of LDL_2. The cholesteryl ester content of both fractions was less than that of human LDL and the particles were very much smaller with mean diameters of 14.2 and 11.6 nm, respectively. The HDL fraction was remarkable for its low triglyceride (0.5%) content compared with human HDL and appeared as a homogeneous population of relatively small particles (5.1-7.3nm). In five of the six animals, the HDL appeared as a single band and in the other there was a second band early in the HDL density range that the authors reported was chemically heterogeneous to the bulk of HDL, with a lower protein and higher phospholipid content.

In a sequel to the last paper, Le Goff, Pastier, Hannan, Petit, Ayrault-Jarrier and Nouvelot (1989) measured the apoB content of VLDL and LDL using a tetramethylurea precipitation technique and determined the distribution of apolipoproteins by SDS-PAGE. The mass of apoB represented 29.6% of VLDL protein and 84.4% of LDL protein, and presented two clearly separated bands on SDS-PAGE with molecular weights corresponding to human apoB-100, and a third similar to that of apoB-48. A 40k Da protein, thought to be apoE, was reported in LDL but not HDL, where bands of molecular weight 27k Da and 17k Da corresponding to human apoA-I and dimeric apoA-II predominated. Evidence of apoA-IV was found only as a faint band of molecular weight 45k Da in the second fraction beyond HDL at density >1.178 g/ml. Isoelectric focusing gels suggested that there was one major and three minor isoforms of apoA-I and only one isoform each of apoA-II, apoC-II and apoC-III. The identity of the apoA-II band was confirmed by immunoblotting the focused gels with a human anti apoA-II polyclonal antibody. By calculation of theoretical particle masses, the LDL_1 and LDL_2 subfractions were believed to contain 0.8 and 0.6 molecules of apoB-100, respectively, and HDL three molecules of apoA-I.

Finally, two other papers include reference to the equine apolipoproteins. Chen and Albers (1983), in the course of their LCAT study, isolated apoA-I from equine plasma by chromatography on DEAE-sepharose and found a single band on SDS-PAGE with an apparent molecular weight of 27.25k Da $\pm$0.5 (mean $\pm$sd, n=4). The presence of apoA-IV in equine plasma was confirmed by Juneja, Gahne, Lukka and Ehnholm (1989) who used isoelectric focusing and immunoblotting with rabbit

anti-human apoA-IV to show that the majority of the protein existed in a monomeric rather than a dimeric form. The molecular weight and lipoprotein distribution were not assessed. This protein had previously been designated as serum protein 2 and showed a high degree of polymorphism which was explained by four co-dominant alleles acting at a single locus (Juneja, Anderson, Sandberg, Adalsteinsson and Gunnaesson 1984).

6.2. The Plasma Lipoproteins in Ponies with Hyperlipaemia

In the earliest, and most extensive study of the pathogenesis of hyperlipaemia Morris *et al.* (1972) fasted four ponies for 184 hours thereby inducing hyperlipidaemia in three of the animals (plasma triglycerides 3.5, 6.3 and 11.1 mmol/l; cholesterol 3.8, 4.4 and 2.6 mmol/l, respectively). This was accompanied by the appearance of a pronounced pre-ß band on agarose electrophoresis, the intensity of which approximated to the increase in plasma lipids. Plasma FFA concentrations increased from 0.04-0.2 umol/ml in the pre-fasted samples to a mean of 0.6 umol/ml after eight days of fasting. One pony showed no change in plasma triglyceride or cholesterol concentrations over the eight day period. The hyperlipidaemia was subsequently induced in two other ponies, and VLDL isolated by ultracentrifugation shown to consist of 5.5% protein, 5.8% free cholesterol, 1.5% cholesteryl esters, 13.3% phospholipids and 73.8% triglycerides. Unfortunately, the authors did not isolate VLDL from non-fasted ponies but commented that, relative to human and rat VLDL, these lipoproteins were low in cholesterol with an unusual distribution of cholesterol between the free and esterified forms. They called the condition a hyperprebetalipoproteinaemia and suggested that this response to fasting was unique to the equine species.

To estimate the contribution of VLDL secretion to the hypertriglyceridaemia, Morris *et al.* (1972) in the same paper reported attempts to block the activity of LPL by injecting the fasted ponies with Triton WR 1339. Although this precipitated an acute intravascular haemolytic crisis in each of the animals, plasma triglyceride and cholesterol concentrations continued to rise in a linear fashion for between 6 and 42.4 hours. The triglycerides increased at a rate about 10 times higher than that of cholesterol compatible with the secretion of VLDL with the ratio of triglyceride to cholesterol found in the isolated VLDL (11.7:1). However, the rate of secretion of triglyceride did not appear to be related to the magnitude of the fasting hypertriglyceridaemia, which tempted the authors to postulate that the "mechanism of the hyperprebetalipoproteinaemia resides in the suppression of lipoprotein degradation".

Wensing, Van Gent, Schotman and Kroneman (1975) isolated lipoproteins from nine ponies with hyperlipaemia by a combination of ultracentrifugation (chylomicrons and VLDL) and precipitation (LDL and HDL). In addition to marked increases in the concentration of VLDL, they described the appearance of considerable concentrations of chylomicrons in some animals, including one that had not eaten for 13 days. On the basis of this, Schotman and Wensing (1977) attempted to define three phenotypes of hyperlipaemia; the first was characterised by increased VLDL, and the second and third by increases in both chylomicrons and VLDL with the contribution of chylomicrons much less in the third. Although the chylomicron class apparently presented a band well separated from that of the pre-ß lipoproteins (Wensing *et al.* 1975), neither the fact that this fraction was present in animals that had "very poor appetites", nor the lipid composition of this class (34.8-75.5% triglyceride, 9.8-19.9% cholesterol) supported their conclusion that these lipoproteins were chylomicrons. This aside, Wensing *et al.* (1975) reported a high percentage of triglyceride in the VLDL and LDL fractions relative to those from a single healthy control animal, and that the phospholipid content of HDL was reduced.

In a study of identical design to that of Morris *et al.* (1972), Bauer (1983) induced triglyceride levels of between 7.5 and 20.3 mmol/l in three ponies that were fasted for 144 hours, and failed to identify any chylomicrons on ultracentrifugation. In addition, he found no lipoproteins at the origin on agarose electrophoresis and reported that the hyperlipidaemia was entirely accounted for by the appearance of a pre-ß band. The concentrations of VLDL (density <1.006 g/ml) and LDL (density <1.064 g/ml) isolated by flotation ultracentrifugation were increased compared with two fed ponies by five to 19 fold and 1.6 to 2.4 fold, respectively, while that of HDL (density <1.21 g/ml) was unchanged. Although the triglyceride content of the VLDL was no different to that from the controls, the relative amounts of cholesterol and protein in this lipoprotein fraction were reduced and the triglyceride to protein ratio increased.

In a study recently published, Freestone, Wolfsheimer, Ford, Church and Bessin (1991) attempted to show that insulin insensitivity is indeed at the metabolic heart of equine hyperlipaemia. They defined two groups of ponies as normal or hyperinsulinaemic on the basis of differential responses to an oral glucose load and induced hypertriglyceridaemia by withholding food for 72 hours. There was, however, no difference between the normal and hyperinsulinaemic ponies in plasma triglyceride, cholesterol and lipoprotein (VLDL, LDL and HDL) triglyceride concentrations at any time point during this period. This was perhaps due to the fact that insulin levels in both groups declined to the limits of detection, so that there were then negligible differences in insulin and glucose concentrations between the two

groups. After 72 hours, half the ponies in each group were given dexamethasone and the other half placebo and the fast continued for a further 48 hours. The dexamethasone led to suppressed plasma cortisol levels for 24 hours and increased glucose concentrations for 8 hours, but had no effect on any of the lipid parameters in either the normal or hyperinsulinaemic groups compared with each other or with the placebo-treated animals. The authors admitted that the differences in insulin concentration between the two groups were perhaps inadequate to define the presence or absence of insulin resistance, and that the single bolus of dexamethasone was insufficient to antagonise insulin. One pony, who was neither obese or hyperinsulinaemic, died from hyperlipaemia during the course of the study and the authors concluded that "Consideration should be given to the possibility that these ponies may have a defect in their ability to clear the VLDL triglyceride fraction from plasma".

1.7. AIMS OF THE STUDY

The work described in this thesis was driven by the desire to reduce the threat that hyperlipaemia poses to the health and welfare of ponies. The aim of the study was to identify the underlying defect in lipoprotein metabolism so that effective preventive and therapeutic strategies could be developed.

At the outset, it was clear that these objectives could not be met without a better understanding of plasma lipid transport in the equine species. To this end, the physical and chemical properties of the lipid transport vehicles, that is the plasma lipoproteins, were first examined in healthy horses and ponies (Chapter III). This was followed by an assessment of the enzymes that are responsible for modelling and mediating the metabolism of the lipoproteins in the circulation (Chapter IV). These studies were carried out in Shetland ponies and Thoroughbred horses, and repeated in pregnant and lactating ponies (Chapter V), with the hope of identifying differences in lipoprotein metabolism that might explain the almost exclusive occurrence of hyperlipaemia in the pony breeds, and in pregnancy and lactation in particular. The description of lipoprotein metabolism in the horse was completed by examining the plasma transport of dietary fat and analysing the plasma lipoproteins in suckling foals (Chapter V).

Each of the above components provided essential baseline data for showing how the structure and metabolism of the plasma lipoproteins is perturbed in ponies with hyperlipaemia (Chapters VI). On this basis, the metabolism of VLDL triglyceride was quantified *in vivo* to show that hepatic overproduction, rather than defective catabolism, of these lipoproteins is responsible for the disease (Chapter

VII). In the final section (Chapter VIII), the significant contributions from this thesis are highlighted, a plan of the pathogenesis of equine hyperlipaemia proposed, and the implications of this work for improving the therapy of the condition discussed.

CHAPTER II

GENERAL METHODS

The suppliers of reagents and equipment are listed in Appendix 1.

1. ROUTINE BLOOD CHEMISTRIES

1.1. Lipid and Lipoprotein Concentrations

Blood was collected into potassium EDTA (final concentration 1.2-2.0 mg/ml blood), plasma separated by centrifugation at 2400 rpm for 15 mins. and kept at 4°C until analysis. Plasma cholesterol and triglyceride concentrations were measured using reagent kits (704161 and 237574; Boehringer Mannheim GmbH) on an automated discrete analyser (BM/Hitachi 717). Quality control was monitored using Seronorm Lipid (Nycomed Pharma AS) and Precipath U (Boehringer Mannheim GmbH).

Plasma lipoprotein cholesterol concentrations were measured by a combined ultracentrifugation precipitation technique. Five ml of plasma was placed in a 6.5 ml thermoplastic ultracentrifuge tube (Ultra-Clear; Beckman Instruments, Inc.) and overlayered with normal saline until a meniscus appeared at the top. The tubes were sealed, placed in a Ti50.4 rotor (Beckman Instruments, Inc.) and centrifuged at 37k rpm, 4°C for 18 hours. The supernatant was then removed by tube slicing and transferred to a 3 ml volumetric flask, the cutter was washed with normal saline and the washings used to make up the volume to 3 ml. The infranatant was transferred to a 5 ml volumetric flask and made up to volume with normal saline. One ml of the infranatant was then transferred to a second ultracentrifuge tube and the apoB containing lipoproteins were precipitated by adding 50 ul of 92 mM heparin-manganese chloride reagent. This was made up mixing 9.56 g $MnCl_2.4H_2O$ in 6 ml distilled water (dH_2O) with 1.05 g heparin ($5x10^5$ units; Sigma H-3125) dissolved in 12.5 ml normal saline, made up to 25 ml with dH_2O and stored at 4°C. The tubes were mixed on vortex and incubated at 4°C for 15 mins., then spun at 10k rpm for 30 mins. in the Ti50.4 rotor with the vacuum off to pellet the precipitate. Aliquots of the VLDL fraction, the original infranatant (LDL+HDL) and the second supernatant (HDL) were transferred to autoanalyser cups and their cholesterol concentrations determined as above. The LDL cholesterol concentration was calculated as the difference between that in the infranatant and the second supernatant.

Free fatty acid and free glycerol concentrations were determined manually using colorimetric kits (Wako NEFA C test kit; Wako Chemicals GmbH and Glycerol GY105; Randox Laboratories Ltd).

1.2. Clinical Chemistries

Plasma (collected into lithium heparin) concentrations of urea (urease method), creatinine (Jaffé method) and alkaline phosphatase were measured using Roche kits (Roche) and gamma glutamyl transferase (carboxy'NA method) using a BCL kit (Boehringer Mannheim GmbH), on a Cobas Mira (Roche) autoanalyser.

2. LIPOPROTEINS ISOLATION AND CHARACTERISATION

2.1. Gel Filtration

Gel filtration (gel chromatography, molecular sieve chromatography) was used to separate the lipoprotein classes from plasma on the basis of their differences in particle size (Rudel, Marzetta and Johnson 1986). A 6% agarose gel (Bio-Gel A-5m, 200-400 mesh; Bio-Rad Laboratories Ltd) was prepared as the support medium according to the manufacturers' instructions and packed into a 1.6x100 cm glass column which was maintained at 4°C.

The solvent density of 10 ml plasma was raised to 1.225 g/ml by adding 3.517 g NaBr, placed in a 26.3 ml polycarbonate centrifuge bottle (Beckman Instruments, Inc.) and overlayered with 15 ml of density 1.225 g/ml solution (36.7 g KBr in 100 ml dH_2O). The density of this and all other density solutions used in the following methods was checked using a digital density meter (DMA 35; Paar Scientific Ltd). After centrifugation at 39k rpm, 15°C for 60 hours, the lipoprotein fraction was removed in the top 2 ml and applied directly to the column or stored at 4°C for no longer than 72 hours. The column was eluted by gravity with phosphate buffered saline containing 0.01% NaN_3 at a flow rate of 6 ml/hr. Fractions were collected every 20 mins. (Ultrovac 7000 Fraction Collector; LKB Bromma) and their elution volume plotted against protein content monitored at OD_{280nm} (on a DU-70 Spectrophotometer; Beckman Instruments, Inc.). The fractions comprising the peaks were pooled and concentrated using the Diaflo Ultrafiltration system (Amicon Ltd) with ultrafilters of molecular weight limits of 300k Da (Diaflo XM300) for VLDL and LDL, and 50k Da (Diaflo XM50) for HDL.

2.2. Rate-zonal Ultracentrifugation

The major lipoprotein classes were isolated from plasma according to flotation rate by rate-zonal ultracentrifugation using a two step procedure described by Patsch, Sailer, Kostner, Sandhofer, Holasek and Braunsteiner (1974). In the first step, HDL was separated from the lower density lipoproteins in a stepped gradient of density of 1.0-1.4 g/ml, which were collected, concentrated and applied in the second step to a linear

gradient of 1.0-1.3 g/ml to resolve LDL from VLDL and IDL. The density solutions were made up as follows: density 1.00 g/ml, 10 ml 1 M Tris pH 7.6, 0.1 g Na_2EDTA made up to 1 l with dH_2O; density 1.3 g/ml, 400 g NaBr, 10 ml 1M Tris pH 7.6, 0.1 g Na_2EDTA made up to 1 l with dH_2O; density 1.4 g/ml, 450 g NaBr, 10 ml Tris pH 7.6, 0.1 g Na_2EDTA made up to 1 l with dH_2O. The gradients were formed in a zonal rotor (Ti-14; Beckman Instruments, Inc.) at 3.5k rpm over 30 mins. using a gradient mixer (11300; LKB Bromma) and peristaltic pump (2115; LKB Bromma) set at a rate of 21 ml/min.

The density of 50 ml of plasma was raised to 1.4 g/ml by adding 26.6 g NaBr and loaded onto the first gradient through a rotating seal assembly (Beckman Instruments, Inc.) and centrifuged at 45k rpm, 15°C for 18 hours. The rotor was unloaded at 3.5k rpm by pumping density 1.4 g/ml solution through the peripheral lead of the filling head. The discharge was monitored at OD_{280nm} (Type 6 Optical Unit connected to a Model U-A Absorbance Monitor; Isco Instrument Specialities Co.) and collected into 14 ml fractions. Those fractions (1-10) that contained the lower density lipoproteins were concentrated as above to a volume of 25 ml, adjusted to density 1.3 g/ml by adding 7.5 g NaBr, and loaded onto the second gradient. The VLDL and LDL were then separated by centrifugation at 45k rpm, 10°C for 110 mins. and unloaded with density 1.3 g/ml solution.

2.3. Sequential Flotation Ultracentrifugation

As the plasma lipoproteins have lower hydrated densities than the other plasma proteins, and because the densities of each class are discrete from one another, progressively raising the solvent density of plasma between ultracentrifugation steps allows the isolation of defined lipoprotein classes. This provides the simplest method of simultaneously isolating lipoproteins from a number of samples and in a form that does not require further concentration.

VLDL, IDL, LDL and HDL were isolated at the density limits of 1.006, 1.019, 1.063 and 1.210 g/ml as originally described for man by Havel, Eder and Bragdon (1955). Four ml of plasma was placed in a 6.5 ml thermoplastic ultracentrifuge tube (Ultra-Clear; Beckman Instruments, Inc.) overlayered with 2 ml of density 1.006 g/ml solution (11.4 g NaCl, 0.1g Na_2EDTA in 1 l dH_2O to which was added 1 ml of 1 N NaOH and 3 ml dH_2O) and centrifuged in a Beckman Ti50.3 rotor at 39k rpm, 4°C for 18 hours. The VLDL was then removed in the top 1 ml with a fine bore pipette, the next 1 ml was discarded and 0.32 ml of density 1.182 g/ml solution (24.98 g NaBr in 100 ml density 1.006 g/ml solution) was added to the remaining 4 ml. The sample was mixed, overlayered with 1.68 ml density 1.019 g/ml solution (8 ml 1.182 plus 100 ml normal saline) and centrifuged as above. The IDL was then removed in the top

1 ml and, after disposing the next 1 ml, 1.47 ml of density 1.182 g/ml solution was added, the solution mixed and overlayered with 0.53 ml density 1.063 g/ml solution (made up of a 2:1 mix of 1.006 and 1.182 g/ml solutions). Centrifugation was repeated, the LDL removed in the top 1ml, and 0.944 g NaBr added to the sample after discarding a further 1 ml. The contents of the tube were mixed thoroughly, overlayered with 2 ml density 1.21 g/ml solution (made up of a 2:1 mix of 1.006 and 1.478 g/ml solutions; the latter made from 78.32 g NaBr in 100 ml density 1.006 g/ml) and centrifuged for 24 hours after which the HDL was removed in the top 1 ml.

When necessary, chylomicrons were first isolated from the samples by overlayering 4ml plasma with 2 ml normal saline and centrifuging at 20k rpm, 4°C for 30 mins. The chylomicrons were removed in the top 2 ml and cleaned of plasma proteins by twice overlayering with 4 ml normal saline and repeating the centrifugation.

2.4. Measurement of Lipoprotein Particle Size

The size of VLDL, LDL and HDL was estimated by visualisation of intact lipoproteins by **negative contrast electron microscopy**. Lipoprotein fractions were extensively dialysed in Spectrapor membrane tubing (Spectrum Medical Industries Inc.) against 0.125M ammonium acetate, 2.6 mM ammonium carbonate, 0.26 mM tetra sodium EDTA at pH 7.4 for 24 hours at 4°C. The samples were negatively stained with sodium phosphotungstate at pH 7.0-7.5 and applied to Formvar-carbon-coated grids as described by Forte and Nordhausen (1986).

The particle size distribution of LDL and HDL was also examined by **non-denaturing polyacrylamide gel electrophoresis** on pre-prepared polyacrylamide gradient gels of 2-16% for LDL (PAA 2/16; Pharmacia AB) and 4-30% for HDL (PAA 4/30; Pharmacia AB) using the GE4 apparatus (Pharmacia AB). The gels were first pre-equilibrated in the electrophoresis buffer (0.09M Tris, 0.08M Boric acid, 2.5mM EDTA, pH 8.4) for 20 mins. at 70 volts. The samples were mixed in a 2:1 ratio with tracking dye made up of 4 g sucrose and 1 mg bromophenol blue in 10 ml of the electrophoresis buffer. Approximately 10 ug of protein was loaded on to the gel in an application volume of 8-12 ul and electrophoresed at 20 volts for 20 mins., 70 volts for 30 mins., after which time the buffer was circulated to both the upper and lower tanks and electrophoresis carried out at 120 volts for at least 24 hours. A high molecular weight marker (HMW; Pharmacia AB), containing thyroglobin (radius 8.5 nm), apoferritin (6.1 nm), lactate dehydrogenase (4.08 nm) and bovine serum albumin (3.55 nm), and a 10% solution of latex beads (diameter 38 nm; Dow Chemical Ltd), were used to obtain Rf values for each band. The gels were fixed in 10% sulphosalicylic acid for 30 mins., stained with 0.1% w/v Coomassie Brilliant Blue G-

250 (Bio-Rad Laboratories Ltd) for 60 mins. and destained in 7% acetic acid before being scanned by computer assisted video densitometry (Model 620, Bio-Rad Laboratories Ltd).

2.5. Chemical Composition of Lipoproteins

The triglyceride and total cholesterol content of the isolated lipoprotein fractions were measured as described for plasma in section 1.1., and free cholesterol and phospholipids were determined using enzymatic colorimetric kits (310328 and 691844; Boehringer Mannheim GmbH) on a centrifugal analyser (Encore Chemistry System; Baker Systems). The masses of triglyceride and free cholesterol in mg/dl were calculated by multiplying their molar concentrations by 88.5 and 38.6, respectively. The mass of cholesteryl esters was calculated as the difference between the molar concentration of total and free cholesterol multiplied by 38.6 and again by 1.68 to correct for the mass of the esters. Total protein content was determined by the modified method of Lowry *et al.* (Peterson 1977) using human serum albumin as the standard. When dealing with turbid fractions, *i.e.* chylomicrons and VLDL, 1 mg/ml SDS was added to the Biuret reagent. The contribution of each component to lipoprotein mass was then calculated by dividing their respective masses by their combined masses.

2.6. Resolution of Apolipoproteins

The apolipoprotein content of the isolated lipoprotein fractions was resolved by SDS-PAGE as described by Maguire, Lee and Connelly (1989). Electrophoresis was performed using a vertical slab gel apparatus (SE600; Hoefer Scientific Instruments), 1.5 mm thick gels and a 15 tooth well former. The gel consisted of 3.5% acrylamide with an acrylamide-bisacrylamide ratio of 20:1, 18% glycerol (v/v), 0.1% SDS, 0.075% TEMED, 0.01% ammonium persulphate, 0.1M tris/phosphate, pH 6.8 and was made up according to the authors' protocol. Lipoprotein fractions were freeze dried in aliquots of 50 ug and stored at -70°C prior to analysis. The samples were resolubilised, reduced and applied to the gels as described. The lower buffer chamber was filled with 4 l of 0.1 M sodium phosphate, pH 7.0, and the upper with the same buffer containing 0.1% SDS. Electrophoresis was carried out at a constant current of 20 mA for 30 mins. and then 60 mA for 4.5 hours. The gels were fixed in 4% formaldehyde for 30 mins., stained overnight in 0.025% Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories Ltd) in 45.4% methanol, 9.2% acetic acid, then destained with 7.5% acetic acid, 5% methanol and stored in 5% acetic acid.

High and low molecular weight markers (LMW and HMW-SDS; Pharmacia AB) were included in each run. ApoA-I, apoA-II and the C peptides were identified

using the proteins partially purified by high performance liquid chromatography, and apoB by Western blot analysis.

The apolipoprotein content of each lipoprotein class was estimated by scanning the stained gels and calculating the area under each protein peak by computer assisted video densitometry using the Model 620, Bio-Rad Laboratories Ltd. In doing so, no attempt was made to correct for differing chromogenicities between the various proteins. Quantification of apoB-100 and apoB-48 by gel scanning has been shown to be linear and accurate over the range 0.35-35 ug of apoB (Zilversmit and Shea 1989). Although the percentage contribution of the the non-apoB apolipoproteins to protein mass can not be guaranteed true, the method provided the means by which inter-animal comparisons could be made in the absence of other apolipoprotein quantification methodologies.

2.7. Preparation of Apolipoproteins A-I, A-II, C

Human and equine HDL were isolated by rate-zonal ultracentrifugation, concentrated to 20 ml and dialysed against 0.15 M NaCl, 0.01 M Tris, 0.01% EDTA, pH 7.6 for two days at 4°C. This was lyophilised and then sequentially delipidated with 3:1 ethanol:ether, 3:2 ethanol:ether, and finally ether, over a two day period. The freeze dried powder was combined with the solvent in a rounded bottom glass flask, mixed on rollers (Spiramix 5; Denley-Tech Ltd) at 4°C, and the supernatant removed following centrifugation at 3000rpm, 4°C for 10 mins. The delipidated powder (apoHDL) was dried in a gentle stream of nitrogen and stored at -20°C until use.

Approximately 1 g of apoHDL was dissolved in 2.5 ml of 1.0 M NaCl and 1.8 g of urea (cyanide removed by ion exchange chromatography on Bio-Rad AG501-X8D; Bio-Rad Laboratories Ltd) added with stirring. The pH was adjusted to 3.15 with formic acid and the volume bought up to 5 ml with dH_2O. The solution was then passed through a 0.45 um filter (Minisart NML; Sartorius Ltd) and applied to 2.15x60 cm HPLC column (G3000SW; TSK-Gel) coupled to a 2.15x7.5 cm guard column of the same material on a Gilson HPLC system consisting of a model 303 pump, 802 Manometric Module and Halochrome monitor. The column was eluted with 6.0 M urea, 0.5 M NaCl, pH 3.15 at a flow rate of 3ml/min. and monitored at OD_{280nm} and fractions collected every minute. The peaks that corresponded to those reported for human apoA-I, apoA-II and the C peptides (Polacek, Edelstein and Scanu 1981) were dialysed extensively against 0.05 M NH_4HCO_3, lyophilised and stored at -20 °C. The fractions corresponding to apoA-I and apoA-II were then further purified by repeating the process.

2.8. Western Blotting of Apolipoprotein B

Antisera to equine apoB was raised in two New Zealand White rabbits using LDL isolated from plasma by rate-zonal ultracentrifugation as the antigen. This was concentrated to give a protein content of approximately 1 mg/ml, and was emulsified with complete Freund's adjuvant and injected intradermally (1 mg/rabbit). This was boosted at two week intervals with 0.2 mg of fresh LDL protein in incomplete Freund's adjuvant and the specificity of the antisera was checked against equine plasma, VLDL, LDL and HDL by immunodiffusion in 1% agarose (Ouchterlony 1967). After four booster immunisations the rabbits were sedated and exsanguinated by cardiac puncture and the serum stored in 0.5 ml aliquots at -20°C.

For blotting, the SDS-PAGE gels, nitrocellulose membranes (Whatmann 3MM) and Scotch-Brite pads were soaked in transfer buffer (192 mM glycine, 25 mM Tris 20% methanol, pH 7.9) for 30 mins., assembled, and blotted overnight at 80 mA using the Trans-Blot transfer cell (Bio-Rad Laboratories Ltd). The blot was then incubated in blocking buffer (5 g milk powder in 100 ml transfer buffer) for 60 mins. on an orbital mixer (Denley-Tech Ltd) and washed in three changes of transfer buffer for 20 mins. each. The blot was incubated with the anti-LDL (1ml serum in 5 ml 10 mM Tris-HCl, 0.05% v/v Tween 20 (Sigma P-1379), 0.15 M NaCl, pH 7.4) for 3 hours, washed as before and incubated with HRP donkey anti-rabbit IgG (S083-201; Scottish Antibody Production Unit) for 2 hours. The blot was then developed for 10 mins. in a substrate solution containing 50 ml 0.1 M phosphate pH 7.4, 60 ul hydrogen peroxide and 25 mg 3,4,3',4'-Tetra-aminobiphenyl hydrochloride (BDH Laboratory Supplies).

3. ANALYSIS OF POST-HEPARIN LIPOLYTIC ACTIVITY

3.1. Principle

Lipoprotein and hepatic lipase are released into the circulation following an intravenous injection of heparin and harvested in plasma. The post-heparin plasma (PHP) is incubated with an emulsion containing triglycerides radiolabelled in the fatty acid moiety. The free fatty acid products of lipolysis are captured by albumin, separated from the glycerides and counted by liquid scintillation. Lipolytic activity is expressed as umol fatty acids released per ml of PHP per hour (umolFA/ml/h).

3.2. Collection of Post-heparin Plasma

Sodium heparin (Heparin (Mucous) Injection BP; Leo Laboratories Ltd) was administered by intravenous injection (70 iU/kg body weight) and blood samples

collected into potassium EDTA, placed on ice and the plasma separated at 4°C. This was stored at -20°C for no longer than 12 weeks.

3.3. Preparation of Serum Cofactor

Pooled horse serum was used as the source of apoC-II for the activation of LPL. A total of 250 ml blood was collected in to potassium EDTA from four ponies, the plasma separated and 0.1% w/v $CaCl_2$ and 1 U/ml bovine thrombin (Sigma T-4265) added. The clot was removed after incubation at 37°C for 120 mins. and the serum dialysed against 0.15 M NaCl at 4°C overnight. Any endogenous lipase activity was removed by heating the serum at 56° C for 30 mins., the serum was then dialysed overnight against PBS at 4°C and stored in aliquots of 1 ml at -50° C.

3.4. Preparation of Substrate

Fifty uCi of glycerol tri[1-^{14} C]oleate (CFA 258; Amersham International plc) was dissolved in 24.5 ml of toluene, divided into seven aliquots of 3.5 ml and placed in round bottomed glass flasks. To each was added 3.5 ml of 500 mg triolein (Sigma T-7140) in 25 ml toluene. The solution in each was dried down under nitrogen in a water bath at 55°C, washed three times with 3 ml heptane, and sealed and stored under nitrogen at -20°C. The substrate emulsion was prepared fresh on the day of assay by adding 5.25 ml of 5% gum arabic (Sigma G-9752) in 0.2 M Tris-HCl, pH 8.4. This was mixed on vortex and sonicated on ice at 18 microns for 4 mins. until there were no oily droplets visible on the surface. Finally 5.25 ml of 10% bovine serum albumin (Fraction V; Sigma A-4503) in 0.2 M Tris-HCl, pH 8.4 was added.

3.5. Assay Method

Ten ul of PHP was incubated in duplicate with 200 ul substrate, 250 ul 0.2 M Tris buffer pH 8.6, containing NaCl, and 50 ul serum (for LPL) or 0.15 M NaCl (for HL) at 28°C for 60 mins. The fatty acids were extracted by adding 3.25 ml methanol:chloroform:heptane (1.41:1.25:1 parts) and 0.75 ml 0.14 M K_2CO_3 , 0.14 M H_3BO_3 , pH 10.5 to each tube, which were vortexed and centrifuged at 3,000 rpm, 4° C for 30 mins. One ml of the supernatant was removed and counted in 10 ml liquid scintillant (Ultima Gold; Packard Instrument Co.) and 200 ul acetic acid. Blank incubations contained 10 ul 0.15 M NaCl instead of PHP and were subtracted from the sample counts. The total radioactivity was taken as the counts in 1 ml of the infranatant from the blank incubations. The lipolytic activity was calculated according to the equation derived in Appendix 2.

4. LECITHIN:CHOLESTEROL ACYL TRANSFERASE ASSAY

4.1. Principle

Lipoprotein deficient plasma (LPDP) is incubated with a substrate emulsion containing lecithin and radiolabelled cholesterol in the presence of the LCAT cofactor apoA-I. Free and esterified cholesterol are then extracted, separated by thin layer chromatography on silica sheets and the radioactivity in each counted by liquid scintillation. LCAT activity is expressed in nmol cholesterol esterified per ml LPDP per hour (nmolCE/ml/h).

4.2. Preparation of Lipoprotein Deficient Plasma

Blood was collected into EDTA, placed on ice and plasma harvested at 4°C, and used immediately or stored at -20°C for not more than 6 weeks. The density of 4 ml was raised to 1.215 g/ml by adding 1.346 g KBr, placed in a 6.5 ml ultracentrifuge tube (Ultra-Clear; Beckman Instruments, Inc.) and overlayered with 2 ml of KBr solution of the same density (3.49 g KBr in 10 ml dH_20). This was centrifuged for 24 hours at 50k rpm, 4°C, the lipoproteins were removed in the top 2 ml and the LPDP in the infranatant dialysed overnight against 0.15 M NaCl, 10 mM Tris, 1 mM EDTA, 0.05% NaN_3, pH 7.4 at 4°C.

4.3. Assay Method

Substrate micelles were prepared fresh on the day of assay by drying down 8 ul of a 100 mg/ml solution of phosphatidyl choline (Sigma P-4279) in toluene and 0.15 umol (8.4 uCi) of [4-^{14}C]cholesterol (CFA 128; Amersham International plc) under a stream of nitrogen in a polypropylene tube. To this, 1 ml of phosphate buffer (50 mM KH_2PO_4 , 0.025% EDTA, 2 mM NaN_3 pH 7.4) was added, the tube mixed and sonicated on ice by four 5 mins. bursts at 2 microns to give a final activity of approximately 70k dpm/5 ul.

Twenty five ul of substrate vesicles were mixed with 45 ul of the assay buffer (0.015% apoA-I, 2% bovine serum albumin, 8 mM 2-mercaptoethanol made up in the phosphate buffer) in a 1.5 ml tube, flushed with nitrogen, capped and equilibrated at 37°C for 30 mins. The LPDP was diluted 1:1 with the phosphate buffer and 20 ul added at 30 second intervals to the tubes in triplicate, which were mixed, flushed with nitrogen, capped and incubated for 45 mins. at 37°C. Blank incubations included buffer instead of LPDP.

The reaction was stopped by adding 750 ul of hexane/isopropanol (3:2 v/v) containing 50 ug of unlabelled cholesterol (Sigma C-8253) and cholesteryl linoleate (Sigma C-0289) followed by 100 ul of 60% ethanol containing 0.02% Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories Ltd). The tubes were mixed on vortex

and centrifuged at 1750 rpm for 3 mins. The upper organic (clear) phase was removed, evaporated to dryness at room temperature, and dissolved in 100 ul of chloroform. Twenty five ul of this was spotted onto silica sheets (Empore; Analytichem International), separated by thin layer chromatography in petroleum spirit/diethyl ether (85:15 v/v), and developed by exposure to iodine crystals. Cholesterol and cholesteryl linoleate (5mg in 25 ml chloroform) were used as standards. The regions corresponding to free and esterified cholesterol were cut out, placed in scintillation vials to which were added 0.5 ml toluene and 5 ml liquid scintillant (Opti-fluor O; Packard Instrument Co.).

The fractional enzyme activity was calculated by dividing the counts in the esterified region by the sum of the counts in the free and esterified cholesterol regions. This was converted to molar activity (nmolCE/ml/h) by multiplying by the concentration of cholesterol in the substrate mixture.

5. CHOLESTERYL ESTER TRANSFER PROTEIN ASSAY

5.1. Principle

Lipoprotein deficient plasma is incubated with radiolabelled HDL_3 as donor and VLDL/LDL as acceptor particles for cholesteryl ester transfer. At the end of the reaction the lower density particles are precipitated and the radioactivity remaining in HDL counted to calculate the percentage transfer.

5.2. Preparation of Donor Particles

Human and equine HDL_3 were prepared from 20 ml of pooled plasma collected into potassium EDTA from healthy donors. The plasma was raised to density 1.125 g/ml by adding 3.66 g KBr, placed in a 26.3 ml polycarbonate centrifuge bottle (Beckman Instruments, Inc.) and overlayered with 5 ml of density 1.125 g/ml solution (1.96 g KBr in 10 ml dH_2O). After centrifugation at 50k rpm, 20°C for 24 hours, the less dense lipoproteins were removed in the upper 5 ml and the infranatant dialysed against Tris-saline buffer (0.15 M NaCl, 10 mM Tris, 1mM EDTA, 0.05% NaN_3, pH 7.4) at 4°C overnight. A second batch of equine HDL was prepared from 20 ml plasma by rate-zonal ultracentrifugation, concentrated to 5 ml, and similarly dialysed.

To label the HDL, the following were dried down in a siliconised glass tube under a stream of nitrogen; 9 ul of triolein (Sigma T-7140) in hexane (10mg/ml), 15 ul of L-α-phosphatidylcholine (Sigma P-4279) in hexane (100 mg/ml), 8.8 ul butylated hydroxytoluene (Sigma B-1378) in chloroform (1 mM), 160 ul of [1α-2α(n)-^{3}H]cholesteryl oleate in toluene (160 uCi; Amersham International plc) and 184 ul of cholesteryl oleate (Sigma C-9253) in hexane (1mg/ml). To this was added

0.8 ml of Tris-EDTA buffer (50 mM Tris-HCl, 0.01% Na_2EDTA, pH 7.4), the tube flushed with nitrogen, covered with sealing film (Whatman International Ltd) and mixed on vortex for 10 mins. to give a cloudy suspension. This was then clarified by sonication for two 20 mins. bursts at 2 microns and transferred to a siliconised glass conical flask containing 20 ml of the dialysed HDL_3, 1.66 ml of 14 mM 5,5'-dithio-*bis*-(2-Nitrobenzoic acid) (Sigma D-8130) and 0.2 ml of 0.4 M Na_2EDTA containing 4% NaN_3. The mixture was incubated for 18 hours at 37°C under nitrogen. The [^{3}H]HDL was first isolated by raising the density of the solution to 1.215 g/ml by adding 0.349 g/ml KBr, overlayering with density 1.215 g/ml solution (as prepared for 4.2.) and centrifuging at 50k rpm, 20°C for 24 hours. The HDL was removed in the supernatant, adjusted to density 1.125 g/ml by adding 0.196 g/ml KBr, overlayered with density 1.125 g/ml solution and centrifuged as before to remove any remaining HDL_2. The [^{3}H]HDL_3 was then isolated from the infranatant by ultracentrifugation at density 1.215 g/ml. The supernatant was collected and loaded onto an 80 ml (1.5x44 cm) sepharose gel filtration column (CL-6B; Pharmacia AB) and eluted by gravity with Tris-saline buffer at a flow rate of 11 ml/hour. Fractions were collected every 15 mins., and 50 ul aliquots removed for counting by liquid scintillation (in 5 ml Ultima-Gold; Packard Instrument Co.). The fractions comprising the peak of [^{3}H]HDL_3 were pooled. The protein content of the mixture was assayed by the dye binding method of Bradford (1976), and where necessary concentrated to a protein content of approximately 0.5 mg/ml (5-8000 cpm/10ul), passed through a 0.2 um filter (Minisart NML; Sartorius Ltd) and stored at 4°C.

5.3. Preparation of Acceptor Particles

Plasma collected into potassium EDTA from fasting healthy volunteers was pooled and the density of 20 ml raised to 1.063 g/ml by adding 0.085 g/ml KBr. This was overlayered in a polycarbonate centrifuge bottle (Beckman Instruments, Inc.) with density 1.063 g/ml solution (0.973 g KBr in 10 ml dH_2O) and centrifuged at 45k rpm, 10°C for 20 hours. The supernatant was removed, overlayered with density 1.063 g/ml solution and centrifuged for a second time. The supernatant from the second spin was dialysed overnight at 4°C against Tris-saline, sterilised by passing through a 0.2 um filter (Minisart NML; Sartorius) and stored at 4°C. The protein content of the mixture was assayed by the method of Bradford (1976), and where necessary concentrated to give 0.8 mg/ml protein.

5.4. Assay Method

Polypropylene tubes (3.5 ml capacity) were coated in protein by incubating in 3% bovine serum albumin for one hour at 42°C, shaking off excess solution and leaving

to dry at room temperature overnight. Lipoprotein deficient plasma was prepared as the enzyme source as described in section 4.2. and its protein content assayed by the method described by Bradford (1976).

The assay incubation consisted of 10 ul of [^{3}H]HDL$_3$, 10 ul of VLDL/LDL, 5 ul of 0.7 mM 5,5'-Dithio-*bis*-(2-Nitrobenzoic acid) (Sigma D-8130), with LPDP and Tris-saline buffer up to a volume of 60 ul. In general, three to five tubes were set up for each sample containing volumes of LPDP in the range 1-35 ul so that percentage transfer could be plotted over a range of protein concentrations. Six blank incubations containing no LPDP were included in each assay. The tubes were incubated for 150 mins. at 42°C with shaking (Varishaker-Incubator; Dynatech Laboratories Ltd) and the assay stopped by placing the tubes on ice and adding 150 ul chilled plasma. The VLDL/LDL were precipitated by adding 25 ul of heparin manganese (5000 units heparin (Sigma H-9133) /ml of 0.8 M $MnCl_2$), mixing and leaving the tubes to stand for 25 mins. on ice, and then pelleted by centrifugation at 2800 rpm, 4°C for 25 mins. The radioactivity in 150 ul of the supernatant was then counted (in 5 ml Ultima Gold; Packard Instruments Co.).

The percentage transfer of cholesteryl esters from super- (HDL) to infranatant (VLDL, LDL) was calculated as the difference between the counts in the blanks and each sample tube divided by the counts in the blanks. A curve was then plotted of the LPDP protein concentration against %transfer and the %transfer/ug protein taken from the linear portion of the curve, which was generally between 0 and 25-35 %transfer .

6. ORAL FAT TOLERANCE TEST

6.1. Principle

The assimilation and clearance of lipids of dietary origin are studied by giving a bolus of fat labelled with retinyl palmitate and the magnitude of the post-prandial lipaemia is monitored by measuring plasma triglyceride concentrations. Retinyl esters are hydrolysed in the small intestine, absorbed into the adjacent mucosal cells along with cholesterol and monoglycerides and co-secreted in the chylomicron particle. Following chylomicron catabolism, these esters are taken up by the liver where they enter a storage pool and do not re-enter the circulation in VLDL, and are therefore truthful markers of the synthesis and clearance of chylomicrons.

6.2. Method

The oral fat load, for a 200 kg pony, consisted of 700 ml fresh cream, 50 g sucrose, 50 g of dried skim milk, made up to 1.25 l with water. To this was added 750,000 iU of retinyl palmitate (Ro-A-Vit; Roche) and the mixture homogenised with a kitchen

blender. This was given through a nasogastric tube to animals that had been fasted for 16 hours and blood samples (10 ml) collected into potassium EDTA at 0, 2, 4, 6, 8 and 10 or 24 hours. In all subsequent manipulations, the plasma was protected as far as possible from light and stored at 4°C.

Plasma triglyceride concentrations were measured as in section 1.1. Chylomicrons and VLDL were isolated together from 4 ml plasma by flotation ultracentrifugation at density <1.006 g/ml. The retinyl palmitate in these fractions was quantified by HPLC as follows; 500 ul of sample was mixed with 500 ul normal saline, 50 ul acetic acid, and 50 ul retinyl acetate (10 ug/ml) as an internal standard. This was extracted into 1 ml diethyl ether and then a further 2 ml of diethyl ether and blown to dryness under a gentle stream of nitrogen. This was redissolved in 250 ul of isopropanol and the retinyl esters in 100 ul separated on a Brownlea Aquapore RP-300 reversed phase column (4.6x250 mm) with a guard of the same (4.6x30 mm) using a gradient of 50-100% acetonitrile in water . The column was eluted at a flow rate of 2 ml/min. and monitored at 326 nm (Gilson HPLC system consisting of a model 303 pump, 802 Manometric Module and Halochrome monitor). The retinoid absorption peaks were quantified by a HP 3396A Integrator (Hewlett-Packard Ltd) and the concentration of retinyl palmitate calculated by reference to those of the internal standards and five runs of retinyl acetate and palmitate (each 0.5 ug) external standards.

7. KINETIC ANALYSIS OF FREE FATTY ACID AND VERY LOW DENSITY LIPOPROTEIN TRIGLYCERIDE METABOLISM

7.1. Principle

Radiolabelled FFA and glycerol are injected into the patient as tracers of the synthesis, secretion and catabolism of triglyceride. Blood samples are collected at increasing intervals from this time point and the radioactivity in plasma FFA and VLDL triglycerides (VLDL-TG) counted. Percutaneous liver biopsies taken during the course of the study add information on the hepatic pools. The disappearance from plasma of the of FFA tracer, and the appearance and disappearance and of the tracers in VLDL-TG are analysed by a computerised multicompartmental modeling programme that provides values for the kinetic parameters that describe plasma FFA and VLDL-TG metabolism.

7.2. Preparation of Tracers

[2-^{3}H]glycerol (TRA-118) and [1-^{14}C]palmitic acid (CFA-23) were purchased from Amersham International plc and stored at -20° C. The tracers were either made up

fresh on the day of study or frozen at -20°C. Aliquots of 500 or 1000 uCi of [^{3}H]glycerol were made up in a total volume of 10 ml sterile normal saline and sterilised by filtration. Aliquots of 100 or 200 uCi of [^{14}C]palmitic acid were placed in polypropylene tubes and the toluene base blown off under a gentle stream of nitrogen. The fatty acids were converted to the potassium salt by adding 1 ml ethanol:water (1:1 v/v) containing 1 M K_2CO_3 and heating in a boiling water bath for 2 mins., and prepared for injection by adding 2.5 ml of 25% bovine serum albumin and the solution sterilised by filtration (0.45 um Acrodisc; Gelman Science). The containers were weighed before and after administration of their contents so that the injection volume could be accurately determined. Approximately 5 ul of each tracer was retained for counting by liquid scintillation.

7.3. Extraction of Free Fatty Acids and Triglycerides

Blood samples were collected into EDTA, placed on ice and plasma harvested at 4°C. A five ml aliquot of this was used to spin up VLDL as described in section 2.3. Percutaneous liver biopsies were obtained via a right flank approach on a line between the tuber coxa and the point of the shoulder at the thirteenth intercostal space. The area was surgically prepared and infiltrated with 2% lignocaine. An 11.4 cm, 14 gauge Tru-Cut biopsy cannula with a 20 mm specimen notch (Baxter Health Care Ltd) was inserted through a stab incision in the skin and directed towards the contralateral elbow. The biopsy material was placed immediately in chilled sterile normal saline, transported to the laboratory, blotted dry and frozen under nitrogen at -20°C.

The lipids were extracted from plasma, VLDL and the liver specimens by the method of Folch, Lees and Sloane Stanely (1957). To 0.5 ml plasma or 0.9 ml VLDL was added 15 ml chloroform:methanol (2:1), which was mixed on rollers (Spiramix 5; Denley-Tech Ltd) for 30 mins. at 4°C and filtered through paper (No 1; Whatman International Ltd) into glass graduated tubes. The solid residue was washed with 5 ml methanol and 10 ml chloroform. The liver samples were weighed (10-30 mg wet weight) and homogenised in 1 ml methanol for 1 min., then 2 ml chloroform was added and homogenisation continued for a further 2 mins. This solution was filtered as for plasma/VLDL, the solid residue was resuspended in 3 ml chloroform:ether, filtered and washed with 1 ml methanol and 2 ml chloroform. To the combined filtrates of each extraction, a quarter volume of 0.88% KCl was added, the solution shaken and allowed to settle. The upper layer was removed and a quarter of the volume of the lower layer of water:methanol (1:1) added, the tube shaken and allowed to settle. This was washed with a second quarter volume of 0.88% KCl, shaken,

allowed to settle and the upper aqueous layer discarded. The infranatant was then blown to dryness under nitrogen in a water bath at 56°C.

The free fatty acids and triglycerides were separated by thin layer chromatography. The extracted lipids were dissolved in 100 ul chloroform and spotted onto silica sheets (Empore; Analytichem International) which were run in a mixture of petroleum ether:diethyl ether:acetic acid (82:18:1 parts). Standards of 200 mg/ml in chloroform of oleic acid (Sigma O-0750) and triolein (Sigma T-7140) stored in 25 ul aliquots at -20°C were used. The sheets were developed by exposure to iodine crystals, the FFA (plasma) and triglyceride (VLDL and liver) regions were cut out, placed in liquid scintillation vials to which was added 1 ml toluene and 10 ml liquid scintillant (Ultima-Gold; Packard Instrument Co.) and counted for ^{14}C and ^{3}H activity. The recovery of activity by this method was 71% (n=5), necessitating that the measured cpm be multiplied by 1/0.71 in the calculation of specific activity as the FFA and triglyceride masses were measured before extraction.

Plasma FFA and VLDL-TG concentrations were determined as in section 1.1. Only 50 ul of the extracted hepatic lipids were applied to the TLC sheets, the remainder was dried down and re-dissolved in 250 ul isopropanol and 250 ul water and used to measure FFA and triglyceride concentrations.

7.4. Modification of the Extraction Procedure

Because of the high lipid content of plasma and VLDL from ponies with hyperlipiaemia, the separation of triglycerides from FFA on the TLC plates was poor as the spotted samples streaked along the length of the plate. VLDL-TG was therefore prepared free of the major sources of tracer contamination, *i.e.* phosholipids and FFA, by using a modification (Zech, Grundy, Steinberg and Berman 1979) of the Zeolite absorption method of Fletcher (1968). In this, 0.9 ml of VLDL was added dropwise to 12 ml isopropanol in polypropylene tubes, which were shaken and 2 g of Zeolite mixture (Sigma 990-2) added that had been activated at 110°C overnight. The tubes were then mixed on rollers at room temperature for 30 mins., the supernatant was recovered after centrifugation at 3500 rpm for 15 mins. and dried down under nitrogen at 84°C (DB-3 Dri-Block, SC-3 Sample Concentrator; Techne). The extract was redissolved in 1.2 ml isopropanol and 1 ml removed for counting by liquid scintillation (in 10 ml Ultima-Gold; Packard Instruments Co). The remainder was assayed for triglyceride, as in section 1.1., using isopropanol as a blank.

7.5. Data Analysis

A multicompartmental computer model describing the relationships between the plasma precursor pools, the hepatic triglyceride synthetic pools and the plasma

VLDL-TG pools was developed using the CONSAM 30 programme (Resource Facility for Kinetic Analysis, Seattle, USA) and is described in detail in Chapter VII.

8. STATISTICAL METHODS

Non-parametric tests of significance were used throughout this thesis using the Minitab release 7 statistical package (Ryan, Joiner and Ryan 1985). Two group comparisons were resolved by the Mann-Whitney test and three or more groups compared by the Kruskal-Wallis test. The Sign test was used to analyse paired data where derived from the same individual. The associations between variables were assessed using linear regression analysis. The level of significance, unless stated otherwise, was taken as $p < 0.05$.

CHAPTER III

THE ISOLATION, CHARACTERISATION, AND QUANTIFICATION OF THE EQUINE PLASMA LIPOPROTEINS

1. INTRODUCTION

Much of the available knowledge of lipid transport in the horse concerns the actual transport vehicles, *viz.* the plasma lipoproteins, and was extensively reviewed in Chapter I. In brief, early studies that used paper electrophoresis showed that the horse possessed α-lipoproteins (HDL) as the predominant class and identified a ß-migrating class typical of LDL. Subsequently, analytical and density gradient ultracentrifugation were used to demonstrate a third class corresponding to VLDL, and showed that LDL was disperse comprising two subfractions. These studies indicated that, in contrast to man, HDL was homogeneous with respect to particle size, density and composition. It is only recently that the distribution of apolipoproteins amongst these classes has been considered. In addition, these latter studies comprised a total of 11 subjects, all Thoroughbred horses, and with the exception of those of Le Goff *et al.* (1987, 1989) included one or two horses amongst a variety of laboratory and domestic species.

The investigations described in this and the following two chapters were therefore planned to provide more detailed and comprehensive information on the structure and function of the equine plasma lipoproteins. This was accomplished using techniques novel to the species, a much larger number of animals than before, and by paying attention to the fact that differences in lipid transport might exist between breeds that are resistant to, and those that are susceptible to, equine hyperlipaemia. Lipoproteins were first isolated from plasma using independent techniques that allowed direct visualisation of their size and density distribution, and provided material for more exact definition of structural heterogeneity within each class. The chemical composition of each class was then analysed and the distribution of the apolipoproteins defined and quantified. Finally the plasma concentrations of the major lipoprotein classes were measured. The results provided not only original and fundamental information on the equine lipoprotein transport system, but were the foundation for the investigations of plasma lipoprotein metabolism in ponies with hyperlipaemia described in Chapters VI and VII.

2. SUBJECTS, SAMPLES AND METHODS

Blood samples were collected from healthy adult (>2 years old) Thoroughbred horses and Shetland ponies, either geldings or non-pregnant mares. The animals were housed and fed a maintenance diet of hay and concentrates, which was withdrawn for the 16 hours prior to sampling. Plasma was separated within two hours of collection by centrifugation at 2,400 rpm for 15 mins., kept at 4°C and used that day. Where indicated, proteolysis of apolipoproteins was blocked by placing the blood samples on ice immediately after collection, separating the plasma at 4°C after which chloramphenicol (0.1 mg/ml), gentamicin (0.1 mg/ml), NaN_3 (0.05 mg/ml) and aprotinin (200 KiU/ml) were added.

The methods employed are described in their entirety in Chapter II. Plasma lipoproteins were isolated by gel filtration from eight Thoroughbred horses and 12 Shetland ponies and the size distribution of particles within each class examined by electron microscopy. Lipoproteins were also separated from eight Thoroughbred horses and eight Shetland ponies by rate-zonal ultracentrifugation and heterogeneity within the LDL and HDL classes defined using non-denaturing polyacrylamide gradient gel electrophoresis. The identity of the isolated classes was confirmed by reference to the elution characteristics and chemical composition of the human lipoproteins isolated by similar techniques (Rudel, Lee, Morris and Felts 1974; Patsch *et al.* 1974). Size heterogeneity within VLDL was investigated by gel filtration, as detailed in section II.2.1, but the matrix of the columns was replaced with 2% agarose (BioGel A50m; Bio-Rad Laboratories Ltd) which resolves two populations of VLDL in man, one peak at the void volume and another broad peak in the included volume (Sata *et al.* 1972).

The lipid and protein compositions of VLDL, IDL, LDL and HDL isolated by sequential flotation ultracentrifugation from 18 Thoroughbred horses and 18 Shetland ponies were then determined. The distribution of the apolipoproteins in these classes was examined by SDS-PAGE, with reference to apoA-I, apoA-II and apoC partially purified by HPLC, and by Western blotting of apoB. The quantitative distribution of the proteins was determined by computer assisted densitometry of the stained gels. Plasma lipid and lipoprotein concentrations were measured in these 36 animals as outlined in section II.1.1.

3. RESULTS

3.1. Gel Filtration

A typical protein absorbance profile obtained following the elution of lipoproteins from the agarose gel column is shown in Fig. 4. Three well separated peaks were identified in each of the animals examined, with elution characteristics and compositional analyses that were similar to those of human VLDL, LDL and HDL. The first (VLDL) peak eluted soon after the void volume (calculated using dextran blue) and was typically triglyceride rich, and poor in cholesterol, phospholipids and protein, relative to the other classes (Table 4; Appendix 3) Examination of these fractions under the electron microscope revealed that the particles spanned a considerable size range, from 30-60 nm in diameter, with a mean of 45 nm (Fig. 5). The second peak (LDL) comprised particles in the range 20-30 nm that were depleted of triglyceride and enriched in each of the other components, especially cholesteryl esters relative to VLDL. The third peak (HDL) was composed of a more homogeneous population of particles than the first two, in the range 5-12 nm, and was rich in protein and poor in neutral lipids relative to VLDL and LDL.

Two other minor peaks were variably present in the profiles; the first extended the tail of the VLDL curve and was taken to represent IDL. The second followed HDL eluting near the total volume of the column at approximately 200 ml, it had a maximum OD_{280nm} of 0.20 and was almost entirely composed of protein and therefore represented trace contamination of the initial lipoprotein preparation by plasma proteins.

The elution profiles for each animal were analysed and compared to provide information on the size distribution of lipoproteins between the two breeds of horse (Appendix 4). The peak elution volume for each curve was determined as a meter of the modal particle size, and the width of each peak was taken as a measure of the dispersity of particle size within each lipoprotein class. In order to reduce variation between runs, the same column was used for the duration of the study (6 weeks); however, there was an indication of some settling of the packing bed over the course of the initial runs and in these cases the elution volumes were corrected with respect to that of the albumin peak. The results (Table 5) showed only small variations in particle size and dispersity between individuals of the same breed, and between animals of the two breed groups. Measurement of the amplitude of the peaks provided an assessment of the plasma concentration of each lipoprotein and showed that quantitative differences might exist between the two breeds, as the VLDL peak was significantly higher in the Shetland ponies than the Thoroughbreds, a result that would later be confirmed by fuller quantitative analyses.

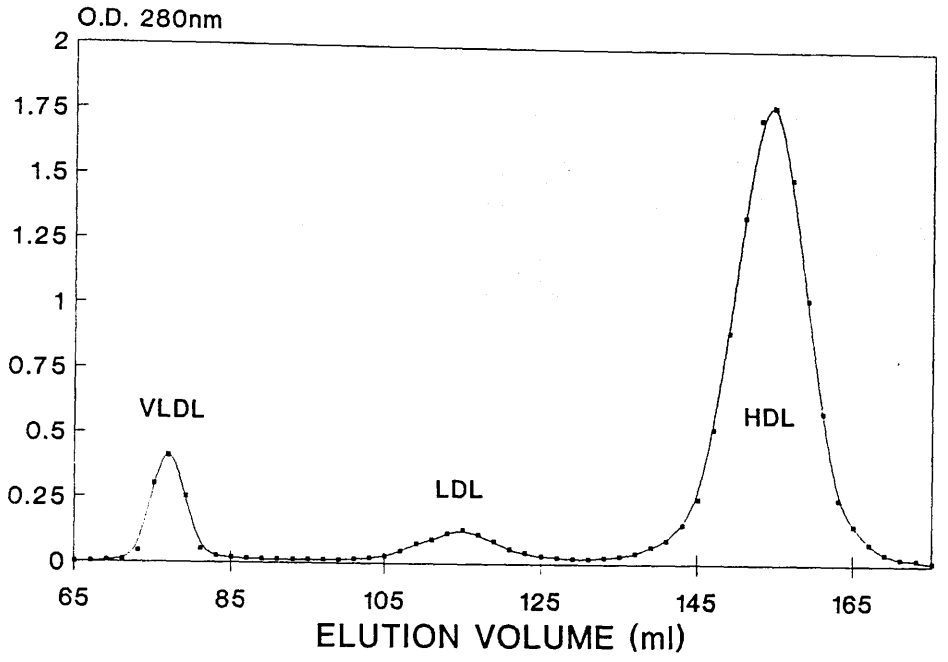


Figure 4. Profile of density <1.225 g/ml lipoproteins eluted from 6% agarose gel.

	Percentage Particle Mass			
Lipoprotein	Cholesterol	Triglyceride	Phospholipid	Protein
VLDL	15.2 ±3.6	53.5 ±5.6	14.2 ±3.2	15.6 ±3.9
LDL	41.1 ±6.8	5.5 ±1.6	23.4 ±3.3	28.1 ±4.2
HDL[a]	22.7 ±4.2	5.8 ±4.4	26.7 ±3.8	44.6 ±5.4

[a]n=19

Table 4. Chemical composition of lipoprotein classes isolated from eight Thoroughbred horses and 12 Shetland ponies by gel filtration (mean ±sd).

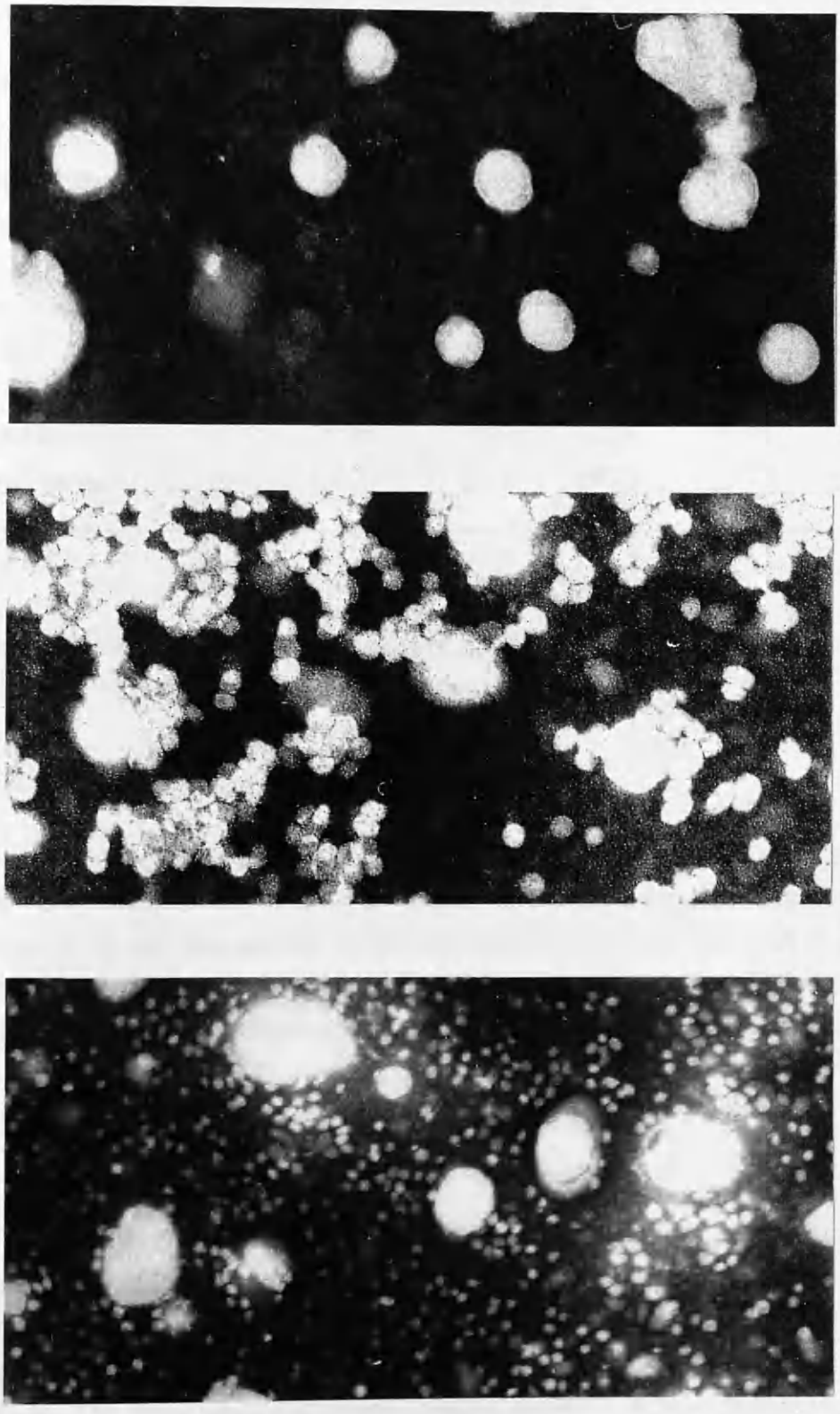


Figure 5. Electron micrographs of intact lipoproteins negatively stained with phosphotunstic acid: top VLDL; middle LDL; bottom HDL (x200,000).

Parameter	VLDL		LDL		HDL	
	TB	SP	TB	SP	TB	SP
Elution Volume (ml)	80.4 ±2.1	78.9 ±1.2	119.4 ±2.5	111.0 ±3.9	157.6 ±2.6	154.7 ±3.5
Peak Width (ml)	10.4 ±0.7	11.1 ±0.8	28.4 ±2.8	31.6 ±3.5	34.8 ±3.0	36.2 ±2.9
Peak Height (OD_{280nm})	0.17 ±0.11	0.46* ±0.23	0.13 ±0.15	0.16 ±0.11	1.80 ±0.23	1.37 ±0.40

* $p < 0.01$

Table 5. Comparison of elution profiles of lipoproteins from agarose gel from eight Thoroughbred horses (TB) and 12 Shetland ponies (SP) (mean ±sd).

All three of the major lipoprotein classes had symmetrical elution profiles. The VLDL and HDL curves had sharp peaks, but that of LDL was shallow and broad hinting at the size heterogeneity within that class. Despite the wide span of particle sizes seen in VLDL under the electron microscope and although the VLDL peak from four ponies appeared broader in the columns of 2% agarose and extended further into the included volume, there were no maxima or minima in the elution profiles to indicate that discrete subpopulations of VLDL exist in the equine species.

3.2. Rate-zonal Ultracentrifugation

On unloading the HDL zonal gradient, the emergence of the lower density lipoproteins (VLDL, IDL, LDL) early in the effluent was marked by visible turbidity of the first fraction collected. The profile then returned to baseline by the sixth or seven fraction after which there was a rapid rise in absorbance as the HDL emerged (Fig. 6). The HDL presented a single sharply peaked Gaussian profile in all the animals that suggested that the particles in this class were homogeneous with respect to flotation density. In each case, the HDL eluted between fractions 10 (±1) and 25 (±1) and was cleanly separated from both the lower density lipoproteins and the plasma proteins that succeeded it in the gradient effluent. The fractions comprising

the HDL peak were concentrated from four Thoroughbred horses and four Shetland ponies and their compositional analysis is shown in Table 6 (Appendix 5).

The possibility that size heterogeneity might exist within the HDL class, although apparently homogeneous with respect to flotation density, was refuted by the by the single band presented on polyacrylamide gradient gels by the concentrated zonal material (Fig. 7). This band represented a mean particle diameter of 8.37 nm (sd ±0.08, range 8.24-8.48 nm; Appendix 5).

Separation of the lower density lipoproteins in the second gradient led to the appearance of VLDL in the first two fractions signalled by its turbidity and confirmed by its compositional analysis (Table 6; Appendix 5). Thereafter the trace declined to baseline by the tenth fraction with a shallow slope that was assumed to contain IDL. The LDL elution profile was markedly asymmetric and was resolved into three overlapping curves labelled LDL_1, LDL_2 and LDL_3 in order of increasing particle density (Fig. 6). The elution characteristics of each subfraction were a constant feature of each of the 16 animals examined; LDL_1 appeared between fractions 10.5 (±1) and 13 (±1), LDL_2 between fractions 14 (±1) and 18 (±1), and LDL_3 between fractions 19 (±1) and 24.5 (±1). Attempts were made to improve the resolution of these subfractions by increasing the sample volume and by using the greater gradient length provided by the Beckman Ti15 rotor, but only succeeded in widening the LDL profile without enhancing the separation of the subfractions.

In the eight animals for which HDL composition and size were established, the LDL profile was divided into its constituent subfractions at the points of inflection and similarly analysed (Table 6; Appendix 5). The results indicated that as particle flotation density increased, there was an increase in cholesterol and progressive reduction in triglyceride content. The contributions of protein and phospholipids to particle mass remained relatively constant along the gradient. Although the mean cholesteryl ester contents of LDL_1 and LDL_2 and were similar (43.1% and 42.8%, respectively), LDL_2 contained a greater mean mass of free cholesterol (4.4%) than either LDL_1 (1.3%) or LDL_3 (1.3%).

Application of these fractions to polyacrylamide gradient gel electrophoresis showed that the changes in core composition along the LDL density range were accompanied by a progressive decrease in particle diameter represented by a pattern of three independently migrating bands (Fig. 7) Although there was some overlap between adjacent fractions, discrete maxima were identifiable both visually and by densitometric scanning, and the mean (±sd, range) particle diameters at each maximum calculated from their Rf were statistically independent of one another; LDL_1 26.4 nm (±1.1, 24.4-27.8), LDL_2 24.5 nm (±1.0, 23.6-25.8), LDL_3 22.4 (±0.7, 21.4-23.4) (Appendix 5).

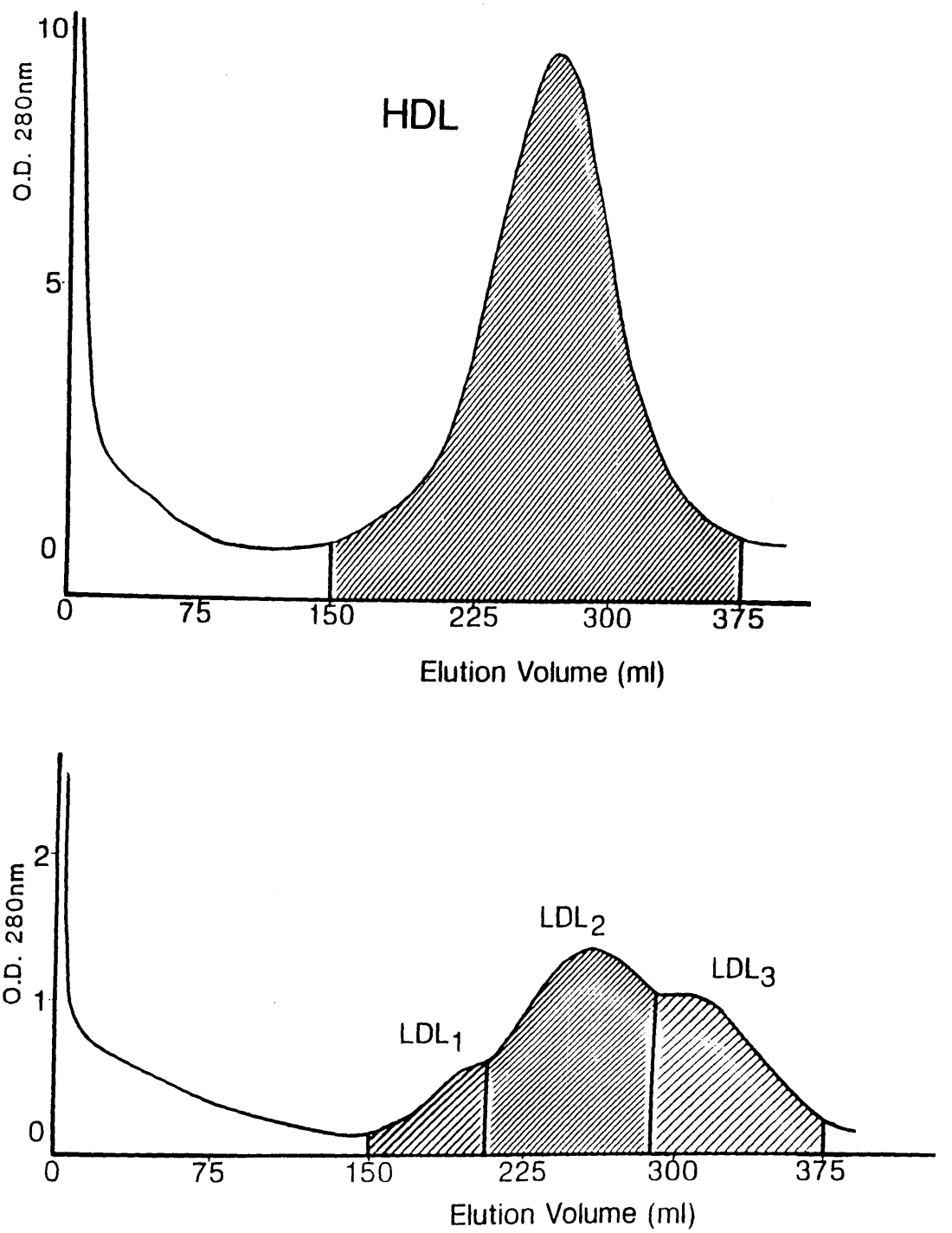


Figure 6. Elution profiles of equine HDL (top) and LDL (bottom) isolated by rate-zonal ultracentrifugation. The fractions within the shaded areas were pooled and concentrated for compositional analysis and gradient gel electrophoresis.

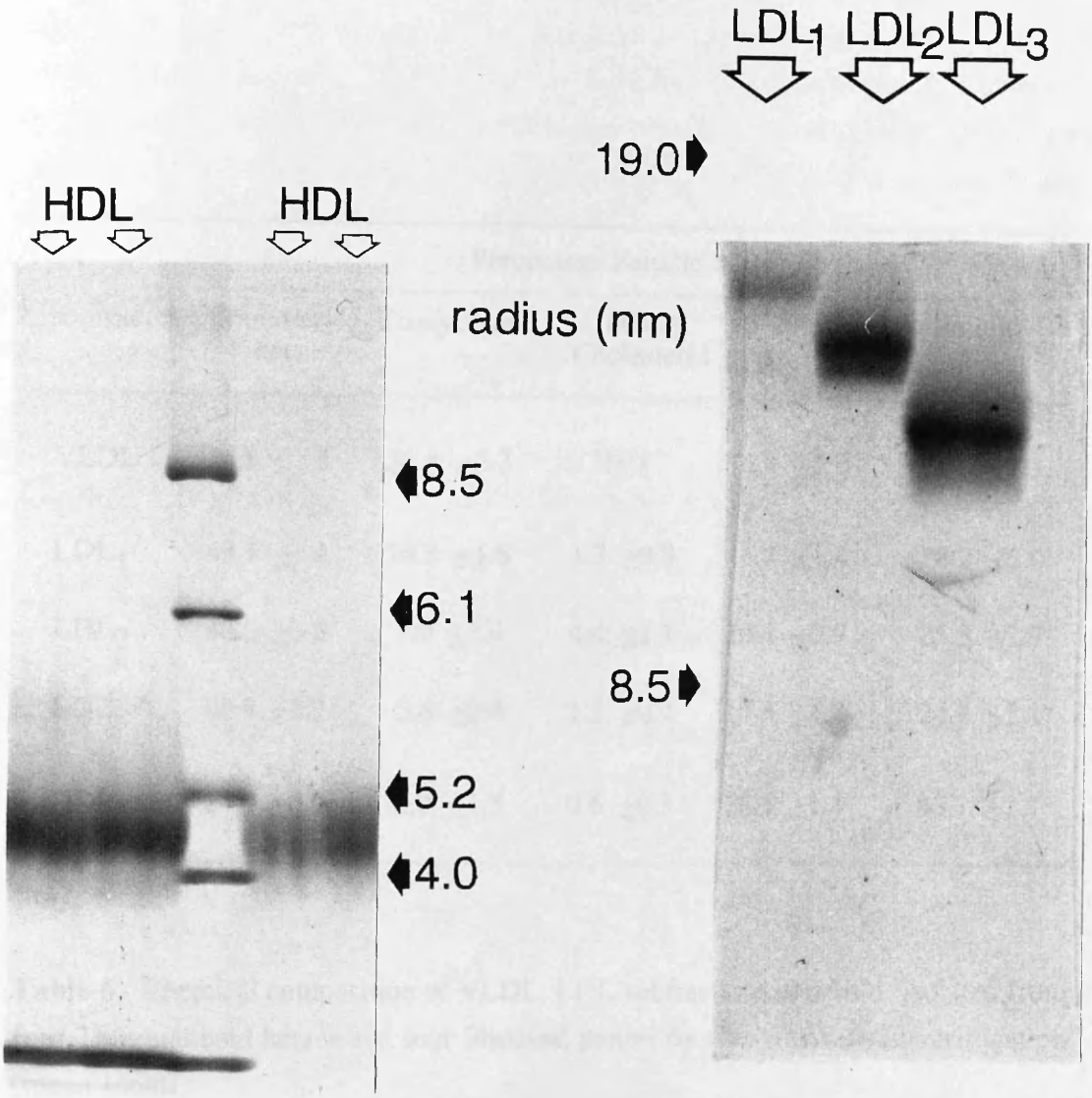


Figure 7. Electrophoresis of HDL (left) on a 2-16% polyacrylamide gradient gel, and LDL subfractions (right) on a 4-30% polyacrylamide gradient gel. The lipoproteins were prepared by rate-zonal ultracentrifugation and the positions of the molecular weight markers are shown in the centre.

Lipoprotein	Percentage Particle Mass				
	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
VLDL	14.8 ±1.8	53.8 ±3.2	ND	12.4 ±1.0	20.0 ±1.3
LDL_1	43.1 ±1.4	10.3 ±1.6	1.3 ±0.8	16.8 ±1.4	28.2 ±2.0
LDL_2	42.8 ±3.2	7.9 ±1.4	4.4 ±1.1	19.4 ±0.9	25.3 ±2.7
LDL_3[a]	49.9 ±2.9	5.6 ±0.8	1.3 ±0.7	17.5 ±1.2	24.7 ±2.1
HDL	24.0 ±0.9	3.9 ±1.3	0.6 ±0.3	28.5 ±1.1	43.1 ±1.7

[a]n=7

Table 6. Chemical composition of VLDL, LDL subfractions and HDL isolated from four Thoroughbred horses and four Shetland ponies by rate-zonal ultracentrifugation (mean ±sem).

3.3. Chemical Composition of the Lipoproteins

The chemical composition of VLDL, LDL and HDL fractionated by sequential flotation ultracentrifugation is shown in Table 7 (Appendix 6). Free cholesterol was below the level of detection in VLDL, and these lipoproteins contained more triglyceride and less cholesteryl esters than those prepared by gel filtration or rate-zonal ultracentrifugation. The lipid in the IDL fractions was below the level of detection, precluding analysis of lipoprotein composition. The triglyceride content of HDL was lower and the mass of free cholesterol higher than in that isolated by gel filtration and rate-zonal ultracentrifugation.

In VLDL from the Shetland ponies, the mass of cholesteryl esters was significantly higher and the protein content significantly lower than in the Thoroughbred horses. The mean mass of protein in LDL was also lower in the Shetland pony, but was not associated with any difference in the ratio of core to shell mass as the phospholipid mass of these lipoproteins was higher than that of the Thoroughbreds.

Lipoprotein		Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
VLDL	TB	7.8 ±1.7	57.5 ±3.9	ND	13.8 ±2.0	20.9 ±4.1
	SP	10.1* ±1.7	58.9 ±3.9	ND	15.3 ±2.3	16.6* ±2.4
LDL	TB	41.9 ±5.1	5.4 ±1.9	4.7 ±2.1	21.2 ±3.5	27.0 ±4.0
	SP	41.0 ±5.2	5.7 ±2.2	4.0 ±2.4	24.0 ±3.6	24.0 ±6.6
HDL	TB	20.2 ±3.3	0.7 ±0.6	2.6 ±0.9	26.9 ±4.0	49.6 ±5.0
	SP[a]	19.7 ±4.1	0.7 ±0.4	2.8 ±1.0	27.2 ±4.4	49.5 ±7.7

* $p < 0.01$, [a] $n = 17$

Table 7. Chemical composition of VLDL, LDL, and HDL isolated from 18 Thoroughbred horses (TB) and 18 Shetland ponies (SP) by sequential flotation ultracentrifugation (mean ±sd).

3.4. Distribution of the Apolipoproteins

Equine VLDL and LDL presented two clearly resolved bands on SDS-PAGE with molecular weights of 542.5k Da (±15.3; mean ±sd, n=12) and 492k Da (±10.1), that migrated close to the apoB-100 component of human LDL. A third band with was found in VLDL (Fig. 8) but not LDL. The molecular weight 273.5k Da (±7.2) of this protein and the ratio of its mass to that of the larger apoB-100 band (50.4% mean, range 48-52%) were similar to those of human apoB-48.

The identity of all three bands as species of apoB was first confirmed by Western blot analysis of VLDL using rabbit anti-horse LDL as the first antibody (Fig. 9; lanes 1 and 2). The possibility that the second and third bands might result from the proteolysis of apoB-100 was eliminated by demonstrating their persistence in VLDL after a cocktail of antiproteases had been added to the plasma immediately after separation (Fig. 9; lanes 3 and 4). Nor did they appear an artefact of the SDS-PAGE system used, as human VLDL and LDL similarly prepared present only a single apoB-100 band. Finally, the apoB-48 band was present in VLDL from two ponies that had been fasted for 48 hours (Fig. 9; lanes 5 and 6) allaying concerns that this might represent the persistence in the density <1.006 g/ml fraction of chylomicrons or their remnants. The two larger apoB bands contributed equally to the total protein mass of both VLDL and LDL, and are collectively referred to as apoB-100 as they appeared to be co-transferred from VLDL to LDL just as human apoB-100, although it may be argued that on the centile system used to name apoB, the second band in VLDL and LDL should be called apoB-91 and the third in VLDL apoB-50.

Three protein peaks were partially purified from equine HDL by HPLC. The first and dominant peak eluted between 12 and 27 ml with a retention time similar to that of human apoA-I and presented a single band on SDS-PAGE with a molecular weight of 29.5k Da (Fig. 10). The second peak eluted in the tail of the apoA-I between 30 and 54 ml in a position similar to apoA-II and contained apoA-I and a trace component with molecular weight 7.5k Da when electrophoresed under reducing conditions. This was purified free of apoA-I by a second HPLC run (Fig. 10) The third peak was well separated from the first two, co-eluting with the human C peptides between 54 and 78 ml and contained two bands of molecular weight 14.5k and 12k Da that were assumed to represent the equine equivalent of apoC-II and apoC-III (Fig. 10).

The distribution of these proteins amongst VLDL, LDL and HDL is also shown in Fig. 10. Their relative contributions to lipoprotein protein mass was quantified in eight Thoroughbred horses and 18 Shetland ponies (Table 8; Appendix 7). Apolipoprotein B was confined to VLDL and LDL where it was the major protein. Apolipoprotein A-I was the major protein of HDL and was also in VLDL.

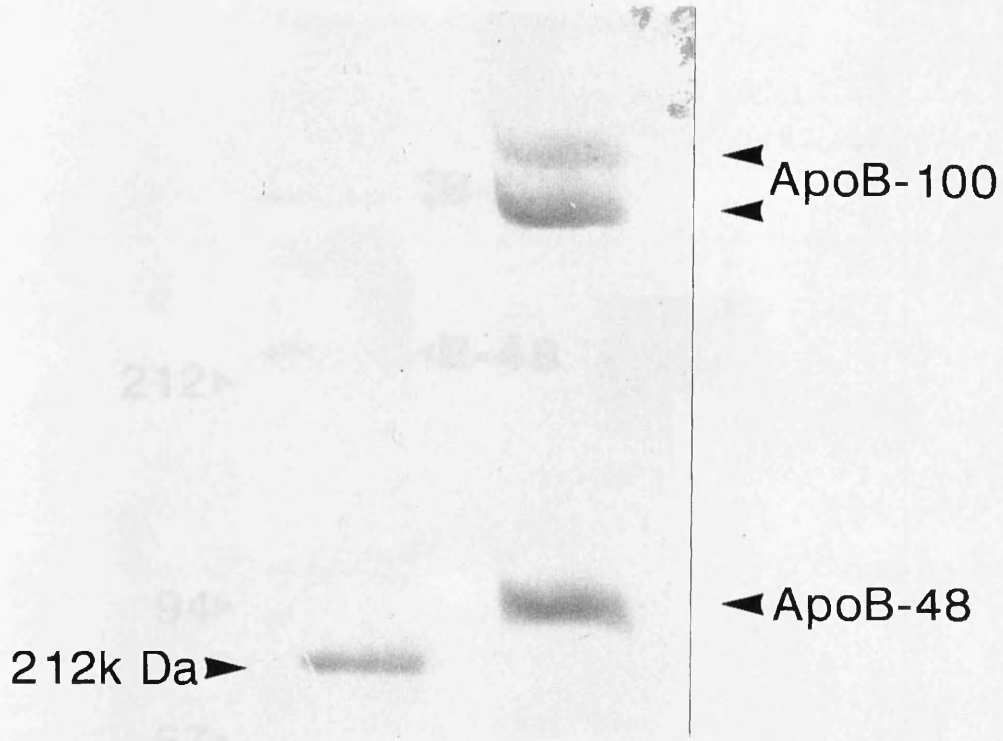


Figure 8. Separation of VLDL apoB by SDS-PAGE. The photograph shows the top third of the Coomassie stained gel with the position of a 212k Da marker on the left.

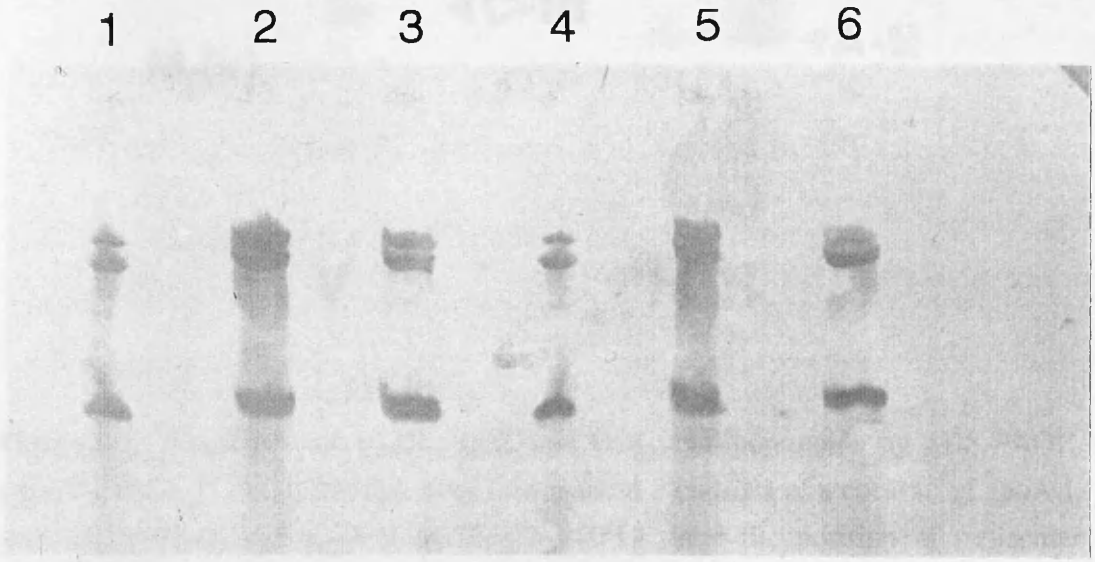


Figure 9. Western blot of VLDL apoB from SDS-PAGE. Lanes 1 and 2, native plasma VLDL; lanes 3 and 4, VLDL prepared from plasma treated with antiproteases; lanes 5 and 6, density <1.006 g/ml fraction from two ponies fasted for 48 hours.

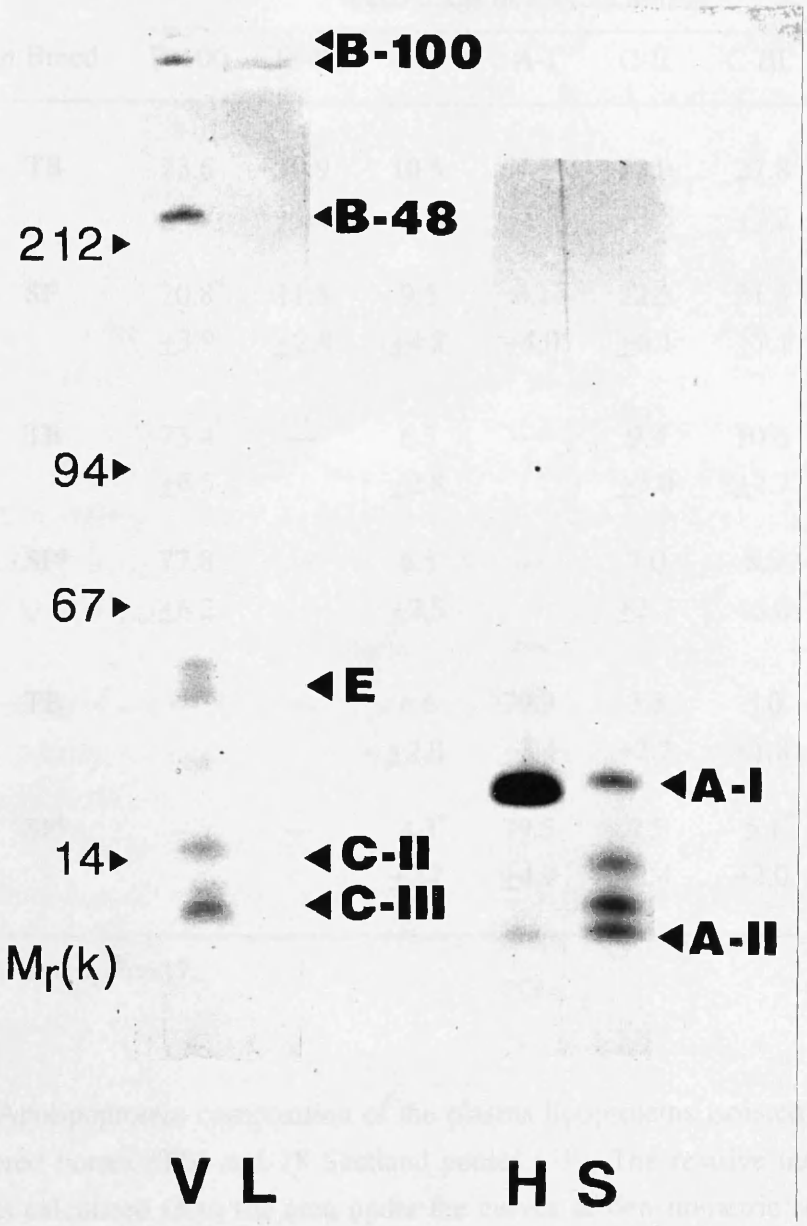


Figure 10. Resolution of VLDL, LDL and HDL apolipoproteins by SDS-PAGE. Lane V, VLDL; L, LDL; H HDL. The lane marked S consists of a cocktail of apoA-I, apoC-II, apoC-III and apoA-II purified by HPLC, and the position of molecular weight markers is shown on the left.

Lipoprotein	Breed	Percentage of Protein Mass						
		B-100	B-48	E	A-I	C-II	C-III	A-II
VLDL	TB	23.6 ±2.7	10.9 ±1.6	10.5 ±4.3	4.3 ±1.5	22.9 ±3.8	27.8 ±2.2	-
	SP	20.8* ±3.9	11.5 ±2.8	9.5 ±4.8	8.1* ±4.0	22.5 ±6.1	31.3 ±7.1	---
LDL	TB	73.4 ±6.5	---	6.5 ±2.8	---	9.4 ±3.0	10.6 ±2.7	---
	SP[a]	77.8 ±6.2	---	6.5 ±2.5	---	7.0 ±2.3	8.9 +5.0	---
HDL	TB	---	---	6.6 ±2.0	79.9 ±3.4	3.3 +2.7	3.0 +1.8	7.3 ±2.5
	SP[b]	---	---	4.3* ±2.7	79.5 ±4.9	4.5 ±2.4	5.1* +2.0	7.1 ±3.5

* $p < 0.05$; [a]n=16, [b]n=17

Table 8. Apolipoprotein composition of the plasma lipoproteins isolated from eight Thoroughbred horses (TB) and 18 Shetland ponies (SP). The relative mass of each protein was calculated from the area under the curves of densitometric scans of the Coomassie stained gels.

Apolipoprotein A-II was confined to HDL where it was very much the minor protein. The C peptides, apoC-II and apoC-III were present in significant amounts in VLDL and to a much lesser extent in LDL and HDL. In some gels, apoC-III appeared to be resolved into two bands, although it was not determined whether the variable upper band represented an isoform of apoC-III or another of the C peptides, and where present its mass was included in that of apoC-III. In addition, a broad band of molecular weight 39k Da was identified in VLDL, LDL and HDL and taken to represent apoE. A faint band with a molecular weight of 45k Da was occasionally identified in some HDL preparations, but was not quantified and may have represented apoA-IV that has remained with the HDL despite ultracentrifugation.

In VLDL, the relative mass of apoB-100 appeared significantly higher in the Thoroughbred horses than in the Shetland ponies so that the ratio of apoB-100 to apoB-48 was 2.2:1 in the former and 1.8:1 in the latter. There was a significantly greater mass of apoA-I in VLDL from the Shetland ponies than the Thoroughbreds, and the Thoroughbred HDL contained on average 53% more apoE and 41% less apoC-III than that of the Shetland ponies. The protein composition of LDL was similar in both breed groups.

3.5. Plasma Lipid and Lipoprotein Concentrations

Plasma cholesterol, triglyceride and lipoprotein cholesterol concentrations for the 18 Thoroughbred horses and 18 Shetland ponies are summarised in Table 9 (Appendix 8). There was no difference between the two groups in total, LDL and HDL cholesterol concentrations. The concentrations of triglyceride and VLDL cholesterol were significantly higher in the Shetland ponies than the Thoroughbred horses.

Animal	Cholesterol	Triglyceride	VLDL	LDL	HDL
Thoroughbred	2.23 ±0.27	0.34 ±0.16	0.20 ±0.10	0.61 ±0.21	1.41 ±0.21
Shetland pony	2.13 ±0.41	0.48* ±0.17	0.29* ±0.14	0.30 ±0.22	1.32 ±0.29

* $p < 0.05$

Table 9. Plasma cholesterol, triglyceride and lipoprotein cholesterol concentrations (mmol/l) in 18 Thoroughbred horses and 18 Shetland ponies (mean ±sd).

4. DISCUSSION

4.1. Physical Properties of the Equine Plasma Lipoproteins

Gel filtration and rate-zonal ultracentrifugation were used in the initial isolation of lipoproteins as they do not presume knowledge of the density limits of the lipoprotein classes. While both techniques have been widely used to examine the plasma lipoproteins of other species, neither had been applied to the horse. Agarose gel filtration is renowned as a simple, gentle, and non-destructive means of fractionating lipoproteins from both healthy and hyperlipidaemic subjects (Margolis 1967; Sata, Estrich, Wood and Kinsell 1970; Rudel *et al.* 1986). In addition, the rate-zonal ultracentrifugation method has a high sample volume capacity that provided for the separation and analysis of lipoprotein subfractions. By monitoring of the protein content of the eluants at OD_{280nm}, both techniques provided ready visualisation of the size and density distribution of the lipoprotein particles. The previous studies that had defined the density distribution of the equine lipoproteins, with the exception of the single animal examined by Hollanders *et al.* (1986), had relied upon lipid prestaining of the sample to provide visual identification of lipoprotein classes that were then removed manually from the gradient. The potential that such techniques possess for disturbing the gradient and contaminating the lipoprotein fractions was eliminated in the present study by collecting the freely flowing eluant.

Columns of 6% agarose gel were used on the recommendation of Rudel *et al.* (1986) and provided good separation of the major equine lipoprotein classes from one another and from the other plasma proteins. Preliminary ultracentrifugation of the plasma samples was performed to concentrate the lipoproteins so that a smaller sample volume could be applied to the column to maximise its resolution. Visualisation of individual lipoproteins under the electron microscope indicated that both the VLDL and LDL peaks contained particles spanning a wide range in size. The apparent diameters of both VLDL (30-60 nm) and LDL (20-30 nm) are considerably larger than those of 10-48 nm for VLDL and 11-15 nm for LDL reported by Le Goff *et al.* (1987). The source of this discrepancy is not clear, as the masses of neutral lipids, the determinants of core volume and therefore particle size, were similar in both classes to those found here. In support of the present data, the size of LDL was independently calculated in the range 21.4-27.8 nm using non-denaturing polyacrylamide gradient gel electrophoresis.

The failure to demonstrate heterogeneity within the equine HDL class, despite using a high resolution stepped gradient designed to maximise the fractionation of HDL by rate-zonal ultracentrifugation, was consistent with previous reports. Although this technique might be criticised on the basis that the particles do not reach equilibrium within the gradient, the single HDL band found on non-denaturing

polyacrylamide gradient gel electrophoresis justified the conclusion that this class was homogeneous with respect to particle size and density. Equine HDL occupied a position in the rate-zonal gradient similar to that reported for human HDL_3 (150-300 ml) rather than that of HDL_2 (90-140 ml) (Patsch *et al.* 1974). This alliance with the smaller, denser human subfraction was also borne out by the small size of the particles, their low triglyceride content and the predominance of apoA-I as their protein. Le Goff *et al.* (1987) commented that the size range of particles in HDL was particularly homogeneous (5.1-7.3 nm), and although they found a second HDL band in one animal, this was later shown to contain apoB (Le Goff *et al.* 1989) and may therefore have resulted from minor contamination of the HDL fraction by LDL.

The resolution of LDL provided by rate-zonal ultracentrifugation reinforced previous reports of its heterogeneity from analytical and density gradient ultracentrifugation and allowed us to demonstrate the existence of a third population of particles in this class. The size and lipid content of the subfractions support the conclusion that they represent discrete, thermodynamically stable subpopulations of LDL, rather than the presence of small IDL or large HDL particles in the LDL density range. The density distributions and lipid compositions of the two subfractions LDL_2 and LDL_3, rather than those of LDL_1, agree with the characteristics of the two subfractions previously described (Le Goff *et al.* 1987) indicating that LDL_1 is the novel species.

Heterogeneity of LDL is now well established in man (Shen *et al.* 1981) and animal models of atherosclerosis including primates (Puppione, Nicolosi, Kowala and Schumaker 1989) and swine (Checovich, Fitch, Krauss, Smith, Rapacz, Smith and Attie 1988). In contrast with HDL, the metabolic origins of heterogeneity within this class are not understood (Rudel, Parks, Johnson and Babiak 1986), but are clearly of interest because of the apparent link between a preponderance of certain cholesteryl ester rich LDL subfractions and the incidence of CAD in man and the animal models. Possible mechanisms for this heterogeneity may include the remodelling of LDL by the agencies of LPL, CETP and HL (Gambert *et al.* 1990), and the interaction of genetic (Austin and Krauss 1986) and environmental factors (Pownall, Jackson, Roth, Gotto, Patsch and Kummerow 1980). To what extent the individual subfractions represent LDL that arises from VLDL precursors or direct synthesis is not clear, but there is evidence that the subfractions differ in their kinetic behaviour (Swinkels, Demacker, Hak-Lemmers, Mol, Yap and van't Laar 1988; Marzetta *et al.* 1989).

The data presented here may help in understanding the origins of LDL heterogeneity. First, it should be noted that such heterogeneity exists even though LDL levels are much lower in the horse than in the other species for which this feature is described. Secondly, the pattern of subfraction size and density was

constant between individuals and might indicate that the underlying mechanism is an inherent, basic feature of the equine lipoprotein system rather than the product of an acquired or environmental factor. And thirdly, as shown in the following chapter, the horse has no significant CETP activity indicating that the heterogeneity arises in the absence of neutral lipid exchange. Indeed, the changes in neutral lipid content of LDL along the density gradient could be held consistent with the step wise hydrolysis of core triglyceride by the endothelial lipases. More exact interpretation of the compositional data is hampered by the poor resolution of the fractions and the large standard errors in the data. However, comparison of the triglyceride and cholesteryl ester contents of LDL_1 and LDL_3 suggests that these subfractions may represent the two extremes of the LDL delipidation pathway. On the other hand, the reduction in particle size from LDL_1 to LDL_2 is accompanied by only minor changes in the neutral lipid content, while the surface is relatively enriched in free cholesterol, and it is therefore possible that these particles may originate independent of VLDL precursors.

Despite their obvious merits, gel filtration and rate-zonal ultracentrifugation suffered as preparative techniques in that their analytical capacity was low and at best restricted to four samples per week. In addition, both resulted in dilution of the samples that necessitated considerable concentration of the fractions prior to analyses. The unavoidable precipitation, contamination and loss of lipoprotein components during ultrafiltration was perhaps the source of the large standard errors in the compositions presented in Tables 4 and 6. For these reasons sequential flotation ultracentrifugation was used to isolate the lipoprotein fractions in a ready concentrated form from a number of animals with considerably less demands on technical time. Initial concerns that the human density ranges would be unsuitable for the equine lipoprotein classes were allayed by the absences of apoB from HDL and apoA-I from LDL on SDS-PAGE. This refuted a previous suggestion that 1.070 g/ml should serve as the upper density limit for equine LDL made on the basis that the density >1.063 g/ml fraction displayed a minor ß-migrating component on gel electrophoresis (Robie *et al.* 1975b).

4.2. Chemical Composition of the Lipoproteins

The compositions of VLDL, LDL and HDL isolated by the three methods here are very similar to those previously reported from density gradient ultracentrifugation (Le Goff *et al.* 1987), with the notable exception that these authors detected free cholesterol in VLDL (approximately 5% particle mass). There was good agreement in the compositions of the lipoprotein classes isolated by each of the three methods used here, with the notable exception that the masses of cholesteryl esters in VLDL, and

triglyceride in HDL, were higher when isolated by gel filtration and rate-zonal ultracentrifugation. These differences are not readily explicable, but may have resulted from contamination of the VLDL with LDL, and HDL with LDL during concentration. The lower protein content of the VLDL isolated by gel filtration might be due to superior resolution of this lipoprotein from other plasma proteins in the gel matrix or have resulted from the loss of apolipoproteins from the surface of VLDL in the preliminary ultracentrifugation step or during the passage of particles through the gel. In refute of the former, the VLDL prepared by flotation ultracentrifugation was found to be free of contaminating proteins on SDS-PAGE. The protein content of VLDL reported here, and by Le Goff *et al.* (1987), is high relative to that of human VLDL, but again does not appear to represent contamination of the density <1.006 g/ml fraction with other plasma proteins.

The existence of multiple species of apoB in the horse is a peculiar and fascinating feature of its lipid transport system. The pattern presented on SDS-PAGE has previously been reported although not characterised by Le Goff *et al.* (1989), and is clearly at odds with the concepts of apoB metabolism in man outlined in Chapter I. In summary of our findings, equine VLDL contains three species of apoB, two that are in the molecular weight range of human apoB-100, and a third almost identical in size to human apoB-48 that is present in VLDL in the fasting state suggesting that it derives from hepatic synthesis. An analogous pattern is seen in the rat, where three species of apoB (B-100, B-95 and B-48) are synthesised in the liver and secreted in VLDL (Wu and Windmueller 1981). In the rat, the synthesis of apoB-48 in the liver involves an editing mechanism identical to that described in human enterocytes, but the origins of the two larger proteins are unclear. It is possible that these, and those described here in the horse, arise from post-translational modifications of apoB-100 that might include glycosylation, acylation and phosphorylation.

If, as with human VLDL, the equine particles contain only a single molecule of apoB, the data presented here indicates that the horse liver secretes two types of VLDL particle. The first, containing either of the larger apoB proteins, would appear to proceed along the lipolytic cascade and enter the LDL density range. The second type would contain the smaller protein and are probably cleared directly from the circulation after lipolysis by receptor recognition of their apoE component as apoB-48 does not appear in LDL. The masses of apoB-100 and apoB-48 are present in equine VLDL in an approximate ratio of 2:1 which, given that the mass of apoB-100 is twice that of apoB-48, indicates that the two types of particles are present in approximately equimolar concentrations. It is also likely that these particles do not reside in the circulation as discrete populations, in line with the findings from gel filtration, but that the liver retains the capacity to secrete a spectrum of sized particles depending

upon its metabolic demands. In the rat, the smaller VLDL particles are relatively enriched with the larger apoB proteins, and increased hepatic triglyceride secretion is accomplished by producing larger particles that contain apoB-48 rather than apoB-100 or apoB-95 (Windmueller and Spaeth 1985). It is possible, especially given the evidence presented in Chapter VI, that the equine liver is also capable of regulating apoB synthesis and the activity of the editing mechanism in response to metabolic demands.

The molecular size and distribution of the equine apolipoproteins were largely similar to those found in man and a variety of animal species, with the exception that the C peptides were a little larger than those reported. The origin of the small amounts of apoA-I in VLDL are unclear as in man this protein is largely confined to HDL, with a small amount found in chylomicrons. It is possible that in the horse, this protein is secreted from the liver with the VLDL particle or transferred from HDL along with apoE and the C peptides in the circulation. The presence of apoE in HDL might well access a fraction those particles to direct hepatic clearance by the receptors that recognise this protein.

4.3. Differences in the Composition and Plasma Concentrations of the Lipoproteins between the Thoroughbred Horse and the Shetland pony.

There were few differences in either the composition or concentration of the lipoproteins, or their physical attributes, between the two breeds. The most significant divergence related to the composition of VLDL, which in the Shetland ponies, contained more cholesteryl esters and less protein, and was slightly enriched in triglyceride. This was associated with a reduction in the ratio of apoB-100 to apoB-48 (1.8:1 compared with 2.2:1 in the Thoroughbreds) which would be compatible with a relative preponderance in the ponies of larger VLDL particles that have apoB-48 rather than apoB-100 as their structural protein.

This alone might not be sufficient to explain the higher plasma triglyceride and VLDL cholesterol concentrations noted in the pony group here and in four ponies by van Dijk and Wensing (1989). It is therefore possible that the circulating numbers of VLDL are higher in ponies than Thoroughbred horses. If this is the case, there might exist a discrepancy between the two breeds in the rate of synthesis of VLDL and/or the rate of its clearance from the circulation that renders the pony breed susceptible to hyperlipaemia. As hepatic triglyceride synthesis, in other species, is enhanced by the supply of precursors, the higher plasma FFA levels found in the Shetland pony by Robie *et al.* (1975a) might provide a basis for increased synthesis of this lipoprotein.

CHAPTER IV

THE ACTIVITY OF LIPOPROTEIN LIPASE, HEPATIC LIPASE, LECITHIN:CHOLESTEROL ACYL TRANSFERASE AND CHOLESTERYL ESTER TRANSFER PROTEIN IN THE HORSE

1. INTRODUCTION

Once in the circulation, the plasma lipoproteins are subject to the activities of an alliance of enzymes that model their composition and mediate their catabolism. The roles that these enzymes play in integrating and coordinating lipoprotein metabolism are reviewed in Chapter I and summarised here. Lipoprotein lipase (LPL) is responsible for the catabolism of triglyceride rich lipoproteins and thereby releases surface remnants that contribute to the conception and maturation of HDL. Its close relative hepatic lipase (HL) removes triglyceride from smaller particles so mediating the conversion of IDL to LDL, of HDL_2 to HDL_3, and the remodelling of large buoyant LDL to smaller denser particles. The activity of lecithin:cholesterol acyl transferase (LCAT) is essential for the generation of cholesterol esters and the maturation of HDL from small discoidal precursors. The lipid transfer proteins, chiefly cholesteryl ester transfer protein (CETP), promote the net transfer and exchange of cholesteryl esters for triglyceride between HDL and the lower density lipoproteins. This is largely responsible for the conversion of HDL_3 to HDL_2 and, in concert with LCAT, supports the reverse transport of cholesterol from peripheral tissues to the liver.

Much of the understanding of the roles that these enzymes play in lipoprotein metabolism has come from the aberrancies of particle size, composition and plasma concentration observed in human patients with inherited deficiencies of the enzymes. The study of animals that are naturally deficient in one or another enzyme has also made a significant contribution. Yet, of the abundance of species examined the horse has largely been ignored. Notable exceptions are the four papers that examined equine LCAT activity (Yamamoto *et al.* 1979a, 1979b, 1987; Chen and Albers 1983) and an abstract describing the partial purification and quantification of LPL (Bauer, Miller, Beauchamp, and Sciscent 1987).

Any comprehensive study of lipid transport in the horse clearly requires that the function and activity of these enzymes be examined. In the work described here, LPL and HL were isolated from plasma and their enzymatic characteristics established. An assay system that selectively measured the activities of LPL and HL in equine PHP was then developed and validated. The cofactor dependence of LCAT

was defined and the impact of the enzyme on the plasma concentration and composition of HDL determined. Preliminary analysis of transfer protein activity suggested that the horse was deficient in CETP and subsequent investigations focused on whether this was a true deficiency of the protein or the product of a plasma inhibitor of the enzyme. The results of these studies provided an insight into the pathways that govern the metabolism of the equine plasma lipoproteins and allowed the structural characteristics defined in Chapter III to be rationalised.

2. MATERIALS AND METHODS

The methods used for the measurement of LPL, HL, LCAT and CETP activities were described in Chapter II.

2.1. Subjects and Samples

Post-heparin plasma was collected as detailed in section II.3.2, and lipoprotein deficient plasma (LPDP) prepared as in section II.4.2. The subjects were healthy adult Thoroughbred horses or Shetland ponies (geldings and non-pregnant mares) that were housed and fed a maintenance diet of hay and proprietary concentrates that was withdrawn for 16 hours prior to sampling. HDL was prepared by flotation ultracentrifugation as described in section II.2.3.

2.2. Heparin Sepharose Affinity Chromatography

The two lipases were separated from PHP by affinity chromatography on heparin sepharose, using the protocol described by Iverius and Östlund-Lindqvist (1986). PHP was collected from four ponies, pooled (total volume 250 ml) and adjusted to 0.4 M NaCl by adding solid NaCl (5.844 g). This was mixed with 30 ml Heparin Sepharose CL-6B (Pharmacia AB) at 4°C for 30 mins. and then washed with 800 ml 0.4 M NaCl in 30% glycerol (v/v), 0.01 M phosphate, pH 7.5 on a scintered glass funnel. The mixture was resuspended in this buffer, poured into a 2.0x20 cm glass column and washed with one bed volume of buffer. The column was maintained at 4°C and eluted with a 145 ml linear gradient of 0.4 to 1.5 M NaCl in 30% glycerol (v/v), 0.01 M phosphate, pH 7.5, followed by 25 ml of the higher salt buffer. This was mixed with a gradient mixer (11300; LKB Bromma) and pumped through the column at 25 ml/hr using a peristaltic pump (2115; LKB Bromma). Fractions of 5 ml were collected and analysed for lipase activity using the standard method described in section II.3.5, with the Tris buffer containing 0.2 M NaCl, and in the presence of serum activator. The protein content of the fractions was monitored at OD_{280nm}.

The fractions comprising the peaks of activity were pooled and dialysed against 3.6 M ammonium sulphate, 0.01 M phosphate, pH 7.5 overnight at 4°C. The precipitate was retrieved by centrifugation, dissolved in 50% glycerol (v/v), 0.01 M phosphate, pH 7.5 and stored at -20°C. The protein content of the isolates was determined by the modified method of Lowry *et al.* (Peterson 1977). The identity of the isolates was confirmed by examining their activities when assayed (10 ul of a 1:10 dilution in the glycerol/phosphate buffer) in the presence of high (2.0 M) and low (0.2 M) concentrations of NaCl, and in the presence and absence of serum activator.

2.3. Phenyl Sepharose Affinity Chromatography

Chromatography of LPDP on phenyl sepharose has been shown to enhance human CETP activity and unmask lipid transfer activity in species (rat and pig) reportedly devoid of CETP (Tollefson *et al.* 1988a). Freshly prepared LPDP (20 ml) was pooled from four ponies, adjusted to 4 M NaCl, added to an equal volume of Phenyl Sepharose CL-4B (Pharmacia AB) and equilibrated in a total volume of 200 ml of 4 M NaCl, 10 mM Tris, pH 7.4 at 4°C overnight. The mixture was then poured into a 4.6x20 cm column and washed with 0.15 M NaCl, 10 mM Tris, pH 7.4 until the absorbance of the eluant at OD_{280nm} was less than 0.1. The bound material that is reported to contain transfer protein activity was eluted with distilled water containing 0.02% NaN_3 at a rate of 2.5 ml/2mins and collected in 5 ml fractions until the OD_{280nm} was less than 0.05. Aliquots from each fraction (35 ul) were assayed immediately for CETP activity.

Inhibitor protein was then eluted with either 15% ethanol (Son and Zilversmit 1984), or a linear gradient of water to 100% ethanol (Tollefson *et al.* 1988a), until the absorbance was less than 0.05. These fractions were dialysed against 0.15 M NaCl, 10 mM Tris, pH 7.4 at 4°C overnight and assayed directly for CETP activity. Inhibitory activity was assessed by adding an aliquot of each fraction to assay tubes containing human LPDP as a source of CETP.

3. RESULTS

3.1. Isolation and Characterisation of Lipoprotein Lipase and Hepatic Lipase

Two peaks of lipolytic activity were eluted from the heparin sepharose column (Fig. 11), the first (A) with approximately 0.58 M NaCl and the second (B) at approximately 1.02 M NaCl. The fractions under these peaks (A, 16-22 and B, 28-37) were pooled and concentrated. The activity in A was similar when assayed in the presence of 0.2 M and 2.0 M NaCl and did not require serum for the maximal expression of activity at either concentration (Table 10). This was typical of the salt

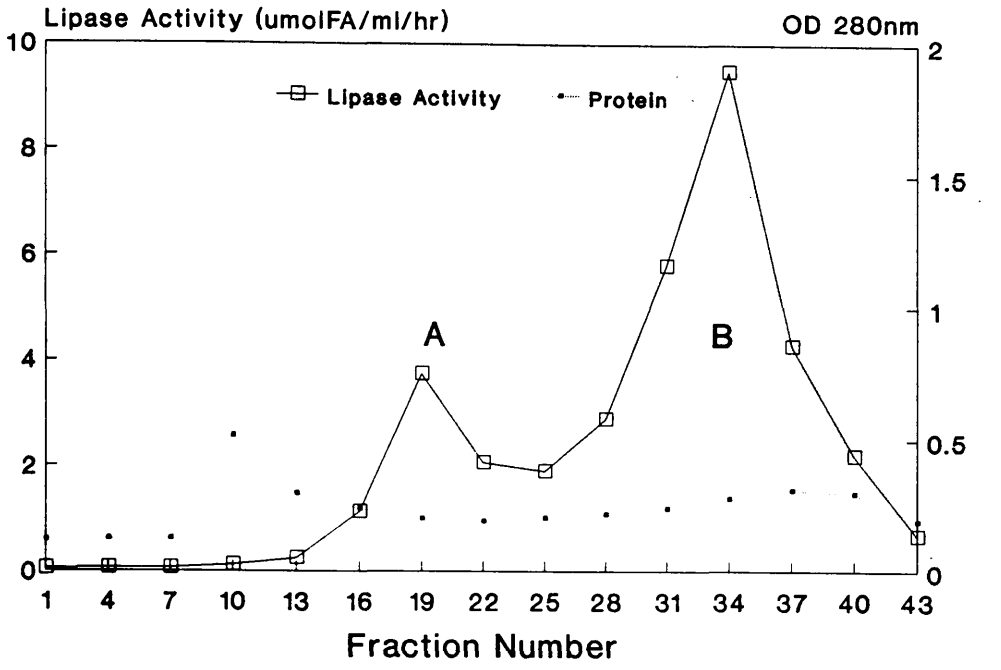


Figure 11. Elution of post-heparin plasma lipase activity and protein (OD_{280nm}) from heparin sepharose. The column was eluted with a linear gradient of 0.4 M to 1.5 M NaCl.

		Activity (% maximal)	
Peak	Serum	0.2 M NaCl	2.0 M NaCl
A	-	89	100
	+	92	100
B	-	52	4
	+	100	35

Table 10. Characterisation of lipase activities separated from equine post-heparin plasma by affinity chromatography. The two peaks of activity were assayed in 0.2 M and 2.0 M NaCl with (+) and without (-) 50 ul of serum as a source of lipoprotein lipase activator. Enzyme activity is expressed as a percentage of the maximum obtained, that is for A at 2.0 M NaCl and for B at 0.2 M NaCl with serum.

resistant lipase of hepatic origin that is now called HL. In contrast, the enzyme activity in peak B was greatest at 0.2 M NaCl, it was reduced by 48% when serum was excluded from the assay, and was negligible when assayed at 2.0 M NaCl without serum (Table 10). The sensitivity of this enzyme to inactivation at high salt concentrations and the requirement of a serum cofactor were characteristic of LPL. Details of the purification and yield of each enzyme are given in Table 11.

3.2. Development of an Assay System for the Selective Measurement of Lipoprotein Lipase and Hepatic Lipase in Post-heparin Plasma

The partially purified enzymes were incubated in a series of Tris buffer NaCl concentrations as shown in Fig. 12. The activity of LPL was maximal at 0.1 M NaCl and declined rapidly thereafter. The HL isolate was characteristically resistant to changes in salt concentration and was maximally active at 1.0 M NaCl. These two salt concentrations were therefore used for all subsequent assays of activity in the isolates or PHP. Both enzymes were active over the pH range 8.5-9.0, with optima of 8.5 and 9.0 for HL and LPL, respectively (Fig. 13).

The design of conditions that discriminated the activities of LPL and HL was the next step in the development of a selective assay system. The negligible (<8%) LPL activity found at the salt concentration required for maximal HL activity when serum was excluded from the assay presented an uncomplicated means of measuring HL. However, in an effort to improve upon the inhibition of LPL in these assay conditions, protamine sulphate (final concentration 1.67 mg/ml) was added to the incubation mixture. This failed to abolish LPL activity, which remained at 6%, and was abandoned as it caused an 11% reduction in the activity of HL.

As HL retained approximately 67% of its activity when assayed under conditions optimal for LPL, there was clearly a need to remove this interference. Prior incubation of the isolates with an equal volume of 50 mM SDS in 0.2 M Tris-HCl at 26°C for 60 mins., was found to reduce the activity of the HL isolate by 93% while leaving that of LPL unaffected (actually increased by 9%) when assayed under conditions optimal for LPL.

The volume of serum required for maximal LPL activity was confirmed as 50 ul (Fig. 14) and addition of volumes greater than this reduced the activity of the enzyme. An assay system was therefore adopted in which HL was measured in 10 ul of PHP in the presence of 1.0 M NaCl and without serum, and LPL was analysed in 20 ul of the incubated PHP/SDS solution, at 0.1 M NaCl in the presence of 50 ul serum.

The dose of heparin required for maximal enzyme recovery was determined in PHP collected from a pony injected with 0, 25, 50, 70, 90 and 120iU/kg

Enzyme	Source	Protein (mg)	Activity Specific	Activity Total	Purification - fold	Yield %
HL	Plasma	13,000	0.30	395.0	-	-
	Peak A	2	105.39	211.2	347	54
LPL	Plasma	13,000	0.19	297.5	-	-
	Peak B	2	84.8	172.3	442	58

Table 11. Purification and yield of lipoprotein lipase (LPL) and hepatic lipase (HL) isolated from equine post-heparin plasma by affinity chromatography on heparin sepharose. Specific activity is expressed in umolFA/mg protein/h and total activity in umolFA/h.

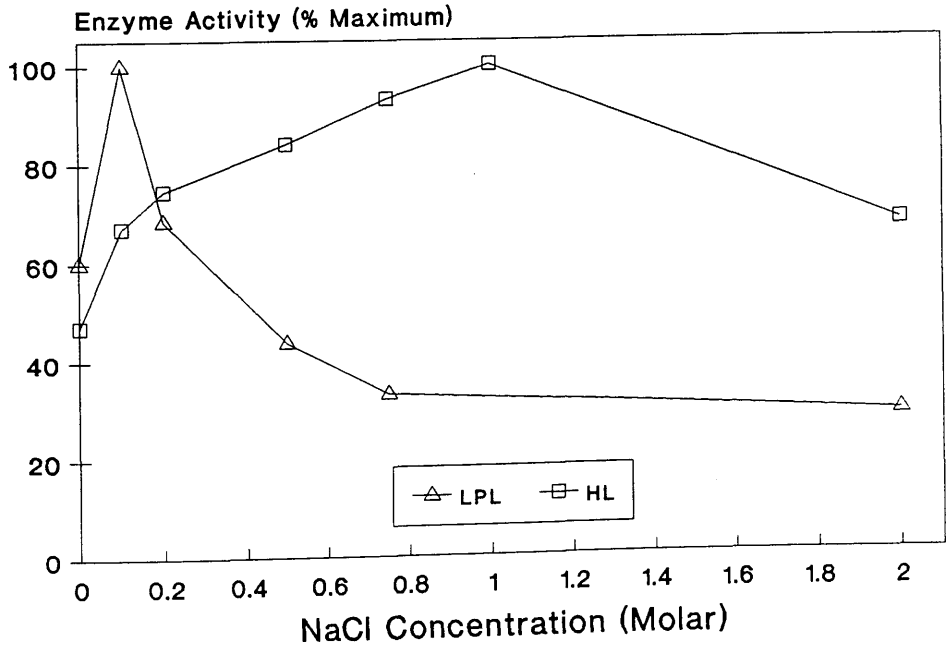


Figure 12. Effect of NaCl on the activity of lipoprotein lipase (LPL) and hepatic lipase (HL) isolated by affinity chromatography on heparin sepharose. The partially purified isolates were assayed in the presence of the NaCl concentrations indicated.

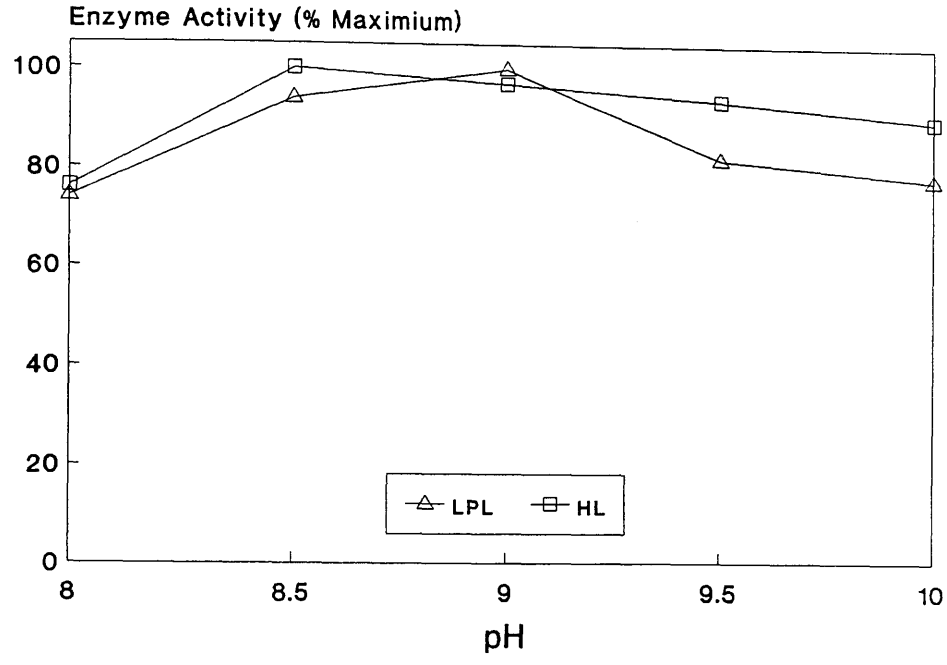


Figure 13. Effect of assay pH on the activity of lipoprotein lipase (LPL) and hepatic lipase (HL) isolated by affinity chromatography on heparin sepharose. The partially purified isolates were assayed in the buffer pH indicated.

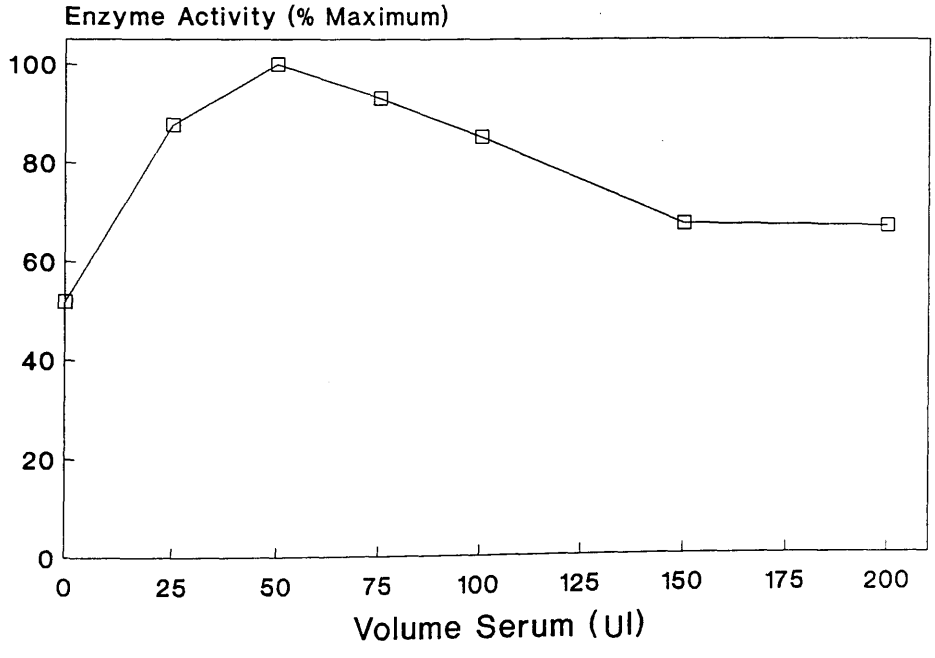


Figure 14. Volume of serum cofactor required to give maximal activity of lipoprotein lipase. Serum was added to the assay of LPL in the amounts indicated.

at weekly intervals. The samples were analysed in a single assay run and the results (Fig. 15) confirmed that 70 iU/kg was the optimum dose, beyond which the activity of both enzymes declined. The appearance of the two enzyme activities in the PHP from a pony is shown in Fig. 16. Neither enzyme was detectable prior to injection of heparin; the activity of LPL peaked 5 minutes after injection and slowly declined over the next 60 mins., while the peak of HL was found at 10 mins.. As it would have been impractical to take and process separate blood samples for each enzyme, a ten minute interval between injection and sampling was adopted to ensure maximal recovery of HL with minimal loss of LPL activity.

The performance of the assay system was evaluated by selecting samples from healthy ponies with low and high activities of each enzyme (Appendix 9). Ten aliquots of each pool were analysed in duplicate in a single assay run to establish the intra-assay coefficients of variation, which ranged between 3.4% and 8.7% (Table 12). The interassay coefficients of variation were determined from seven consecutive assays of each aliquot performed over a seven week period, and varied between 5.2% and 7.1% (Table 12).

The activities of LPL and HL were measured in PHP from eight Thoroughbred horses (four mares and four geldings) and 16 Shetland ponies (10 mares and six geldings) (Appendix 10) and no differences were found in the activity of either enzyme between the two breeds (Table 13).

3.3. Lecithin:Cholesterol Acyl Transferase Activity

Lecithin:cholesterol acyl transferase activity was found in LPDP from two ponies (SP-L, 24.89; SP-7, 25.36 nmolCE/ml/h) in a similar magnitude to that present in LPDP from a healthy human volunteer (TW, 39.16 nmolCE/ml/h). This activity was entirely dependent upon the inclusion of apoA-I in the assay incubation, being reduced by 79.8% and 88.5% to 5.02 and 2.93 nmolCE/ml/h, respectively, when equine apoA-I was excluded from the buffer. This cofactor dependency was not restricted to apoA-I of equine origin, as there was no statistical difference in the activity of LCAT in LPDP from five Shetland ponies (mean $\pm$sd) when apoA-I of human (21.75 $\pm$1.62 nmolCE/ml/h) or equine (22.52 $\pm$1.34 nmolCE/ml/h) origin was used (Appendix 11).

The activity of LCAT was found to be significantly higher in 12 Shetland ponies (three geldings and nine non-pregnant mares) than in 12 Thoroughbred horses (Table 13; Appendix 12).

The impact of LCAT activity on HDL composition and concentration was examined in 12 healthy adult Shetland ponies (nine mares and three geldings) (Appendix 13). The mean ($\pm$sd) activity of LCAT in this group was 21.48 ($\pm$3.94)

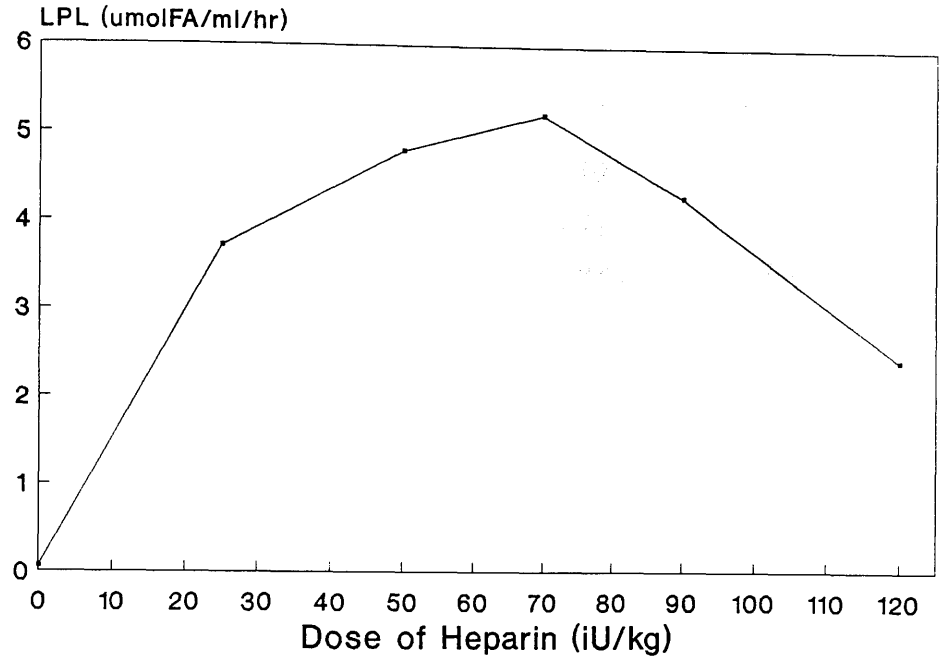


Figure 15. Dose of heparin required for maximal recovery of lipoprotein lipase (LPL) and hepatic lipase (HL). Post-heparin plasma was collected at weekly intervals from a Shetland pony injected with the dosages indicated.

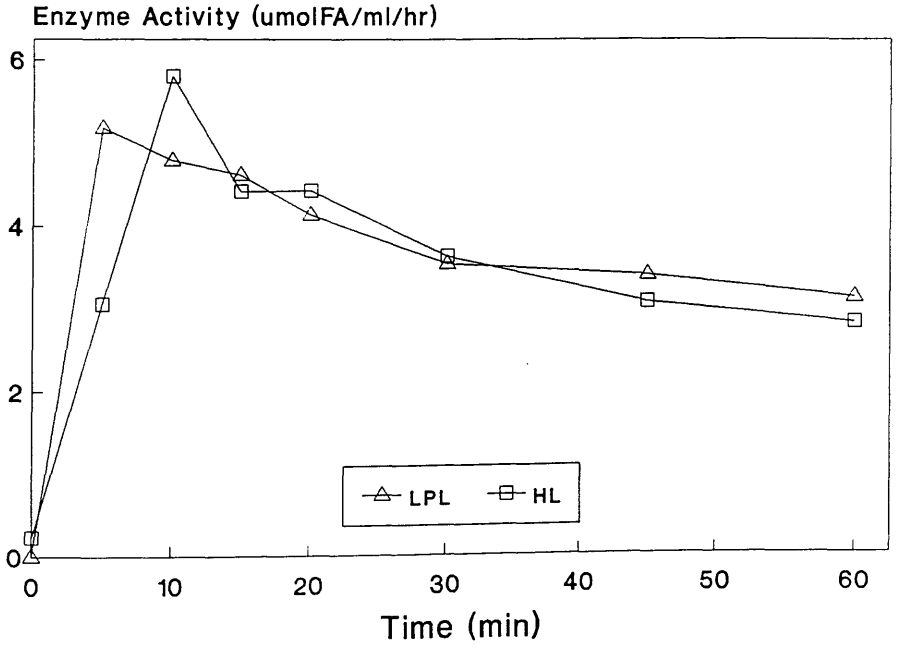


Figure 16. Appearance of lipoprotein lipase (LPL) and hepatic lipase (HL) in equine plasma from injection of 70 iU/kg heparin. A healthy Shetland pony was injected with 70 IU/kg of heparin and blood samples collected over the following hour.

Precision		Lipoprotein Lipase		Hepatic Lipase	
		Low	High	Low	High
Intra-assay	Mean Activity	2.21	6.10	2.78	7.04
(n=10)	sd	0.19	0.21	0.19	0.43
	cv	8.7%	3.4%	6.8%	6.0%
Interassay	Mean Activity	2.05	6.05	2.67	6.70
(n=7)	sd	0.22	0.54	0.19	0.35
	cv	10.7%	8.9%	7.1%	5.2%

Table 12. Performance of the assay for the selective measurement of lipoprotein lipase and hepatic triglyceride lipase in equine post-heparin plasma. Aliquots of plasma with high and low activities (umolFA/ml/h) of each enzyme were analysed in ten sets of duplicates in a single assay run to estimate the intra-assay error, and in duplicate in seven consecutive assays run over a seven week period (n, number of samples; sd, standard deviation; cv, coefficient of variance).

Breed	LPL		HL	LCAT	
	n	umolFA/ml/h		n	nmolCE/ml/h
TB	8	3.31 ±1.17	3.22 ±1.40	12	20.21 ±3.39
SP	16	3.54 ±1.62	4.46 ±1.73	12	23.76* ±4.22

* $p < 0.05$

Table 13. Activities of lipoprotein lipase (LPL), hepatic lipase (HL) and lecithin:cholesterol acyl transferase (LCAT) in healthy Thoroughbred horses(TB) and Shetland ponies (SP).

nmolCE/ml/h and was correlated with the relative mass of cholesteryl esters in HDL (r = 0.47). There was no correlation between the activity of LCAT and the mass of triglyceride (r = -0.04), free cholesterol (r = -0.14) and phospholipid (r = 0.16) in HDL.

3.4. Cholesteryl Ester Transfer Protein Activity

The protein (OD_{280nm}) and radioactivity in the labelled human and equine HDL eluted from the sepharose column is shown in Fig. 17. In both cases, tubes 15-21 were taken as the peak. The human [^{3}H]HDL_3 was preceded by a small peak of radioactivity and protein (fractions 9-12) that represented trace amounts of HDL_2 which was discarded. The equine [^{3}H]HDL_3 peak was relatively poor in counts and was preceded by a larger peak of non-protein associated radioactivity (fractions 7-11). The nature of this peak was not determined, it eluted earlier than the human HDL_2 peak in the void volume of the column and may have consisted of aggregates of the labelling materials.

The assay method used gave results for CETP activity in LPDP from three healthy human volunteers of 0.150, 0.126 and 0.143 %T/ug that were compatible with values reported elsewhere (Freeman, Packard, Shepherd and Gaffney 1990). A single human sample was therefore included in each of the subsequent assays of equine CETP as a positive control. Initial analysis of activity in LPDP from two Thoroughbred horses and Shetland ponies failed to demonstrate any significant CETP activity (Table 14). Transfer of cholesteryl esters ranged from -4.5% to 5.8% (-0.050 to 0.006 %T/ug) and was largely contained within the coefficient of variation of the five blank incubations (±4.2%T). In a second assay where LPDP from four Shetland ponies and a further four Thoroughbred horses was analysed (Appendix 14), percentage transfer outwith the blank range (±5.6%T) was found in only two ponies (11.1%, 6.1%) and two Thoroughbreds (11.2%, 7.0%), giving transfer activities of 0.007, 0.004, 0.007 and 0.077 %T/ug, respectively.

At this point, there were concerns that the poor recovery and inefficient labelling of the equine, compared with the human, substrate might have contributed to this failure to demonstrate any significant CETP activity. A second batch of substrate was then prepared for which HDL was isolated from 37.5 ml of pooled equine plasma by rate-zonal ultracentrifugation as previously described and the fractions comprising the HDL peak concentrated to a volume of 4 ml. As the labelling procedure itself is in part dependent upon lipid transfer, 2 ml of the equine HDL was mixed with 18 ml ofhuman LPDP, as a source of CETP, and incubated with [^{3}H]cholesteryl oleate as before. The HDL (fractions 13-21) that eluted from the sepharose column (Fig. 18) was as rich in radioactivity as the human [^{3}H]HDL_3previously prepared.

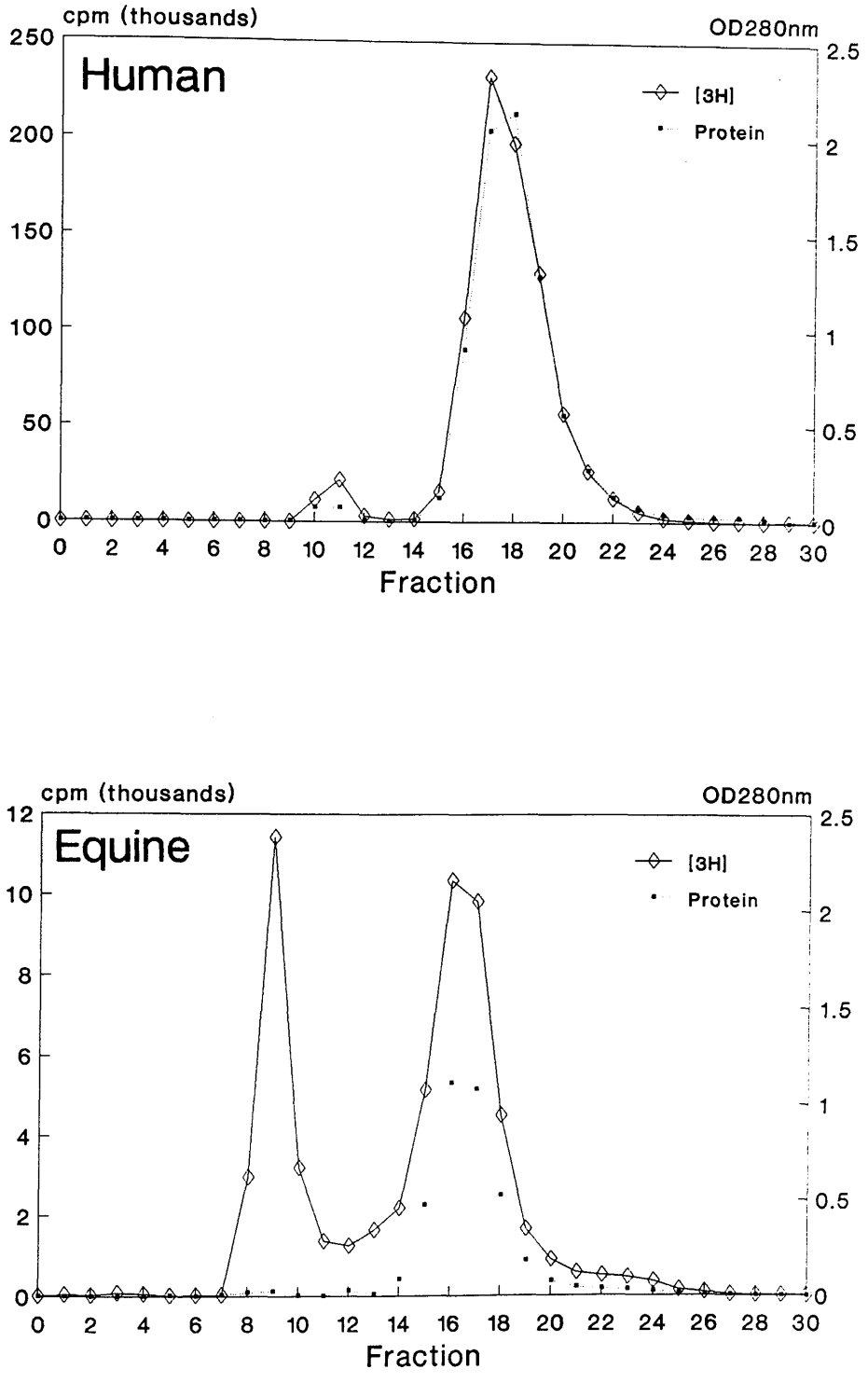


Figure 17. Elution of protein and radioactivity from sepharose of human (top) and equine (bottom) [^{3} H]HDL preparations. The density >1.125 g/ml fraction of plasma was incubated with [^{3} H]cholesteryl oleate, the HDL was re-isolated at density <1.215 g/ml, applied to sepharose columns and eluted with Tris-saline.

Animal	% Transfer/2.5h Volume of LPDP 1 ul	2 ul	4 ul	20 ul	35 ul	Protein ug/ml	%T/ug
TB-13	-2.8	-2.7	3.9	-1.6	-0.8	44.5	-
TB-14	-2.4	0.7	-2.4	-1.0	0.9	41.5	-
SP-2	-2.3	-0.9	-0.1	2.0	2.5	41.0	-
SP-3	0.9	-4.5	0.1	5.2*	5.8*	41.0	*0.006
Human	6.8	10.9	19.5	62.8	68.5	44.5	0.125

Table 14. Cholesteryl ester transfer protein activity in two Thoroughbred horses, two Shetland ponies and a healthy human volunteer (*Tubes with % transfer greater then the coefficient of variation (±4.2%) of the five blanks were used to calculate %T/ug).

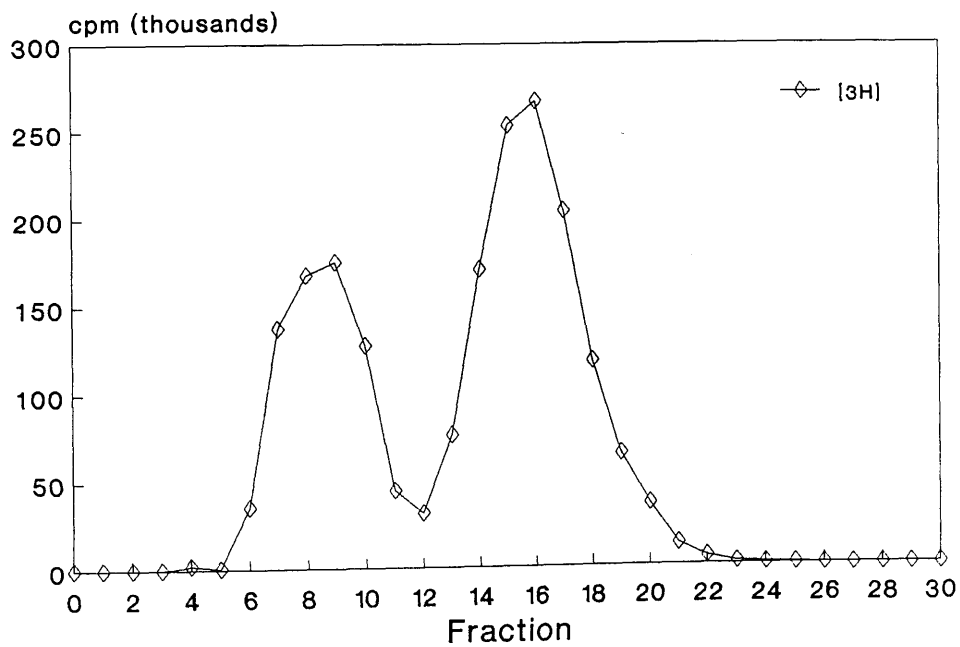


Figure 18. Elution of radioactivity from sepharose of a second equine [^{3}H]HDL preparation. HDL was isolated by rate-zonal ultracentrifugation and incubated with [^{3}H]cholesteryl oleate in the presence of human LPDP. The incubation mixture was then applied to a sepharose column and eluted with Tris-saline.

The efficacy of this substrate and an equine VLDL/LDL acceptor preparation to detect CETP activity was first tested using human LPDP as a source of CETP. The activity of CETP was reduced by only 13% when equine instead of human [^{3}H]HDL$_3$ was used (Table 15). The equine acceptor preparation gave slightly higher activities when assayed with donor particles of human (+14%) or equine (+2%) origin. The equine substrate was therefore used with the equine VLDL/LDL preparation to measure CETP activity in LPDP from a further 12 Shetland ponies (Appendix 15). Percentage transfer beyond the coefficient of variation of blanks (±3.1%T) was found in 11 of the animals and varied between 3.7% and 18.2%, giving transfer activities of 0.004-0.013 %T/ug (0.007 ±0.004; mean ±sd). However, as with the previous animals examined, these values represented single isolated results from a series of incubations containing 10-35 ul of LPDP rather than part of a linear relationship between volume of LPDP and percentage transfer as seen with human samples.

In a final attempt to demonstrate significant transfer activity in the horse, [^{3}H]HDL$_3$ and VLDL/LDL of human origin were used in the assay of LPDP from a Shetland pony. The results are summarised in Table 15 and show that no significant activity was obtained when any of the combinations of human and equine donor and acceptor particles were used.

Activity in human LPDP (%T/ug)			Activity in equine LPDP (%T)		
	VLDL/LDL			VLDL/LDL	
[^{3}H]HDL$_3$	Human	Equine	[^{3}H]HDL$_3$	Human	Equine
Human	0.145	0.165	Human	3.7%	7.9%
Equine	0.126	0.129	Equine	4.2%	5.1%

Table 15. Activities of cholesteryl ester transfer protein (CETP) in human and equine lipoprotein deficient plasma (LPDP) when assayed with donor and acceptor particles of human and equine origin.

3.5. Separation of Transfer Proteins from Inhibitor Protein

The elution of protein by water from the phenyl sepharose column is shown in Fig. 19 together with the percentage transfer present in 35 ul of the fractions comprising the protein peak. Two separate activities were identified. The first was in fractions 10 (35.8%) and 11 (11.8%) and the second in fractions 14 (9.6%) and 15 (22.9%). The protein contents of fractions 10 and 15 were 2.9 mg/ml and 0.58 mg/ml giving transfer activities of 0.353 and 1.138 %T/ug, respectively. However, the column fractions were not assayed in duplicate and two subsequent analyses using 10-35 ul of the fractions failed to demonstrate any significant transfer in either. This suggested that the two activities may have represented spurious results. The procedure was therefore repeated using fresh LPDP and phenyl sepharose; the fractions were assayed in duplicate and no significant activity was detected.

The first of the columns was eluted with 15% ethanol and the elution of protein is shown in Fig. 20. The fractions comprising the protein peak were tested for CETP inhibitory activity by adding 25 ul to assay incubations containing 3.3 ul of human LPDP that gave 40.4% transfer of cholesteryl esters (0.269 %T/ug) (Appendix 16). Contrary to the expected pattern, each of the fractions appeared to enhance the transfer activity in the LPDP by between 7% and 50%. However, when 35 ul aliquots of each fraction were assayed for CETP activity, no significant transfer activity was detected in any of the samples. The second column was eluted with a gradient of water to 100% ethanol and the fractions were assayed for transfer activity and inhibition of CETP as with the first column. A similar pattern was obtained; there was a consistent augmentation of human LPDP in the inhibition assay which was not due to the presence of CETP.

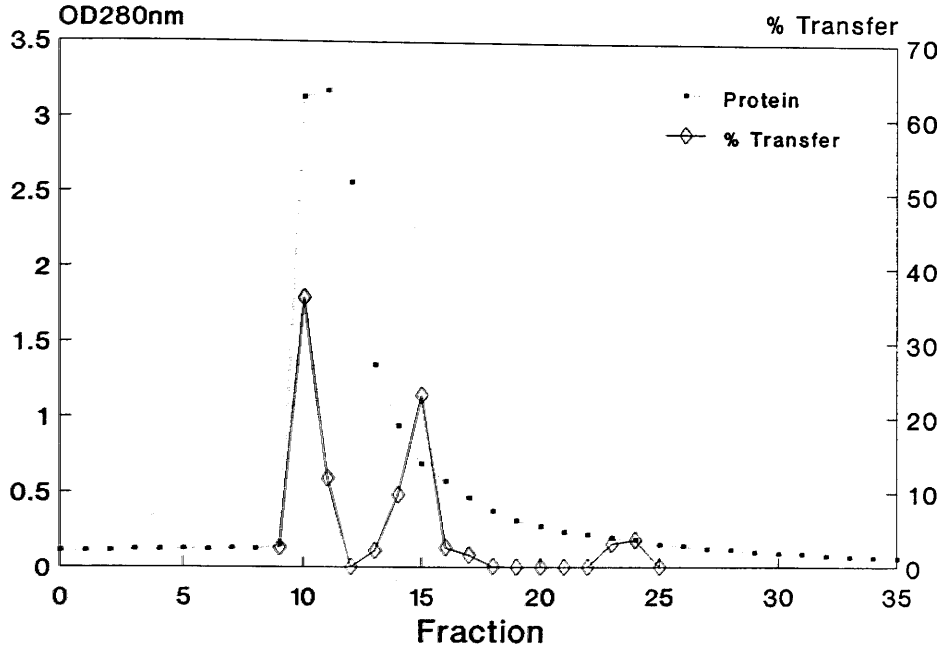


Figure 19. Elution of protein and cholesteryl ester transfer activity from phenyl sepharose. Equine LPDP was applied to a phenyl sepharose column and eluted with distilled water, a single 35 ul aliquot from each 5 ml fraction was then analysed for cholesteryl ester transfer activity.

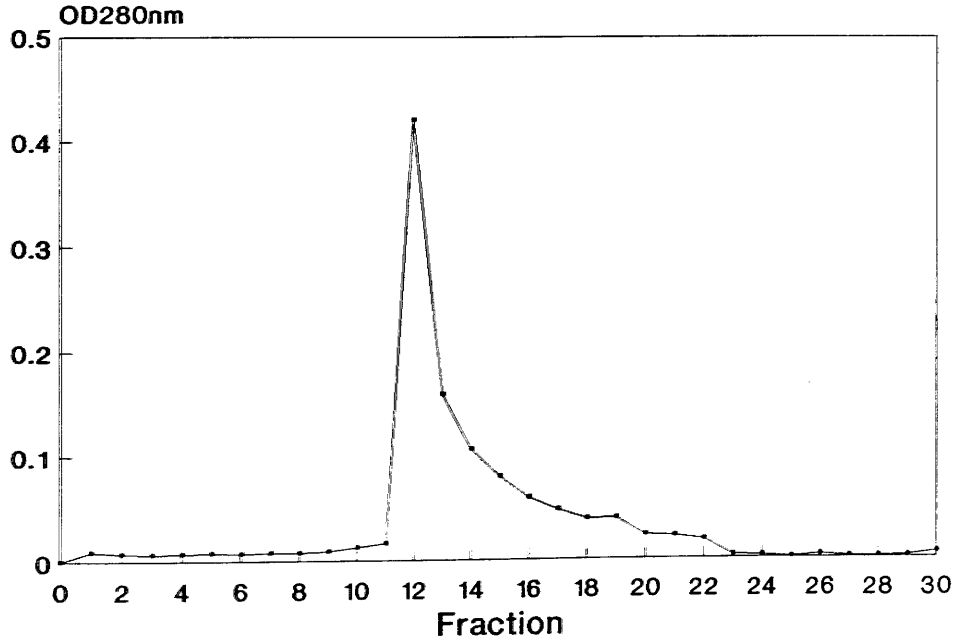


Figure 20. Elution of protein with 15% ethanol from phenyl sepharose. The column used in Fig. 17 was eluted with 15% ethanol and the protein content of the fractions measured at OD_{280}nm.

4. DISCUSSION

4.1. Lipoprotein Lipase and Hepatic Lipase

Heparin sepharose chromatography defined two lipolytic enzymes that were discrete with respect to their column affinities in the face of increasing salt concentrations. The first was typical of HL in that it eluted at a concentration of NaCl (0.58 M) similar to that reported for HL from human (0.62 M NaCl; Östlund-Lindqvist and Boberg 1977) and pig (0.75 M; Ehnholm, Bensadoun and Brown 1977) PHP. The peak of activity in the second eluted with a salt concentration (1.02 M) that corresponded to that of LPL from man (0.94; Östlund-Lindqvist and Boberg 1977) and the pig (1.2 M; Ehnholm *et al.* 1977). These identities were confirmed by the characteristic salt resistance of the HL isolate, and by the sensitivity of the LPL isolate to inactivation at high salt concentrations together with its dependence on a serum cofactor for maximal activity. The purification and yield of the two enzymes were compatible with those reported using similar methodologies for human HL (2943 fold, 72%; Augustin, Freeze, Tejada and Brown 1978) and LPL from human (329 fold, 31%; Ehnholm *et al.* 1975) and equine (260 fold, 75% Bauer *et al.* 1987) PHP. The equine LPL reported by Bauer *et al.* (1987) had an apparent molecular weight of 67k Da similar to that of human LPL (67k Da; Augustin *et al.* 1978) but, in contrast to the isolate described here, was only partially inhibited (by 50%) in the presence of 1 M NaCl. Although they did not isolate HL, the authors reported that some lipase activity was eluted with 0.72 M NaCl buffer prior to the collection of LPL, which may have been HL. It is therefore possible that contamination of LPL activity with this enzyme might explain why their LPL was only partially inhibited at 1.0 M NaCl. In addition, the authors did not state whether the inhibition of LPL was detected in the presence or absence of serum cofactor, which might also explain the discrepancy with the present data as the LPL isolate described here retained 40% of its activity when assayed at 1.0M NaCl in the presence of serum.

The presence of significant (65%) HL activity at 0.1 M NaCl presented problems in the measurement of LPL. Because the activity of HL was not the same at 0.1 and 1.0 M NaCl, any attempt to calculate LPL activity as the difference between "total" lipolytic activity at 0.1 M NaCl and HL activity at 1.0 M NaCl was invalid. In man, this has been overcome by using reagents that selectively inhibit one or both of the enzymes, such as protamine sulphate which inhibits LPL (Krauss, Levy and Fredrickson 1974), SDS that inactivates HL (Baginsky and Brown 1977) and inhibitory antisera directed to HL (Huttunen, Ehnholm, Kinnunen and Nikkila 1975). In the absence of antisera to either human or equine HL, the SDS method was tested against the equine HL isolate and shown to be a specific and highly effective inhibitor of the enzyme. The slight enhancement of LPL activity was not considered

significant, but may have been due to the effects of SDS on the stability of the substrate or the efficiency of fatty acid partitioning during the retrieval of reaction products.

In their abstract, Bauer *et al.* (1987) based an assay of equine LPL on the almost complete inhibition (90-95%) of the enzyme in the presence of 3.3 mg/ml protamine sulphate, and calculated LPL activity as the difference between total and protamine resistant lipolytic activity. Although no details of the assay conditions or measured activities were given, the authors used the assay to show that LPL activity was increased three fold over controls in seven fasted ponies. This deserves caution as the effects of protamine sulphate on HL were not examined; when protamine sulphate was used here at 50% of their final concentration, the activity of HL was reduced by 11%.

The inhibition of LPL activity by volumes of serum in excess of 50 ul might have been due to inhibition of LPL by unphysiological amounts apoC-III in the incubation mixture, or an effect of the serum on the stability of the assay substrate or the efficiency of fatty acid extraction. The time courses of the appearance and clearance of LPL and HL activity following heparin injection were similar to the human where HL peaks at 5-10 mins. and LPL at 15-20 mins. (Huttunen *et al.* 1975). However, the reduction in activities of both LPL and HL at doses of heparin greater than 90 iU/kg is not apparent in man where they plateau between 50 and 200 iU/kg (Huttunen *et al.* 1975). The reason for this discrepancy is not clear.

The assay system developed for PHP performed well with intra- and interassay precisions that are compatible with those reported using SDS in man (2-5% and 11%, respectively; Baginsky and Brown 1977). One criticism of post-heparin lipase assays of this type is the lack of a robust external standard with which to test the accuracy of, and correct for any variation between, assay runs. While the assay presented here was not validated against any other, it was shown to be reproducible and the inclusion of a "standard" PHP sample in each run helped keep a check on any drift encountered when using a new batch of substrate, serum or buffers. The assay showed that there were no significant differences in the activity of LPL between the Thoroughbred and the Shetland pony, and as such suggested that the higher plasma triglyceride and VLDL cholesterol concentrations found in the ponies in Chapter III do not result from differences in LPL activity.

Given that the characteristics of the two enzymes isolated were *in vitro* almost identical to those ascribed to human LPL and HL, it is hard not to conclude that the equine enzymes do not perform similar functions *in vivo*. Thus the activity of LPL would explain the decrease in particle triglyceride mass from VLDL through to IDL, and in concert with HL, the progression of IDL through to LDL. The presence of

significant HL activity in the horse indicates that this enzyme, in contrast to CETP, is available to participate in the generation of discrete subfractions of LDL. It is harder to define any role for HL in the modelling of HDL as the horse does not appear to possess a suitable substrate *i.e.* HDL_2 .

4.2. Lecithin:Cholesterol Acyl Transferase

The studies of LCAT activity described here differed from those previously published in the respect that LPDP, rather than serum (Yamamoto *et al.* 1979a; 1979b) or plasma (Chen and Albers 1983), was used as the source of enzyme. By using ultracentrifugation to prepare LPDP, the enzyme was dissociated from HDL and divorced from the apolipoproteins that modulate its activity. This enabled the cofactor requirements of equine LCAT to be truly defined. The equine enzyme, in common with that of other species, was almost entirely dependent upon apoA-I for activity and the small amount of esterification that occurred in the absence of the protein could be attributable to free apoA-I present in the d >1.215 g/ml fraction after ultracentrifugation. The LCAT activity in LPDP did not demonstrate any preference for apoA-I of equine or human origin. Although Chen and Albers (1983) found that there was good cross species activation of LCAT, their assay used plasma in which there would have been endogenous apoA-I present.

The equine LCAT activity here was somewhat lower than that of 90.7 ±10.6 nmolCE/ml/h (mean ±sd, n=8) reported by Chen and Albers (1983). These authors used substrate vesicles of almost identical composition and specific activity, but the apoA-I was incorporated into the liposomes rather than being added separately to the assay tubes, which they commented led to higher values for LCAT activity in both human and rabbit plasma. This might explain the discrepancy with the present data as the expression of activity in the assay used here does not appear to have been limited by the availability of cofactor, as the amount of apoA-I used (67.5 ug/10 ul LPDP) was slightly higher than that used by Chen and Albers (27.5 ug of purified apoA-I plus approximately 22.5 ug native apoA-I/15 ul plasma). Yamamoto *et al.* (1979a) reported serum LCAT activities in the range 30-90 nmolCE/ml/h in seven horses using very different methodologies that were based on a Tween 20 substrate or one prepared from heat inactivated plasma, neither of which used exogenous apoA-I.

The origins and significance of the differences in LCAT activity found between the two breeds of horse here are not clear. First, the increased activity in the Shetland ponies was not reflected by any differences in the HDL masses of cholesteryl esters and free cholesterol in Chapter III. Secondly, the mean activity of LCAT when measured in the same 12 ponies almost a year later during the course of the correlation study was not statistically different to the Thoroughbred values. This

may reflect genuine changes in LCAT activity over the intervening period, or been due to drift in the precision of the assay. The limited size of the groups might also prevent any concrete conclusions at this point. Interestingly, in Chapter III the mass of cholesteryl ester in VLDL from the ponies was found to be significantly higher than that from the Thoroughbred horses. Although equine LCAT has been shown capable of esterifying cholesterol in VLDL *in vitro* (Yamamoto *et al.* 1979b) the degree of esterification was only one-twentieth of that in its preferred substrate HDL. It is therefore likely that the difference in VLDL composition reflected the neutral lipid content of the liver and the activity of acyl:CoA cholesterol acyl transferase (ACAT) in particular.

The affinity of equine LCAT for HDL *in vitro* (Yamamoto *et al.* 1979b) was shown here *in vivo* by the positive correlation between LCAT activity and the mass of cholesteryl ester in HDL. Although Yamamoto *et al.* (1979b) reported that LCAT reduced the masses of free cholesterol and lecithin in HDL, no relationship was found here between the activity of the enzyme and these masses. This was not surprising, as *in vivo* the free cholesterol lost to LCAT would be rapidly replaced by the flux of fresh material into HDL from cell membranes and other lipoproteins. In man, this would be accompanied by the transfer or exchange of cholesteryl esters away from HDL, so that the composition of these lipoproteins in fact bear little relationship to LCAT activity. In the horse, the mass of cholesteryl ester in HDL was correlated with the activity of LCAT suggesting that the esters are "trapped" in HDL, providing further evidence that the horse is deficient in transfer protein activity.

4.3. Cholesteryl Ester Transfer Protein

A number of methodologies exist for the measurement of lipid transfer protein activity, differing in the nature of substrate (LDL, HDL, or lecithin/cholesterol liposomes, labelled in the triglyceride, cholesteryl ester or phospholipid moieties) and whether mass transfer or the rate of exchange of lipid are measured. The multitude of systems reported undoubtedly reflects the difficulties that exist in assaying transfer activity, and are likely to be superseded in the future by immunoassays that detect CETP mass rather than activity The assay used here was developed by Freeman (1990) and measured exchange activity. LPDP plasma was used as the source of enzyme because of an increasing weight of evidence that the proteins that inhibit lipid transfer activity are associated with the HDL fraction of plasma (Tollefson *et al.* 1988a; Nishide *et al.* 1989). Cholesteryl ester labelled HDL_3 was used as the substrate to reflect the net function CETP and bovine serum albumin appears was included in the incubation to maintain the stability of the substrate and ensure minimal background activity. Cold plasma was added to the assay mixture after

incubation to increase the mass of lipoproteins precipitated by the heparin-manganese and improve the pelleting of the lower density lipoproteins.

Although this methodology was designed for use with human LPDP, there was no obvious reason why, if donor and acceptor particles of equine origin were prepared, it should not detect CETP activity in the horse. Initial concerns over the quality of the equine donor and acceptor preparations were proved to be unfounded. The decision to use rate-zonal ultracentrifugation to prepare a second batch of HDL was based on the evidence presented in the previous chapter that equine HDL was analogous with human HDL_3 in terms of physical and chemical structure. This substrate was proven capable of supporting CETP activity in human LPDP, and gave results for equine LPDP identical to the first batch. It is therefore hard to blame the inability to demonstrate significant CETP activity in the horse on the assay methodology employed. When activity was detected, it represented between 1% and 6% of the human activity measured in that assay. This was significantly lower than the 14-27% range quoted for species (rat, sheep, cow, pig, dog) defined as low in CETP activity by Ha and Barter (1982).

The CETP activity in two of these "low" species has subsequently been unmasked by chromatography on phenyl sepharose, to the extent that activities approaching (rat) and in excess (pig) of the activity found in human LPDP were recovered (Tollefson *et al.* 1988a). This posed the question as to whether the negligible equine activity reflected a true deficiency of CETP or the masking of its activity by an inhibitor. The initial attempt to isolate transfer protein activity yielded equivocal results. Although transfer was found in two peaks, repeat analysis of the two most potent fractions failed to confirm their activity suggesting that the original results were erroneous. This was supported by the fact that no inhibitory activity was identified when the column was eluted with ethanol, and by the deficiency of transfer activity in fractions eluted from a second column.

Tollefson *et al.* (1988a) reported that they were unable to elute an inhibitor of CETP (rat or human) from pig LPDP using 15% ethanol, but showed that a 0-100% ethanol gradient eluted a nondialysable component that produced greater than 30% inhibition of CETP activity. For this reason the second of the columns was eluted with such a gradient, which also failed to identify any inhibitory activity. Rather, the eluted fractions from both columns enhanced CETP activity in human LPDP. The reason for this was not clear as the fractions did not possess transfer activity of their own. The conclusion therefore remains that the horse is deficient in CETP activity.

Species that have low levels of CETP activity classically have a prominent class of large cholesteryl ester and apoE rich HDL_1 particles (Tall 1986). These particles are formed by the agency of LCAT and the transfer of apoE from

triglyceride rich lipoproteins undergoing lipolysis. However, as their conversion to HDL_2 and HDL_3 is dependent upon lipid transfer activity (Gavish, Oschry and Eisenberg 1987), they accumulate in species that lack CETP. Although the horse satisfies these criteria in having LCAT but not CETP, it has no recognisable HDL_1 and it is hard to understand how it retains a single homogeneous population of small HDL_3 like particles. In the following Chapter it is shown that post-prandial lipaemia, which is largely responsible for the generation of triglyceride rich HDL_2, does not occur in the adult horse as it does in man and the dog. This might explain the deficiency of both HDL_1 and HDL_2 in the horse. As the horse lacks CETP activity, how does it accomplish reverse cholesterol transfer? In those other species deficient in CETP, the prominence of apoE rich population of HDL provides the route for the transfer of cholesterol from peripheral tissues in the liver. Given that equine HDL contains less than 7% of its protein mass as apoE, compared with more than 22% in human HDL_1 (Kostner and Laggner 1989), this is unlikely to provide a significant contribution to reverse cholesterol transport.

CHAPTER V

LIPOPROTEIN METABOLISM IN THE FED ANIMAL, THE SUCKLING FOAL, AND IN PONY MARES DURING PREGNANCY AND LACTATION

1. INTRODUCTION

The information currently available concerning lipid transport in the horse is confined to the lipoproteins of endogenous, *i.e.* those present in the circulation when the animal is fasting, rather than exogenous origin. In other monogastric species, including man (Krasinski, Cohn, Russell and Schaefer 1990) and the dog (Melchoir, Mahley and Buckhold 1981), the digestion and absorption of dietary fat has profound effects on both the concentration and composition of the plasma lipoproteins. Most prominently, the plasma concentrations of triglyceride and VLDL cholesterol, and the triglyceride and apoB-48 content of the density <1.006 g/ml fraction of plasma, increase as chylomicrons appear in the circulation (Rifai, Merrill and Holly 1990). This is accompanied by increases in the activity of LPL and in the plasma concentration and triglyceride content of the HDL_2 subfraction (Patsch *et al.*). The latter results from the transfer of surface remnants to HDL, which first expand under the agency of LCAT and then become enriched with triglyceride as neutral lipid exchange, mediated by CETP, takes place.

While the lipid content of the adult equine diet is low compared with those of man and the dog, it is possible that the continued ingestion of grass or hay throughout the day may alter the lipoprotein profile. This was examined by analysing plasma lipoprotein concentrations, the composition of the density <1.006 g/ml fraction, and the activities of LPL, HL, and LCAT in plasma from ponies that were sampled with, and then without, access to food. The transport of dietary fat was subsequently studied using an oral fat tolerance test in which the metabolic fate of chylomicrons was traced with retinyl palmitate. Retinyl esters are a convenient marker for exogenous fat metabolism as they are taken up by enterocytes and incorporated in to the core of chylomicrons, where they remain until the remnants of these particles are taken up by the liver. In addition, these esters do not appear to be re-secreted by the liver and their exchange amongst lipoproteins is minimal (Berr and Kern 1984). The results suggested that the adult pony does not handle dietary fat in the way that humans and dogs do. However, in contrast to the adult animal, fat represents a major dietary component for the suckling foal and their lipoprotein system was therefore examined.

A wealth of epidemiological evidence indicates that pregnancy and lactation represent significant risk factors for the occurrence of hyperlipaemia in ponies. The reasons for this are unclear. The demands placed on maternal energy metabolism by foetal growth and milk synthesis have been blamed for inciting the mobilisation of FFA from adipose stores (Jeffcott and Field 1985b). In late pregnancy this may be exacerbated by the peripheral insulin insensitivity that develops so that the supply of glucose to the foetus is maintained (Fowden *et al.* 1984). To examine how these two states affect lipoprotein metabolism in the horse, the concentrations and composition of the plasma lipoproteins, and the activities of the two endothelial lipases were determined in six ponies in the last trimester of pregnacy and again soon after foaling. Hypertriglyceridaemia was found to be a significant feature of late gestation and was accompanied by changes in VLDL composition that establish a mechanistic basis for the risk posed by pregnancy.

2. SUBJECTS AND SAMPLES

Plasma cholesterol, triglyceride and lipoprotein concentrations were determined in 12 healthy, non-pregnant Shetland ponies as described in section II.1.1. Blood samples were collected at 9am when the animals were at grass, the ponies were then taken inside and given access to hay until 5pm, from which time they were fasted, and sampled at 9am the next day. The density <1.006 g/ml fraction was isolated from the plasma of four animals by flotation ultracentrifugation (section II.2.3.) and its lipid and apolipoprotein composition determined as described in sections II.2.5. and II.2.6. These four ponies were then kept inside and fed a maintenance diet of hay and concentrates. After an acclimatisation period of two weeks, blood samples for the analysis of LPL and HL, and LCAT were collected following an overnight fast and again one week later when the animals had been left with free access to hay in the period prior to sampling. The activities of LPL and HL were determined using the selective assay conditions developed in Chapter IV, and that of LCAT by the method described in section II.4.

Oral fat tolerance tests were performed according to the protocol described in section II.6 on two non-pregnant ponies and two ponies that were four and eight months pregnant, respectively. The animals were fasted from 16 hours prior to the test through to the 8 hour sample, and then allowed free access to hay.

Six pregnant Shetland ponies were housed approximately one month prior to their estimated foaling dates, which was two to six weeks before they actually foaled. Plasma cholesterol, triglyceride, FFA and lipoprotein cholesterol concentrations were determined, lipoprotein fractions were isolated by sequential flotation

ultracentrifugation and their composition analysed, and the activities of LPL and HL measured. The animals were sampled again between one and four weeks after foaling and these analyses repeated. On the second occasion that the mares were sampled, non-fasting blood was collected from their foals and, in addition to the analyses performed on the maternal samples, the apolipoprotein content of the isolated fractions were determined and the activity of LCAT measured.

3. RESULTS

3.1. Post-prandial Lipoprotein Metabolism

The mean plasma concentrations of triglyceride and VLDL cholesterol were significantly higher in the ponies sampled at grass than when fasted, while those of total and HDL cholesterol were significantly lower (Table 16; Appendix 17). There was no change in the LDL cholesterol concentration. In the fed state, the density <1.006 g/ml fraction was significantly enriched in triglyceride and depleted of cholesteryl esters relative to that isolated from the ponies when fasted, but was unchanged in respect of the masses of phospholipid and protein (Table 17; Appendix 18). There were no significant differences in the apolipoprotein content of this fraction (Table 18; Appendix 18), and the ratio of the masses of apoB-100 and apoB-48 in the fed animals (1.8:1) was the same as that when the ponies were fasted (1.8:1). Although the fraction isolated from the fed ponies contained approximately twice as much apoE and apoA-I as that from the fasted animals, the differences were not significant. There were no significant differences in the activities of LPL, HL and LCAT (Table 19; Appendix 19).

The plasma triglyceride responses to the oral fat load are shown in Fig. 21. There was no peak of lipaemia in any of the four ponies examined and the plasma triglyceride concentrations of the two non-pregnant animals remained constant over the ten hours following the fat meal. Retinyl palmitate was detected in only one sample, at 24 hours from SP-R (0.64 ug/ml). In the two pregnant ponies, the plasma triglycerides rose in a linear fashion during the initial eight hours of the test and then declined after the animals were refed at that time point. This presumably represented a response to the deprivation of food in the period prior to the test as retinyl palmitate was detected in one pony (SP-L) only at 24 hours (0.43 ug/ml) and in the other (SP-B) at eight (0.41 ug/ml) and 24 hours (1.27 ug/ml).

Animal	Plasma Concentration (mmol/l)				
	Cholesterol	Triglyceride	VLDL	LDL	HDL
Fed	2.13* ±0.45	0.53* ±0.19	0.43** ±0.07	0.56 ±0.24	1.28** ±0.32
Fasted	2.43 ±0.33	0.42 ±0.16	0.25 ±0.06	0.48 ±0.21	1.70 ±0.19

* $p < 0.05$, ** $p < 0.01$

Table 16. Plasma cholesterol, triglyceride and lipoprotein cholesterol concentrations in 12 Shetland ponies sampled at grass (Fed) and after being fasted overnight (Fasted) (mean ±sd; Fed VLDL, LDL, HDL n=8).

Animal	Percentage Particle Mass				
	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
Fed	5.7* ±0.4	66.8* ±3.4	ND	11.1 ±0.9	16.4 ±3.6
Fasted	9.1 ±0.7	59.9 ±1.5	ND	13.5 ±0.5	17.6 ±1.2

* $p < 0.01$; ND, not detected

Table 17. Chemical composition of density <1.006 g/ml lipoprotein fraction isolated from four Shetland ponies sampled at grass (Fed) and after being fasted overnight (Fasted) (mean ±sd).

Animal	Percentage of Protein Mass					
	B-100	B-48	E	A-I	C-II	C-III
Fed	16.5 ±2.5	9.1 ±1.0	15.3 ±2.6	5.0 ±4.6	18.6 ±5.4	35.7 ±2.9
Fasted	23.8 ±10.2	13.1 ±3.5	7.7 ±5.1	2.5 ±2.3	19.5 ±5.9	33.4 ±6.7

Table 18. Apolipoprotein composition of density <1.006 g/ml lipoprotein fraction isolated from four Shetland ponies sampled at grass (Fed) and after being fasted overnight (Fasted). The proteins were separated by SDS-PAGE and their masses calculated by densitometric scanning of the stained gels (mean ±sd).

Animal	LPL (umolFA/ml/h)	HL (umolFA/ml/h)	LCAT (nmolCE/ml/h)
Fasted	2.39 ±0.57	3.12 ±0.71	20.17 ±2.37
Fed	2.59 ±0.42	3.10 ±0.88	23.90 ±5.65

Table 19. Activities of lipoprotein lipase (LPL), hepatic lipase (HL) and lecithin:cholesterol acyl transferase (LCAT) in four Shetland ponies sampled after being fasted overnight (Fasted) and after access to hay (Fed) (mean ±sd).

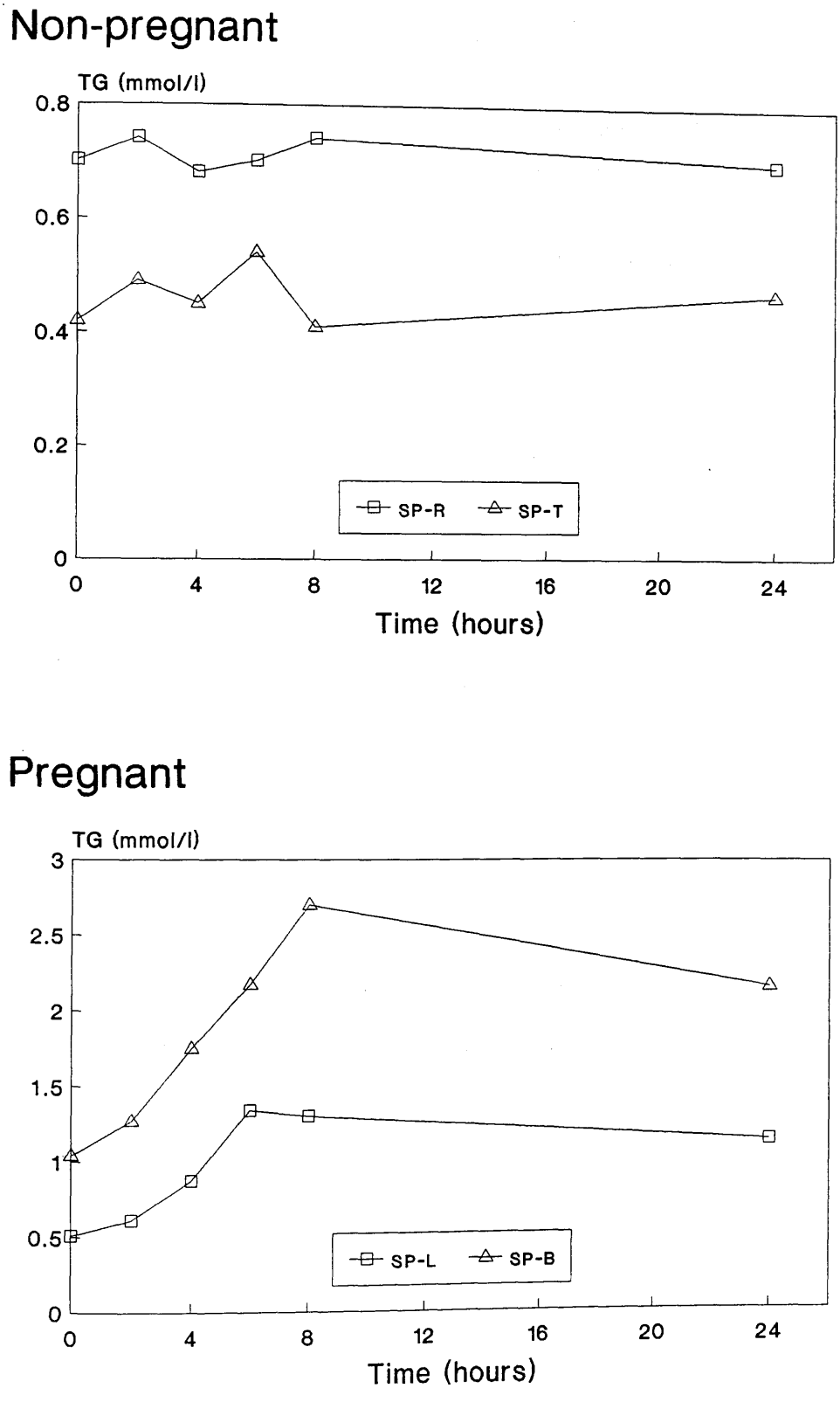


Figure 21. Plasma triglyceride concentrations (TG) in two non-pregnant (top) and two pregnant (bottom) Shetland ponies following an oral fat tolerance test.

3.2. Lipids, Lipoproteins and Enzymes in the Suckling Foal

The non-fasting plasma samples collected from each of the six foals were noticeably lipaemic compared with those from adult animals (Fig. 22). The plasma concentrations of total cholesterol, triglyceride, VLDL and LDL cholesterol were significantly higher than those of their parents in both the fasted and fed states (Table 20; Appendix 20). The concentrations of HDL cholesterol were similar to those of the fed adults.

The plasma samples were cleared of turbidity following flotation ultracentrifugation at density <1.006 g/ml, 20k rpm for 20 mins. when a cloudy supernatant appeared (Fig. 22). This was presumed to contain chylomicrons, and was collected and purified of contaminating plasma proteins by two further spins at density <1.006 g/ml, 20k rpm for 20 mins. The chemical compositions of this, and the VLDL, LDL and HDL fractions are summarised in Table 21 (Appendix 21). There was insufficient material in the IDL fractions for compositional analysis. The composition of the chylomicron fraction was almost identical to that of the intestinally derived lipoproteins found in man as the particles were composed almost entirely of triglyceride, with small contributions from cholesteryl esters and protein. Despite the low cholesterol content, a significant mass of free cholesterol was detected and represented an average 25% of the total cholesterol mass. Phospholipids were below the level of detection.

	Plasma Concentration (mmol/l)				
Animal	Cholesterol	Triglyceride	VLDL	LDL	HDL
Foals	3.77** ±0.84	0.87* ±0.20	0.23 ±0.11	2.06** ±0.62	1.48 ±0.25
Mothers (Fed)	2.15 ±0.14	0.52 ±0.15	0.48 ±0.14	0.43 ±0.23	1.43 ±0.18

* $p < 0.05$; ** $p < 0.01$

Table 20. Plasma cholesterol, triglyceride and lipoprotein cholesterol concentrations in non-fasting samples from six Shetland pony foals and their mothers (mean ±sd).

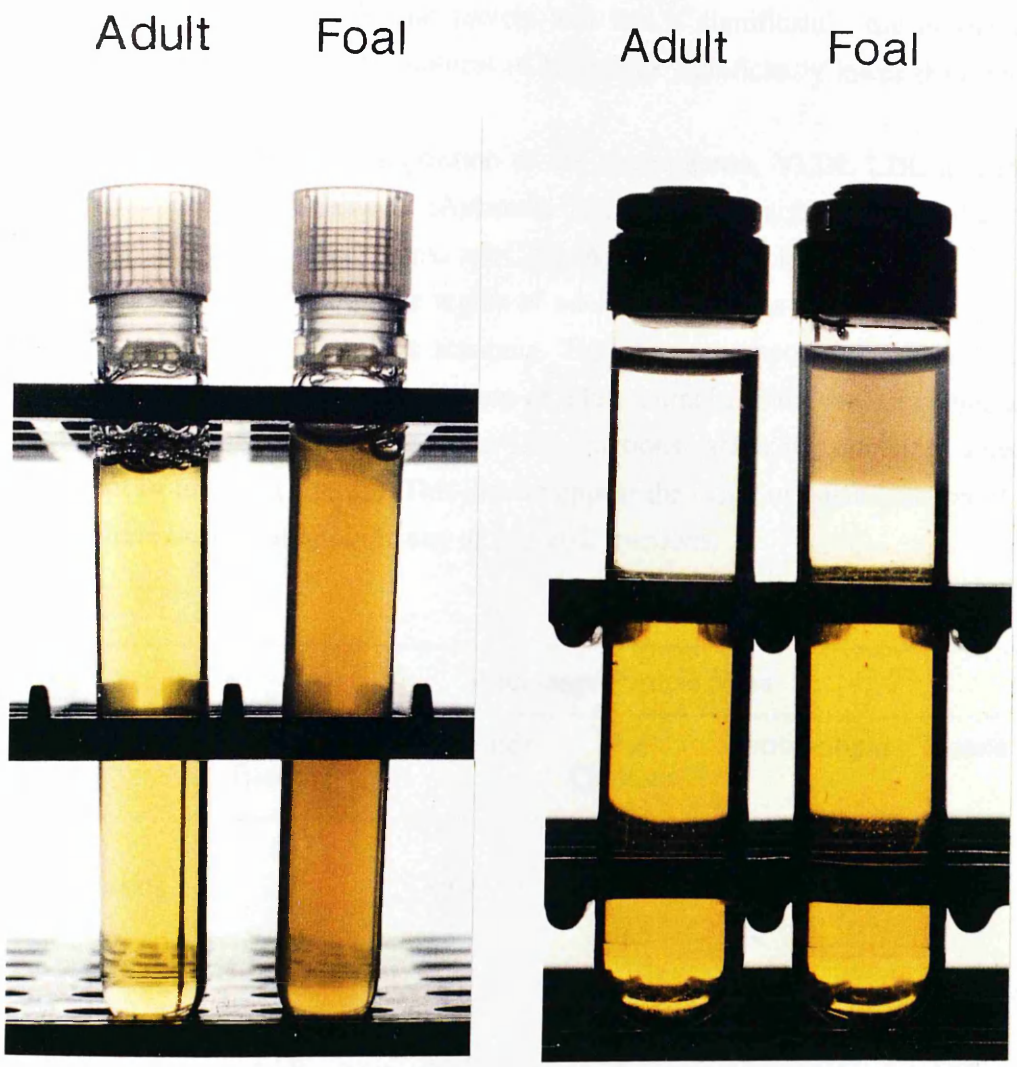


Figure 22. Appearance of a non-fasting plasma sample from a suckling foal. Note the opacity of the plasma compared with a sample from a fasted adult pony on the left. After ultracentrifugation at density <1.006 g/ml, 20k rpm for 20 mins., the plasma has cleared as the chylomicron fraction has floated to the surface.

The foal VLDL was distinct from that of adult ponies in that the mass of triglyceride was significantly lower and the protein content significantly higher. Free cholesterol was also detectable and represented 19% of the total cholesterol mass. The composition of foal LDL was bizarre as a far greater proportion of the cholesterol was present in the unesterified form such that the ratio of esterified to free cholesterol was approximately 1.5:1 compared with 24:1 in the adults. These particles also contained significantly less triglyceride and protein and had a significantly higher mass of phospholipids. The triglyceride content of HDL was significantly lower than that of the adults.

The apolipoprotein composition of the chylomicron, VLDL LDL and HDL fractions are detailed in Table 22 (Appendix 22). The chylomicrons consisted almost exclusively of apoA-I, apoC-II and apoC-III. Apolipoprotein E was detected in one sample and very faint band in the region of adult apoB-48 was visible on the gel but not quantifiable by densitometric scanning. The protein compositions of the VLDL and HDL fractions were similar to those of adult animals. However, an appreciable amount of albumin was present in all the LDL fractions, which also contained a minor component of apoA-I (Fig. 23). This did not appear the result of contamination of the HDL as there was no albumin in any of the HDL fractions.

Lipoprotein	Percentage Particle Mass				
	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
Chylomicrons	2.7 ±1.5	89.7 ±3.1	0.9 ±0.5	ND	7.6 ±1.9
VLDL	9.4 ±2.6	52.6 ±4.6	2.2 ±1.1	13.0 ±1.8	22.7 ±2.4
LDL	26.8 ±6.4	1.8 ±0.4	18.5 ±4.5	35.1 ±3.5	17.1 ±2.8
HDL	18.6 ±3.3	0.4 ±0.2	4.6 ±0.7	28.9 ±1.3	47.5 ±3.7

Table 21. Chemical composition of lipoproteins isolated from six Shetland foals (mean ±sd).

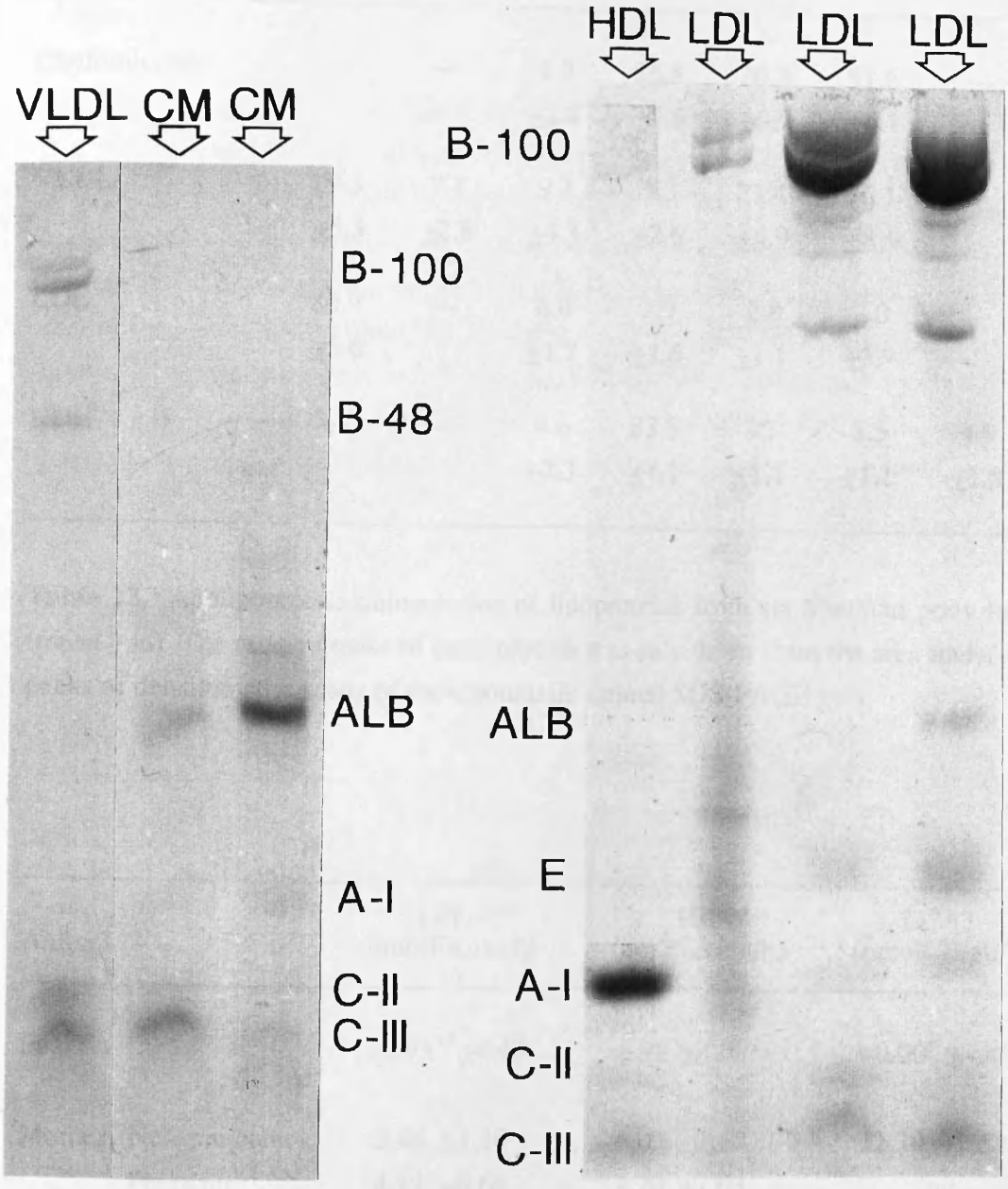


Figure 23. Resolution of foal apolipoproteins by SDS-PAGE. CM, chylomicrons; ALB, albumin. (The second and third LDL tracks have been overloaded to demonstrate the albumin and apoA-I bands present in this lipoprotein class, with the result that there has been some degredation of the protein).

Lipoprotein	Percentage of Protein Mass						
	B-100	B-48	E	A-I	C-II	C-III	A-II
Chylomicrons	---	---	1.2 ±2.8	26.8 ±7.6	20.5 ±6.7	51.6 ±6.1	---
VLDL	19.3 ±5.3	7.1 ±2.8	9.7 ±4.3	7.3 ±2.6	21.4 ±4.9	36.1 ±8.6	---
LDL	80.9 ±5.6	---	6.4 ±1.7	2.9 ±1.6	2.8 ±1.1	7.0 ±3.7	---
HDL	---	---	4.6 ±2.3	83.3 ±1.1	3.7 ±1.1	3.5 ±1.1	4.9 ±1.8

Table 22. Apolipoprotein composition of lipoproteins from six Shetland pony foals (mean ±sd). The relative mass of each protein was calculated from the area under the peaks of densitometric scans of the Coomassie stained SDS-PAGE gels.

Animal	LPL (umolFA/ml/h)	HL (umolFA/ml/h)	LCAT (nmolCE/ml/h)
Foals	10.93** ±4.49	6.94 ±4.08	40.00* ±4.40[a]
Mothers: Non-pregnant	3.44 ±1.10	5.05 ±1.88	22.29 ±2.52
Pregnant	4.12 ±0.68	4.20 ±1.03	nd
Lactating	14.76* ±4.44	9.78 ±3.83	nd

* $p < 0.05$, ** $p < 0.01$, [a] n=5; nd, not determined

Table 23. Lipoprotein lipase (LPL), hepatic lipase (HL), and lecithin:cholesterol acyl transferase (LCAT) activities in six Shetland pony foals and their mothers when non-pregnant, in late gestation and in early lactation (mean ±sd; the foal data has been compared with that of the non-pregnant mares).

The activities of LPL and LCAT were significantly higher in the foals than their mothers (Table 23), being increased by approximately three and two fold, respectively. Although the activity of HL was also increased this was not significant (Appendix 23).

3.3. Lipids, Lipoproteins and Enzymes in Pregnancy and Lactation

The plasma concentrations of total cholesterol, triglyceride, and VLDL and HDL cholesterol were significantly higher in the mares in late pregnancy than they were when the animals were not in foal (Table 24; Appendix 20). The LDL cholesterol and FFA concentrations were unchanged. Once the animals had foaled, the concentrations of triglyceride returned to normal, but those of VLDL cholesterol were significantly lower than the non-pregnant values and the HDL cholesterol concentrations remained significantly elevated. Although plasma FFA concentrations were higher when the ponies were lactating, the differences were not of statistical significance.

	Plasma Concentration (mmol/l)					
Animal	Cholesterol	Triglyceride	VLDL	LDL	HDL	FFA
Non-pregnant	2.02 ±0.33	0.49 ±0.06	0.42 ±0.05	0.52 ±0.24	1.08 ±0.21	0.55 ±0.10
Pregnant	2.91** ±0.25	1.36** ±0.49	0.55* ±0.11	0.63 ±0.12	1.73** ±0.22	0.43 ±0.20
Lactating	2.61 ±0.74	0.41 ±0.27	0.22** ±0.08	0.64 ±0.53	1.75** ±0.27	1.10 ±0.59

* $p < 0.05$; ** $p < 0.01$ compared with non-pregnant data.

Table 24. Plasma lipid and lipoprotein concentrations in six Shetland pony mares when non pregnant, in late gestation, and in early lactation (mean ±sd).

There were significant changes in the composition of VLDL in the pregnant mares (Table 25; Appendix 24), which contained on average 13% more triglyceride and 51% less protein than the particles isolated prior to pregnancy. Although the total cholesterol content was unchanged, the mass of cholesteryl esters was significantly reduced such that free cholesterol represented 92% of the cholesterol mass. After foaling, the VLDL remained significantly enriched in triglyceride while the protein content was normal and the mass of phospholipid was significantly lower. Free cholesterol was not detected in the VLDL from the lactating mares and the mass of cholesteryl esters was similar to that found prior to pregnancy. These differences retained their statistical significance when the groups were compared with the data for 12 non-pregnant Shetland pony mares in Chapter III.

Lipoprotein		Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
VLDL	NP	8.8 ±0.9	60.4 ±1.7	ND	16.6 ±2.3	16.4 ±2.0
	Preg[a]	0.7** ±0.6	68.5** ±2.1	7.8 ±2.0	15.0 ±1.0	8.0** ±1.5
	Lact	6.6* ±1.8	67.8* ±4.7	ND	9.5** ±1.4	16.2 ±3.6
LDL	NP	41.2 ±2.6	5.9 ±2.5	1.2 ±1.9	21.4 ±1.6	29.1 ±3.8
	Preg[a]	37.8 ±3.5	5.9 ±1.2	8.0** ±4.3	24.6 ±1.9	23.1* ±2.8
	Lact	34.5 ±9.1	4.5 ±2.0	9.8** ±5.6	27.3 ±8.1	25.5* ±6.3
HDL	NP	19.5 ±3.4	1.0 ±0.4	3.3 ±4.1	31.0 ±4.1	45.3 ±6.8
	Preg[a]	20.9 ±3.4	0.4* ±0.1	4.1 ±0.8	30.4 ±1.4	44.2 ±3.2
	Lact	20.7 ±2.2	0.6 ±0.2	4.0 ±0.4	28.1 ±3.4	50.0 ±4.8

* $p < 0.05$, ** $p < 0.01$ compared with non-pregnant data; ND, not detected, [a]n=5.

Table 25. Chemical composition of VLDL, LDL and HDL isolated from Shetland pony mares when not pregnant (NP), in late pregnancy (Preg) and early lactation (Lact) (mean ±sd).

The masses of free cholesterol and phospholipid in LDL were significantly higher in pregnancy and lactation and the mass of protein significantly lower in both states. There was a significant reduction in the triglyceride content of HDL in the pregnant ponies. However when these data were compared with the values for the 12 non-pregnant Shetland pony mares, none of these changes in LDL or HDL composition were of statistical significance.

There were no significant differences in the activities of LPL and HL in pregnant, and HL in lactating ponies (Table 23). However, the activity of LPL was significantly higher in the lactating ponies, with individual values increased by between three and six fold over the mean of healthy ponies (Appendix 23).

4. DISCUSSION

4.1. Exogenous Lipoprotein Metabolism in the Horse

The comparison of lipoprotein profiles in fed ponies with those fasted prior to sample collection was conceived as a means of assessing how the equine lipoprotein profile might change when lipids of dietary origin enter the system. The results did not provide any concrete evidence for a post-prandial phenomenon, such as that seen in other monogastrics, for the reasons discussed below. The plasma concentrations of triglyceride and VLDL cholesterol were indeed higher in the fed ponies and might have reflected the presence of triglyceride rich chylomicrons in the circulation. For this is the reason why, in man, plasma triglyceride levels are increased by 30-50% approximately three hours after a normal meal (Durrington, Whicher, Warner, Bolton and Hartog 1976). The presence of chylomicrons in plasma of the fed ponies would also explain the enrichment of the <1.006 g/ml fraction with triglyceride, and account for its reduced cholesteryl ester content as chylomicrons are poor in cholesterol relative to VLDL. However, in conflict with this was a body of data that indicated that feeding had no effect on the lipoprotein system. First, although the protein content of the chylomicrons isolated from the foals was less than 50% of that of VLDL, there was no reduction in the protein mass of the density <1.006 g/ml fraction of the fed ponies. Secondly, the ratio of apoB-100 to apoB-48 was unchanged. Thirdly, while the presence of chylomicrons in foal plasma accounted for moderate hypertriglyceridaemia, the cholesterol concentrations of VLDL were lower, not higher, than those found in adult ponies due to the inferior contribution of cholesterol to the mass of these particles. Finally, the activities of neither LPL or LCAT were increased in response to feeding.

Some of the variation between the fed and fasted animals may have due to the fact that the groups were small in size and that single samples were taken on each

occasion. The day to day variation in serum triglycerides in man, after laboratory errors have been excluded, is in the region of 25% (Hammond, Wentz, Statland, Philips and Winkel 1976) and similar fluctuations may have accounted for some of the observed differences. However, the changes in plasma triglyceride concentration and VLDL composition might be better explained by consideration of the fact that in the rat, a species whose lipid transport system has much in common with that of the horse, the hepatic secretion of VLDL triglyceride is greater in the fed than the fasted state (Wilcox and Heimberg 1987). Thus it is possible that in the horse, the absorption of short and medium chain fatty acids in the small intestine, and volatile fatty acids in the large intestine, leads to increased output of triglyceride rich VLDL from the liver. In the absence of post-prandial chylomicronaemia, as shown by the retinyl palmitate studies, this would cause the differences observed in the concentration and lipid composition of VLDL, and no significant change in the ratio of apoB-100 to apoB-48 in the density < 1.006 g/ml fraction.

The uncertainties raised by the above data were compounded by the results from the oral fat tolerance tests. In man, plasma triglycerides increase following an oral fat load peaking at 2 hours and return to fasting values within 8 hours of the meal (Cohen 1989). The magnitude of this response in healthy volunteers is proportional to the fat content of the meal, such that 100 g fat given to a 70 kg person produces an approximate three fold increase in plasma triglyceride concentrations (Demise, Hauptman and Dunton 1989). The fat content of the oral load used here approximated to 120 g fat/70 kg and yet provoked no such lipaemia or incorporation of retinyl palmitate into the density <1.006 g/ml fraction. In man, plasma retinyl ester concentrations rise after the meal, peaking at 6 hours, and then decline but remain greater than baseline at 12 hours (Krasinski *et al.* 1990). In contrast, retinyl palmitate was found in the density <1.006 g/ml fraction of one the ponies at 8 hours and in two others only at 24 hours. This would not appear to be the result of the hay offered after the eight hour sample as the vitamin A content of sun dried forages is negligible (Cunha 1991).

Why should the horse differ so greatly in its response to oral fat from that of other monogastrics? Although there are no records of oral fat tolerance tests of this type being performed in the horse, the protocol was based on that used for oral glucose tolerance testing, a common clinical practice which in healthy horses gives rise to a peak of plasma glucose at 2-4 hours that declines to baseline by 8-10 hours. A delay in gastric emptying, or slow small intestinal transit, would therefore appear unlikely to account for the flat response to the fat load. The explanation must therefore reside in the events that start with the digestion of fat in the small intestine and culminate in the appearance of chylomicrons in the circulation. Although the

horse does not possess a gall bladder and has a bile pool approximately 10% the size of that of man, mature animals appear capable of digesting diets containing up to 18% animal fat (Hintz, Schryver and Lowe 1973). This capability is far in excess of the fat content of grass, hay and proprietary concentrates which is in the region of 2-5 g/100g dry weight (Givens and Moss 1990) with a total sterol content 10-50 mg/100g fresh weight (Weinrauch and Gardner 1978).

While the primary site for the absorption of dietary fatty acids is presumed to be the small intestine (Hintz *et al.* 1973), the appearance of retinyl palmitate in the later samples might indicate that the label, together with the fatty acid products of triglyceride hydrolysis, were absorbed in the large intestine. If this was the case, then why were the products not absorbed through the small intestine? One possible explanation may reside in an adaptation of the adult horse to its diet which is low in fat and rich in short and medium chain fatty acids that are absorbed without the need for chylomicron synthesis. Such adaptation would, however, need to be age related as lipoprotein particles with the physical and chemical features of chylomicrons were found in the plasma of suckling foals at a time when the fat content of their diet was high (approximately 2 g/100ml, 18% of the total solids; Ullrey, Struthers, Hendricks, and Brent).

4.2. Lipoprotein Metabolism in the Suckling Foal

The lipoprotein profile of foals contrasted with that of adult horses in that LDL was the predominant class in terms of cholesterol and accounted for the higher plasma total cholesterol concentration. As discussed above, the lipaemic appearance of the plasma and the decreased VLDL cholesterol concentrations were explained by the presence of lipoproteins with the features of chylomicrons. As in humans, the presence of chylomicrons in the circulation was the likely stimulus for the higher activities of LPL and LCAT seen in the foals compared with the adults, further evidence of a more typical monogastric response to dietary fat.

Despite washing the chylomicron fractions twice, there still remained slight albumin contamination which will have exaggerated the protein content of these particles. In common with human chylomicrons, the C peptides and apoA-I were prominent apolipoprotein components. The presence of apoB in trace and unquantifiable amounts was puzzling as this protein is absolutely essential for the synthesis of chylomicrons in man.

As the neutral lipid content of VLDL reflects that of the liver, the greater mass of cholesterol in this fraction was probably the result of an increased flux of cholesterol to the liver in the form of chylomicron remnants. The significant mass of free cholesterol may have been due to differences in the hepatic activity of ACAT

between the foal and the adult animal. The composition of foal LDL was unusual because the ratio of esterified to free cholesterol was out of proportion with that in the VLDL precursors, the proportions of phospholipids to protein were reversed compared with those in adult LDL, and there were significant contributions to protein mass from albumin and apoA-I. It is therefore likely that certain particles in this density range were derived independent of VLDL precursors. In fact the composition of this fraction resembles a mix of that of adult equine LDL and Lp-X, the latter of which contains significantly higher amounts of phospholipids (61%) and free cholesterol (25%) and has albumin and apoA-I representing 30% and 2% of its protein mass, respectively (Kostner and Lagger 1989). The origins of such particles in apparently healthy foals is unclear, but would not appear to be related to defective hepatic function or LCAT activity.

4.3. Lipoprotein Metabolism in Pregnancy and Lactation

The results from the six ponies clearly showed that moderate hyperlipidaemia is prevalent in the last trimester of pregnancy. The changes in VLDL composition were strikingly similar to those seen in ponies with hyperlipaemia in the following Chapter. Thus it is possible that this might represent a subclinical form of hyperlipaemia, and that any additional disruption in the control of lipid metabolism could rapidly lead to the development of clinical disease. This was perhaps evident in the responses of the two pregnant ponies to fasting prior too the oral fat tolerance tests where plasma triglyceride levels had rose steeply after only a 16 hour period of food withdrawal in marked contrast to the non-pregnant animals.

There is little in the literature to confirm or deny the significance of these findings. Forenbacher *et al.* (1960) commented that the concentration of neutral fats was increased by nearly four fold in a single pregnant mare and Stammers, Silver and Fowden (1989) reported triglyceride levels of 1.3, 0.6, 5.5, 12.7 and 1.9 mmol/l in the uterine vein of five pony mares between 250 and 300 days of gestation, and showed that the latter value rose to 10.6 mmol/l when the pony concerned was fasted for 30 hours. Prior to this, Fowden *et al.* (1984) demonstrated that there are marked changes in carbohydrate metabolism during pregnancy in the mare which included hyperinsulinaemia, enhanced resistance to the action of insulin and exaggerated response to feeding and fasting. The net effect of these changes is to divert glucose to the uterus, with the result that up to 75% of the available maternal glucose pool is lost to the rapidly developing foetus (Silver and Fowden 1982). Together with the reduction in voluntary feed intake that is an universal feature of pregnancy, this clearly places severe demands on maternal energy metabolism with the consequence that adipose stores are drawn upon to meet any deficit. In support of this, Stammers *et*

al. (1989) showed that plasma FFA concentrations were normal (0.71 and 0.54 mmol/l) in two pregnant pony mares that were well nourished, but elevated (1.3, 1.5 and 2.27 mmol/l) in three others that were on a low plane of nutrition.

Although lactation is also recognised as a risk factor for hyperlipaemia, the plasma concentrations of triglyceride returned to normal after foaling as the composition of VLDL tended towards normality. In common with lactation in other species, the activity of LPL was increased presumably as the result of induction of the enzyme in mammary tissue by prolactin (Scow and Chernick 1987). This higher level of activity probably accounts for the decrease in the levels of VLDL in the lactating mares, as triglyceride is channelled to the mammary gland for milk production. The increases in plasma FFA concentration, although not statistically significant, might be due to the mobilisation of fatty acids from adipose tissue to help meet the demands of lactation, or alternatively reflect the increased activity of LPL. If the former is the case, then any interruption in the supply of dietary energy coupled with the factors that lead to peripheral insulin insensitivity might well precipitate massive mobilisation of FFA and explain the incidence of hyperlipaemia in lactating pony mares. However, the activity of LPL in lactation was approximately 50% greater than in the animals with hyperlipaemia, suggesting that lactating mares might be better able to cope with any increase in VLDL production and are therefore protected to some extent against hyperlipaemia.

In conclusion the data presented here clearly shows that the risk of hyperlipaemia in pregnancy is related to underlying changes in lipid and lipoprotein metabolism and indicate that there may be a place for the routine screening of plasma triglyceride concentrations in the management of pregnancy in the pony mare. Animals then identified as at risk of developing hyperlipaemia might be candidates for lipid lowering therapies as a preventive strategy, which is discussed in Chapter VIII.

CHAPTER VI

PLASMA LIPOPROTEIN CONCENTRATION AND COMPOSITION, AND ENZYME ACTIVITY IN PONIES WITH HYPERLIPAEMIA

1. INTRODUCTION

The early evidence for the biochemical mechanisms behind hypertriglyceridaemia in man came from examining how the concentration and composition of the plasma lipoproteins were altered in affected subjects (Fredrickson *et al.* 1967). It is therefore reasonable to expect that the lipoproteins in ponies with hyperlipaemia might also hold similar aetiological clues. The equine condition was defined as hyperprebetalipoproteinaemia by Morris *et al.* (1972) and subsequent studies have confirmed the predominance of VLDL in ponies with naturally occurring disease (Wensing *et al.* 1975) and experimental reproductions of the condition (Bauer 1983, Freestone *et al.* 1991). However, analysis of the composition of the lipoproteins has not been rewarding. Morris *et al.* (1972) reported that the VLDL in two hyperlipidaemic ponies was depleted of cholesterol, particularly of the esterified form, relative to human and rat VLDL but presented no data from healthy ponies for comparison. Wensing *et al.* (1975) commented that the triglyceride content of VLDL and LDL was increased in the more severely affected of nine ponies relative to a single control, but Bauer (1983) found no significant change in the composition of VLDL in four hyperlipidaemic ponies compared with two controls.

More recently, progress in understanding the fundamental biology of lipoprotein metabolism has led to the unravelling of the genetic, molecular and metabolic aetiologies of hyperlipidaemia in man and certain experimental species. Despite this, the mechanisms that underlie equine hyperlipaemia are not known. The consensus of opinion believes that affected animals mobilise fatty acids from adipose stores in response to an energy imbalance. The mobilisation is thought to proceed in an uncontrolled fashion, because of peripheral insulin insensitivity, so that the FFA are re-esterified in the liver and secreted in VLDL as triglyceride (Jeffcott and Field 1985b). However, this has not been supported by experimental data to date. Rather, attempts to quantify VLDL secretion by blocking its hydrolysis with Triton led Morris *et al.* (1975) to conclude that the "mechanism resides in the suppression of lipoprotein degradation". Bauer (1983) made a similar speculation on the basis that he found little change in the chemical composition of VLDL, and Freestone *et al.* (1991) concluded that their failure to show differences in the hyperlipidaemia induced in

normo- and hyperinsulinaemic ponies by fasting was because "some aberration in the degradation of VLDL was responsible" for the disease.

In the studies described here, the plasma concentration and chemical composition of the lipoproteins from 18 ponies with hyperlipaemia were examined. This defined the lipid phenotype associated with the disease and showed that, in contrast to previous work, there are significant changes in the composition of the lipoproteins. The activities of LPL and HL were measured for the first time in ponies with hyperlipaemia and, in an attempt to explain some of the changes in lipoprotein composition, the activities of LCAT and CETP were analysed. This series of cases constitutes the first report of the disease in the United Kingdom and the clinical picture presented is rationalised with previous records from other parts of the World. The findings provided important clues to the aetiology of equine hyperlipaemia and established the focus for metabolic studies described in the following Chapter.

2. MATERIALS AND METHODS

2.1. Subjects and Samples

The clinical details of the 18 cases of hyperlipaemia are summarised in Appendix 25. Five cases were diagnosed at Glasgow University Veterinary School. The remainder were recruited following notice of the study in the *Veterinary Record* (1989, **144**, 474), a newsletter of the British Equine Veterinary Association, and *Equine Veterinary Education* (1990, **2**, 230). In this, attending veterinarians were invited to submit blood samples from cases in which they suspected hyperlipaemia, which was then confirmed or excluded by measurement of triglyceride concentrations. A routine blood chemistry panel was also run to identify hepatic and renal dysfunction and to help in offering a prognosis for each case. Three cases were subsequently referred to Glasgow University Veterinary School and the author visited another at the Royal (Dick) School of Veterinary Studies, University of Edinburgh. Blood for lipid and lipoprotein analysis, including PHP and plasma for the preparation of LPDP, was collected from these cases prior to the institution of therapy as described in Chapter II. The nine other cases were managed in general practice and the practitioners submitted further blood samples prior to therapy; these were collected into potassium EDTA and the plasma was either separated before dispatch or immediately upon arrival in Glasgow where it was used within two days of collection.

2.2. Methods

The methods used here were described in Chapter II. Plasma lipid and lipoprotein concentrations were measured and the lipoprotein particle size distribution examined

by gel filtration on columns of 6% and 2% agarose gel. The size of particles in VLDL was measured by electron microscopy. Rate-zonal ultracentrifugation was not used for the reason that insufficient plasma was available from all the cases, and where it was obtained from an early case (HLP-2) there was ineffective fractionation of HDL and LDL because of the massive amounts of VLDL. The size of HDL particles was therefore estimated by electrophoresing HDL prepared by sequential flotation ultracentrifugation on non-denaturing polyacrylamide gels. However, the concentration of LDL prepared by this route was inadequate for the loading of the gels so that the size of these particles was not examined.

Lipoprotein fractions were prepared by sequential flotation ultracentrifugation for analysis of lipid and apolipoprotein composition with the following modification. It was found that by initial ultracentrifugation at density <1.006 g/ml, 20k rpm for 20 mins. the samples could be cleared of their lipaemia. Complete clearance required between one and four spins; the supernatants were pooled, labelled $VLDL_1$, and cleaned of plasma lipoprotein contamination by gel filtration on 6% agarose. The conventional VLDL fraction collected by the subsequent spin at d <1.006 g/ml, 39k rpm for 18 hours was then called $VLDL_2$. The plasma concentrations of $VLDL_1$ was estimated by dividing the total cholesterol concentration of this 1 ml fraction by 4 (the original volume of plasma) and subtracting from the plasma VLDL cholesterol concentration. The $VLDL_2$ concentration was taken as the difference between the two. These data were compared with that from 18 healthy Shetland ponies in Chapter III.

The activities of LPL and HL were determined in PHP using the selective assay conditions developed in Chapter IV, and the activities of LCAT and CETP were measured using the assay conditions detailed in Chapter II. The enzyme activities were compared with those presented for healthy Shetland ponies in Chapter IV.

3. RESULTS

3.1. The Clinical Disease

The mean (±sd, range) age of the affected ponies was 8.9 years (±4.6, 18 months to 20 years). Eleven of the cases were Shetland ponies (61%), four were of Welsh Mountain pony stock (22%), there was one Fell pony (6%), and the remainder were described as riding ponies of mixed breeding. Four of the animals were geldings (22%), the rest were mares of which nine (50%) were known to be in foal (gestation 5-10 months, mean 8.4) and two (11%) were lactating. The geographic spread of cases was from Cornwall in the South to Shetland in the North of the United Kingdom. Underlying or concurrent diseases were identified in six animals (33%);

these were laminitis (two cases), chronic diarrhoea due to intestinal lymphosarcoma (one case) and large intestinal parasitism (two cases), and dysphagia caused by oesophagitis of undetermined aetiology (one case). Veterinary attention was sought for the remaining 12 cases (67%) because of inappetance and/or dullness, depression and lethargy. Over the three year period of the study, there was no seasonal pattern of incidence with six cases in the Spring months (March to May), five in the Summer (June to August), four in the Autumn (September to November) and three in the Winter (December to February).

Blood chemistry showed that liver enzymes (alkaline phosphatase and gamma-glutamyl transferase) were markedly elevated in 13 cases (72%) and evidence of renal dysfunction (elevated urea and creatinine concentrations) in seven (39%) animals. Four cases (22%) were euthanased without therapy, three because of a grave prognosis for an underlying complaint and the fourth (HLP-18) on humane grounds. Therapy for the remaining 14 animals centred on maintaining energy balance with 4% glucose solutions or Lectade (Beecham), giving five litres four times daily *per os* or via a nasogastric tube. Insulin (50-100 iU *im bid*) and heparin (100 iU/kg *iv bid*) were used in combination in four animals, and insulin alone in two cases. Six animals (33%) recovered and eight died (44%), giving an overall mortality rate of 67%. The outcome was not dependent upon the type of therapy, as only one each of the cases given insulin/heparin or insulin recovered, whereas four of the eight cases supported solely with glucose survived. The length of time from the first recognition of clinical signs by the owner to death or euthanasia ranged from 3 to 24 days, with a mean ($\pm$sd) of 10 ($\pm$7) days. Somewhat surprisingly, the outcome of therapy was not related to the severity of hyperlipidaemia as the plasma triglyceride concentrations at the time of diagnosis in the six animals that recovered (27.24 $\pm$17.11 mmol/l, mean $\pm$sd) were similar to those in the animals that died (28.76 $\pm$9.95 mmol/l). Nor were there any differences in any of the indices of hepatic or renal pathology between the two groups.

3.2. Plasma Lipid and Lipoprotein Concentrations (Appendix 26)

Bloods samples from all cases were noticeably lipaemic and the plasma was found to clear when left standing at 4°C for one to four days as a creamy supernatant developed at the surface (Fig. 24). The plasma triglyceride concentrations were ten to 165 fold higher than in healthy ponies ($p < 0.001$) and ranged from 4.70 to 78.75 mmol/l (25.43 $\pm$18.09; mean $\pm$sd). Plasma cholesterol concentrations were increased by approximately two to eight fold and ranged from 3.45 to 16.90 mmol/l (7.51 $\pm$3.44; $p < 0.001$). These changes were accompanied by VLDL cholesterol concentrations that were increased by five to 52 fold (5.55 $\pm$3.45; $p < 0.001$). The

concentrations (mmol/l) of LDL (0.60 ±0.20) and HDL (1.36 ±0.50) cholesterol were similar to those in the control groups (0.54 ±0.22 and 1.32 ±0.29, respectively).

Plasma FFA concentrations were higher than the control group (0.39 ±0.16 mmol/l) in all but one of the animals where they ranged from 0.94 to 3.32 mmol/l (1.67 ±0.80), representing a 2.4 to 8.5 fold increase ($p < 0.001$). The FFA concentration in the exceptional case was 0.41 mmol/l. In the entire group of 18 animals, there were significant correlations between concentrations of FFA and total cholesterol ($r = 0.51$), triglyceride ($r = 0.44$), and VLDL cholesterol ($r = 0.49$). The plasma concentrations of $VLDL_1$ ranged from 0.50 to 14.65 mmol/l and were positively correlated with those of total VLDL cholesterol ($r = 1.0$, $p < 0.001$), and represented a percentage of the VLDL fraction that increased with the degree of hyperlipidaemia, from between 33% in the case with the lowest plasma triglyceride to nearly 100% in those with severe hypertriglyceridaemia. In contrast, the $VLDL_2$ cholesterol concentration was relatively constant with a mean (±sd) for the 18 cases of 0.40 (±0.30) that was similar to the VLDL cholesterol concentration of the control group (0.29 ±0.14), and which was not correlated with the degree of hyperlipidaemia.

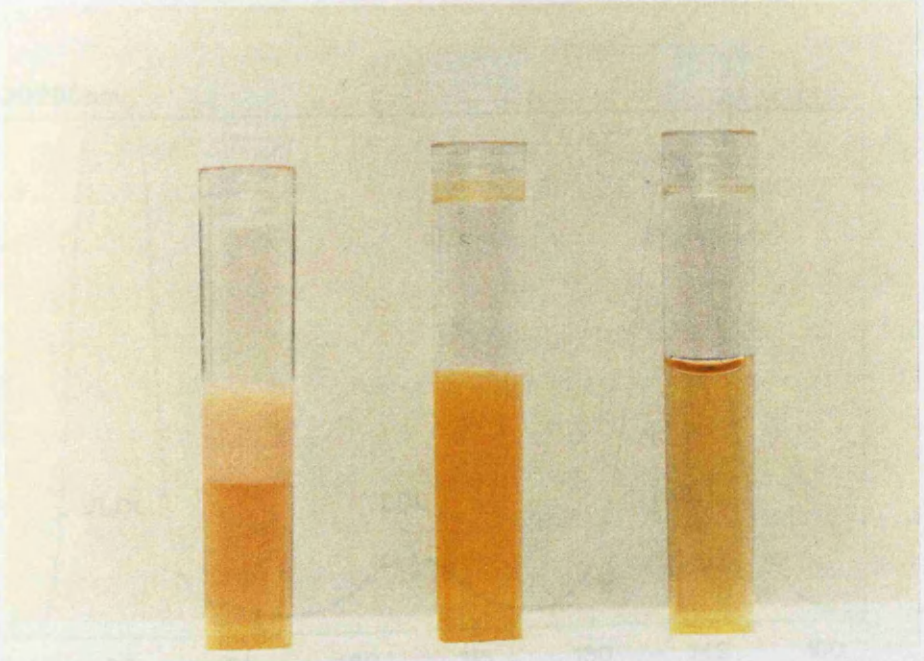


Figure 24. The appearance of plasma from a pony with hyperlipaemia (centre) compared with that from a healthy pony on the right. The sample on the left was left standing at 4° C for two days, after which time the plasma cleared as a creamy supernatant accumulated at the surface.

3.3. Lipoprotein Size Distributions

A typical elution profile of density <1.225 g/ml lipoproteins from a 6% agarose gel column is shown in Fig. 25. The pattern for each of nine hyperlipaemic ponies examined was similar and striking in the predominance of the VLDL peak. In fact, the fractions contained within this required to be diluted by five to ten fold to achieve an absorbance within the limits of the spectrophotometer. The elution profiles (Appendix 27) generally showed greater variability between individuals in the elution volume and width of each peak compared with those from the healthy animals. Much of this might have been due to changes in the column's properties over the 16 month period that encompassed the analysis of the nine samples. Although there was a tendency for VLDL and HDL to elute earlier than in the healthy ponies, there were no statistical differences between the elution volumes (mean ±sd) of VLDL (73.1 ±6.3; normal 78.9 ±1.2 ml), LDL (109.7 ±9.2; normal 110 ±3.9 ml), and HDL (145.3 ±11.9; normal 154.7 ±3.5 ml). The VLDL peak was significantly wider in the hyperlipaemic ponies (19.8 ±4.3; normal 11.1 ±0.8 ml), while those of LDL and HDL were similar to the healthy ponies (LDL 29.9 ±3.3; normal 31.6 ±3.5 ml, HDL 37.8 ±5.3; normal 36.2 ±2.9 ml).

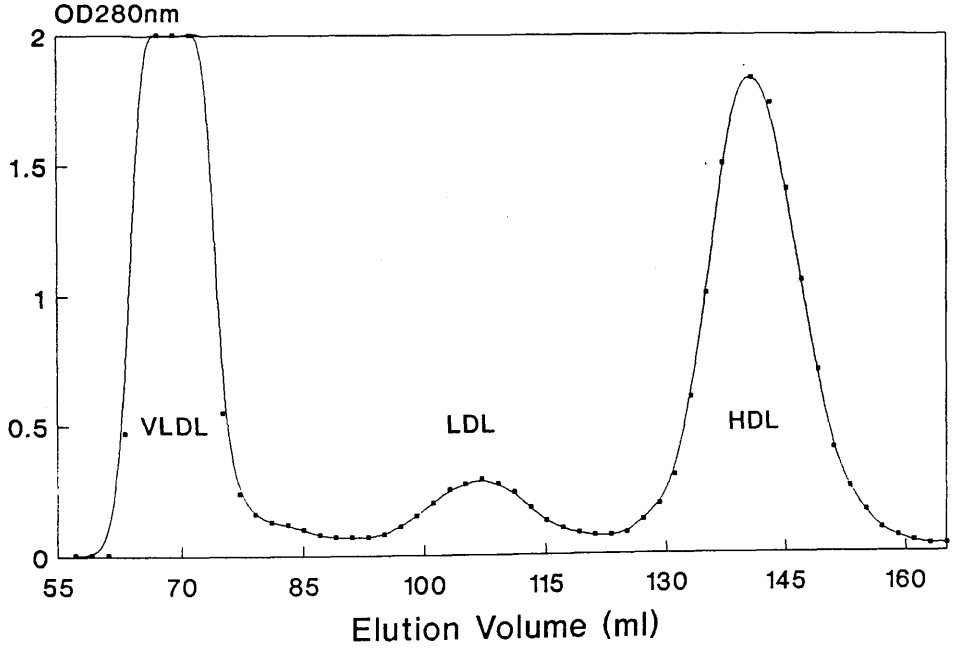


Figure 25. Distribution of plasma lipoproteins in a pony with hyperlipaemia. Lipoproteins were isolated from plasma at density < 1.225 g/ml and eluted from a column of 6% agarose with phosphate buffered saline.

Despite the heterogeneity in VLDL noted in the ultracentrifuge, discrete VLDL subpopulations were not found when the density <1.225 g/ml fraction of plasma was applied to columns of 2% agarose gel.

The average particle size in $VLDL_1$ and $VLDL_2$ from four animals, assessed by electron microscopy, was 65 nm and 50 nm, respectively, representing increases in diameter of 44% and 11% over the mean of VLDL in healthy ponies. The diameter of HDL, measured by non-denaturing polyacrylamide gradient gel electrophoresis, was 8.61 $\pm$0.20 nm (n=9), which was similar to that of HDL prepared from healthy ponies by either flotation ultracentrifugation (8.43 $\pm$0.17 nm, n=9) (Appendix 28) or by rate-zonal ultracentrifugation (8.37 $\pm$0.08 nm, n=8) in Chapter III (Appendix 5).

3.4. Lipoprotein Composition

The chemical compositions of the $VLDL_1$, $VLDL_2$, IDL, LDL and HDL fractions isolated by sequential flotation ultracentrifugation (Appendix 29) are compared with those from healthy ponies in Table 26. Both $VLDL_1$ and $VLDL_2$ were significantly enriched in triglyceride (by 25% and 10%, respectively) and depleted of protein (by 59% and 30%, respectively) relative to VLDL from the healthy ponies. The phospholipid content of $VLDL_1$ was significantly reduced (by 27%) while that of $VLDL_2$ was similar to the control group. The masses of phospholipid and protein were significantly correlated with that of triglyceride in both $VLDL_1$ ($r = -0.57$ and $r = -0.59$, respectively) and $VLDL_2$ ($r = -0.47$ and $r = -0.60$, respectively). The total cholesterol contents of both $VLDL_1$ (8.3%) and $VLDL_2$ (9.7%) were similar to the controls (10.1%) but, in contrast to the healthy animals, free cholesterol was detected in both $VLDL_1$ and $VLDL_2$ where it comprised 63.3% and 32.9% of the total cholesterol mass, respectively. The mass of free cholesterol was significantly higher in $VLDL_1$ than $VLDL_2$, while that of cholesteryl esters was significantly greater in $VLDL_2$ than $VLDL_1$. The masses of cholesteryl esters and free cholesterol were significantly correlated in $VLDL_2$ ($r = -0.75$, $p < 0.001$) but not in $VLDL_1$ ($r = -0.39$).

The LDL fraction had a significantly reduced cholesteryl ester content (by 30%) and the triglyceride mass of these particles was significantly higher (by approximately three fold) than that of the controls. The masses of free cholesterol, phospholipids and protein in LDL and HDL were similar to those in healthy animals. The triglyceride content of the HDL fraction was significantly increased (by three fold).

The apolipoprotein composition of each lipoprotein fraction (Table 27; Appendix 30). In the $VLDL_1$ fraction, the mass of apoB-100 was reduced by an average of 46% while that of apoB-48 was unaltered, so that the ratio of apoB-100 to apoB-48 was approximately 0.9:1 compared with 1.8:1 in VLDL from healthy ponies.

Lipoprotein		Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
$VLDL_1$	HLP	2.5 ** ±2.4	73.9 ** ±4.6	5.3 ±1.6	11.1 ** ±2.7	6.8 ** ±2.6
$VLDL_2$	HLP	6.5 * ±4.3	64.5 * ±6.3	3.2 ±2.7	14.8 ±2.3	11.7 ** ±4.3
VLDL	SP	10.1 ±1.7	58.9 ±3.9	ND	15.3 ±2.3	16.6 ±2.4
LDL	HLP	29.0 ** ±9.2	19.1 ** ±8.5	4.1 ±4.4	21.3 ±3.6	27.9 ±5.4
	SP	41.0 ±5.2	5.7 ±2.2	4.0 ±2.4	24.0 ±3.6	24.0 ±6.6
HDL	HLP[a]	19.4 ±3.3	2.3 ** ±1.6	2.5 ±1.4	25.4 ±5.4	50.5 ±8.5
	SP	19.7 ±4.1	0.7 ±0.4	2.8 ±1.0	27.2 ±4.4	49.5 ±7.7

* $p < 0.01$, ** $p < 0.001$; [a]n=17; ND, not detected

Table 26. Chemical composition of plasma lipoproteins isolated by flotation ultracentrifugation from 18 ponies with hyperlipaemia (HLP) compared with the results from 18 healthy Shetland ponies (SP) (the data for $VLDL_1$ has been compared with that of VLDL from the healthy ponies) (mean ±sd).

Both $VLDL_1$ and $VLDL_2$ were significantly enriched in apoE, but were similar to normal VLDL in respect of their apoA-I, apoC-II and apoC-III content. The protein composition of $VLDL_2$ was almost identical to that of healthy ponies and the percentage masses of apoB-100 and apoB-48 were unaltered and present in a ratio of 1.9:1. The protein composition of LDL was not statistically different to that from healthy ponies. In HDL, there were significant increases in the masses of apoE, apoC-II and apoC-III, while that of apoA-I was significantly lower.

		Percentage of Protein Mass						
Lipoprotein		B-100	B-48	E	A-I	C-II	C-III	A-II
$VLDL_1$	HLP	11.4 ** ±3.6	12.4 ±2.8	15.5 * ±5.7	8.6 ±3.8	23.8 ±8.1	29.8 ±8.1	---
$VLDL_2$	HLP	19.3 ±4.4	10.2 ±3.0	10.2 ±4.8	5.4 ±4.2	21.7 ±5.2	31.4 ±5.3	---
	SP	20.8 ±3.9	11.5 ±2.8	9.5 ±4.8	8.1 ±4.0	22.5 ±6.1	31.3 ±7.1	---
LDL	HLP[a]	69.1 ±12.5	---	10.0 ±5.9	---	10.9 ±6.0	10.1 ±5.7	---
	SP[b]	77.8 ±6.2	---	6.5 ±2.5	---	7.0 ±2.3	8.9 ±5.0	---
HDL	HLP	---	---	5.1 ±2.8	66.9 * ±9.3	11.1 * ±5.5	11.8 * ±5.5	5.6 * ±3.9
	SP	---	---	4.3 ±2.7	79.5 ±4.9	4.5 ±2.4	5.1 ±2.0	7.1 ±3.5

* $p < 0.01$, ** $p < 0.001$, [a] n=14, [b] n=16

Table 27. Apolipoprotein composition of plasma lipoproteins isolated by flotation ultracentrifugation from 18 ponies with hyperlipaemia (HLP) compared with those from 18 healthy Shetland ponies (SP) (mean ±sd).

3.5. Lipoprotein Enzyme Activities

The activities of LPL, HL and LCAT were measured in 12 ponies with hyperlipaemia (Appendix 31) and are compared with those from 12 healthy ponies matched for age and sex in Table 28. The activities of LPL and HL were significantly higher than those of the control group, with the mean values increased by approximately two and three fold, respectively. The activity of LPL was correlated with the plasma concentrations of cholesterol ($r = 0.48$), triglyceride ($r = 0.40$), VLDL cholesterol ($r = 0.45$) and FFA ($r = 0.42$), although these were not statistically significant. The activity of HL was not correlated ($-0.4 > r < 0.40$) with any of these variables. The activities of LPL and HL were significantly correlated ($r = 0.79$).

The activity of LCAT was more variable than in healthy ponies and ranged from 15.09 to 46.33 nmolCE/ml/h, but there was no significant difference in activity between the hyperlipaemic and control ponies (Table 28).

No CETP activity was found in LPDP from nine of these ponies (Appendix 32).

	Lipoprotein Lipase	Hepatic Lipase	Lecithin:Cholesterol Acyl Transferase
	umolFA/ml/h		nmolCE/ml/h
Hyperlipaemic Ponies	8.12* ±2.87	13.20* ±5.13	25.30 ±10.99
Healthy Ponies	3.65 ±1.58	4.99 ±1.45	23.76 ±4.22

* $p < 0.001$

Table 28. Activity of lipoprotein lipase, hepatic lipase and lecithin:cholesterol acyl transferase in 12 ponies with hyperlipaemia, compared with 12 age and sex matched healthy Shetland ponies.

4. DISCUSSION

4.1. The Clinical Disease

This, the first description of the clinical picture presented of hyperlipaemia in the United Kingdom was consistent in many respects with reports of the disease from Holland (Schotman and Wagenaar 1965), Norway (Eriksen and Simesen 1970), Australia (Gay *et al.* 1978) and North America (Naylor *et al.* 1980). The apparent predisposition of the Shetland pony was reiterated, and the disease's associations with pregnancy and lactation were confirmed. However, in contrast with earlier reports, there was no obvious underlying disease in the majority of cases. Of the concurrent diseases that were identified, three (intestinal neoplasia, parasitism, dysphagia) would have had serious consequences for gastro-intestinal function and the energy balance of the affected animals. A role for the other, laminitis, as a cause of hyperlipaemia is less obvious. Certainly affected animals are often inappetant and stressed by the pedal pain. Alternatively, the clustering of laminitis and hyperlipaemia in obese and often pregnant ponies might reflect the role that has been suggested for insulin insensitivity in the aetiology of both conditions (Coffman and Colles 1983, Field and Jeffcott 1989). The fact that there was no clear seasonal incidence in the present report was probaly related to the extended U.K. pony breeding season and the relatively higher number of geldings and non-pregnant mares than in previous case series.

Unfortunately, the mortality associated with hyperlipaemia in the U.K. appears no less than elsewhere in the World. Clearly the outlook for those with untreatable underlying or coexistent diseases is not good, and together with those animals that are in an advanced state of hepatic or renal failure, deserve euthanasia on humane grounds. It was somewhat of a surprise to find that in contrast with Schotman and Wagenaar's (1969) original findings, there was little difference in the severity of hyperlipidaemia between those that survived and those that succumbed to the disease. In addition, the outcome was not predicted by any of the biochemical parameters screened. Although the 18 animals in the present study are an insufficient data base on which to develop predictive models of the disease, it was disappointing to find that the outcome was not predicted by either the severity of hyperlipidaemia or the heaptic/renal pathology.

The efficacy of any therapy is also hard to assess in such a small series and case controlled studies have to date not been presented. While there are arguments in favour of using insulin and heparin, their clinical efficacy has not been borne out by this series or the experiences of others (Gay *et al.* 1978). Considering the degree of hepatopathy and likely coagulopathy encountered with hyperlipaemia, the use of heparin is hard to justify. Any additive effect that this agent might have on the function of LPL is debatable, although the LPL activities measured (4.05-13.51

umolFA/ml/h) were in all but two cases below those found in healthy lactating ponies (10.03-20.20 umolFA/ml/h) in the previous Chapter suggesting that there may be some reserve capacity on which heparin might draw. Given that insulin insensitivity is believed to be central to the pathogenesis of hyperlipaemia, the efficacy of exogenous insulin is questionable. Transient collapsing episodes consistent with hypoglycaemia were reported in two of the present series following the administration of insulin by the attending veterinarians.

4.2. Plasma Lipid and Lipoprotein Concentrations

The severity of the hypertriglyceridaemia reported here was comparable with that recorded from four ponies with spontaneous hyperlipaemia (plasma triglycerides 39.18 ±12.0 mmol/l; Eriksen and Simesen 1970) and from a series of nine cases in which the condition appeared secondary to other diseases (16.5 ±10.0 mmol/l; Naylor *et al.* 1980). It is worthy of note that here, and in both of those reports, plasma triglyceride levels were higher in animals with apparently idiopathic hyperlipaemia than those with obvious underlying disease. Also the degree of hyperlipidaemia seen in the natural disease is in excess of that induced by fasting, where triglyceride levels 2.5 ±1.0 mmol/l (Baetz and Pearson 1972) and 10.1 ±8.3 mmol/l (Bauer 1983) were reported following 184 hours of food deprivation.

The changes in lipoprotein concentration found were not unexpected, as Bauer (1983) and Freestone *et al.* (1991) reported similar findings from their experimental animals. The moderate hypercholesterolaemia seen in ponies with hyperlipaemia is attributable to increased VLDL cholesterol concentrations, as the levels of LDL and HDL are unaltered. Although the normality of LDL and HDL concentrations has not previously attracted comment, they are significant in indicating that VLDL is progressing through its lipolytic cascade. For the generation of LDL from VLDL precursors, and HDL from the surface remnants, is dependent upon the activity of LPL, such that the subnormal levels of LDL and HDL seen in humans with common types hypertriglyceridaemia are blamed on reduced activity of this enzyme (Shepherd and Packard 1987).

Increased plasma FFA concentrations have been previously reported in ponies with hyperlipaemia (Naylor *et al.* 1980). In addition, a number of studies have shown that FFA concentrations rise soon after the onset of fasting to plateau by about 64 hours at levels similar to those in ponies with hyperlipaemia, and return to baseline on refeeding (Baetz and Pearson 1972; Morris *et al.* 1972; Naylor *et al.* 1980; Rose and Sampson 1982). This rise precedes that of triglyceride by approximately 48 hours and is parallelled by free glycerol suggesting that the FFA are coming from adipose tissue (Naylor *et al.* 1980). This, together with the correlations observed between

concentrations of FFA and triglyceride in the latter and the present studies, suggests that plasma FFA might be driving the synthesis and secretion of triglyceride by the liver. However, caution should be exercised as it is possible, given the massive plasma pool of VLDL and increased activity of LPL, that fatty acids released from VLDL triglyceride exceed cellular uptake and spill into the general circulation. In support of this, there was positive correlation between LPL activity and the plasma concentration of FFA, and while not statistically significant, was similar in magnitude to that between FFA and triglyceride concentrations. For this reason, the recycling of FFA from VLDL triglyceride is considered in the following chapter where radiolabelled palmitic acid and glycerol were used as tracers of VLDL triglyceride.

The potential role of FFA in driving hepatic triglyceride synthesis is also supported by the fact that ponies with hyperlipaemia are not ketotic (Eriksen and Simesen 1970; Gay *et al.* 1978; Naylor *et al.* 1980) and the finding that plasma ketone levels do not rise when horses are fasted (Naylor *et al.* 1980; Rose and Sampson 1982). If, as these data suggest, the pathways for ketone synthesis are poorly developed in the horse, this will explain why the mobilisation of FFA in this species results in such massive hypertriglyceridaemia, while parallel conditions in the cow and the sheep result in overt ketoacidosis.

Isolation of the lipoprotein classes by flotation ultracentrifugation showed that much of the lipaemia was due to the presence of buoyant triglyceride rich lipoproteins, such that by repeated short spins the plasma could be cleared. The precise identity of these particles was at first a puzzle, for they demonstrated the buoyancy of chylomicrons when left to stand at 4°C and in the ultracentrifuge, and yet were obtained in profuse quantities from animals that were virtually anorexic. In addition, their chemical composition was quite distinct from that of chylomicrons isolated from suckling foals in the previous Chapter.

The characteristics of this $VLDL_1$ fraction may well explain the confusing data presented by Schotman and Wensing (1977). These authors described three lipid phenotypes in ponies with hyperlipaemia; the first characterised by increased VLDL (analogous with human type IV hyperlipoproteinaemia), and the second (type V) and third by increases in VLDL and chylomicrons, with the contribution of chylomicrons being much less in the third. This was based on the study of Wensing *et al.* (1975) where the chylomicron fraction was isolated by initial ultracentrifugation. However, neither the lipid composition of this fraction or the fact that the majority of these animals were reported as inappetant justified the conclusion that these particles were chylomicrons. Rather, the fraction was composed of particles of similar composition to those identified here as $VLDL_1$. In the present study, the contribution of this fraction to the total VLDL pool increased with the severity of hypertriglyceridaemia,

whereas that of $VLDL_2$ remained constant. Thus in ponies with low levels of VLDL, $VLDL_1$ represented much less of the density <1.006 g/ml fraction than in ponies with higher VLDL concentrations where it was responsible for almost all of the fraction. This gradation would explain how Wensing *et al.* (1975) differentiated their phenotypes, for they found no "chylomicrons" in ponies with low plasma triglyceride concentrations (3.2-7.3 mmol/l) and many in those with high triglycerides (29.4-33.0 mmol/l).

4.3. Lipoprotein Particle Size and Composition

Given that the increase in VLDL concentrations seen in the ponies with hyperlipaemia was accounted for almost entirely by the appearance of $VLDL_1$, it was hoped that analysis of the structure and composition of this lipoprotein would hold information on both its origin, and the aetiology of the disease. It did not disappoint. The particles contained within this fraction were radically different to those in VLDL from healthy ponies; as they were enriched in triglyceride and depleted of phospholipid and protein. This increased ratio of core to shell volume would account for the buoyant behaviour of the $VLDL_1$ fraction and the increased size of the particles visible under the electron microscope. As the lipoprotein shell exists as a single monolayer of constant depth, much of the reduction in phospholipid and approximately one half of the protein mass could also be explained by relative dilution of surface mass by the expanded core of triglyceride.

Analysis of the apolipoprotein content of $VLDL_1$ suggested that the reduction in protein content was also due to a change in the ratio of apoB-100 to apoB-48. The contribution of apoB-100 to total protein mass was reduced by nearly 50% so that the ratio of the masses of apoB-100 and apoB-48 was approximately 1:1, instead of 2:1 as in healthy ponies. Because VLDL particles contain a single molecule of apoB, this would have reflected in $VLDL_1$ an approximate two fold increase in the number of particles that contain apoB-48 rather than apoB-100. It is exciting to speculate that this reflects the ability of the liver to increase its triglyceride secretory capacity by producing VLDL particles that accommodate a greatly expanded core volume, in the way that chylomicrons do, by virtue of having apoB-48 as their structural protein. This would necessitate a fundamental switch in hepatic apoB synthesis, but is not without precedent, as the rat hepatocyte appears able to coordinate the secretion of triglyceride with the expression of its shorter form of apoB (Coleman *et al.* 1988).

If this is indeed a mechanism by which the equine liver can enhance the export of triglyceride, it is further evidence that overproduction of triglyceride is the pathogenic mechanism of the equine hypertriglyceridaemia. For it would be hard to explain how such fundamental changes in lipoprotein structure arise if the disease is

simply the result of defective VLDL catabolism. However, because the apoB-48 containing particles do not reach the LDL density range, one might argue their increased number simply reflects a defect in their (receptor mediated) clearance. If this were so, and given the evidence that VLDL lipolysis does not appear to be compromised in the equine disease, one would expect to find a lipoprotein pattern similar to that seen with the apoE2/E2 phenotype or hepatic lipase deficiency in man which is characterised by the accumulation of cholesteryl ester rich remnants of VLDL and chylomicrons. This is clearly not the case and we are left with the exciting prospects the hypothesised changes in hepatic apoB synthesis present for developing our understanding of the metabolic regulation of the editing of apoB message.

It is hard to attach any significance to the changes in composition of $VLDL_2$ given that, in the majority of animals, this fraction presented a negligible contribution to the total VLDL pool and that the apolipoprotein content was indistinguishable from that of normal VLDL. The cholesterol content of $VLDL_1$ and $VLDL_2$ does however deserve mention because, in contrast to healthy ponies, free cholesterol was detected in both fractions and represented a considerable portion of the total cholesterol mass. This was not simply because more particles were present raising the concentration of free cholesterol to levels detectable by the assay as free cholesterol was identified in the $VLDL_2$ fraction, the plasma concentration of which were similar to those of VLDL in healthy ponies. Rather, because the appearance of free cholesterol was balanced by a reduction in the mass of cholesteryl esters, this appears the result of defective esterification of VLDL cholesterol. This could arise from reduced activity of hepatic ACAT or plasma LCAT. The activity of LCAT is reduced in humans with liver disease and is associated with impaired cholesterol esterification (Riesen and Kloer 1989). While LCAT has been shown capable of esterifying cholesterol in VLDL from healthy ponies (Yamamoto *et al.* 1975b), the activity of the enzyme was normal in the hyperlipaemic ponies examined here and the mass of HDL cholesteryl esters, the prime focus of LCAT activity, unchanged. Therefore reduced ACAT activity is a more likely explanation. Although this could have arisen from defective synthesis of the enzyme by diseased hepatic tissue, there was evidence of defective cholesterol esterification in the cases without sign of significant hepatic pathology. Hence it is likely that the deficiency of esterification reflected failure of the enzyme to keep pace with an increased rate of VLDL assembly and secretion, further evidence that this hyperlipidaemia results from massive overproduction of VLDL by the liver.

The increases in the triglyceride contents of LDL and HDL are less easy to explain. Although triglyceride enrichment of IDL, LDL and HDL_2 is a feature of HL deficiency in man (Breckenridge *et al.* 1982), the plasma concentration and composition of lipoproteins in the density <1.006 g/ml fraction is generally normal in

such patients, in conflict with the lipoprotein changes seen in the ponies here. In addition, HL activity was normal in each of the animals from which PHP was obtained. However, it is possible that the fractional rate of removal of triglyceride from LDL was reduced because of the increased flux of particles from VLDL.

The increase in HDL triglyceride to 2% was associated with a small increment in particle size (to 8.6 nm), but these particles remained distinct from human HDL_2, which are approximately 9.5-12.0 nm in diameter and typically have triglyceride as 8% of their mass. This, in conjunction with the failure to detect significant CETP activity in the animals examined, would indicate that the triglyceride enrichment of HDL was not the consequence of neutral lipid exchange with the lower density lipoproteins. However, given the hugely expanded pool of triglyceride in the circulation, it may be possible that non-protein mediated or chance exchange/transfer of triglyceride was taking place as the probability of collision between HDL and triglyceride rich lipoproteins increased, in both the circulation and in the samples after collection. Alternatively, triglyceride may be excreted by the liver as a minor component of the nascent HDL particle. The increased triglyceride content of HDL did not appear the result of contamination of this fraction by LDL as apoB-100 was absent on SDS-PAGE. However as a single ultracentrifuge tube was used for the preparation of VLDL through to HDL, contamination of the tube surface or the later fractions by VLDL debris might explain some of the triglyceride enrichment of the later fractions.

With regard to the non-apoB components of the isolated lipoproteins, the normal presence of the C peptides in both VLDL fractions was evidence against deficiency of apoC-II or excess of apoC-III as primary mechanisms in the accumulation of VLDL in the circulation. It is interesting that the apoE content of $VLDL_1$ was increased, as it is possible that the apoB-48 VLDL particles can accommodate more apoE and that this is related to their (hypothesised) removal from the circulation by cell surface receptors. Direct tissue uptake of these particles may go some way to explaining the widespread fatty infiltration of body organs found at necropsy in cases of hyperlipaemia. The apoE content of HDL was also increased and this may suggest that the circulating pool of apoE is expanded in a coordinate response to the increased secretion of apoB-48 containing particles by the liver, which may also explain the increased masses of apoC-II and apoC-III in HDL.

4.4. Lipoprotein Lipase and the Pathogenesis of Hyperlipaemia

The activities of both enzymes involved in the lipolysis of triglyceride lipoproteins were found to be increased in ponies with hyperlipaemia in what appeared to represent a physiological response to increased concentrations of their substrates. This

was especially true for the activity of LPL which positively correlated with the degree of hyperlipidaemia. If defective catabolism of VLDL was the primary mechanism of the disease, then one would expect to find a negative correlation between plasma triglyceride concentrations and LPL activity. Although this was not the case here, the possibility remains that the fractional activity of LPL is reduced in the face of the increased VLDL concentrations and that this contributes to the hyperlipidaemia once triglyceride levels reach a certain threshold.

Thus the weight of evidence presented in this Chapter suggests that the massive hypertriglyceridaemia found in ponies with hyperlipaemia results from hepatic overproduction of VLDL. This appears to be driven by increased plasma concentrations of FFA, such that a larger, triglyceride enriched particle is produced to meet the demands of hepatic triglyceride export. In contradiction with previous speculation, the results indicate that defective catabolism of VLDL is not the primary mechanism of the hyperlipidaemia. Attention should be therefore directed to quantifying the rates of VLDL synthesis and its fractional catabolism, with particular focus on the role that FFA play in the pathogenesis of the hyperlipidaemia. This is the subject of the next Chapter.

CHAPTER VII

KINETIC ANALYSIS OF FREE FATTY ACID AND VERY LOW DENSITY LIPOPROTEIN TRIGLYCERIDE METABOLISM IN HEALTHY PONIES AND PONIES WITH EQUINE HYPERLIPAEMIA

1. INTRODUCTION

Hypertriglyceridaemia is a heterogeneous condition that arises from a variety of genetic, molecular and metabolic defects. One of the major approaches to understanding its origins, and thereby selecting therapy, has involved kinetic analysis of the production and catabolism of the triglyceride rich lipoproteins. Thus, elevations in plasma triglyceride concentrations can be defined as the consequence of increased input into, or decreased clearance of these lipoproteins from the circulation, or a combination of the two mechanisms.

In studying the metabolism of VLDL, two separate approaches have been adopted. In the first, VLDL is isolated from the subject and radiolabelled in its protein moiety, by iodination with ^{125}I or ^{131}I (Shepherd, Bedford and Morgan 1976), and then re-injected. Presently, the use of stable isotopes such as [^{13}C]leucine and [^{15}N]glycine to label apolipoproteins *in vivo* is being heralded as a safer and more informative way in which to trace the synthesis and catabolism of the apolipoproteins (Parhofer, Barrett, Bier and Schonfeld 1991). In the second approach, radiolabelled precursors of triglyceride, usually ^{14}C and ^{3}H labelled fatty acids or glycerol, are injected into the subject and re-isolated in VLDL-triglyceride (Kekki and Nikkilä 1975). This provides more reliable information on the metabolism of the plasma precursor pools and on the synthesis and secretion of VLDL-TG from the liver. Also, because the VLDL is labelled in its triglyceride moiety, the disappearance of activity gives direct insight into the function of lipoprotein lipase rather than the receptor and non-receptor mediated mechanisms that clear apoB.

In order to establish the metabolic basis for the changes in VLDL concentration and composition found in ponies with hyperlipaemia, the VLDL-TG approach was chosen here. The turnover of plasma FFA and VLDL-TG were determined in four healthy ponies using radiolabelled palmitic acid and glycerol. The results were analysed by a multicompartmental model, the components of which were justified by fitting the data calculated by the model to match the observed tracer curves. The modelling of the hepatic pathways was strengthened by the measurement of triglyceride activity in small amounts of liver obtained by percutaneous biopsy at one or two time points during the procedure. The model was then used to interpret the

data from identical turnover studies performed in two ponies with hyperlipaemia. The results confirmed the suggestion from Chapter V that hepatic overproduction, rather than defective plasma catabolism, of VLDL is the cause of this hypertriglyceridaemia and show how therapy for the condition could be improved.

2. SUBJECTS AND METHODS

The control group consisted of four healthy normolipidaemic Shetland pony mares, three of which were barren (subjects 1, 2 and 3) and one (subject 4) that was seven months pregnant. The subjects were fasted for 16 hours prior to the test to remove any possible contribution from intestinal triglyceride production and this was continued for the first 8 hours of the procedure, after which they were allowed access to hay. The procedure was carried out according to the protocol described in section II.7.

Two Shetland ponies with hyperlipaemia (subject A was HLP-14 and subject B was HLP-15 in Chapter V) were studied with the modification of the protocol detailed in section II.7.4. One of the hyperlipaemic ponies had access to hay and concentrates prior to the test but had been inappetant, the other was denied access to food and had been given balanced oral electrolyte/glucose solution (Lectade, Beecham; 5 l every 4 hours via nasogastric tube). Both cases were then maintained on Lectade (5 l, *qid*) and received no other therapy, but were given 70 iU/kg heparin *iv* approximately 24 hours after the start of the procedure to obtain PHP for the analysis of LPL and HL activities. Both subjects were pregnant, and recovered from the condition to give birth to healthy foals three and two months after the procedure, respectively.

For the samples separated by extraction and TLC, the recovery of activity was extraction was found to be 71%, necessitating that the measured cpm be multiplied by 1/0.71 in the calculation of SA. The plasma masses of FFA and VLDL-TG were estimated as the product of the plasma volume and the plasma concentration of each, the plasma volume was taken to equal 46.2 ml/kg (Mattheeuws, Kaneko, Loy, Cornelius and Wheat 1966). The data were analysed using a linear, multicompartmental model which can be described mathematically by a simultaneous set of first-order differential equations. These equations were solved and the model parameters adjusted to give a least-squares fit of the calculated to the observed data using the CONSAM 30 programme supplied by the Resource Facility for Kinetic Analysis, Seattle, USA. A fractional standard deviation of $\pm 5\%$ was attached to the observed data in the input file to allow for random variation in the estimation of specific activities. The model development was based on that proposed for the metabolism of FFA and VLDL-TG in man by Shames, Frank, Steinberg and Berman

(1970) and Zech *et al.* (1979). In the model, the fractional transfer of material from one compartment *e.g.* I to another *e.g.* J is represented by L(J,I); the mass of material in I is M(I); the product M(I)*L(J,I) is the rate at which material leaves compartment I for J, which in a steady state is equal to the production rate (PR). The model was used to calculate the residence time of triglyceride in the VLDL fraction of plasma, and the reciprocal of this value was the fractional catabolic rate (FCR) of VLDL-TG.

3. RESULTS

3.1. Model Development

Following injection of the tracers, there was an initial and rapid decay in plasma FFA activity (Fig. 26) that was followed by flattening of the curve.

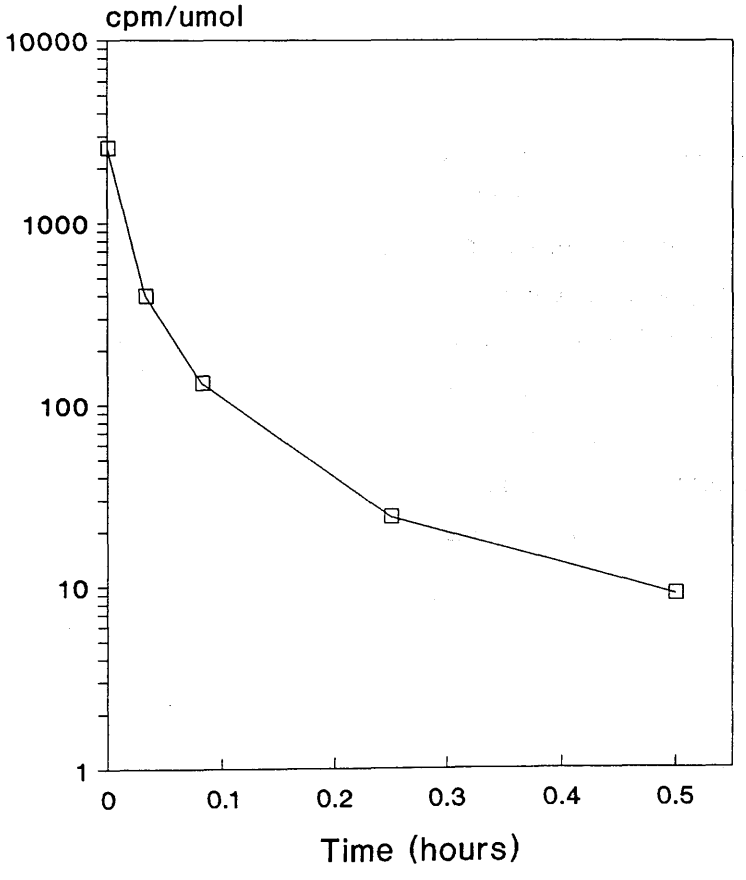


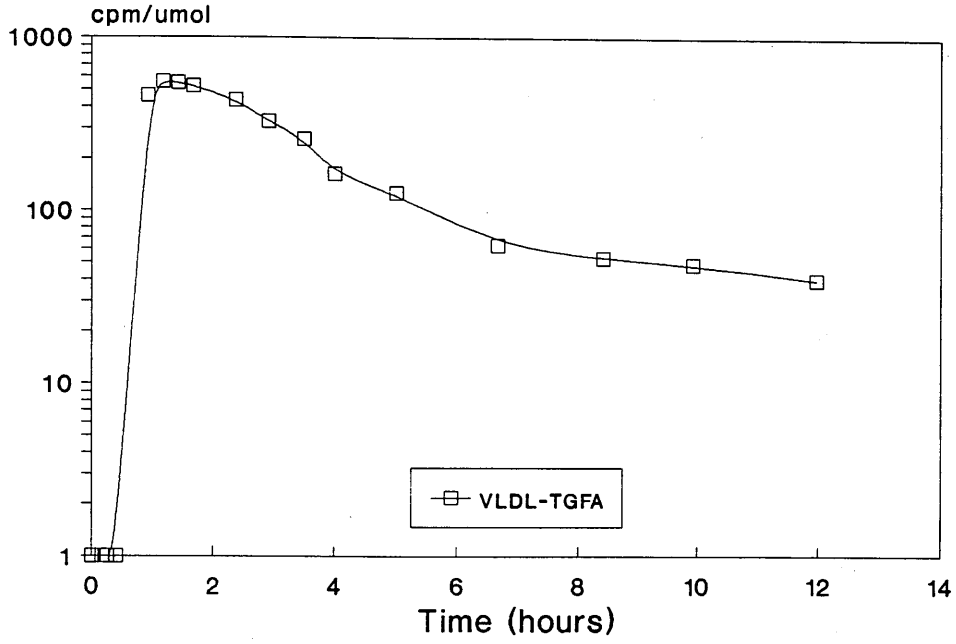
Figure 26. The disappearance of intravenously injected [^{14}C]palmitic acid from plasma of a healthy Shetland pony mare (subject 2).

This curve was fitted with a bi-exponential function in which the steep part of the curve was explained in the model by irreversible loss of the tracer from the plasma compartment (L0,4). The late part was modelled by loss of tracer into and its subsequent re-entry from an extravascular exchange pool (5). The steepness of the slope was proportional to the inverse of L(4,5), such that at slow rates of re-entry (as in subject 4) the slope tended towards the horizontal.

The characteristics of the [^{14}C]VLDL-TGFA and [^{3}H]VLDL-TG curves were similar (Fig. 27). There was an initial time delay so that significant activity was first detected between 0.4 and 1.0 hours after injecting the tracers; the mean (±sd) interval to the appearance of [^{3}H]VLDL-TG was 0.43 (±0.05) hours and for [^{14}C]VLDL-TGFA 0.69 (±0.24) hours (differences not significant). There was then a rapid rise in activity so that both tracers peaked simultaneously in each subject between 0.93 and 1.67 hours (mean ±sd; 1.27 ±0.28 hours). Following the rapid rise, the peak then flattened before decaying with a slope of two parts. The first appeared first-order, but was succeeded by a second slower part that became obvious 5-11 hours after the injection of tracer so that a small amount of activity (approximately 3-10% of peak activity) was present in VLDL one day later.

The features of the VLDL-TG curves were modelled as follows. The initial delay in the appearance of VLDL-TG activity was reproduced by the entry of the tracers into a hepatic component (10) that had an integral delay function before releasing VLDL particles into the circulation (compartment 1). The rapid rise in VLDL-TG activity suggested that this hepatic component was rapidly turning over and was mimicked by giving the L(1,10) function a high value. The slope of the initial decline in VLDL-TG activity was determined by the amount of material lost from compartment 1 *i.e.* L(0,1), as shown by comparison of the slopes of subjects 2 and 3. However, the fitting of this part of the curve did not explain the flattening of the peak or the late slope, as the calculated curve decayed from a single peak value in a mono-exponential fashion.

The flattening of the peak was reproduced by introducing a chain of compartments (6, 7, 8) within the VLDL density range that were linked to compartment 1 to represent a delipidation cascade. Thus, in addition to loss of material from compartment 1 to 0, a fraction of 1 remained in the VLDL density range and moved into compartment 6 where it continued to contribute to the total VLDL-TG activity. The flatness of the peak was then governed by the magnitude of L(6,1), as shown by comparison of subjects 1 and 3. As the activity counted in the density < 1.006/ml fraction was then the sum of compartments 1, 6, 7 and 8, these were joined by a function represented by compartment 9.



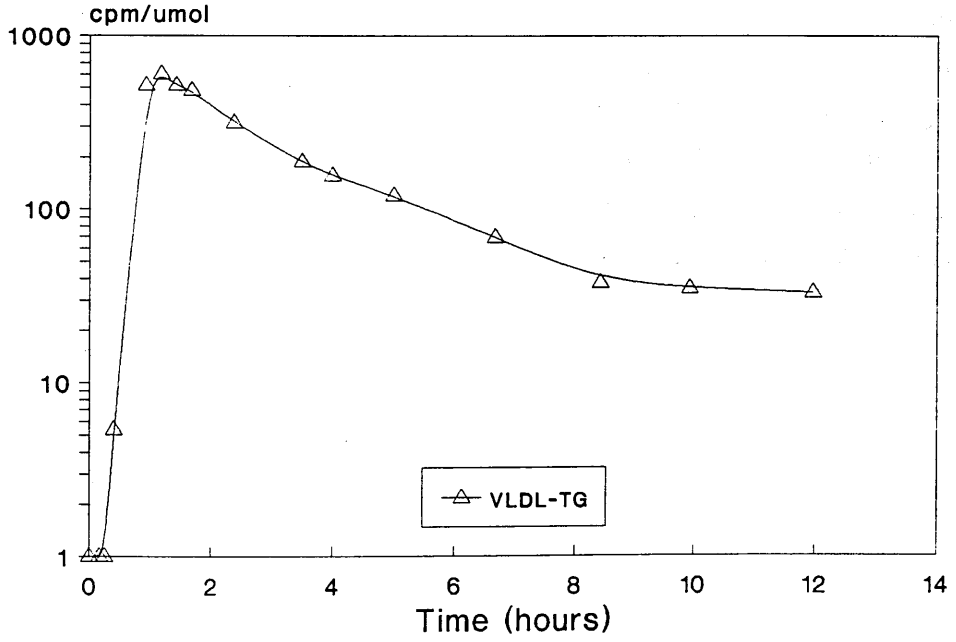


Figure 27. Typical plasma specific activity curves obtained for [^{14}C]VLDL-TGFA (top) and [^{3}H]VLDL-TG (bottom) in a healthy Shetland pony mare (subject 1).

The fractions of material lost and transferred from each of the VLDL compartments to the next were assumed to be equal along the cascade so that L(0,6) and L(0,7) were set to equal L(0,1); L(7,6) and L(8,7) equal to L(6,1); and L(0,8) equal to L(8,7)+L(0,1).

To explain the late part of the VLDL-TG curves, a second, slowly turning over, hepatic compartment (24) was introduced so that L(1,24) was approximately one tenth of the magnitude of that of the rapidly turning over pathway, L(1,10). Thus, as L(1,24) approached zero the late part of the curve became horizontal (see subject 1). The significance of the late part was determined by the fraction of the tracer that was channelled through this pathway, *i.e.* the ratio of L(24,4) to L(10,4), which was 3:1 in subject 1 where the late part predominated compared with 1:3 in subject 4 where the VLDL-TG activity was dominated by the first part of the curve. Although in the model shown in Fig. 3, compartment 24 is linked directly with compartments 4 and 1, the data were equally fitted when 24 was introduced in exchange with compartment 10 or was fed from 4 and fed into 10. As the hepatic biopsy data did not distinguish triglyceride activity in the two synthetic pools, the masses and activities of compartments 10 and 24 were summed and represented by compartment 11.

Two alternative explanations for the late part of the VLDL-TG curves were considered and rejected. In the first, a slowly metabolised species of VLDL, such as ß-VLDL, was introduced in parallel with the cascade of compartments 1, 6, 7, and 8. While this provided some flattening of the tail, the calculated mass of this component represented a major portion of the total VLDL-TG mass and was thus unacceptable in terms of the composition of equine VLDL. In the next, the effect of tracer recycling, *i.e.* that released during the metabolism of VLDL, was assessed by allowing the components of the delipidation cascade to feed back into compartment 4. This function had no discernible impact on the shape of the VLDL-TG curves in any of the four ponies indicating that recycling was not the origin of the tails.
However, as discussed in the previous Chapter, recycling might be important in the generating plasma FFA in ponies with hyperlipaemia and for this reason the function was retained in the model. For the reasons outlined for the parameter L(6,1), the values of L(4,6), L(4,7) and L(4,8) were set equal to L(4,1).

The final version of the model is represented schematically in Figure 28, overleaf.

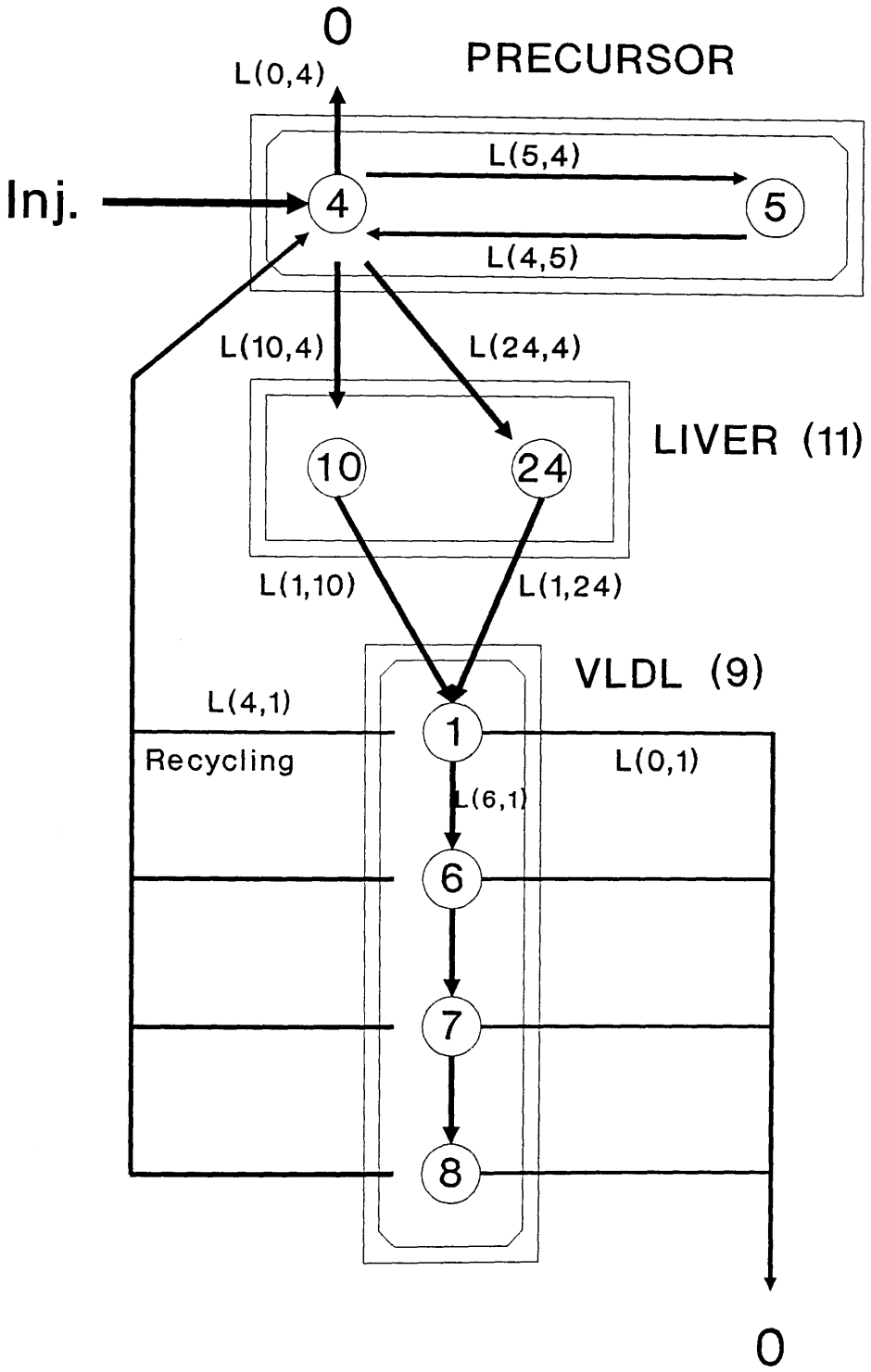


Figure 28. Scheme of the compartmental model developed for the analysis of plasma FFA and VLDL-TG kinetics in healthy ponies. The tracers were injected into compartment 4, and their activities measured in compartments 4, 11 and 9.

3.2. Kinetic Analysis of FFA and VLDL-TG Metabolism in Four Healthy Ponies

The data from each subject were then analysed using the following approach. The [^{14}C]FFA data were fitted first. The FFA curve was fitted by setting parameters L(0,4), L(5,4) and L(4,5) and the input into the liver, L(10,4) and L(24,4), adjusted to give an approximate fit of the hepatic triglyceride activity. The [^{14}C]VLDL-TGFA data were then fitted by adjusting the input and output from the two hepatic compartments, and the fraction of material lost or transferred at each compartment within the delipidation cascade. Once the ratio of the calculated to the observed data was less than 100 for the three solutions (FFA, hepatic- and VLDL-TG), the computer programme was allowed to change each of the parameters in turn, in a process called iterating, until there was no further improvement in the sum of the squares of the residuals. At this point, the values for each parameter were taken as the best fit of the data. The model calculated masses for each of the compartments; that of M(4) was fixed in the input data file while that of M(9) was forced to the observed values by a separate equation.

The [^{3}H]glycerol data were then analysed. As no information was obtained on the kinetics of the glycerol precursor pool, the initial rate constants were set equal to those obtained for the ^{14}C solutions. In fact for each animal, the [^{3}H]glycerol and [^{3}H]VLDL-TG data were fitted following adjustment of L(0,4) only. Therefore to improve the strength of the model, the files for the two tracers were combined and solved simultaneously with the parameters for the ^{3}H data were set equal to those of the ^{14}C data, with the exception of L(0,4). The convergence process was then repeated so that the estimated parameters described the behaviour of both tracers. Typical fits obtained for the FFA, [^{14}C]VLDL-TGFA and [^{3}H]VLDL-TG curves are shown in Figs. 29 and 30(Appendices 33 and 34).

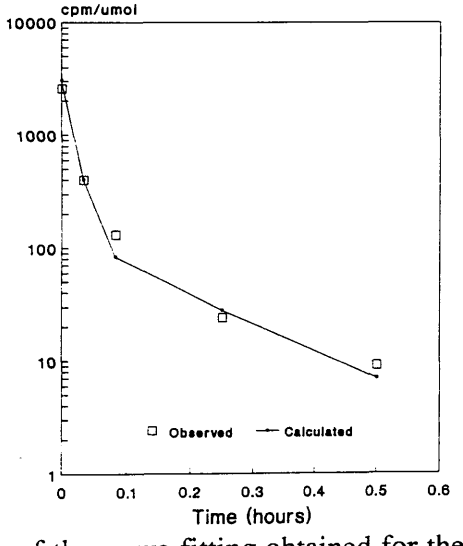
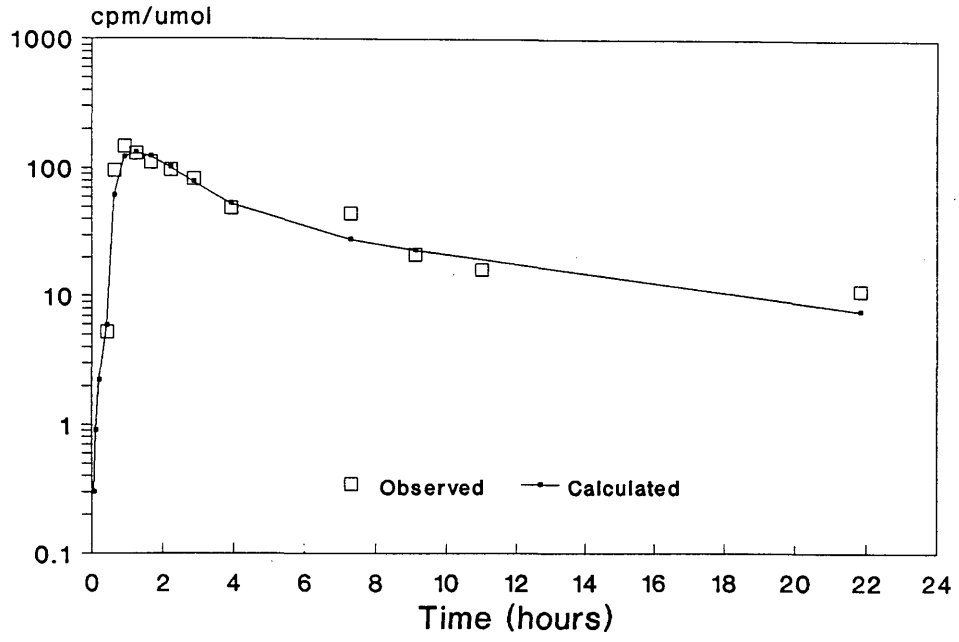
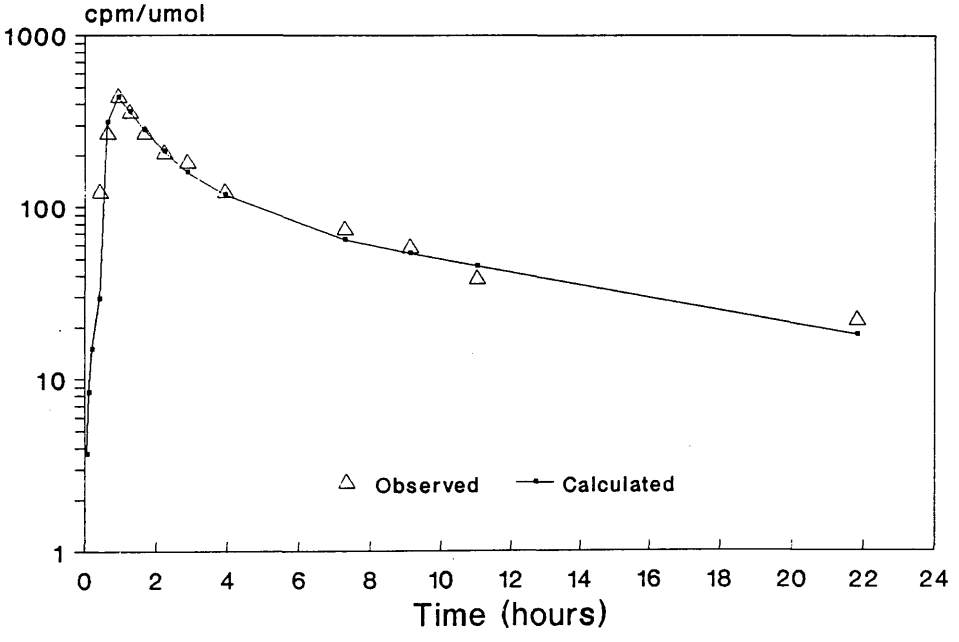


Figure 29. An example of the curve fitting obtained for the disappearance of plasma FFA specific activity in a healthy Shetland pony (subject 2).



VLDL-TGFA



VLDL-TG

Figure 30. An example of the curve fitting obtained for [^{14}C]VLDL-TGFA (top) and [^{3}H]VLDL-TG (bottom) specific activities in a healthy Shetland pony (subject 2).

The parameters describing the precursor systems are detailed in Table 29. Only a small fraction of the injected FFA entered the hepatic triglyceride compartments (4.2 ±1.4%; mean ±sd), with the rest split between the exchangeable (35.0 ±24.7%) and non-exchangeable (60.9 ±25.2%) extravascular pools. In contrast, the vast majority of the glycerol was irreversibly lost (95.3 ±2.4%), and the fractions that entered the exchangeable (4.2 ±2.4%) and hepatic (0.5 ±0.2%) pools were much less. The balance of the input into the two hepatic pools was variable, the slow pathway was favoured in three animals (subjects 1, 2 and 3) in which the transport of precursor into compartment 24 was 2-3 times that into the compartment 10. In subject 4, the transport into the fast was 1.5 times greater than that into the slow pathway.

The parameters describing the hepatic and VLDL-TG systems are detailed in Table 30. In three of the animals (subjects 1, 2 and 3), the slow hepatic pathway accounted for the bulk of the transport of VLDL-TG into compartment 1 (96%, 74% and 91%, respectively), while in the fourth the fast pathway was responsible for 68% of the VLDL-TG entering compartment 1. The parameterL(1,10)was found to hold no constraint on the model as long as it was equal to or greater than L(10,4) and was therefore set to equal this value. The delay time associated with compartment 10 was 0.65 (±0.11) hours. The total production rate of VLDL-TG varied five fold between the four ponies and ranged form 2.45-12.74 mmol/hour.

At each step in the delipidation cascade, 24-75% of the VLDL-TG activity was lost according to the equation L(0,1)/L(0,1)+L(6,1). The residence time for VLDL-TG was between 1.2 and 3.7 hours, giving values for the FCR of between 0.27 and 0.83 pools/hour. The curves for all four ponies were fitted without recycling, *i.e.* L(4,1)=0 and this parameter remained at zero following convergence by the programme.

Parameter	Subject 1	2	3	4	Mean	fsd
Body weight (kg)	237	186	239	234	224	0.11
1. FFA						
M(4) mmol	4.83	8.59	6.21	4.11	5.94	0.33
M(5)	1.33	18.50	17.28	25.18	15.56	0.65
L(0,4) /h	17.76	40.50	7.78	16.31	20.59	0.68
L(5,4)	1.53	16.49	18.16	10.62	11.70	0.64
L(4,5)	5.56	7.66	6.54	1.73	5.38	0.42
L(10,4)	0.16	0.70	0.37	1.30	0.63	0.79
L(24,4)	0.49	1.10	0.74	0.46	0.70	0.43
R(0,4) mmol/h	85.10	348.02	48.34	67.10	137.31	1.03
R(5,4)	7.39	141.70	112.80	43.69	76.40	0.81
R(10,4)	0.77	6.02	2.30	5.35	3.61	0.69
R(24,4)	2.37	9.45	4.60	1.89	4.58	0.76
2. Glycerol						
M(4) mmol	0.90	0.89	0.96	0.65	0.85	0.16
M(5)	0.25	1.92	2.65	3.97	2.20	0.71
L(0,4) /h	157.42	246.59	1255.60	206.40	466.50	1.13
R(0,4) mmol/h	141.68	219.47	1205.38	134.16	407.59	1.17
R(5,4)	1.38	14.67	17.43	6.90	10.10	0.73
R(10,4)	0.16	0.62	0.36	0.85	0.50	0.61
R(24,4)	0.49	0.98	0.71	0.30	0.62	0.47

fsd, fractional standard deviation

Table 29. Kinetic parameter values for FFA and glycerol precursor pools in four healthy Shetland ponies.

Parameter	Subject 1	2	3	4	Mean	fsd
1. Liver						
M(10) mmol	0.56	4.67	1.20	3.11	2.38	0.79
M(24)	131.17	430.83	53.22	44.83	165.15	1.10
DT(10) hours	0.71	0.78	0.53	0.58	0.65	0.18
L(1,10) /h	0.16	0.70	0.37	1.30	0.63	0.79
L(1,24)	0.018	0.022	0.086	0.043	0.04	0.74
R(1,10)	0.09	3.26	0.44	4.04	1.96	1.02
R(1,24)	2.36	9.48	4.58	1.93	4.59	0.73
2. VLDL						
M(1) mmol	1.49	1.48	2.40	4.61	2.50	0.59
M(6)	1.14	0.94	0.59	2.36	1.26	0.61
M(7)	0.81	0.59	0.14	1.03	0.64	0.59
M(8)	0.60	0.38	0.04	0.62	0.41	0.66
M(9)	4.01	3.39	3.17	8.80	4.84	0.55
L(0,1) /h	0.36	0.23	0.83	0.32	0.43	0.63
L(6,1)	1.15	0.39	0.27	0.34	0.54	0.76
L(4,1)	0.00	0.00	0.00	0.00	0.00	---
FCR	0.46	0.27	0.83	0.34	0.48	0.53
R(0,1) mmol/h	0.54	0.33	1.98	1.48	1.08	0.72
R(0,6)	0.41	0.31	0.49	0.76	0.49	0.39
R(0,7)	0.29	0.14	0.12	0.33	0.22	0.48
R(0,8)	0.91	0.24	0.04	0.41	0.40	0.93
R(6,1)	1.72	0.58	0.65	1.55	1.13	0.52
R(7,6)	1.31	0.37	0.16	0.80	0.66	0.77
R(8,7)	0.93	0.34	0.09	0.35	0.43	0.83

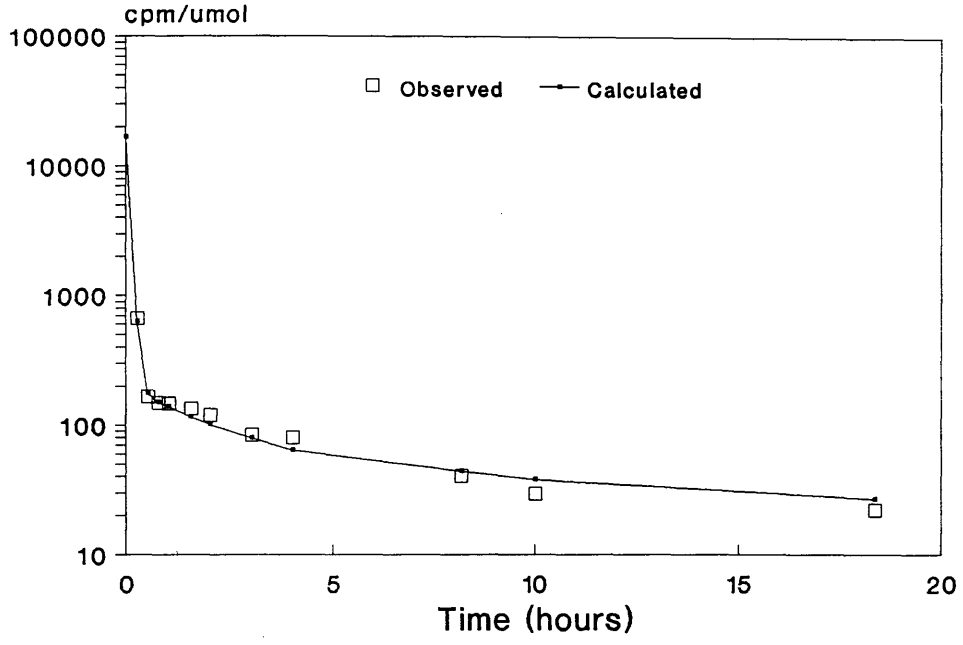
Table 30. Kinetic parameter values for the hepatic and VLDL triglyceride systems in four healthy Shetland ponies.

3.3. Kinetic Analysis of FFA and VLDL-TG Metabolism in Two Ponies with Hyperlipaemia

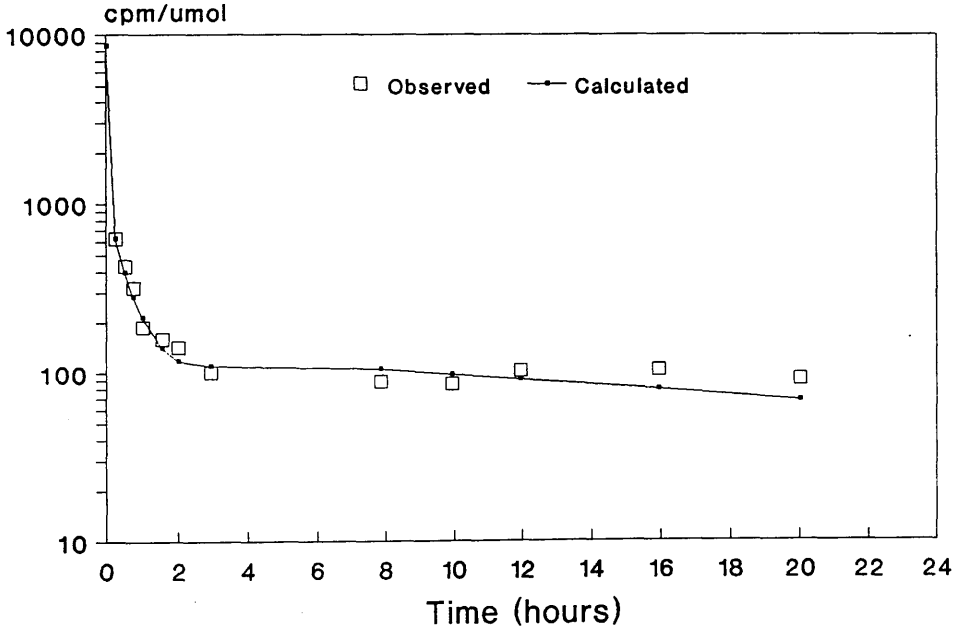
The FFA curves from both hyperlipaemic ponies had long tails that meant that significant activity was present in the plasma 18 and 20 hours after injection of the tracers (open squares in Fig. 31). The VLDL-TG curves (Figs 32 and 33) also contrasted markedly with those of the normal ponies. The rise in activity was very much slower such that the peak was reached between 4 and 5 hours instead of 0.9-1.7 hours in the controls. The flattening of the peak was also very much more pronounced. After this, the curves decayed at a slow rate in an almost mono-exponential fashion so that significant activity (20-30% peak activity) was present nearly two days after the injection of the tracers.

The extended portion of the FFA curves could not be fitted by manipulation of the precursor pool parameters in the way that the slope in the normal animals could. However, a reasonable fit was obtained over the first three hours of the curve that allowed the convergence process to proceed once the VLDL-TG data had been fitted. During this process, the computer explained the slow decay of these curves by significant recycling of tracer from VLDL-TG to compartment 4 to give an excellent fit of the data (Fig. 31). The parameters of the precursor pools are summarised in Table 31. The plasma FFA mass was increased by 2.3 and 3.8 fold in subjects A and B, respectively, over that of the controls. In both cases, the model calculated that none of the FFA were lost to compartment 0, and that the fractions transferred to the hepatic triglyceride compartments were increased from around 8% in the controls to 73% and 61% in subjects A and B, respectively. The fraction of FFA that entered the exchangeable pool was similar to the controls in both cases. The input into the slow hepatic pathway accounted for 99% and 96% of the FFA that entered the hepatic triglyceride compartments in subjects A and B, respectively. The trend for glycerol was similar, with the fraction lost from the precursor system reduced from around 95% in the controls to 86% and 56% in A and B, respectively. The fraction that entered the hepatic triglyceride pools was increased from 0.5% to 10.1% and 5.4%, with 99% and 96% of this input directed to compartment 24. The transport of both precursors into the fast pathway was within the range of the controls, while that into the slow was increased by over 30 fold in both subjects. In subject A, the mass of triglyceride in compartment 10 was higher than that in the controls. The mass of compartment 24 was increased in both subjects to approximately eight times that of the controls.

The VLDL-TG mass was increased by over 100 fold to 511.78 and 603.69 mmol/l in subjects A and B, respectively (Table 32).



Subject A

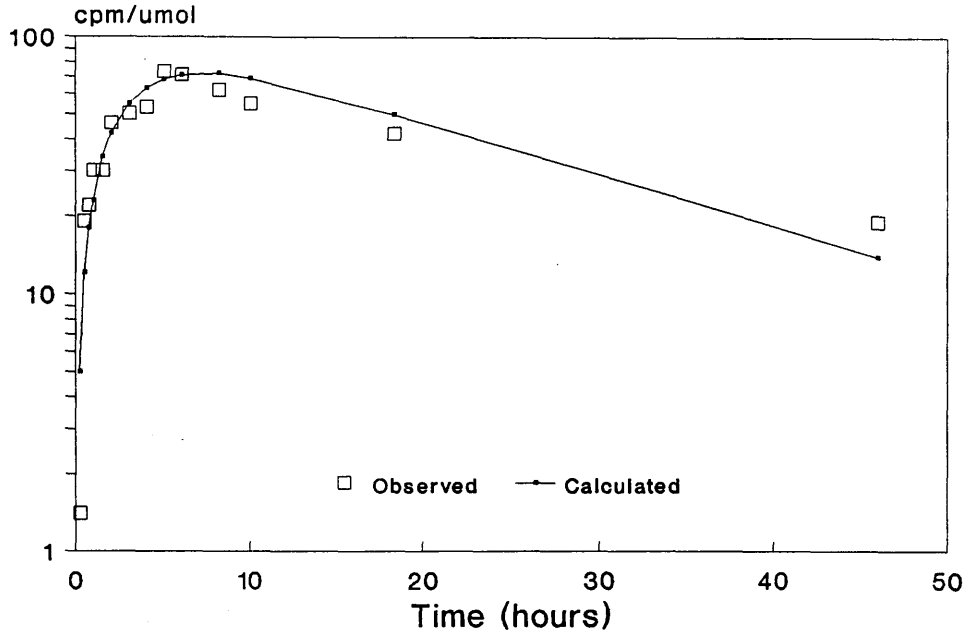


Subject B

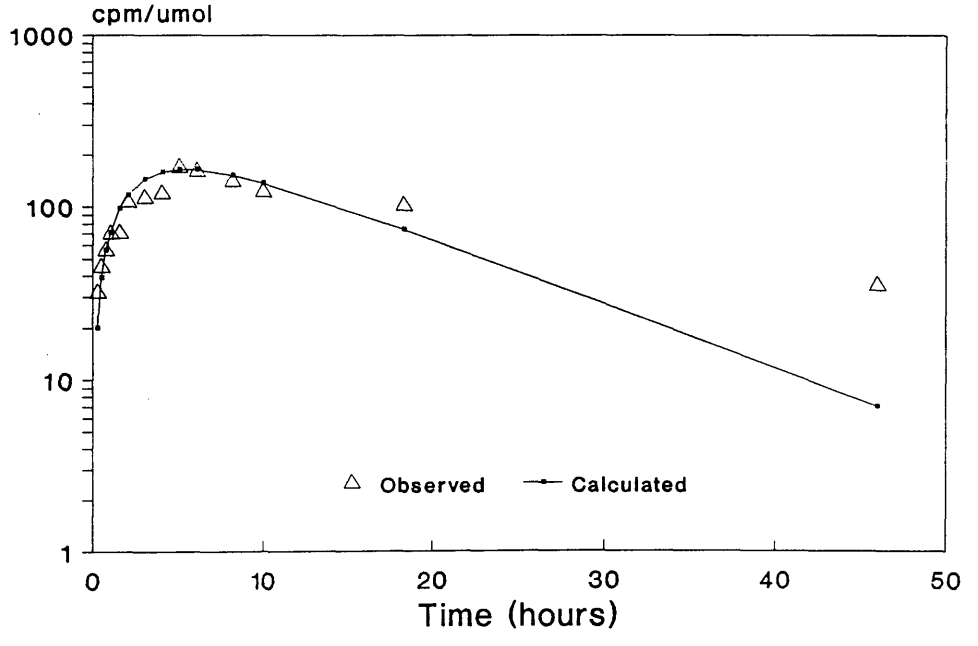
Figure 31. Disappearance of [^{14}C]palmitic acid from the plasma of two ponies with hyperlipaemia (top, subject A; bottom, subject B).

Parameter	Subject A	Subject B	Controls
Body Weight (kg)	234	246	224
1. FFA			
M(4) mmol	13.73	22.85	5.94
M(5)	89.68	66.76	15.56
L(0,4) /hour	0.00	0.00	20.59
L(5,4)	3.87	4.38	11.70
L(4,5)	5.93	1.50	5.38
L(10,4)	0.13	0.28	0.63
L(24,4)	10.23	6.46	0.70
R(0,4) mmol/hour	0.00	0.00	137.31
R(5,4)	53.14	100.08	11.70
R(10,4)	1.73	6.49	3.61
R(24,4)	140.46	147.61	4.58
2. Glycerol			
M(4) mmol	5.84	6.12	0.85
M(5)	38.10	17.89	2.20
L(0,4)	87.58	14.35	466.50
R(0,4)	511.46	87.82	407.59
R(5,4)	22.60	26.81	10.10
R(10,4)	0.74	1.74	0.50
R(24,4)	59.74	39.53	0.62

Table 31. Kinetic parameter values for FFA and glycerol precursor pools in two Shetland ponies with hyperlipaemia compared with the mean of four healthy controls.

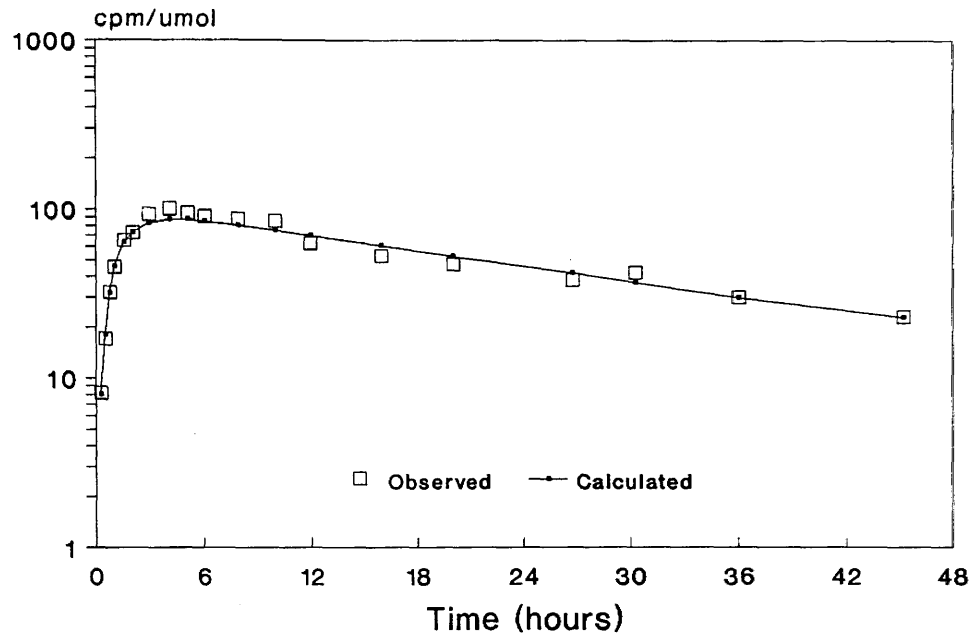


VLDL-TGFA

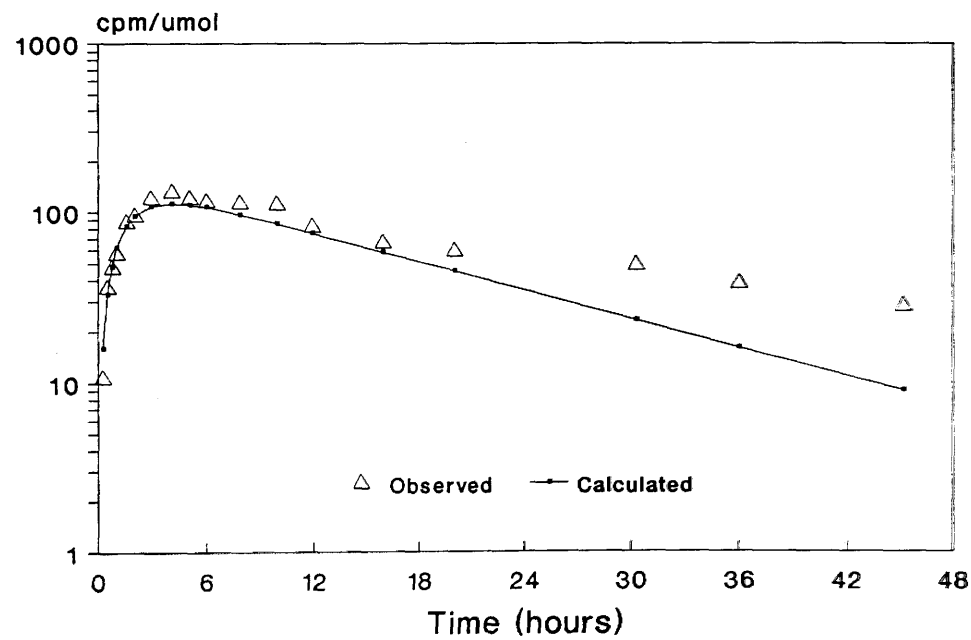


VLDL-TG

Figure 32. Specific activities and curve fitting of [^{14}C]VLDL-TGFA (top) and [^{3}H]VLDL-TG (bottom) in a hyperlipaemic pony (subject A).



VLDL-TGFA



VLDL-TG

Figure 33. Specific activities and curve fitting of [^{14}C]VLDL-TGFA (Top) and [^{3}H]VLDL-TG (Bottom) in a hyperlipaemic pony (subject B).

Parameter	Subject A	Subject B	Controls
1. Liver			
M(10)	0.00	5.92	2.38
M(24)	1998.40	752.00	165.15
DT(10)	0.00	0.41	
L(1,10)	0.00	0.04	0.63
L(1,24)	0.09	0.26	0.04
R(1,10)	0.00	0.24	1.96
R(1,24)	187.85	193.30	4.59
2. VLDL			
M(1)	511.78	410.25	2.50
M(6)	---	128.62	1.26
M(7)	---	40.33	0.64
M(8)	---	28.36	0.41
M(9)	511.78	607.56	4.04
L(0,1) /h	0.25	0.11	0.43
L(4,1)	0.15	0.44	0.00
L(6,1)	0.00	0.25	0.54
FCR	0.39	0.56	0.48

Table 32. Kinetic parameter values for hepatic and VLDL-triglyceride systems in two ponies with hyperlipaemia, compared with the mean of four healthy controls.

The rate constant L(1,24) was ten fold greater than the controls in both cases so that the transport of triglyceride was increased by over 30 fold to 187.85 and 193.30 mmol/h, respectively (Table 32). The rate constants describing the loss of triglyceride from each compartment in the delipidation cascade, 0.35 in each case, were similar to the control animals (0.43 $\pm$0.23), although 40% in subject A and 71% in subject B of the products of lipolysis were recycled to the plasma precursor pool (Table 32). The residence time of VLDL-TG in the two hyperlipaemic ponies was 2.9 and 2.4 hours, giving values for the FCR of 0.35 and 0.42 /hour that were within the range of the control ponies (0.48 $\pm$0.25 /hour).

4. DISCUSSION

These studies have shown, for the first time, the qualitative and quantitative metabolic behaviour of VLDL in the horse. The results are of significance for their contribution to the interpretation of VLDL-TG kinetics and for providing a direct insight into the pathogenesis of equine hyperlipaemia.

4.1. Plasma FFA and VLDL-TG Metabolism in Healthy Ponies

One of the constraints placed upon the analysis of lipoprotein kinetics is that the model must be compatible with data collected from a number of sources. Here, and in contrast to the majority of human studies, the strength or uniqueness of the model was maximised by using two tracers of VLDL-TG in the same individual and measuring their activity in all three of the model's systems. The activity was traced in VLDL rather than plasma because the latter reflects the kinetics of triglyceride in lipoprotein species other than VLDL which have very different metabolic characteristics (approximately 30% of the plasma triglyceride of the Shetland pony is carried in LDL and HDL). Although the model did not consider the possible exchange of triglyceride between VLDL and the higher density lipoproteins, such a feature was not required for accurate fitting of the data consistent with the fact that the horse, as shown in Chapter IV, lacks CETP activity. The final version of the model appears similar to that proposed for the analysis of human VLDL-TG kinetics by Zech *et al.* (1979), suggesting that the pathways of triglyceride metabolism are similar in both species. Although the human model is 12 years old, it has withstood rigourous testing (Phair 1982) and remains widely used in studies of VLDL-TG metabolism (Harris, Connor, Illingworth, Rothrock and Foster 1990).

Several features of the model deserve mention. The two compartment portrayal of the precursor system was identical to that arrived at to explain the behaviour of palmitic acid in the rat (Baker and Schotz 1967) and man (Shames *et al.* 1970). The fraction of

material irreversibly lost from plasma represents that which enters extravascular tissues, predominantly the liver, where it is oxidised to CO_2 or ketone bodies. The nature of the exchangeable pool is unclear; it may represent FFA that are esterified to triglyceride and then hydrolysed in adipose tissues, or the uptake and release of fatty acids from the capillary endothelium, interstitial fluid, or extrahepatic cells. In this study, the fractional rates at which palmitic acid and glycerol entered and left the exchange pool (*i.e.* L(5,4)) were identical suggesting that exchange mechanism is common to both molecules and as such may well represent a rapidly turning over pool of triglyceride. The fractional and mass transport (on a per kg basis) of the precursors found here were similar to those reported for human FFA (Eaton, Berman and Steinberg 1969) and glycerol (Zech *et al.* 1972).

The biochemical nature of the time delay incorporated in compartment 10 was not resolved by the model, but conceivably resides in the time taken to synthesis triglyceride from the precursors. However, this might not be the case as Baker and Schotz (1967) found virtually no delay in the appearance of labelled triglyceride in the livers of rats injected with [^{14}C]palmitic acid. However, the magnitude of the delay was consistent with the 30 minutes required for the synthesis and secretion of VLDL-apoB by hepatocytes during the course of pulse chase experiments (Siuta-Mangano, Janero and Lane 1982). Thus it is likely that the delay function represents the assembly, intracellular transport and secretion of VLDL proteins rather than the synthesis of its lipids (Janero and Lane 1983).

In contrast to compartment 10, the rate constant for precursor entering compartment 24 was approximately seventeen times greater than that of triglyceride leaving. Thus it is possible that the slow turnover of this compartment was due to newly synthesised triglyceride entering large storage pools within the liver, consistent with the very large calculated mass of compartment 24 relative to that of 10. Although compartment 24 was modelled in parallel with 10, the data were equally fitted when 24 had no direct input from plasma, but exchanged with compartment 10. Thus rather than representing discrete hepatic pools, it is possible that the two pools are linked, with the fast pathway being readily saturable so that excess precursor enters the slowly turning over hepatic pool. Physiologically, this may be explained by the entry of precursor into a common synthetic pathway, the exit from which is limited by the rate of VLDL assembly and secretion, and specifically by the synthesis of apoB. Once this capacity is exceeded, the newly synthesised triglyceride enters a slowly turning over storage pool reflected by the mass of compartment 24.

The hepatic triglyceride masses calculated by the model represented approximately 1.5% of total wet liver weight (assuming that the liver weighs 4% of body weight). This value is similar to that of 2% in man (Shames *et al.* 1970), but

underestimated the mass of triglyceride in the biopsy specimens by two to six fold. This is probably not of concern given the small size of the biopsies and the potential errors in the extraction of lipids, and the likelihood that triglyceride is not distributed equally throughout the liver. Human VLDL-TG studies with direct hepatic data are rare, but Farquhar, Gross, Wagner and Reaven (1965) obtained biopsies from four patients and required a third hepatic "sink" compartment in their model to equate the measured and calculated hepatic triglyceride masses. A similar compartment could conceivably explain the discrepancy in the present data, although the physiological identity of a non-exchangeable pool of this type is hard to rationalise (Phair 1982).

It was perhaps of little surprise that the parameters describing the behaviour of the two precursors in the hepatic and VLDL-TG compartments were identical, considering that the synthesis and catabolism of triglyceride is unlikely to be different if the molecule is labelled in its glycerol or fatty acid moiety. The impact of the tracer VLDL-TG kinetics was addressed by Farquhar *et al.* (1965) who found no difference in the curves obtained with glycerol labelled in the 2 position with [^{3}H] and in the 1 and 3 positions with ^{14}C. However, the VLDL-TG curves for [1-^{14}C]palmitic acid and [2-^{3}H]glycerol differed in that the slow late component was present in the palmitic but not the glycerol curve of the single subject studied. This was later contested by Zech *et al.* (1979) who found significant tails in each of 13 healthy subjects studied.

The metabolic origins of these late tails has been the subject of extensive debate (Zech *et al.* 1979, Phair 1982). In the early human studies (Farquhar *et al.* 1965, Eaton *et al.* 1969) they were attributed to recycling of the FFA precursors, but Zech *et al.* (1979) then showed that recycling of glycerol had very little effect on the shape of the curves. Zech *et al.* (1979) also presented convincing evidence that the tails do not arise form exchange of VLDL-TG with other lipoproteins or extravascular tissues, or the presence in the density <1.006 g/ml fraction of a slowly turning over VLDL species such as ß-VLDL. The authors concluded, as here, that they are the result of the slowly turning over hepatic compartment. Much of the success in describing the origins of these tails in the present study was because the late parts of the VLDL-TG curves are much more significant in the pony than they appear in man, and thus required more exact modelling. Their prominence was reflected by the fact that the transport of triglyceride from the slowly turning over pathway was on average 2.5 times that from the fast, where in man triglyceride synthesis from the fast is approximately three times that from the slow (Zech *et al.* 1979).

Early studies of VLDL-TG kinetics modelled the plasma VLDL-TG pool as a single compartment (Baker and Scotz 1964; Farquhar *et al.* 1965; Eaton *et al.* 1969; Shames *et al.* 1970). Consideration of the physiology of VLDL catabolism and the

need to explain the flattening of the VLDL activity peaks then led to the introduction of a cascade (Phair 1982). The data were fitted here with four compartments, although in reality this is an oversimplification constrained by the insensitivity of the data; if many samples had been taken over the period of the peak more compartments may have been required for a fit. At each step in the cascade, a portion of the VLDL-TG is lost due to the action of LPL and the rest passes onto the next compartment. Thus hydrolysis drives the particles through the cascade until their density is >1.006 g/ml and they are lost from the VLDL pool to IDL and LDL. In interpreting the data, the rate constant L(0,1) was assumed to represent lipolysis although it may also include a small fraction of particles lost from the compartment to cellular uptake.

The model used here paid no attention to heterogeneity in the size and triglyceride content of VLDL, and it was assumed that all newly secreted VLDL entered compartment 1. This was the limitation of the data, although in reality smaller VLDL particles secreted by the liver might enter compartment 6, 7 or 8. If this is the case, then the residence time (and FCR) in plasma of VLDL-TG is not be solely determined by the activity of LPL, but also by the size of the particle when it is secreted. Differences in the kinetics of large and small VLDL-apoB are well established in man (Packard *et al.* 1984, Packard, Demant and Shepherd 1987, Demant *et al.* 1988) and Barrett, Baker and Nestel (1991) have recently shown that this heterogeneity is extended to VLDL-TG. This is clearly of concern to the present studies, given that equine VLDL appears to be composed of particles that contain one or another of two distinct species of apoB that are likely to dictate significant differences in the assembly, secretion and clearance of their host particles.

4.2. The Pathogenesis of Equine Hyperlipaemia

The analysis of VLDL-TG kinetics in two ponies with hyperlipaemia has provided a clear insight into the pathogenesis of the condition and vital information on which to base novel therapies for the disease. In contrast to previous speculation (Morris *et al.* 1972; Bauer 1980; Freestone *et al.* 1991), the hypertriglyceridaemia is not the result of defective clearance of triglyceride rich lipoproteins but arises from massive overproduction of VLDL by the liver stimulated by very significant changes in plasma FFA and hepatic triglyceride metabolism.

Although the size of plasma precursor pool was increased by only three fold, but the fate of the FFA was very different to that in the control subjects. The fractions of FFA that entered the oxidation or hepatic triglyceride pathways were reversed so that virtually none were lost to oxidation and ketone synthesis, and the majority were directed to the hepatic triglyceride synthesis. In addition, the transport into the fast hepatic pathway was reduced to negligible proportions while that into the slow

pathway was increased as a result of increases in the precursor pool size and the fraction of precursor entering compartment 24. The output from this compartment indicated that it was the flux of FFA into the liver that was driving triglyceride synthesis.

The failure of oxidation and ketone synthesis to dispose of the increased flux of FFA into the liver is perhaps a key event in the pathogenesis of this hypertriglyceridaemia. This is reflected in the fact that ketonaemia is not prominent in ponies with hyperlipaemia (Naylor *et al.* 1980) and, as Rose and Sampson (1982) found, blood ketone concentrations do not rise (while FFA do) in horses fasted for 96 hours. Taken together, these findings suggest that the pathways for ketone synthesis are not well developed in the horse. This is in marked contrast to the cow and the sheep where overt ketosis, rather than hyperlipidaemia, is a common sequel to the mobilisation of FFA from adipose tissue during late pregnancy and early lactation. In the absence of this pathway, and faced with an increased FFA load, the equine liver has no option but to channel the FFA into the triglyceride synthetic pathways that lead to the export of VLDL. As the fast pathway is readily saturable, an ever greater proportion of the FFA enter the slow production pathway as shown in the model by the normal mass of triglyceride in compartment 10, while that in 24 is increased many fold. The accumulation of triglyceride in this pool would then explain the massive intracellular fatty infiltration seen in the liver of ponies with hyperlipaemia. The model failed to justify any time delay in subject A and gave a value for this function in B that was below that of the controls. This is likely to have been a reflection of the domination of the VLDL-TG kinetics by the slow pathway, so that the curves held little or no characteristics of the fast pathway for the model to interpret.

The hypertriglyceridaemia was entirely accounted for by the transport of triglyceride into compartment 1 as the rate constants and fractional losses from each compartment in the delipidation chain were within the range of the controls. This meant that the residence time and FCR for VLDL-TG were within the control range, and was consistent with the values for LPL activity obtained in Chapter V. Thus, despite the massive VLDL-TG synthesis the catabolism of these particles does not appear compromised. For this reason, heparin might not provide any therapeutic benefit and it was worthy of note that the heparin given to both subjects for the collection of PHP had no significant affect on the curves of VLDL-TG kinetics.

In contrast to the controls, the recycling of the precursors from VLDL-TG was of qualitative and quantitative significance. This is likely to have resulted from the increased activity of LPL on the expanded VLDL pool, so that the FFA released overwhelmed the normal cellular uptake mechanisms and spilled over into the general circulation. This was of such magnitude that the hourly transport of FFA from VLDL-

TG into compartment 4 represented 6 and 12 times the mass of FFA in that compartment in subjects A and B, respectively, and 38% and 69% of the total FFA leaving that compartment (*i.e.* for the exchange and hepatic compartments), respectively. There are two reasons, why in the face of this recycling, peripheral blood FFA concentrations rose only three fold. First, the transport of FFA out of the plasma pool was increased. The second explanation considers the fact that LPL activity is concentrated in adipose tissue, a large proportion of which in the pony is located in the abdomen so that the FFA released may well have been removed by the liver on their first pass through the portal vein. This latter phenomena would expose the liver to much higher FFA levels than when the hydrolysis occurs elsewhere in the body and would exacerbate the stimulation of triglyceride synthesis. Clearly recycling can only contribute to the pathogenesis of the hyperlipidaemia once a certain threshold has been breached, indicating that the initial stimulus for triglyceride synthesis must come from the mobilisation of FFA from adipose stores. However, it is obvious from the data presented here that recycling creates a vicious circle in which triglyceride synthesis, the mass of hepatic triglyceride and VLDL-TG secretion could be forced even in the absence of adipose lipolysis.

In conclusion, the data presented here has considerably advanced our understanding of the pathogenesis of hyperlipaemia and has shown how the mobilisation of FFA leads to massive hypertriglyceridaemia. The potential that these results hold for improving the therapy of the condition is discussed in the following Chapter.

CHAPTER VIII

CONCLUSIONS AND FUTURE STRATEGIES

The aim of this work was to identify the defect in lipoprotein metabolism that is responsible for the hypertriglyceridaemia in ponies with hyperlipaemia. This objective has largely been met, and in doing so our understanding of lipoprotein metabolism in the horse has advanced considerably. As the biochemical, physiological and comparative implications of these findings have already been discussed, the purpose of this conclusion is to highlight what I feel are the important contributions of this work, to draw attention to the questions that remain, and to suggest investigative strategies that might provide the answers.

1. LIPOPROTEIN METABOLISM IN THE HORSE

The foundation of the studies of lipoprotein metabolism in ponies with hyperlipaemia was the characterisation of lipid transport in healthy horses and ponies. From this, the following features of significance emerged. The first was the confirmation of the absence of heterogeneity within the HDL class, with the particles possessing the character of the denser HDL subfraction found in man. The "trapping" of these particles at the far extreme of the HDL spectrum was later attributed to deficiency of CETP activity. However, in contrast with other species that lack CETP, HDL particles enriched in apoE and cholesteryl esters were not present. The reason for this may reside in the fact that post-prandial lipaemia, which is necessary for the generation of HDL_1 from HDL_3, is not prominent in the adult horse. Given that, it would be of interest to examine the size and density of HDL in the suckling foal, as these animals appear more typical of other monogastric species in their response to dietary fat.

That equine LDL is polydisperse was also confirmed and a third, previously unrecognised, subfraction was identified early in the LDL density range. The subject of LDL heterogeneity in man and some animal species is presently in vogue because of the realisation that certain subfractions are more atherogenic than others. Because coronary heart disease does not occur in the horse, this species has little to contribute to these investigations. However, in discussing the origins of LDL heterogeneity it should be remembered that in the adult horse the subfractions are present in absence of CETP activity and significant post-prandial lipaemia.

The presence of multiple species of apoB in the lower density lipoproteins was perhaps the most exciting feature of the early work. All the evidence indicated the equine liver has the capacity to secrete both large (apoB-100) and small (apoB-48)

forms of this protein. Those particles that possess apoB-48 would appear to behave in a similar fashion to human chylomicrons in that they are removed from the circulation before their density reaches that of LDL. As in the rat, the expression of these proteins appears to be subject to metabolic control such that the secretion of triglyceride is coordinated with the synthesis of the smaller protein. The evidence for this came from the fact that an increase in the ratio of apoB-48 to apoB-100 was associated with higher plasma triglyceride and VLDL concentrations, and triglyceride enrichment of VLDL, in the Shetland ponies compared with the Thoroughbred horses, and in the ponies with hyperlipaemia compared with healthy ponies.

Despite this, a number of questions remain to be answered. That the multiple species of apoB are synthesised and secreted by the liver needs to be proven. This would involve examining the size of apoB in hepatic cell homogenates or preferably in those proteins secreted *in vitro* by an equine hepatocyte cell culture. The molecular basis for the difference in size of the two larger proteins also needs to be established by consideration of the posttranslational modifications to which human apoB-100 is subject. To confirm that the apoB-48 originates from the editing of the apoB-100 message would be technically difficult as the cDNA and mRNA sequences of equine apoB are unknown. To show if the introduction of a stop codon into the message causes the premature end to translation would require the reverse transcription of mRNA, amplification of the cDNA by the polymerase chain reaction and sequence analysis of the product. In the meantime, the arrival of stable isotope tracer technologies offers the ability to study the turnover of these proteins *in vivo* by endogenous labelling of apoB with *e.g.* [^{15}N]glycine and measuring the enrichment of each protein isolated from apoVLDL by SDS-PAGE, as recently described for human apolipoproteins by Patterson, Hachey, Cook, Amann and Klein (1991).

The final feature of the equine lipid transport system that deserves mention is the failure of the adult ponies to show a typical monogastric post-prandial response to feeding or fat loading. This was in part rationalised by considering that the fat content of their diet was low and lacking in long chain fatty acids, and that the adults had "lost" the ability to synthesise chylomicrons. This adaptation was presumed to be age related as particles with the characteristics of chylomicrons were found in suckling foals that were being presented with a high fat load. This assumption needs to be strengthened by performing oral fat tolerance tests in foals. If the adults do indeed fail to produce chylomicrons, then the molecular basis for these would need to be determined. The most obvious mechanism for this would involve a downregulation in, or loss of, the intestine's ability to synthesise apoB. This could easily be examined by visualisation of apoB in sections of small intestine using an immunoperoxidase

technique such as that described for the expression of apolipoproteins in the human intestine (Green, Lefkowitch, Glickman, Riley, Quinet and Blum 1982).

2. THE PATHOGENESIS OF EQUINE HYPERLIPAEMIA AND PROSPECTS FOR THERAPY

Examination of the plasma lipoproteins in ponies with hyperlipaemia confirmed that the massive hypertriglyceridaemia was the result of the accumulation of VLDL in the circulation. Further, it showed for the first time that this was due to the emergence of a novel, triglyceride enriched, variant of VLDL. These particles seemed to be designed to maximise the export of triglyceride from the liver by having apoB-48 rather than apoB-100 as their structural protein. This, together with the observations that LPL was active and its cofactor present, indicated that contrary to previous speculation the hyperlipidaemia was the result of overproduction rather than defective catabolism of VLDL. The synthesis of triglyceride, and hence the secretion of VLDL, appeared to be driven by increased plasma FFA concentrations, but it was unclear whether these originated from adipose lipolysis or the action of LPL on VLDL. Although the absolute activity of LPL was not compromised, it remained possible that the fractional catabolism of VLDL was reduced in the face of the massive increases in substrate concentration.

These remaining questions were resolved by kinetic analysis of plasma FFA and VLDL-TG turnover. This confirmed that the increased triglyceride VLDL concentrations were entirely attributable to increased production of VLDL-TG by the liver, and that this was driven by a greatly increased flux of FFA into hepatic triglyceride synthesis. The absolute and fractional catabolism of VLDL was unaffected, but recycling of FFA from the lipolysis of VLDL-TG was indeed a significant source of plasma FFA and contributed to the input into triglyceride synthesis. Thus it is likely that the origin of the hypertriglyceridaemia lies in the mobilisation of FFA from adipose, but that recycling of precursors from VLDL-TG maintains the stimulus for triglyceride synthesis. These developments are summarised schematically in Figure 34, in which the pathogenesis of this hyperlipidaemia is explained.

Why should the pony respond in this way to the mobilisation of body fat, which afterall is a natural response to an energy deficit? One of the explanations certainly lays in the observation of others that the pony, and especially those animals that are obese and/or pregnant, are resistant to the action of insulin. This would conceivably allow adipose lipolysis to proceed in an uncontrolled fashion such that the flux of FFA to the liver becomes great. This resistance might well reflect the fact that obesity is prevalent in the pony breeds, in which a significant amount of the body

fat is stored within the abdomen. For in man, abdominal rather than gluteal-femoral obesity is associated with insulin resistance and hypertriglyceridaemia (Kissebah and Peiris 1989). The accumulation of fat in the abdomen is associated with hypertrophy of the adipocytes, which renders them more responsive to lipolytic stimuli, *e.g.* by catecholamines, so that they release FFA more readily (Mauriège, Després, Prud'homme, Pouliot, Marcotte, Tremblay and Bouchard 1991). This results in higher systemic FFA concentrations than in non-obese and non-abdominally obese humans, and because of the proximity of omental fat cells to the portal circulation, the liver is exposed to even higher FFA concentrations and responds by increasing triglyceride synthesis and VLDL secretion (Després 1991).

If a similar situation exists in the abdominally obese equine it may well explain the fact that plasma concentrations of FFA, triglyceride and VLDL are higher in ponies than in larger types of horse. In consensus with this, we found in a close relation of the horse, the donkey, that plasma triglyceride and VLDL concentrations were higher in obese animals rather than those of ideal body weight, and that these concentrations were positively correlated with those of FFA (Watson, Packard, Shepherd and Fowler 1990). Thus it is possible that abdominal obesity can explain the increased incidence of hyperlipaemia in ponies and donkeys that are overweight. Interestingly in man, the basal turnover of FFA is higher in abdominally obese women than men (Després 1991), which might also help account for the higher incidence of hyperlipaemia in mares rather than geldings or stallions.

The second feature of the pony that renders its response to adipose mobilisation peculiar is the failure of oxidation and ketone synthesis to dispose of the FFA. This had been suggested by the fact that ketonaemia is neither a feature of hyperlipaemia nor apparent when ponies are fasted, and was confirmed here by kinetic analysis of FFA turnover. In other species that mobilise FFA in response to an energy imbalance, such as the cow and the sheep, the fatty acids released are taken up by the liver and converted to ketones with resulting ketosis (Bruss 1989). Kinetically, this would be shown by an increase in FFA irreversibly lost from the plasma compartment rather than the pattern seen in the two hyperlipaemic ponies where the majority of FFA leaving plasma entered the hepatic triglyceride synthetic pathways. Whether the deficiency of fatty acid oxidation is specific to ponies or is a feature of the equine species as a whole is unclear, and at the moment cannot explain the almost exclusive incidence of hyperlipaemia in the pony breeds.

Whilst the kinetic data presented is convincing, there remain opportunities for building upon the findings of the last two Chapters. The number of turnovers carried out in ponies with hyperlipaemia was restricted to two. This was because it would have been unwise to transport animals whose condition was of concern, and of those

that reached Glasgow or were diagnosed there, four were euthanased almost immediately on humane grounds. Thus it might be more convenient if future turnover studies were based on the experimental reproduction of the disease by fasting ponies for two to three days. Concerns over the use of radioactive tracers would also be allayed if these studies were to use stable isotopes of glycerol or palmitic acid. Indeed, in combination with tracing the metabolism of apoB-48 and apoB-100, such studies would provide a powerful tool with which to examine the pathogenesis of this hyperlipidaemia and to investigate the metabolic regulation and coordination of hepatic apoB and triglyceride synthesis. With the experimental model of the disease, it would be interesting to see how the Thoroughbred horse differs in response to fasting in an attempt to explain the resistance of the larger types of horse to hyperlipaemia.

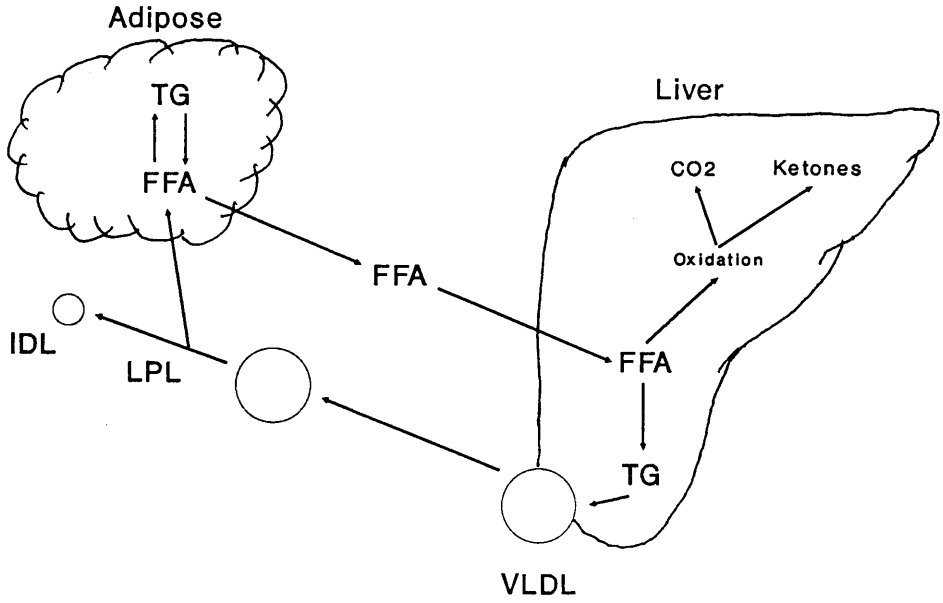
Finally I would like to discuss the prospects that the data presented in this thesis hold for improving the medical management of hyperlipaemia. Of the variety of lipid lowering agents available for the treatment of hyperlipidaemia in man, three groups possess suitable actions. The first of these, the fibric acid derivatives *e.g.* bezafibrate and gemfibrozil, are widely used in the treatment of primary hypertriglyceridaemia in man where reductions in plasma triglyceride concentrations of 54% have been reported (Saku, Gartside, Hynd and Kashyap 1985). The exact mode of action of these agents is unclear, although both decreased hepatic triglyceride synthesis and enhanced VLDL catabolism are thought to occur (Gaw and Shepherd 1991). The former appears secondary to a reduction in peripheral lipolysis and the flux of FFA to the liver, while the latter results from stimulation of lipoprotein lipase activity (Vessby and Lithel 1990) such that the residence time of VLDL-apoB is decreased to one third of pretreatment values (Shepherd, Packard, Stewart, Atmeh, Clark, Boag, Carr, Lorimer, Ballantyne, Morgan and Lawrie 1984). Inhibition of adipose lipolysis is the main mode of action of the second group, nicotinic acid and its derivatives *e.g.* acipimox, which exert their triglyceride lowering effect by reducing VLDL-TG synthesis (Grundy, Mok, Zech and Berman 1981). While reductions in plasma triglycerides in the region of 60% are attainable (Packard, Stewart, Third, Morgan, Lawrie and Shepherd 1980), tolerance can be poor in some patients, particularly those on nicotinic acid, because of cutaneous hyperaemia (Fattore and Sitori 1991). The final group, the fish oils, are also potent in reducing plasma triglyceride concentrations when used as dietary supplements (Sanders, Sullivan, Reeve and Thompson 1985) and appear to act primarily by reducing VLDL-TG synthesis (Harris *et al.* 1990).

Given the role that FFA mobilisation appears to play in the pathogenesis of equine hyperlipaemia, those agents that inhibit peripheral lipolysis and reduce FFA

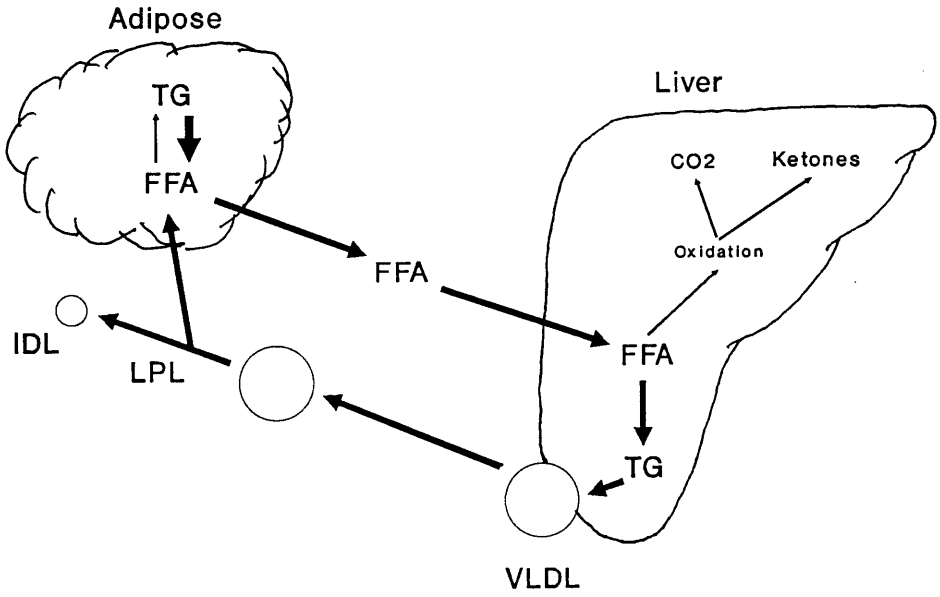
flux are attractive candidates. To test the efficacy of these agents in a clinical situation would be restricted by the number of patients required for a case controlled study. It might therefore be more appropriate to examine their effects on FFA and VLDL metabolism in ponies in which hyperlipidaemia has been induced by fasting. This could be done in two ways. First by analysing FFA and VLDL-TG kinetics in ponies that are fasted while being treated with the appropriate agent and comparing the results with those from the ponies when fasted without therapy. This would provide data on how effective the agents are in preventing FFA mobilisation and triglyceride synthesis in response to fasting and create the possibility of prophylactic therapy for ponies identified as at risk of developing hyperlipaemia, *e.g.* those with mild hypertriglyceridaemia in late pregnancy.

Secondly, the acute effects of these drugs in the hyperlipaemic pony should be examined, as in man both the fibrates and nicotinic acid derivatives are used for longer term management of hypertriglyceridaemia. This could be achieved using a constant infusion turnover protocol in which the drug is administered intravenously and immediate changes in FFA and VLDL-TG kinetics observed. This would provide the rationale on which to base therapy for the clinically hyperlipaemic pony and thus satisfy the longer term objectives of these studies, which are to offer an effective treatment for ponies with hyperlipaemia.

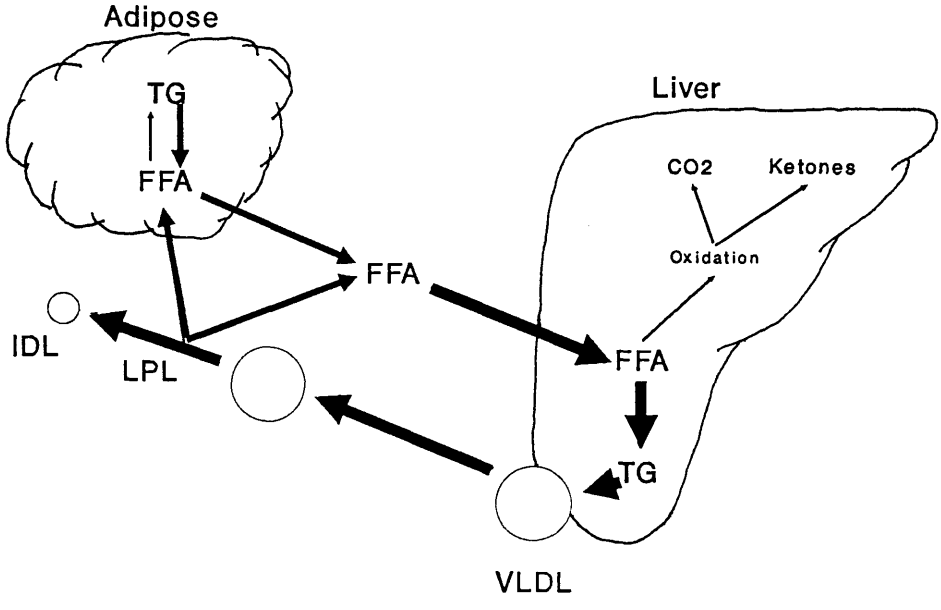
Figure 34. Schematic Representation of the Pathogenesis of Equine Hyperlipaemia.



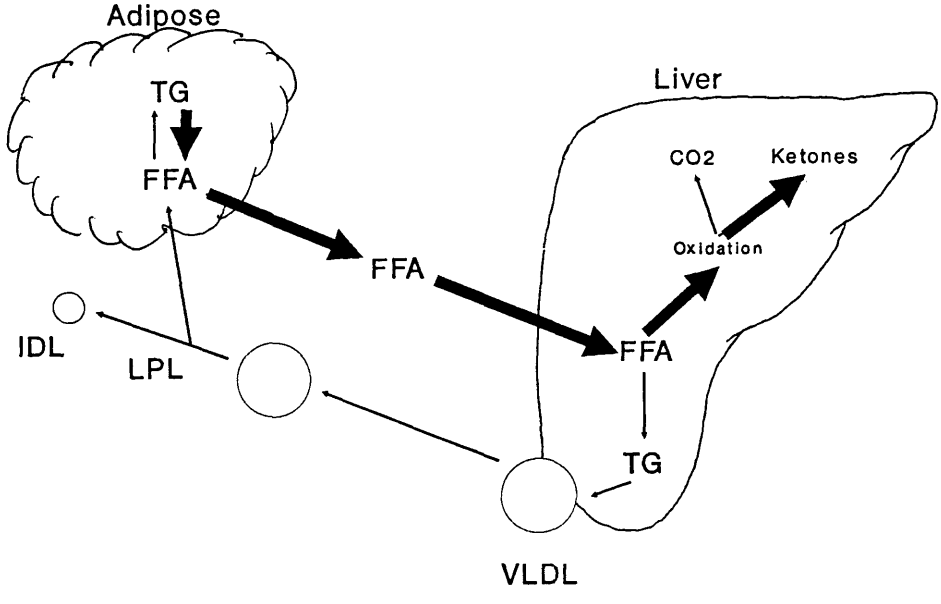
i) In the healthy, fed pony above, there is a low basal rate of adipose triglyceride turnover which feeds FFA into the liver where they are oxidised to CO_2 (and a small amount of ketones) or esterified and secreted as triglyceride in VLDL.



ii) When the animal enters a negative energy balance, or when the hormone sensitive lipase is activated by stress, adipose lipolysis and the flux of FFA to the liver increase. Because of the limited oxidative capacity of the equine liver, these FFA are resecreted as triglyceride, in the form of larger, triglyceride rich particles.



iii). As plasma VLDL concentrations rise, the activity of lipoprotein lipase (LPL) increases to the point that the FFA released overwhelm adipose uptake mechanisms and spill over into the general circulation. This recycling of FFA exacerbates the stimulus for triglyceride synthesis and VLDL concentrations continue to rise.



iv). Contrast the equine situation with that in ruminants, where the mobilised FFA are channelled into the oxidative and ketogenic pathways within the liver rather than those of triglyceride synthesis.

LIST OF APPENDICES

LIST OF APPENDICES (continued)

APPENDIX 1

Suppliers of Reagents and Equipment

Amersham International plc
Amersham Place, Little Chalfont, Amersham, Bucks. HP7 9NA.

Amicon Ltd
Upper Mill, Stonehouse, Glos. GL10 2BJ.

Analytichem International
Jones Chromatography Ltd
Tiry Berth Industrial Estate, New Road, Hengoed, Mid Glamorgan.

Baker Systems
Baker Instruments Ltd
Rusham Park, Whitehall Lane, Egham, Surrey, TW20 9NW.

Baxter Health Care Ltd
8 Tollpark Place, Wardpark East, Cumbernauld G68 0LN.

Beckman Instruments, Inc.
Beckman Instruments (UK) Ltd Analytical Sales and Service Operation
Progress Road, Sands Industrial Estate, High Wycombe, Bucks. HP12 4JL.

BDH Laboratory Supplies
McQuilkin and Co.
21 Polmadie Avenue, Glasgow G5 0BB.

Bio-Rad Laboratories Ltd
Bio-Rad House, Marylands Avenue, Hemel Hempstead, Herts. HP2 7TD.

Boehringer Mannheim GmbH
Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd
Bell Lane, Lewes, East Sussex BN7 1LG.

Brownlea Aquapore
Anachem Ltd
20 Charles Street, Luton, Beds. LU2 0EB

Denley-Tech Ltd
Denley Instruments Ltd
Natts Lane, Billinghurst, Sussex, RH14 9EY.

Dow Chemical Ltd
Uniscience Ltd
Wildmere Road, Banbury, Oxon. OX16 7JU.

Dynatech Laboratories Ltd
Daux Road, Billingshurst, West Sussex, RH14 95J

APPENDIX 1 (continued)

Gelman Sciences Ltd

Brackmill Business Park, Caswell Road, Northampton NN4 0ES.

Gilson HPLC System

Anachem Ltd

20 Charles Street, Luton, Beds. LU2 0EB.

Hewlett-Packard Ltd

Cain Road, Bracknell, Berks. RG12 1HN.

Hoefer Scientific Instruments

Scottish Biotechnology Instrumentation

Blairgowrie Business Centre, 60 High Street, Blairgowrie, PH10 6AF.

Isco Instrument Specialities Co.

Jones Chromatography Ltd

Tiry Berth Industrial Estate, New Road, Hengoed, Mid Glamorgan CF8 8AU.

Leo Laboratories Ltd

Longwick Road, Princes Risborough, Aylesbury, Bucks. HP17 9RR.

LKB Bromma

Pharmacia Ltd, Pharmacia LKB Biotechnology Division

Midsummer Boulevard, Central Milton Keynes, Bucks. MK9 3HP.

Nycomed Pharma AS

Nycomed (UK) Ltd

Nycomed House, 2111 Coventry road, Sheldon, Birmingham B26 3EA.

Paar Scientific Ltd

594 Kington Road, Raynes Park, London SW20 8DN.

Packard Instrument Co.

Canberra Packard Ltd

Brook House, 14 Station Road, Pangbourne, Berks. RG8 7DT.

Pharmacia AB

Pharmacia Ltd, Pharmacia LKB Biotechnology Division

Midsummer Boulevard, Central Milton Keynes, Bucks. MK9 3HP.

Randox Laboratories Ltd

Ardmore, Diamond Road, Crumlin, Co. Antrim BT29 4QY.

Resource Facility for Kinetic Analysis

Centre for Bioengineering

FL-20, University of Washington, Seattle, WA 98195, USA.

Roche

Roche Products Ltd

P.O. Box 8, Welwyn Garden City, Herts. AL7 3AY.

APPENDIX 1 (continued)

Scottish Antibody Production Unit
Law Hospital, Carluke, Lanarkshire ML8 5ES.

Sartorius Ltd
Longmead Business Centre, Blenheim Road, Epsom, Surrey KT19 9QN.

Sigma
Sigma Chemical Company Ltd
Fancy Road, Poole, Dorset BH17 7TG.

Spectrum Medical Industries Inc.
Orme Technology
P.O. Box 3, Stakehill Industrial Park, Middleton, Manchester M24 2RH.

Techne
Scotlab
Kirkshaw Road, Coatbridge, Lanarkshire ML5 8AD

TSK-Gel HPLC Columns and Packings
Laboratory Impex Ltd
111-113 Waldegrave Road, Teddington, Middlesex, TW11 8LL

Wako Chemicals GmbH
Alpha Laboratories Ltd
40 Paman Drive, Eastleigh, Hampshire SO5 4NU.

Whatman International Ltd
Whatman Labsales Ltd
St. Leonard's Road, 20/20 Maidstone, Kent ME16 0LS.

APPENDIX 2

Derivation of Equation for Calculating Post-heparin Lipolytic Activity

$$\text{Activity (umolFA/ml/hr)} = \frac{[\text{Sample cpm} - \text{Blank cpm}] \times 755.1}{\text{Total cpm} - \text{background cpm}}$$

$$\text{Where } 755.1 = \frac{1.5059^{a} \times 3^{b} \times 100^{c} \times 2.45^{d}}{2.05^{e} \times 1^{f} \times 0.715^{g}}$$

[a] is umol triolein/assay tube

[b] three fatty acids are released from one molecule of triolein

[c] to correct 10 ul sample to 1 ml

[d] volume of upper phase after extraction (2.45 ml $\pm$0.05, n=64)

[e] volume of lower phase after extraction (2.05 ml $\pm$0.05, n=64)

[f] incubation time in hours

[g] % oleic acid extracted into upper phase from Belfrage and Vaughan (1969)

APPENDIX 3

Chemical Composition of Lipoprotein Classes Isolated by Gel Filtration

Lipoprotein	Animal	% Particle Mass			
		Cholesterol	Triglyceride	Phospholipid	Protein
VLDL	TB-13	19.9	39.5	18.1	22.6
	TB-14	14.3	46.2	21.4	18.1
	TB-15	13.7	57.0	9.2	10.1
	TB-16	11.3	54.2	14.0	17.5
	TB-23	14.2	55.8	16.8	13.2
	TB-30	10.4	48.6	16.0	25.0
	TB-32	22.5	49.3	8.7	19.5
	TB-51	19.8	49.1	12.9	18.2
	SP-1	12.9	50.9	14.4	21.8
	SP-2	17.8	48.0	15.1	19.2
	SP-7	10.2	64.0	13.6	12.0
	SP-9	12.9	59.8	17.6	9.5
	SP-10	10.0	54.5	11.0	14.5
	SP-11	15.8	55.3	11.9	16.9
	SP-12	18.9	53.4	10.4	17.3
	SP-25	16.6	56.9	12.3	14.2
	SP-26	15.9	57.0	12.5	14.6
	SP-27	12.4	58.4	17.2	12.0
	SP-28	14.5	52.4	16.5	16.6
	SP-31	18.9	59.3	15.0	16.8
LDL	TB-13	40.5	5.4	25.3	30.8
	TB-14	32.3	4.5	28.1	35.1
	TB-15	49.7	5.6	18.3	26.4
	TB-16	38.2	2.7	27.5	31.5
	TB-23	40.8	7.4	21.6	30.2
	TB-30	46.4	6.5	16.3	28.9
	TB-32	45.7	9.4	22.0	22.9
	TB-51	47.8	8.0	21.9	32.3

APPENDIX 3 (continued)

Lipoprotein	Animal	% Particle Mass			
		Cholesterol	Triglyceride	Phospholipid	Protein
LDL	SP-1	42.2	4.7	22.4	30.7
	SP-2	44.7	6.2	24.2	24.9
	SP-7	57.8	3.7	18.3	20.1
	SP-9	40.2	4.7	27.2	27.5
	SP-10	47.2	3.8	24.5	24.5
	SP-11	34.7	6.5	26.4	32.4
	SP-12	44.8	5.0	20.7	29.5
	SP-25	41.3	4.6	26.1	28.4
	SP-26	51.3	4.0	24.9	19.7
	SP-27	37.2	5.5	25.7	32.0
	SP-28	39.2	6.6	23.7	30.4
	SP-31	45.5	5.8	24.4	24.2
HDL	TB-13	22.8	3.4	28.5	45.2
	TB-14	---	---	---	---
	TB-15	27.0	13.8	26.5	42.8
	TB-16	19.2	10.0	22.6	48.2
	TB-23	21.7	4.0	25.0	29.2
	TB-30	27.0	1.6	24.6	46.8
	TB-32	25.2	3.4	25.9	45.5
	TB-51	19.4	12.4	34.8	38.4
	SP-1	21.6	10.1	22.8	45.5
	SP-2	28.9	5.5	34.1	41.4
	SP-7	17.9	10.1	21.2	44.8
	SP-9	23.2	8.0	23.9	42.9
	SP-10	27.9	10.0	24.4	37.2
	SP-11	29.5	0.8	26.2	43.4
	SP-12	19.7	4.1	25.2	51.0
	SP-25	22.0	0.0	30.5	47.3
	SP-26	19.6	2.3	32.8	45.3
	SP-27	24.0	0.9	24.6	50.5
	SP-28	21.0	1.0	27.1	50.8
	SP-31	13.1	9.2	27.1	50.6

APPENDIX 4

Agarose Gel Filtration Elution Profile Parameters

Animal	VLDL			LDL			HDL		
	Vol.[a]	Width[b]	Height[c]	Vol.	Width	Height	Vol.	Width	Height
TB-13	84	10	0.10	118	30	0.13	156	34	1.32
TB-14	82	11	0.42	119	28	0.15	162	38	2.05
TB-15	80	9	0.09	120	24	0.06	157	29	1.77
TB-16	81	10	0.18	117	30	0.17	157	35	1.95
TB-23	78	11	0.18	116	32	0.22	154	32	1.93
TB-30	81	10	0.10	121	31	0.11	161	37	1.69
TB-32	78	11	0.14	120	26	0.08	157	36	1.82
TB-51	79	11	0.16	124	26	0.12	157	37	1.90
SP-1	80	1	0.51	115	32	0.14	154	38	1.97
SP-2	79	11	0.35	121	38	0.09	149	39	1.43
SP-7	79	12	0.92	114	38	0.23	152	33	1.47
SP-9	78	12	0.56	114	32	0.25	156	34	2.42
SP-10	81	11	0.38	125	29	0.16	162	34	2.34
SP-11	79	9	0.45	119	26	0.18	156	30	2.40
SP-12	78	11	0.23	118	31	0.10	155	35	1.87
SP-25	80	11	0.26	118	29	0.12	157	38	1.93
SP-26	77	12	0.81	113	30	0.22	150	38	2.46
SP-27	78	11	0.12	116	30	0.10	154	39	1.37
SP-28	80	11	0.41	111	32	0.15	157	39	2.16
SP-31	78	11	0.48	115	32	0.12	154	37	1.89

[a] peak elution volume (ml), [b]elution width of peak (ml), [c]peak height (OD_{280nm})

APPENDIX 5

Particle Size and Composition of VLDL, LDL Subfractions, and HDL Isolated by Rate-zonal Ultracentrifugation

Animal/ Lipoprotein	Diameter (nm)	Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
VLDL						
TB-13	nd	11.6	55.0	ND	14.4	19.0
TB-14	nd	13.9	55.3	ND	11.9	18.8
TB-30	nd	9.1	64.9	ND	10.2	15.8
TB-32	nd	23.3	40.8	ND	7.2	28.7
SP-1	nd	11.5	55.5	ND	12.7	20.3
SP-8	nd	12.2	66.1	ND	13.8	17.9
SP-9	nd	22.1	44.7	ND	12.3	19.2
SP-10	nd	15.0	48.0	ND	16.9	20.1
LDL_1						
TB-13	26.8	47.4	9.4	5.4	20.2	17.6
TB-14	26.3	42.5	5.2	ND	15.3	26.9
TB-30	31.2	42.6	8.4	ND	19.2	29.8
TB-32	24.4	38.4	9.2	ND	21.4	35.5
SP-1	27.3	38.5	14.0	4.7	16.1	26.7
SP-2	25.4	45.5	0.0	ND	19.6	34.9
SP-9	25.6	42.4	14.5	1.0	17.6	24.5
SP-10	27.8	47.7	11.7	ND	11.1	29.5
LDL_2						
TB-13	25.8	45.5	8.8	5.2	20.2	20.2
TB-14	24.2	48.9	15.9	ND	14.7	20.6
TB-30	25.7	31.2	4.0	7.2	20.2	37.4
TB-32	23.8	36.7	6.2	8.4	23.0	35.7
SP-1	24.1	42.4	11.4	ND	19.6	26.6
SP-8	23.6	48.7	3.5	4.3	22.1	21.4
SP-9	23.6	45.6	9.0	5.1	17.6	22.7
SP-10	25.5	48.7	12.5	2.8	16.2	21.8

APPENDIX 5 (continued)

Animal/ Lipoprotein	Diameter (nm)	Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
LDL_3						
TB-13	23.0	46.9	9.5	ND	15.4	28.2
TB-14	22.3	59.5	7.1	ND	12.8	20.6
TB-30	23.4	40.8	3.9	4.0	17.4	34.1
SP-1	22.4	47.4	5.4	3.9	20.9	22.6
SP-8	21.4	52.8	6.2	ND	17.0	24.0
SP-9	21.5	58.5	3.8	1.1	17.1	19.5
SP-10	22.8	43.5	3.6	ND	22.0	30.9
HDL						
TB-13	8.30	22.7	0.0	ND	24.9	52.4
TB-14	8.24	21.5	8.1	1.7	26.8	41.8
TB-30	8.48	23.1	0.7	ND	31.0	45.2
TB-32	8.36	29.4	7.3	ND	26.3	37.0
SP-1	8.36	22.8	0.0	ND	32.8	44.4
SP-8	8.30	23.5	4.5	1.5	26.0	44.5
SP-9	8.42	25.2	8.5	ND	27.8	38.5
SP-10	8.46	23.4	1.7	1.6	32.4	40.9

APPENDIX 6

Composition of Plasma Lipoproteins Isolated from 18 Thoroughbred Horses and 18 Shetland Ponies by Sequential Flotation Ultracentrifugation

Lipoprotein/ Animal	Sex	Percentage Particle Mass				
		Cholesteryl	Triglyceride	Free Cholesterol	Phospholipid	Protein
VLDL						
TB-53	F	9.0	50.3	---	13.9	26.8
TB-54	F	8.9	59.1	---	11.0	20.9
TB-55	F	8.6	58.8	---	13.7	18.9
TB-56	F	6.0	56.9	---	11.1	26.0
TB-57	F	7.9	55.2	---	16.2	20.3
TB-58	F	8.9	58.8	---	13.7	18.9
TB-62	F	3.9	53.7	---	18.2	24.3
TB-64	F	8.1	58.3	---	15.0	18.6
TB-72	F	7.1	65.1	---	12.6	15.4
TB-13	G	5.9	54.5	---	14.7	24.9
TB-14	G	7.1	54.6	---	14.2	24.1
TB-15	G	11.4	60.7	---	10.4	17.5
TB-59	G	10.7	56.3	---	14.0	19.0
TB-60	G	7.7	61.6	---	14.3	16.4
TB-66	G	8.3	59.6	---	14.3	17.5
TB-67	G	6.4	52.2	---	11.8	29.5
TB-68	G	7.6	55.0	---	16.2	21.1
TB-69	G	7.6	63.6	---	12.9	15.8
SP-1	F	10.0	58.5	---	13.3	18.2
SP-2	F	9.6	58.7	---	14.8	16.9
SP-3	F	10.8	58.7	---	17.6	14.6
SP-7	F	10.0	58.4	---	13.4	18.2
SP-8	F	9.2	59.3	---	13.6	17.9
SP-11	F	13.9	58.8	---	13.9	12.6
SP-37	F	7.2	59.6	---	18.1	14.9
SP-L	F	8.2	61.5	---	16.5	16.4
SP-R	F	9.2	58.6	---	18.9	18.9

APPENDIX 6 (continued)

Lipoprotein Animal	Sex	Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
VLDL						
SP-B	F	9.9	58.7	---	18.4	18.4
SP-T	F	9.1	60.9	---	13.9	16.0
SP-S	F	9.0	63.0	---	13.8	13.8
SP-5	G	11.5	58.9	---	14.9	14.7
SP-9	G	11.5	47.2	---	18.5	22.8
SP-10	G	13.5	54.8	---	14.1	17.5
SP-12	G	10.6	57.4	---	14.8	17.2
SP-41	G	9.0	59.2	---	16.7	16.7
SP-PA	G	8.7	67.1	---	10.8	13.4
LDL						
TB-53		44.6	6.0	6.2	19.0	24.2
TB-54		46.4	9.5	___	13.5	30.7
TB-55		44.0	7.0	5.2	18.3	25.5
TB-56		44.3	6.2	6.3	18.9	24.4
TB-57		46.6	5.5	5.6	17.7	24.6
TB-58		41.4	7.3	4.0	20.8	26.3
TB-62		43.6	3.1	3.5	19.4	30.5
TB-64		46.0	3.7	4.2	19.7	26.4
TB-72		36.9	5.1	4.7	23.5	29.9
TB-13		48.2	3.6	7.4	26.0	14.8
TB-14		40.7	5.1	5.1	22.6	26.6
TB-15		26.2	4.9	7.5	30.0	33.4
TB-59		44.7	6.8	---	21.0	27.7
TB-60		41.3	6.6	4.9	21.4	25.8
TB-66		42.7	3.1	6.0	22.8	25.3
TB-67		40.5	2.9	4.1	21.9	30.9
TB-68		39.6	2.9	6.1	22.6	28.8
TB-69		36.4	7.5	4.6	22.1	29.4
SP-1		35.7	9.0	5.3	24.1	25.6

APPENDIX 6 (continued)

Lipoprotein Animal	Sex	Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
LDL						
SP-2		34.2	3.6	4.7	34.1	23.4
SP-3		34.6	5.7	4.5	24.0	31.2
SP-7		38.8	8.5	3.8	23.3	23.6
SP-8		44.1	6.2	4.7	24.6	20.4
SP-11		40.1	7.2	5.6	25.6	21.5
SP-37		37.9	10.4	---	23.0	24.7
SP-L		41.5	3.8	3.7	22.9	28.1
SP-R		41.7	4.0	---	19.3	34.9
SP-B		44.5	6.7	---	19.8	25.9
SP-T		43.1	5.6	---	22.2	29.1
SP-S		38.5	4.7	3.5	21.4	31.9
SP-5		33.9	5.4	6.7	20.5	25.7
SP-9		51.9	2.5	6.6	26.2	12.9
SP-10		51.6	4.7	5.4	27.2	11.1
SP-12		43.8	8.2	5.1	28.1	12.5
SP-41		43.8	3.9	4.8	19.6	25.9
SP-PA		41.9	2.9	6.9	25.3	23.1
HDL						
TB-53		24.3	1.9	2.1	23.4	48.2
TB-54		22.3	1.7	2.1	24.9	49.0
TB-55		23.1	1.4	2.2	25.0	48.4
TB-56		23.4	1.5	2.1	24.3	50.3
TB-57		24.3	1.3	1.8	22.3	50.3
TB-58		22.8	0.9	2.6	30.8	42.9
TB-62		18.2	0.5	2.1	26.2	53.1
TB-64		21.8	0.4	4.2	35.6	38.6
TB-72		14.8	0.3	3.5	29.5	51.9
TB-13		17.6	0.4	1.9	21.9	56.3
TB-14		17.5	0.4	2.0	22.0	56.2
TB-15		20.7	0.4	2.8	23.8	52.7

APPENDIX 6 (continued)

Animal	Percentage Particle Mass				
	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
HDL					
TB-59	22.4	0.0	2.1	30.6	44.8
TB-60	24.0	0.6	4.8	33.7	40.4
TB-66	16.7	5.5	2.4	27.3	53.0
TB-67	16.2	0.3	3.5	27.9	52.0
TB-68	17.4	0.5	2.1	27.3	52.6
TB-69	16.8	0.3	3.3	27.4	52.1
SP-1	22.4	0.9	3.0	28.5	45.1
SP-2	18.0	0.7	1.9	25.3	54.1
SP-3	14.2	0.4	1.5	20.2	63.9
SP-7	19.7	0.7	3.0	27.5	49.2
SP-8	20.8	0.6	2.9	26.6	46.2
SP-11	30.3	0.3	2.5	23.2	45.7
SP-37	21.5	0.8	3.1	36.8	38.6
SP-L	20.7	0.9	2.5	28.1	47.7
SP-R	13.4	1.1	3.2	30.8	51.6
SP-B	19.7	0.8	2.4	26.5	51.5
SP-T	19.2	1.7	2.8	28.9	47.3
SP-S	23.3	0.5	5.9	35.0	35.3
SP-5	16.8	0.3	2.2	24.2	56.5
SP-9	20.3	0.3	3.1	27.7	48.5
SP-10	21.4	0.6	3.2	24.9	49.9
SP-12	13.4	0.4	1.2	19.6	65.4
SP-41	20.6	0.6	3.1	28.2	47.5

APPENDIX 7

Apolipoprotein Composition of Lipoproteins Isolated from Eight Thoroughbred Horses and Shetland Ponies by Flotation Ultracentrifugation

Lipoprotein/ Animal	Percentage of Protein Mass						
	B-100	B-48	E	A-I	C-II	C-III	A-II
VLDL							
TB-13	20.5	10.6	9.9	7.3	23.8	28.0	---
TB-14	27.4	10.1	7.2	3.2	25.3	26.7	---
TB-15	20.2	9.4	17.4	3.5	19.3	30.3	---
TB-62	23.2	11.7	11.9	4.4	21.6	27.2	---
TB-64	23.6	10.9	14.4	4.7	22.2	24.2	---
TB-66	27.6	14.0	7.2	3.5	19.9	27.8	---
TB-68	23.1	8.7	11.6	5.2	20.3	31.2	---
TB-72	23.4	11.4	4.3	2.8	31.0	27.1	---
SP-1	19.2	13.1	10.6	12.0	26.6	29.4	---
SP-2	22.9	9.9	3.9	8.4	22.2	28.2	---
SP-3	23.0	6.8	11.8	3.9	18.9	41.6	---
SP-5	17.1	12.5	13.6	11.2	29.0	27.6	---
SP-7	19.2	11.3	11.2	13.6	29.0	35.5	---
SP-8	27.8	13.1	18.1	11.8	11.2	34.4	---
SP-9	19.2	16.4	3.6	11.2	9.7	39.9	---
SP-10	12.7	9.1	12.9	10.9	32.2	23.1	---
SP-11	28.3	9.2	18.1	9.7	20.8	27.6	---
SP-12	18.7	13.1	6.6	6.9	22.7	33.5	---
SP-37	22.1	14.7	12.2	12.1	15.3	19.1	---
SP-41	20.0	13.2	9.8	8.3	18.2	28.8	---
SP-L	20.5	10.5	10.6	10.1	25.6	20.0	---
SP-R	21.0	16.4	11.3	6.4	20.6	25.0	---
SP-B	17.0	9.7	3.2	1.4	26.2	45.5	---
SP-T	19.8	7.7	6.6	2.0	24.8	39.2	---
SP-S	20.0	11.6	3.0	4.9	27.5	33.0	---
SP-P	26.3	8.6	4.3	1.2	25.1	34.6	---

APPENDIX 7 (continued)

Lipoprotein/ Animal	Percentage of Protein Mass						
	B-100	B-48	E	A-I	C-II	C-III	A-II
LDL							
TB-13	70.3	---	11.4	---	11.3	7.1	---
TB-14	70.2	---	4.9	---	11.5	13.4	---
TB-15	83.8	---	3.3	---	4.4	8.5	---
TB-62	67.1	---	8.6	---	12.1	12.1	---
TB-64	79.5	---	2.9	---	6.1	11.6	---
TB-66	73.0	---	5.9	---	10.3	10.7	---
TB-68	77.8	---	7.9	---	7.2	7.1	---
TB-72	65.2	---	6.8	---	12.1	14.0	---
SP-1	73.1	---	6.8	---	9.1	9.1	---
SP-2	74.2	---	5.1	---	10.3	5.7	---
SP-3	67.8	---	8.9	---	10.6	10.1	---
SP-5	83.2	---	3.9	---	7.5	11.0	---
SP-7	71.5	---	4.2	---	5.5	12.8	---
SP-8	79.0	---	7.9	---	5.1	12.9-	---
SP-9	76.5	---	10.7	---	6.4	6.3	---
SP-10	79.1	---	3.9	---	9.0	8.0	---
SP-37	87.2	---	7.0	---	3.0	2.9	---
SP-41	82.7	---	4.7	---	6.8	5.8	---
SP-L	84.7	---	5.9	---	6.0	3.4	---
SP-R	75.1	---	2.2	---	7.8	14.9	---
SP-B	73.0	---	8.4	---	8.5	10.1	---
SP-T	67.1	---	12.3	---	7.0	13.5	---
SP-S	81.9	---	9.3	---	3.2	15.5	---
SP-P	84.9	---	2.9	---	5.0	7.2	---

Appendix 7 (continued)

Lipoprotein/ Animal	Percentage of Protein Mass						
	B-100	B-48	E	A-I	C-II	C-III	A-II
HDL							
TB-13	---	---	6.3	78.2	5.6	2.6	7.3
TB-14	---	---	13.8	73.8	3.7	4.2	4.5
TB-15	---	---	4.5	81.5	1.2	2.0	10.8
TB-62	---	---	6.1	85.2	1.9	1.5	5.3
TB-64	---	---	7.3	80.6	1.9	2.5	7.6
TB-66	---	---	10.3	82.4	0.9	1.1	5.3
TB-68	---	---	12.2	70.8	2.5	3.6	10.9
TB-72	---	---	10.1	68.3	8.7	8.7	6.7
SP-1	---	---	0.7	87.3	1.8	4.8	5.4
SP-2	---	---	0.4	79.8	8.8	9.3	1.8
SP-3	---	---	2.6	71.9	9.4	5.0	11.1
SP-5	---	---	2.5	85.3	6.6	6.1	12.2
SP-7	---	---	3.9	74.5	6.0	6.0	9.6
SP-8	---	---	1.9	75.2	7.9	5.1	10.0
SP-9	---	---	4.7	77.4	3.7	3.7	9.5
SP-10	---	---	2.4	77.6	5.8	5.8	9.7
SP-12	---	---	5.5	79.7	5.0	6.4	3.3
SP-37	---	---	6.9	79.8	2.8	3.3	7.2
SP-41	---	---	3.9	86.4	0.9	6.8	2.0
SP-L	---	---	3.7	84.4	3.1	4.7	4.1
SP-R	---	---	8.7	77.8	3.2	3.5	6.8
SP-B	---	---	4.2	83.8	2.2	5.5	4.4
SP-T	---	---	11.0	76.5	3.6	1.1	7.9
SP-S	---	---	3.8	84.9	3.6	2.8	4.9
SP-P	---	---	4.1	76.4	3.0	3.5	13.1

APPENDIX 8

Plasma Cholesterol, Triglyceride, Free Fatty Acid (FFA) and Lipoprotein Cholesterol Concentrations in Healthy Thoroughbred Horses (TB) and Shetland Ponies (SP)

Animal	Plasma Concentration (mmol/l)					
	Cholesterol	Triglyceride	VLDL	LDL	HDL	FFA
TB-13	2.50	0.30	0.10	1.00	1.40	nd
TB-14	2.00	0.25	0.20	0.60	1.20	nd
TB-15	2.00	0.20	0.05	0.50	1.45	nd
TB-53	2.65	0.35	0.30	1.10	1.25	nd
TB-54	2.20	0.65	0.35	0.45	1.04	nd
TB-55	2.45	0.35	0.25	0.65	1.55	nd
TB-56	2.50	0.30	0.20	0.70	1.60	nd
TB-57	2.35	0.30	0.30	0.90	1.15	nd
TB-58	1.75	0.35	0.30	0.40	1.05	nd
TB-59	1.90	0.20	0.10	0.50	1.30	nd
TB-60	2.45	0.40	0.20	0.65	1.60	nd
TB-62	2.00	0.15	0.15	0.40	1.45	nd
TB-64	2.00	0.30	0.20	0.65	1.15	nd
TB-66	2.20	0.70	0.20	0.70	1.30	nd
TB-67	2.15	0.15	0.05	0.45	1.65	nd
TB-68	1.90	0.15	0.10	0.45	1.35	nd
TB-69	2.55	0.50	0.25	0.45	1.85	nd
TB-72	2.50	0.45	0.35	0.50	1.65	nd
SP-1	2.05	0.40	0.15	0.45	1.40	0.36
SP-2	1.95	0.15	0.20	0.40	1.35	0.41
SP-3	1.65	0.15	0.10	0.30	1.25	0.30
SP-5	2.00	0.70	0.35	0.20	1.45	0.15
SP-7	2.15	0.55	0.10	0.65	1.40	0.22
SP-8	2.95	0.70	0.25	0.65	2.05	0.24
SP-9	2.80	0.25	0.25	1.05	1.50	0.52
SP-10	2.55	0.45	0.30	0.75	1.50	0.33

nd, not determined

APPENDIX 8 (continued)

Animal	Plasma Concentration (mmol/l)					
	Cholesterol	Triglyceride	VLDL	LDL	HDL	FFA
SP-11	2.50	0.70	0.30	0.55	1.65	0.31
SP-12	1.40	0.58	0.15	0.45	0.80	0.55
SP-37	2.35	0.55	0.45	0.70	1.20	0.32
SP-41	2.15	0.40	0.10	0.65	1.40	0.36
SP-L	2.20	0.45	0.35	0.70	1.15	0.58
SP-R	1.85	0.40	0.35	0.35	1.15	0.62
SP-B	1.75	0.50	0.45	0.30	1.00	0.55
SP-T	1.60	0.50	0.45	0.25	0.90	0.62
SP-S	2.35	0.55	0.45	0.80	1.10	0.11
SP-P	2.10	0.70	0.55	0.50	1.50	0.48

APPENDIX 9

Selective Measurement of Lipoprotein Lipase and Hepatic Lipase Activity

Intra-assay performance: Post-heparin plasma with low and high LPL and HL activity was analysed in duplicate in a single assay run.

	Activity (umolFA/ml/h)			
	LPL		HL	
Aliquots	Low	High	Low	High
A	2.10	5.68	2.52	7.28
B	2.34	5.82	2.51	7.28
C	2.16	5.16	2.58	7.42
D	2.06	6.24	2.70	7.69
E	2.02	6.37	2.78	6.24
F	2.27	6.13	2.86	7.08
G	2.14	6.25	2.95	7.31
H	2.65	6.04	2.91	6.72
I	2.04	6.16	3.05	6.80
J	2.31	6.18	2.95	7.42

Interassay Performance: Post-heparin plasma with low and high LPL and HL activity were analysed in duplicate in seven consecutive assays.

	Activity (umolFA/ml/h)			
	LPL		HL	
Date of Assay	Low	High	Low	High
23/10/90	2.00	5.52	2.30	6.42
30/10/90	1.76	5.59	2.68	6.39
31/10/90	2.21	6.10	2.78	7.12
05/11/90	2.05	6.47	2.67	6.74
08/11/90	2.30	7.02	2.87	7.20
09/11/90	2.26	5.90	2.78	6.72
11/12/90	1.76	5.73	2.59	6.34

APPENDIX 10

Lipoprotein Lipase and Hepatic Lipase Activities in Post-heparin Plasma from Eight Thoroughbred Horses (TB) and 16 Shetland Ponies (SP)

Animal	Sex	umolFA/ml/h LPL	umolFA/ml/h HL	Animal	Sex	umolFA/ml/h LPL	umolFA/ml/h HL
TB-K	F	2.71	2.92	SP-L	F	5.42	6.47
TB-20	F	2.75	3.53	SP-R	F	3.85	5.66
TB-34	F	4.87	3.18	SP-B	F	2.95	4.45
TB-64	F	4.78	5.50	SP-T	F	2.88	1.97
TB-14	G	1.73	1.78	SP-7	F	3.43	6.46
TB-15	G	2.12	1.38	SP-S	F	2.27	4.43
TB-16	G	3.96	2.63	SP-8	F	2.28	5.25
TB-D	G	3.53	4.81	SP-37	F	3.25	7.29
				SP-C	F	2.00	2.30
				SP-2	F	7.76	5.09
				SP-9	G	4.28	4.60
				SP-10	G	3.13	5.03
				SP-BA	G	2.33	3.22
				SP-12	G	2.58	1.04
				SP-41	G	6.07	4.78
				SP-PA	G	2.11	3.25

APPENDIX 11

Activation of Equine Lecithin:Cholesterol Acyl Transferase by Equine and Human ApoA-I

Animal	LCAT Activity (nmolCE/ml/hr)	
	+ Equine ApoA-I	+ Human ApoA-I
TB-13	22.61	22.20
TB-14	21.48	20.92
TB-15	17.84	21.00
SP-L	24.38	20.29
SP-R	21.59	24.36

APPENDIX 12

Lecithin:Cholesterol Acyl Transferase Activity in 12 Thoroughbred Horses (TB) and 12 Shetland Ponies (SP)

Animal	Sex	LCAT nmolCE/ml/h	Animal	Sex	LCAT nmolCE/ml/h
TB-34	F	18.62	SP-L	F	20.05
TB-B	F	22.80	SP-R	F	29.71
TB-C	F	20.86	SP-B	F	23.14
TB-K	F	15.54	SP-7	F	22.86
TB-P	F	25.95	SP-T	F	18.26
TB-S	F	25.64	SP-8	F	29.26
TB-13	G	16.96	SP-37	F	23.65
TB-14	G	21.48	SP-C	F	18.09
TB-15	G	15.82	SP-F	F	26.30
TB-16	G	19.53	SP-9	G	23.63
TB-D	G	19.79	SP-10	G	29.34
TB-E	G	19.51	SP-B	G	21.47

APPENDIX 13

Lecithin:Cholesterol Acyl Transferase Activity and HDL Composition in 12 Shetland Ponies

Animal	LCAT nmolCE/ml/h	% Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
SP-L	14.74	17.6	1.0	3.9	29.7	47.9
SP-R	18.71	17.9	1.0	3.0	28.8	49.3
SP-B	20.76	19.2	1.1	3.1	29.2	47.4
SP-7	23.96	20.5	1.1	3.0	29.3	46.1
SP-T	18.79	20.3	1.1	2.8	27.9	47.9
SP-8	23.12	19.6	1.3	2.3	27.1	49.8
SP-37	19.30	18.7	1.2	3.4	30.1	46.6
SP-9	25.63	19.1	1.0	3.4	28.8	47.7
SP-10	27.64	20.1	1.2	3.1	29.9	45.8
SP-B	21.62	18.2	0.7	3.9	33.3	43.9
SP-F	26.30	19.6	0.9	3.9	31.0	44.6
SP-C	17.25	19.0	0.6	4.2	33.8	42.4
Mean	21.49	19.15	1.0	3.3	29.9	46.6
SD	3.94	0.9	0.2	0.6	2.0	2.2
Correlation with LCAT (r)		0.47	-0.04	-0.14	0.16	-0.30

APPENDIX 14

Cholesteryl Ester Transfer Protein Activity in Four Thoroughbred Horses (TB) and Four Shetland Ponies (SP)

	% Transfer/2.5h						
	Volume of LPDP					Protein	
Animal	1 ul	2.5 ul	5 ul	10 ul	35 ul	ug/ul	%T/ug
SP-1	1.4	-1.7	0.5	-1.9	4.0	42.0	-
SP-4	-4.9	-4.5	-1.5	2.9	11.1*	45.0	*0.007
SP-5	-0.9	-1.7	4.9	3.9	0.7	47.5	-
SP-6	2.0	3.6	3.3	1.5	6.1*	44.5	*0.004
TB-14	0.7	-1.1	4.3	4.3	11.2*	44.3	*0.007
TB-C	-10.2	3.0	1.5	-0.9	4.8	47.5	-
TB-D	0.5	7.0*	4.9	-1.6	2.6	41.5	*0.077
TB-16	2.4	-8.0	-15.2	-9.4	-3.7	44.0	-

cv of blanks was $\pm$5.6%, values greater than this are marked * and used to give %T/ug.

APPENDIX 15

Cholesteryl Ester Transfer Protein Activity in 12 Shetland Ponies (SP)

Animal	% Transfer					Protein	
	Volume of LPDP						
	1 ul	5 ul	10 ul	20 ul	35 ul	ug/ml	%T/ug
SP-L	-	-	3.0	4.5*	4.5*	49.0	*0.004
SP-R	-	-	4.8*	1.7	7.5*	56.5	*0.006
SP-B	-	-	-0.4	2.1	-2.3	55.5	-
SP-7	-	-	1.7	9.6*	-2.7	38.5	*0.012
SP-T	-	-	1.8	12.3*	-2.4	52.5	*0.012
SP-8	-	-	1.8	3.8*	-4.5	62.0	*0.004
SP-37	-	-	0.8	4.7*	0.5	66.5	*0.004
SP-9	-	-	3.7*	4.9*	3.5*	51.5	*0.005
SP-10	-	-	4.0*	4.6*	2.5	56.0	*0.005
SP-B	-	-	1.8	5.6*	-0.8	30.0	*0.009
SP-C	-	-	2.0	1.4	18.2*	39.0	*0.013
SP-F	-	-	-18.8	9.0*	-2.9	56.0	*0.008

cv of blanks was $\pm$3.1%, values greater than this are marked *, the %T/ug calculated from the mean of these values.

APPENDIX 16

Cholesteryl Ester Transfer Activity and CETP Inhibitory Activity in LPDP Eluted from Phenyl Sepharose

	Eluant		
	15% Ethanol		0-100% Ethanol
Fraction	% Transfer[a]	% Inhibition[b]	% Inhibition[c]
1	-7.4	nd	+29.9
2	-3.2	nd	+52.1
3	-6.0	nd	+23.4
4	-4.6	nd	+18.0
5	-5.4	nd	-2.4
6	-10.7	nd	+16.2
7	8.8	nd	+37.1
8	-3.1	nd	+4.2
9	-8.2	nd	-9.6
10	-8.6	+34.2	+47.3
11	-6.0	+44.6	+33.5
12	-6.3	+49.4	+10.2
13	-2.7	+41.6	+30.5
14	-5.2	+42.8	-1.8
15	1.2	+49.8	+15.6
16	-2.0	+16.7	-13.2
17	-3.1	+6.7	+19.2

[a] 35 ul of each fraction from column one was assayed for CETP activity.

[b] 25 ul of each fraction was added in duplicate to the assay of 10 ul of a 1:3 dilution of human LPDP that gave 40.4% transfer (0.269 5T/ug).

[c] 50 ul of each fraction was added to 10 ul of a 1:10 dilution of human LPDP that gave 10.0% transfer (0.167 %T/ug).

N.B. The data for both inhibition assays show the percentage change in CETP activity, with negative results representing inhibition (nd; not determined).

APPENDIX 17

Plasma Cholesterol, Triglyceride and Lipoprotein Cholesterol Concentrations In 12 Shetland Ponies at Grasss (Fed) and Following an Overnight Fast (Fasted)

Animal		Plasma Concentration (mmol/l)				
		Cholesterol	Triglyceride	VLDL	LDL	HDL
Fed	SP-42	1.60	0.50	0.45	0.25	0.90
	SP-43	2.20	0.45	0.35	0.70	1.15
	SP-44	1.85	0.40	0.35	0.35	1.15
	SP-45	1.75	0.50	0.45	0.30	1.00
	SP-46	2.80	0.95	---	---	---
	SP-47	2.00	0.35	---	---	---
	SP-48	1.55	0.35	---	---	---
	SP-49	1.90	0.35	---	---	---
	SP-L	2.35	0.55	0.45	0.80	1.10
	SP-R	2.10	0.70	0.55	0.50	1.05
	SP-B	2.75	0.55	0.40	0.85	1.50
	SP-37	2.75	0.75	0.45	0.75	1.55
Fasted	SP-42	2.70	0.35	0.20	0.50	2.00
	SP-43	2.35	0.30	0.20	0.45	1.70
	SP-44	2.10	0.40	0.30	0.20	1.60
	SP-45	2.00	0.25	0.20	0.30	1.50
	SP-46	2.75	0.40	0.20	0.45	2.10
	SP-47	2.15	0.35	0.20	0.35	1.60
	SP-48	2.15	0.30	0.30	0.30	1.55
	SP-49	2.15	0.20	0.20	0.35	1.60
	SP-L	2.50	0.50	0.20	0.65	1.65
	SP-R	2.65	0.60	0.30	0.55	1.80
	SP-B	3.05	0.70	0.35	0.95	1.75
	SP-37	2.60	0.65	0.30	0.75	1.55

APPENDIX 18

Chemical Composition of Density <1.006 g/ml Lipoproteins in Four Shetland Ponies at Grass (Fed) and Following an Overnight Fast (Fasted)

1. Chemical Composition

Animal		Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
Fed	SP-L	5.9	71.1	ND	11.0	11.9
	SP-R	5.2	63.3	ND	12.0	19.4
	SP-B	6.2	67.4	ND	11.4	14.9
	SP-37	5.6	65.2	ND	9.9	19.2
Fasted	SP-L	8.2	61.5	ND	13.9	16.4
	SP-R	9.2	58.6	ND	13.2	18.9
	SP-B	9.1	60.9	ND	13.9	16.0
	SP-37	9.9	58.7	ND	13.0	18.4

2. Apolipoprotein Composition

Animal		Percentage of Protein Mass					
		B-100	B-48	E	A-I	C-II	C-III
Fed	SP-L	14.8	9.3	11.4	8.9	19.0	36.7
	SP-R	14.5	9.1	17.0	0.0	26.1	33.2
	SP-B	16.9	10.2	16.0	8.9	14.6	33.5
	DP-37	19.8	7.7	16.6	2.0	14.8	39.2
Fasted	SP-L	24.7	15.0	5.6	1.0	20.2	33.5
	SP-R	37.8	10.8	6.9	1.7	11.9	30.9
	SP-B	15.5	17.0	14.9	6.7	19.9	26.7
	SP-37	17.0	9.7	3.2	1.4	26.2	42.5

APPENDIX 19

Lipoprotein Lipase, Hepatic Lipase, and Lecithin:Cholesterol Acyl Transferase Activities in Four Shetland Ponies Sampled after an Overnight Fast and after Access to Hay (Fed)

Animal		LPL umolFA/ml/h	HL umolFA/ml/h	LCAT nmolCE/ml/h
Fast	SP-L	1.96	2.16	18.09
	SP-R	1.85	3.03	21.47
	SP-B	2.86	3.62	18.26
	SP-37	2.90	3.68	22.86
Fed	SP-L	2.82	3.08	19.07
	SP-R	2.43	2.63	23.79
	SP-B	2.86	2.34	20.88
	SP-M	3.02	4.34	31.86

APPENDIX 20

Plasma Cholesterol, Triglyceride, Free Fatty Acid (FFA) and Lipoprotein Cholesterol Concentrations in Six Shetland Pony Mares in Late Pregnancy and Early Lactation, and in their Foals Aged One to Four Weeks

	Plasma Concentration (mmol/l)					
Animal	Cholesterol	Triglyceride	VLDL	LDL	HDL	FFA
Foals						
F-L	3.70	0.65	0.45	1.80	1.45	0.14
F-R	3.85	1.05	0.20	2.10	1.55	0.12
F-B	3.10	0.60	0.20	1.50	1.40	0.09
F-M	5.00	0.95	0.20	3.00	1.80	0.14
F-S	2.65	0.90	0.20	2.55	1.60	0.07
F-T	4.30	1.05	0.15	2.55	1.60	0.09
Pregnant Mares						
SP-L	2.55	1.20	0.40	0.60	1.55	0.23
SP-R	2.75	0.95	0.55	0.80	1.40	0.43
SP-B	3.25	2.15	0.75	0.65	1.85	0.79
SP-M	3.00	1.20	0.55	0.70	1.75	0.43
SP-S	2.85	1.75	0.55	0.45	1.85	0.27
SP-T	3.05	0.90	0.50	0.55	2.00	0.45
Lactating Mares						
SP-L	1.85	0.10	0.20	0.20	1.45	0.50
SP-R	2.05	0.15	0.10	0.30	1.65	0.49
SP-B	3.25	0.30	0.25	0.15	1.85	1.06
SP-M	3.85	0.50	0.20	1.45	2.20	1.78
SP-S	2.60	0.80	0.35	0.70	1.55	0.96
SP-T	3.05	0.60	1.05	1.80	1.82	

APPENDIX 21

Chemical Composition of Lipoproteins from Six Suckling Shetland Pony Foals

Lipoprotein/ Animal	Percentage Particle Mass				
	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
Chylomicrons					
F-L	5.8	88.8	0.4	ND	5.1
F-R	1.5	89.9	1.1	ND	7.6
F-B	2.6	84.8	1.6	ND	10.9
F-M	2.2	90.7	0.3	ND	6.8
F-S	2.0	89.2	1.1	ND	7.7
VLDL					
F-L	5.6	60.7	1.1	13.7	18.9
F-R	7.6	55.2	1.5	13.1	22.6
F-B	12.1	48.5	3.6	11.4	24.3
F-M	8.9	50.7	3.5	11.7	25.2
F-S	12.5	49.4	1.8	12.1	24.2
F-T	9.4	51.3	1.9	16.2	21.2
LDL					
F-L	20.6	1.9	23.8	34.9	18.9
F-R	33.9	1.3	16.3	30.3	18.2
F-B	24.4	1.9	21.9	32.4	19.5
F-M	21.8	2.5	21.6	40.4	13.7
F-S	24.3	1.8	13.1	35.8	19.0
F-T	35.6	1.4	14.3	36.6	13.3
HDL					
F-L	15.8	0.3	4.9	26.5	52.5
F-R	21.9	0.4	4.1	28.5	45.1
F-B	21.9	0.4	4.4	28.9	44.3
F-M	15.4	0.1	5.2	29.7	49.1
F-S	15.5	0.6	3.4	30.2	50.4
F-T	20.8	0.7	5.3	29.5	43.6

APPENDIX 22

Apolipoprotein Composition of Foal Lipoproteins.

Lipoprotein	Foal	Percentage of Protein Mass						
		B-100	B-48	E	A-I	C-II	C-III	A-II
Chylomicron	F-L	---	---	---	18.5	28.6	52.8	---
	F-R	---	---	7.0	36.9	11.1	45.0	---
	F-B	---	---	---	20.1	17.8	62.1	---
	F-M	---	---	---	30.1	17.2	52.8	---
	F-S	---	---	---	33.1	20.7	46.2	---
	F-T	---	---	---	22.0	27.6	50.4	---
VLDL	F-L	18.6	6.5	7.1	6.9	20.4	40.5	---
	F-R	17.6	10.1	9.3	7.8	23.4	31.9	---
	F-B	12.4	4.9	7.3	3.5	28.9	43.1	---
	F-M	16.3	3.2	5.2	5.3	23.1	46.8	
	F-S	27.0	10.4	12.0	9.3	18.0	28.3	---
	F-T	24.1	7.3	17.2	10.8	14.8	25.8	
LDL	F-L	80.6	---	7.6	2.4	3.1	6.4	---
	F-R	90.3	---	3.4	0.7	1.5	4.0	---
	F-B	80.7	---	7.7	1.7	3.5	6.4	---
	F-M	81.8	---	6.5	5.1	1.3	5.3	---
	F-S	73.0	---	5.6	3.2	3.9	14.4	---
	F-T	79.1	---	7.4	4.2	3.7	5.5	---
HDL	F-L	---	---	4.5	81.6	4.5	5.4	4.0
	F-R	---	---	3.6	82.9	5.2	3.3	5.0
	F-B	---	---	8.8	82.9	2.7	2.8	2.8
	F-M	---	---	2.0	84.2	3.3	2.7	7.9
	F-S	---	---	4.2	84.8	2.3	2.7	6.0
	F-T	---	---	4.7	83.4	4.0	4.2	3.7

APPENDIX 23

Lipoprotein Lipase, Hepatic Lipase and Lecithin:Cholesterol Acyl Transferase Activities in Six Shetland Pony Mares in Late Pregnancy and Early Lactation and in Their Foals Aged One to Four Weeks

Animal	LPL umolFA/ml/h	HL umolFA/ml/h	LCAT nmolCE/ml/h
Foals			
F-L	18.44	12.83	44.60
F-R	9.64	7.23	35.74
F-B	12.69	7.48	42.11
F-M	9.81	1.18	nd
F-S	10.20	9.15	42.69
F-T	4.77	3.76	34.85
Pregnant Mares			
SP-L	2.95	3.21	nd
SP-R	3.99	2.84	nd
SP-B	4.45	4.04	nd
SP-M	4.29	4.27	nd
SP-S	4.01	4.04	nd
SP-T	5.01	5.19	nd
Lactating Mares			
SP-L	14.53	8.04	nd
SP-R	10.03	3.67	nd
SP-B	20.20	8.69	nd
SP-M	13.68	13.02	nd
SP-S	10.31	11.02	nd
SP-T	19.82	14.24	nd

nd, not determined

APPENDIX 24

Chemical Composition of Lipoproteins from Shetland Pony Mares in Late Pregnancy and Early Lactation

Lipoprotein/ Animal		Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
VLDL						
Pregnant	SP-L	1.5	67.9	5.4	15.9	9.3
	SP-R	1.2	65.9	10.0	13.3	9.6
	SP-B	0.7	71.5	6.8	15.0	6.0
	SP-S	0.0	67.9	9.6	15.3	7.3
	SP-T	0.3	69.2	7.0	15.5	8.0
Lactation	SP-L	6.4	67.3	ND	9.8	16.5
	SP-R	8.6	62.0	ND	9.5	19.9
	SP-B	5.7	65.1	ND	8.2	21.0
	SP-M	8.2	66.5	ND	11.0	14.3
	SP-S	6.4	70.1	ND	10.9	12.5
	SP-T	4.2	75.8	ND	7.5	12.7
LDL						
Pregnant	SP-L	35.5	5.4	6.7	26.6	25.7
	SP-R	38.6	6.1	5.3	23.7	23.5
	SP-B	43.6	6.1	3.9	21.9	24.5
	SP-S	36.4	4.2	14.8	26.2	18.4
	SP-T	35.1	7.6	9.1	24.5	23.6
Lactation	SP-L	40.9	3.2	8.0	21.0	36.9
	SP-R	41.9	2.9	6.9	25.3	23.1
	SP-B	43.6	7.8	4.8	18.9	24.8
	SP-M	28.5	3.1	16.6	24.9	26.8
	SP-S	20.7	6.1	17.1	33.2	23.0
	SP-T	31.5	4.0	5.5	40.5	18.6

APPENDIX 24 (continued)

Lipoprotein/ Animal		Percentage Particle Mass				
		Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
HDL						
Pregnant	SP-L	17.5	0.4	4.0	31.0	47.2
	SP-R	24.5	0.4	4.6	28.5	42.1
	SP-B	19.2	0.4	3.6	32.2	44.4
	SP-S	24.6	0.4	5.2	30.0	39.9
	SP-T	18.8	0.6	3.2	30.1	47.3
Lactation	SP-L	18.3	0.3	3.6	26.3	51.4
	SP-R	21.4	0.5	4.7	34.6	58.5
	SP-B	24.1	0.8	4.2	25.9	45.0
	SP-M	20.0	0.5	3.9	25.9	49.7
	SP-S	18.4	0.8	3.8	29.1	47.9
	SP-T	21.8	0.8	4.0	26.7	46.6

Case	Age yrs	Sex	Breed	Location	Pregnant	Primary Disease	Urea/ Creat.	SAP/ GGT	Treatment	Outcome	Disease Duration
HLP-1	9	F	Welsh	Stroud	No	None	4.6/188	13528/395	Gluc+Ins+Hep	Recovered	
HLP-2	7	F	Fell Pony	Newcastle	10 months	None	12.3/333	2796/35	Gluc+Ins+Hep	Died	3 days
HLP-3	9	F	Welsh	Cornwall	9 months	Laminitis	15.8/153	2059/45	Gluc+Ins+Hep	Died	24 days
HLP-4	7	F	Shetland	Winchester	9 months	Laminitis	4.1/112	1217/62	Gluc only	Recovered	
HLP-5	13	F	Riding pony	Newmarket	9 months	None	21.3/1721	3365/277	Gluc only	Died	5 days
HLP-6	8	G	Shetland	Cornwall	No	None	9.7/-	450/32	Gluc+Ins	Recovered	
HLP-7	11	F	Shetland	Perth	5 months	None	8.5/-		Gluc+Ins+Hep	Died	13 days
HLP-8	20	G	Welsh x	Kent	No	None	2.8/139	399/33	Gluc only	Died	18 days
HLP-9	15	G	Riding Pony	Glasgow	Lactating	Oesophagitis	7.8/-	548/19	None	Euthanased	4 days
HLP-10	8	F	Shetland	Kent	No	Lymphosarcoma	6.0/154	240/99	None	Euthanased	8 days
HLP-11	8	G	Welsh	Edinburgh	No	None	5.9/140	4231/165	Gluc only	Died	7 days
HLP-12	2	F	Shetland	Glasgow	No	Cyathostomiasis	5.7/70	953/30	Gluc+Ins	Died	6 days
HLP-13	1.5	F	Shetland	Glasgow	No	Cyathostomiasis	10.9/206	1807/69	None	Euthanased	3 days
HLP-14	6	F	Shetland	Glasgow	8 months	None	24.1/-	1964/32	Gluc only	Recovered	
HLP-15	10	F	Shetland	Aberdeen	9 months	None	3.6/113	2548/228	Gluc only	Recovered	
HLP-16	4	F	Shetland	Perth	10 months	None	16.1/569	2894/72	Gluc only	Recovered	
HLP-17	14	F	Shetland	Shetland	7 months	None	10.8/271	2036/92	Gluc only	Died	18 days
HLP-18	7	F	Shetland	Glasgow	Lactating	None	12.4/700	5070/623	None	Euthanased	6 days

Abbreviations and normal ranges: Urea (3.5-8 mmol/l), Creat. (Creatinine 110-170 umol/l), SAP (Serum Alkaline Phosphatase <400 iU/l) GGT (Gamma-Glutamyl Transferase <50 iU/l), Gluc (Glucose), Ins (Insulin), Hep (Heparin)

APPENDIX 26

Plasma Cholesterol, Triglyceride, Lipoprotein Cholesterol and Free Fatty Acid (FFA) Concentrations in 18 Ponies with Hyperlipaemia

	Plasma Concnetration (mmol/l)						
Animal	Chol.	Trig.	VLDL	LDL	HDL	FFA	$VLDL_1$[a]
HLP-1	4.80	10.70	2.65	0.50	1.65	1.45	2.25
HLP-2	8.90	39.10	7.90	0.55	0.45	1.71	7.60
HLP-3	8.75	27.50	6.75	0.45	1.55	2.56	6.65
HLP-4	5.45	15.80	3.40	0.35	1.70	2.03	3.00
HLP-5	6.65	24.65	5.90	0.45	0.30	1.91	5.65
HLP-6	8.15	24.60	6.15	0.70	1.30	2.20	6.00
HLP-7	7.60	17.20	4.90	1.00	1.70	1.27	4.75
HLP-8	5.05	15.15	2.95	0.85	1.25	0.41	2.90
HLP-9	3.45	4.70	1.50	0.50	1.45	1.23	0.50
HLP-10	4.30	8.25	2.45	0.45	1.40	1.64	2.15
HLP-11	6.45	21.00	4.80	0.90	0.75	0.98	4.65
HLP-12	5.10	16.15	3.25	0.50	1.35	0.94	2.90
HLP-13	7.95	22.00	5.60	0.85	1.50	1.15	4.70
HLP-14	4.85	16.89	2.75	0.60	1.50	1.27	1.75
HLP-15	13.65	55.00	11.15	0.35	2.15	3.32	10.60
HLP-16	10.85	40.45	8.45	0.45	1.95	3.31	8.40
HLP-17	16.90	78.75	15.20	0.80	0.90	1.44	14.65
HLP-18	6.90	19.91	4.20	0.50	1.70	1.70	3.85

Chol., cholesterol; Trig., triglyceride

[a] The concentration of cholesterol in the $VLDL_1$ fraction was calculated by subtracting the cholesterol concentration of the $VLDL_2$ fraction (divided by 4, as it was isolated from 4 ml plasma) from the total VLDL cholesterol concentration.

APPENDIX 27

Elution Profiles from 6% Agarose Gel of Density >1.225 g/ml Lipoproteins from Nine Ponies with Hyperlipaemia

	VLDL			LDL			HDL		
Animal	Vol.[a]	Width[b]	Height[c]	Vol.	Width	Height	Vol.	Width	Height
HLP-1	82	16	>3.5	115	25	0.20	154	50	1.90
HLP-2	80	16	>3.5	118	31	0.23	159	38	1.97
HLP-3	71	18	>3.5	105	36	0.35	141	38	1.83
HLP-4	73	19	>3.5	107	31	0.28	141	38	1.48
HLP-5	67	20	>3.5	90	32	0.40	122	38	2.36
HLP-6	70	16	>3.5	104	30	0.20	140	36	1.84
HLP-7	80	24	>3.5	118	28	0.23	159	34	1.97
HLP-8	64	29	>3.5	115	30	0.11	152	30	1.97
HLP-9	71	20	>3.5	115	26	0.20	140	38	1.84

[a] peak elution volume (ml); [b] width of peak (ml); [c] peak height (OD_{280nm})

APPENDIX 28

Gradient Gel Electrophoresis of HDL Prepared by Flotation Ultracentrifugation from Nine Ponies with Hyperlipaemia and Nine Healthy Shetland Ponies

Animal	Diameter (nm)	Animal	Diameter (nm)
HLP-2	8.42	SP-5	8.76
HLP-3	8.66	SP-6	8.56
HLP-4	8.24	SP-7	8.60
HLP-5	8.54	SP-8	8.48
HLP-6	8.84	SP-9	8.42
HLP-7	8.68	SP-10	8.46
HLP-8	8.60	SP-L	8.20
HLP-9	8.88	SP-R	8.30
HLP-10	8.61	SP-B	8.38

APPENDIX 29

Chemical Composition of Lipoproteins Isolated by Flotation Ultracentrifugation from 18 Ponies with Hyperlipaemia

Lipoprotein/ Animal	Percentage Particle Mass				
	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
$VLDL_1$					
HLP-1	0.0	77.1	6.5	11.5	4.9
HLP-2	3.0	70.8	3.3	9.0	13.9
HLP-3	3.9	73.7	4.1	14.0	4.3
HLP-4	2.0	70.4	6.9	13.1	7.6
HLP-5	1.5	71.4	6.1	12.7	8.3
HLP-6	1.7	72.5	7.4	13.0	5.3
HLP-7	5.2	70.1	4.5	11.0	9.8
HLP-8	5.9	70.5	4.1	10.9	8.6
HLP-9	5.7	68.1	5.3	13.1	7.7
HLP-10	7.9	73.5	2.7	10.9	5.1
HLP-11	0.7	69.5	4.5	7.8	9.3
HLP-12	0.4	78.5	7.1	6.4	7.5
HLP-13	3.7	74.9	4.0	11.4	6.0
HLP-14	1.3	86.8	3.1	4.7	4.1
HLP-15	0.0	71.3	7.6	15.2	6.0
HLP-16	0.0	75.0	6.6	13.7	4.7
HLP-17	1.7	76.2	5.1	11.5	5.5
HLP-18	0.5	80.1	6.0	9.5	3.9
$VLDL_2$					
HLP-1	2.3	75.7	4.1	8.7	9.2
HLP-2	11.8	6.06	0.0	14.6	12.9
HLP-3	8.2	66.3	2.7	14.7	10.8
HLP-4	2.9	65.3	2.9	12.6	16.3
HLP-5	4.0	73.0	1.6	13.0	8.5
HLP-6	13.7	62.3	0.0	12.7	11.3
HLP-7	6.8	52.9	4.6	14.6	21.0

APPENDIX 29 (continued)

Animal	Percentage Particle Mass				
	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
$VLDL_2$					
HLP-8	11.2	58.5	0.0	15.2	15.2
HLP-9	13.6	53.2	0.0	15.2	18.1
HLP-10	3.1	61.0	7.1	18.0	10.8
HLP-11	9.4	65.5	0.0	16.2	9.0
HLP-12	5.0	71.6	2.1	14.1	17.2
HLP-13	6.0	67.8	3.3	15.3	7.7
HLP-14	1.8	67.5	8.1	14.0	8.5
HLP-15	3.8	65.4	5.0	18.0	7.8
HLP-16	4.0	63.6	3.4	16.6	12.3
HLP-17	6.1	59.8	7.3	18.4	8.4
HLP-18	3.5	71.2	5.2	14.7	5.4
LDL					
HLP-1	22.7	16.7	11.4	23.6	26.2
HLP-2	29.0	16.1	4.7	21.9	28.3
HLP-3	41.2	11.1	6.0	25.0	16.8
HLP-4	33.1	17.9	0.0	24.5	24.4
HLP-5	36.7	29.5	11.8	18.2	30.3
HLP-6	39.4	15.8	0.0	20.8	24.0
HLP-7	37.4	6.2	3.9	15.7	36.9
HLP-8	39.5	13.5	0.0	22.3	22.3
HLP-9	36.5	8.7	5.5	21.6	27.8
HLP-10	26.0	30.1	0.0	20.4	23.8
HLP-11	22.7	27.5	0.0	18.7	31.1
HLP-12	35.2	24.0	0.0	19.0	21.9
HLP-13	14.6	33.3	0.0	15.1	37.0
HLP-14	13.2	31.8	8.3	16.8	29.9
HLP-15	19.6	13.4	12.3	28.6	26.1
HLP-16	34.6	10.0	3.8	28.5	4.7
HLP-17	20.6.	21.7	3.5	24.5	29.8
HLP-18	20.1	15.7	3.4	24.4	36.4

APPENDIX 29 (continued)

Animal	Percentage Particle Mass				
	Cholesteryl Esters	Triglyceride	Free Cholesterol	Phospholipid	Protein
HDL					
HLP-1	21.9	1.4	4.7	33.3	38.5
HLP-2	15.0	2.6	1.3	18.4	61.0
HLP-3	26.5	1.9	3.0	35.1	33.0
HLP-4	-	-	-	-	-
HLP-5	17.2	1.4	1.3	19.7	60.4
HLP-6	17.2	1.4	1.5	17.7	64.5
HLP-7	21.8	2.1	2.6	24.8	48.7
HLP-8	19.1	0.9	2.4	25.9	51.6
HLP-9	17.6	0.9	2.4	24.6	54.5
HLP-10	21.7	5.5	1.1	26.2	45.5
HLP-11	14.1	5.3	4.3	25.4	50.9
HLP-12	23.7	4.5	1.5	20.0	50.4
HLP-13	17.9	3.6	1.1	20.2	57.2
HLP-14	19.1	2.5	5.6	31.1	41.7
HLP-15	19.1	1.4	4.2	31.1	44.2
HLP-16	23.0	0.0	1.3	30.4	45.3
HLP-17	18.2	2.8	1.3	21.6	56.0
HLP-18	16.4	0.6	2.1	24.4	55.4

APPENDIX 30

Apolipoprotein Composition of Lipoproteins from 18 Ponies With Hyperlipaemia

Lipoprotein/ Animal	Percentage Protein Mass					
	apoB-100	apoB-48	apoE	apoA-I	apoC-II	apoC-III
$VLDL_1$						
HLP-1	6.6	14.6	14.4	12.4	29.8	22.1
HLP-2	12.6	15.0	10.0	13.9	28.3	20.2
HLP-3	10.0	11.0	13.9	7.0	26.7	31.4
HLP-4	8.0	13.1	13.9	11.4	29.8	24.8
HLP-5	4.3	8.3	12.6	6.0	46.7	23.4
HLP-6	16.9	13.8	25.2	9.7	12.8	21.6
HLP-7	11.6	9.0	16.6	12.6	19.5	30.7
HLP-8	9.2	10.0	10.0	3.5	28.6	39.6
HLP-9	13.4	13.0	9.3	11.6	18.8	44.0
HLP-10	12.8	14.7	8.5	5.4	29.0	29.6
HLP-11	17.2	12.6	15.4	7.9	21.2	25.1
HLP-12	11.5	13.0	24.4	2.5	15.1	33.5
HLP-13	13.5	14.3	8.4	4.5	20.6	38.7
HLP-14	13.6	16.9	14.8	8.6	23.4	22.8
HLP-15	8.8	8.5	27.4	14.5	13.4	27.4
HLP-16	14.1	14.7	16.9	3.4	16.4	34.7
HLP-17	6.7	7.2	17.6	8.3	26.8	33.3
HLP-18	14.5	13.7	19.5	11.6	21.4	19.3
$VLDL_2$						
HLP-1	12.6	9.0	16.6	12.6	19.5	31.0
HLP-2	22.5	12.7	14.2	7.4	21.6	21.6
HLP-3	20.7	6.9	10.3	3.4	25.7	33.1
HLP-4	28.9	8.7	8.7	4.4	18.1	31.0
HLP-5	14.1	10.2	12.4	13.1	27.1	23.0
HLP-6	21.6	9.2	4.6	3.5	25.0	30.1
HLP-7	13.2	12.9	12.5	0.0	20.0	25.6
HLP-8	21.0	4.0	9.9	2.1	28.0	35.0

APPENDIX 30 (continued)

Lipoprotein/ Animal	Percentage Protein Mass					
	apoB-100	apoB-48	apoE	apoA-I	apoC-II	apoC-III
$VLDL_2$						
HLP-9	22.5	14.8	4.2	1.7	22.2	34.6
HLP-10	22.6	14.6	4.0	0.5	21.5	36.8
HLP-11	17.6	12.4	4.1	2.6	31.8	31.5
HLP-12	19.2	8.8	10.5	11.0	20.2	26.7
HLP-13	20.6	12.8	12.5	8.6	15.0	33.5
HLP-14	18.4	10.8	7.9	5.1	22.7	35.1
HLP-15	11.6	11.5	23.0	6.7	10.9	36.3
HLP-16	19.2	8.8	10.2	1.0	13.5	42.6
HLP-17	17.2	5.4	13.0	9.4	22.9	32.1
HLP-18	23.6	10.8	10.6	3.7	25.1	26.3
LDL						
HLP-1	58.2	---	7.5	---	17.8	16.6
HLP-2	56.9	---	9.8	---	10.6	22.7
HLP-3	58.4	---	17.6	---	10.8	13.2
HLP-4	83.6	---	2.4	---	5.1	8.9
HLP-5	88.7	---	4.3	---	4.5	2.5
HLP-6	59.6	---	20.2	---	13.0	7.4
HLP-7	73.8	---	5.1	---	10.3	10.8
HLP-8	82.2	---	3.8	---	6.9	7.2
HLP-9	57.0	---	10.6	---	25.4	7.1
HLP-10	82.0	---	8.1	---	7.8	2.1
HLP-11	73.5	---	14.9	---	2.9	8.7
HLP-14	52.9	---	17.5	---	13.9	15.7
HLP-15	71.5	---	7.2	---	12.4	9.0
HLP-18	79.4	---	7.7	---	4.1	8.9

APPENDIX 30 (continued)

Animal	Percentage of Protein Mass				
	apoE	apoA-I	apoC-II	apoC-III	apoA-II
HDL					
HLP-1	2.0	75.2	7.9	5.1	10.0
HLP-2	0.0	82.0	8.1	7.8	2.1
HLP-3	6.3	75.3	5.7	11.2	1.5
HLP-4	11.7	59.9	10.9	7.2	10.2
HLP-5	6.4	65.3	8.6	19.7	4.0
HLP-6	3.4	61.6	17.1	16.2	1.7
HLP-7	4.0	66.9	12.8	10.3	6.4
HLP-8	5.2	60.3	12.0	13.9	13.8
HLP-9	4.0	62.8	18.7	11.6	3.0
HLP-10	4.0	59.7	10.2	15.5	10.6
HLP-11	4.2	51.7	8.4	26.9	8.9
HLP-12	6.0	75.4	3.2	10.2	5.2
HLP-13	2.1	54.1	25.1	10.9	7.7
HLP-14	7.0	66.1	12.7	12.6	1.6
HLP-15	9.7	64.7	10.4	12.0	3.2
HLP-16	2.2	64.3	15.4	10.7	7.5
HLP-17	6.1	86.6	2.1	3.1	1.9
HLP-18	6.8	72.5	10.0	7.9	2.3

APPENDIX 31

Lipoprotein Lipase, Hepatic Lipase and Lecithin:Cholesterol Acyl Transferase Activities in 12 Ponies with Hyperlipaemia.

Animal	LPL umolFA/ml/h	HL umolFA/ml/h	LCAT nmolCE/ml/h
HLP-1	6.02	5.10	nd
HLP-2	7.43	7.90	15.82
HLP-3	nd	nd	15.09
HLP-4	nd	nd	nd
HLP-5	nd	nd	nd
HLP-6	nd	nd	22.23
HLP-7	9.80	21.41	44.41
HLP-8	6.36	9.02	nd
HLP-9	9.95	14.04	15.96
HLP-10	7.60	16.72	18.43
HLP-11	5.85	11.07	33.08
HLP-12	12.00	18.76	21.98
HLP-13	9.17	14.51	31.48
HLP-14	5.68	11.35	20.76
HLP-15	13.51	19.50	18.05
HLP-16	nd	nd	nd
HLP-17	nd	nd	nd
HLP-18	4.05	9.03	46.33

nd, not determined

APPENDIX 32

Cholestery Ester Transfer Protein Activity in Nine Ponies with Hyperlipaemia

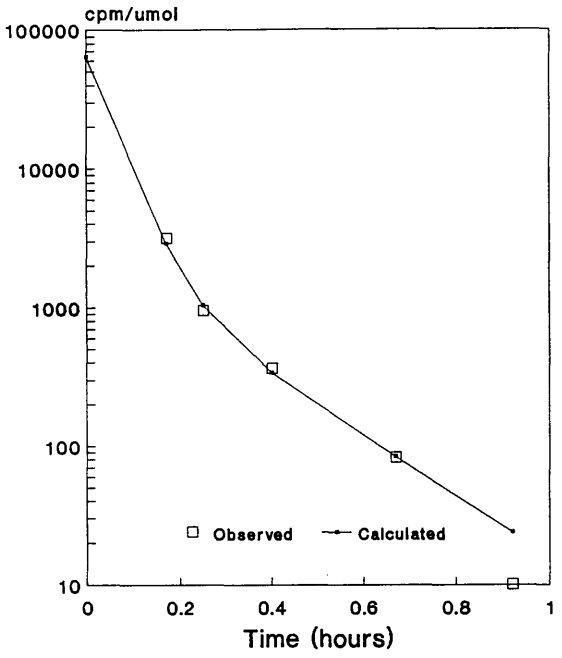
Animal	%Transfer/2.5h Volume of LPDP 10 ul	35 ul	Protein ug/ml	%T/ug
HLP-1	7.9*	1.4	18.3	*0.043
HLP-6	3.1	1.7	18.5	-
HLP-7	4.7	8.7*	25.5	*0.010
HLP-9	-5.0	0.0	19.0	-
HLP-10	-1.6	-1.0	14.3	-
HLP-11	6.4	11.4*	17.0	*0.018
HLP-12	6.3	1.8	13.0	-
HLP-13	0.7	6.3	10.5	-
HLP-14	4.6	0.2	14.0	-
Human (TW)	24.0	47.0	11.4	0.180

cv of blanks was ±7.6%, values greater than this are marked * and used to calculate %T/ug.

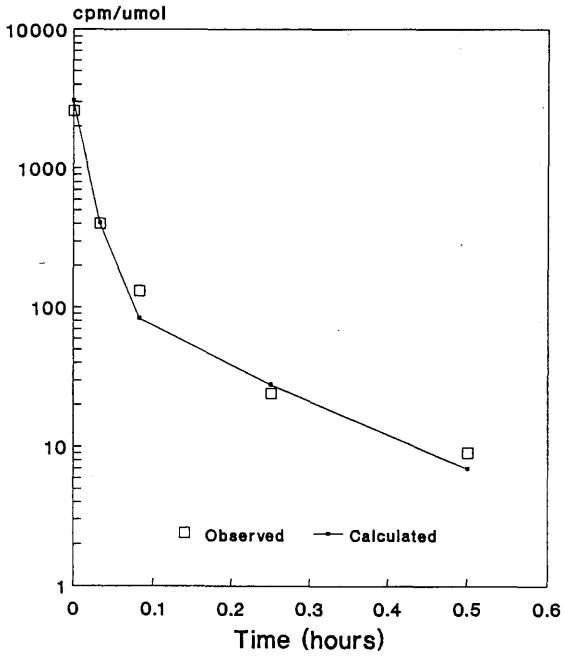
APPENDIX 33

Plasma Decay Curves of [^{14}C]FFA Activity in Four Healthy Shetland Ponies

Subject 1

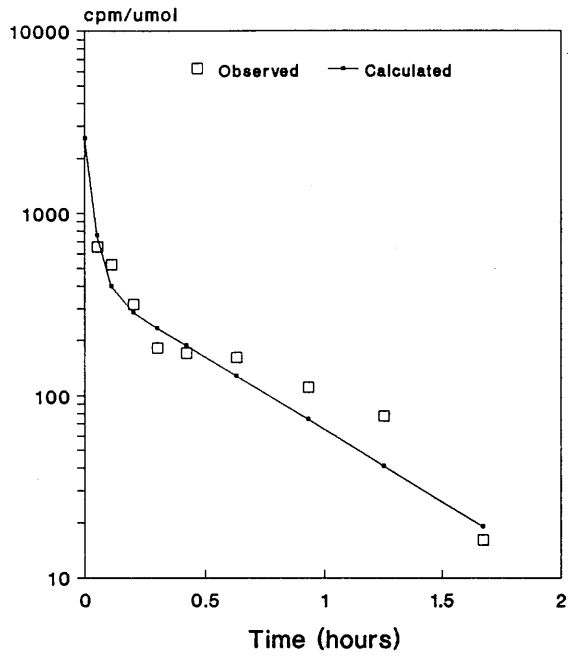


Subject 2

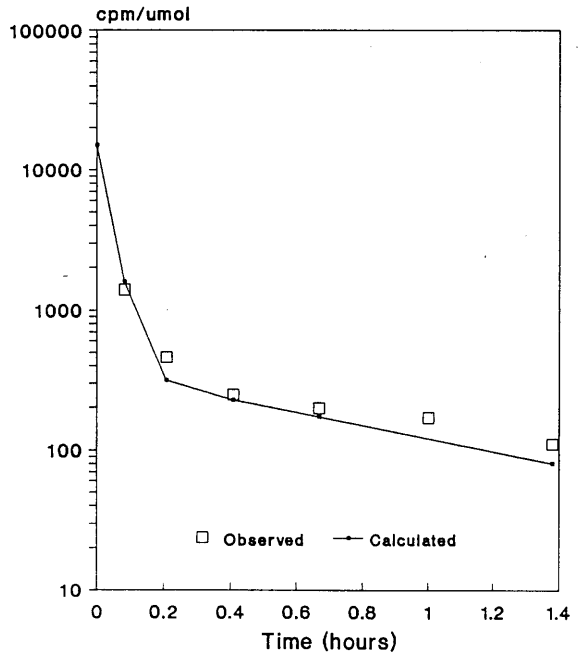


APPENDIX 33 (continued)

Subject 3

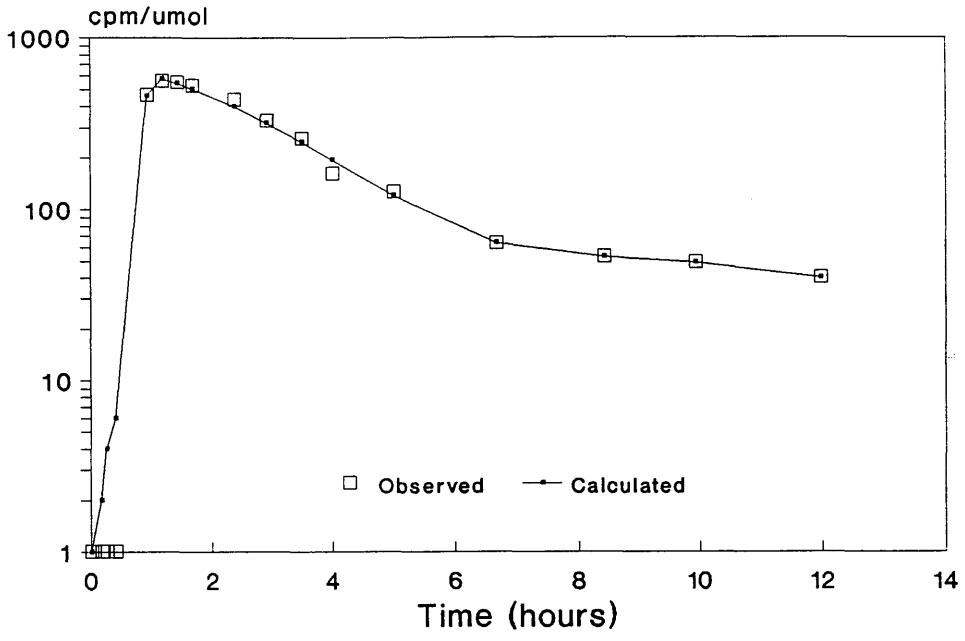


Subject 4

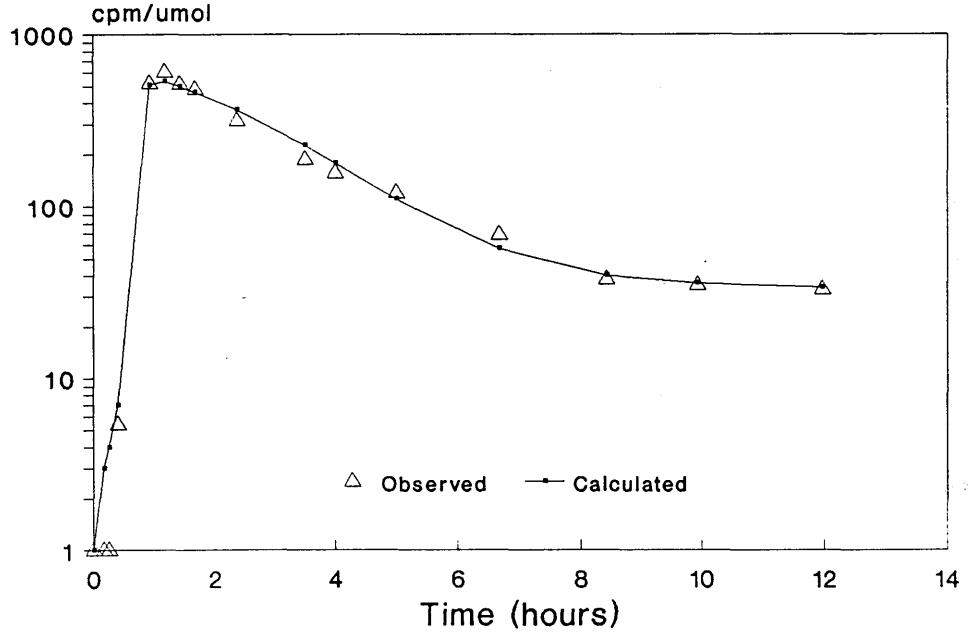


APPENDIX 34

Plasma [^{14}C]VLDL-TGFA and [^{3}H]VLDL-TG Specific Activity Curves in Four Healthy Shetland Ponies



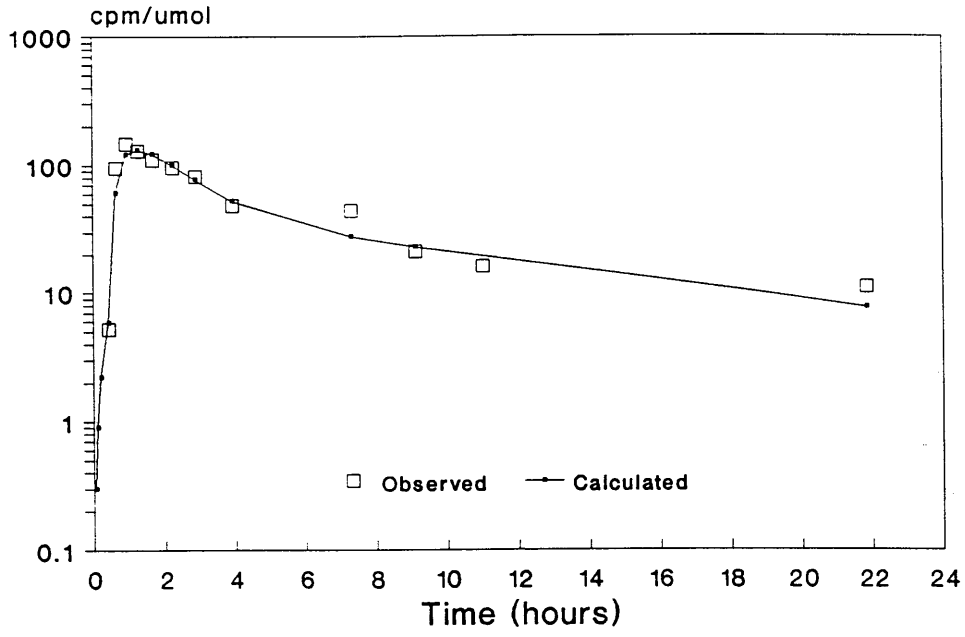
VLDL-TGFA



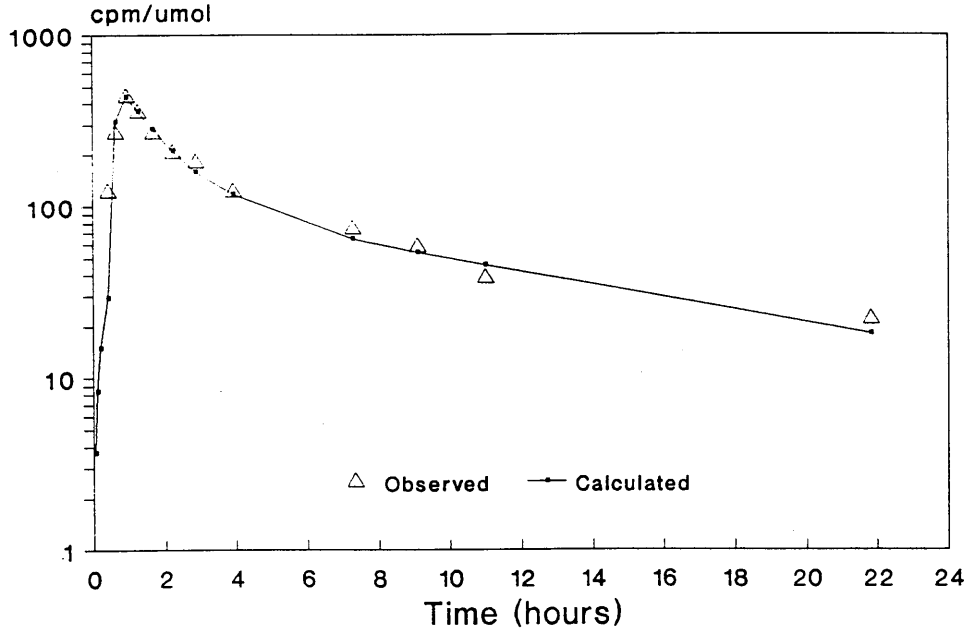
VLDL-TG

Subject 1. Top [^{14}C]VLDL-TGFA
Bottom [^{3}H]VLDL-TG

APPENDIX 34 (continued)



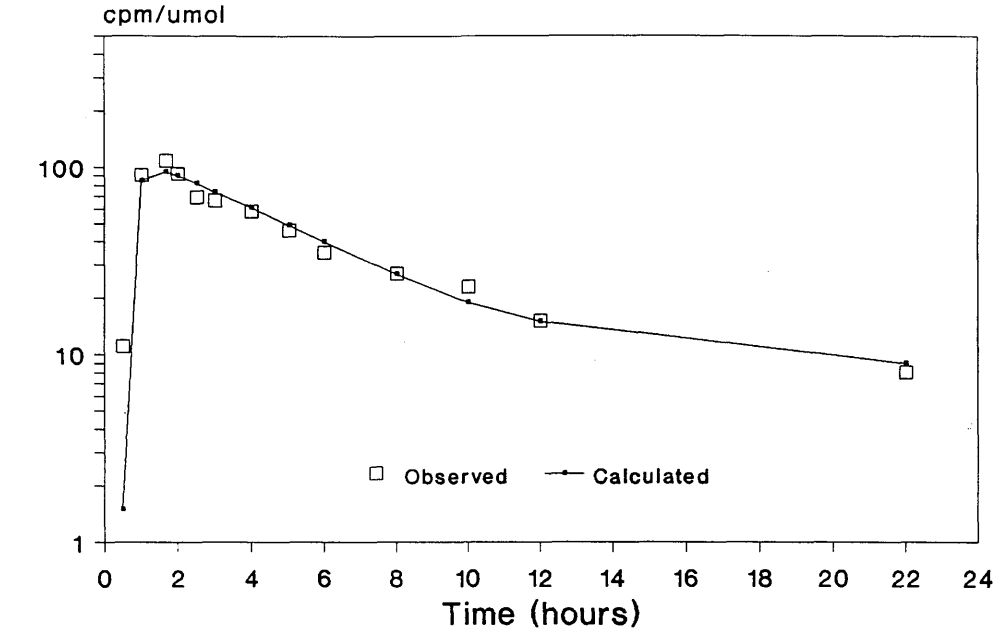
VLDL-TGFA



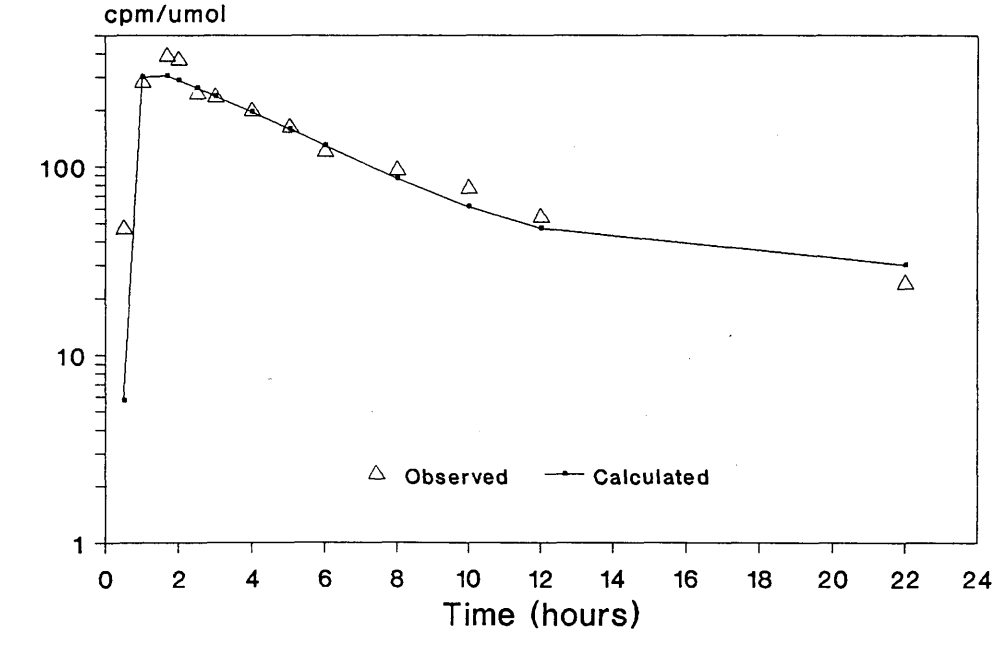
VLDL-TG

Subject 2. Top [^{14}C]VLDL-TGFA
Bottom [^{3}H]VLDL-TG

APPENDIX 34 (continued)



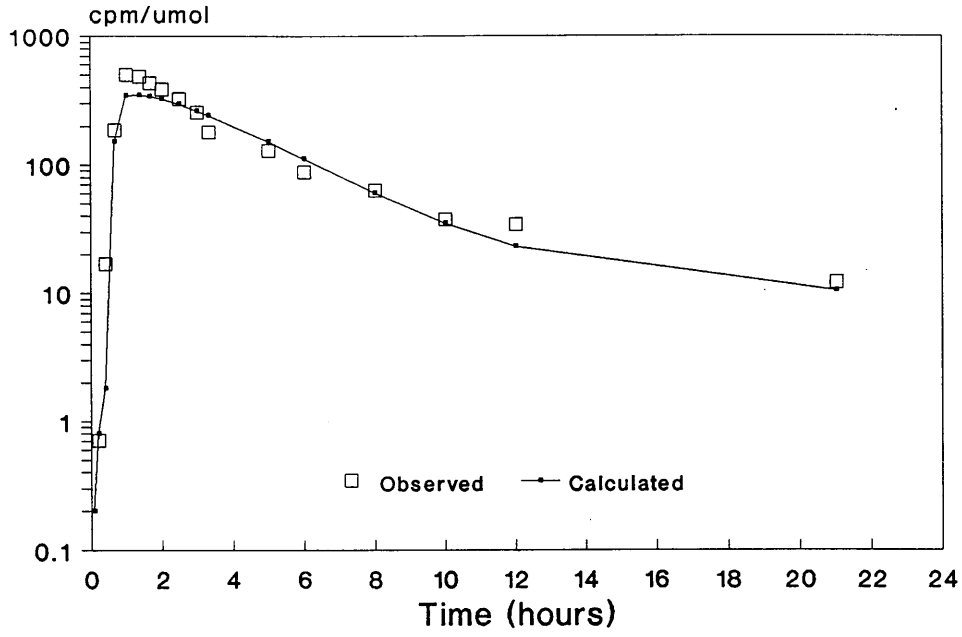
VLDL-FA



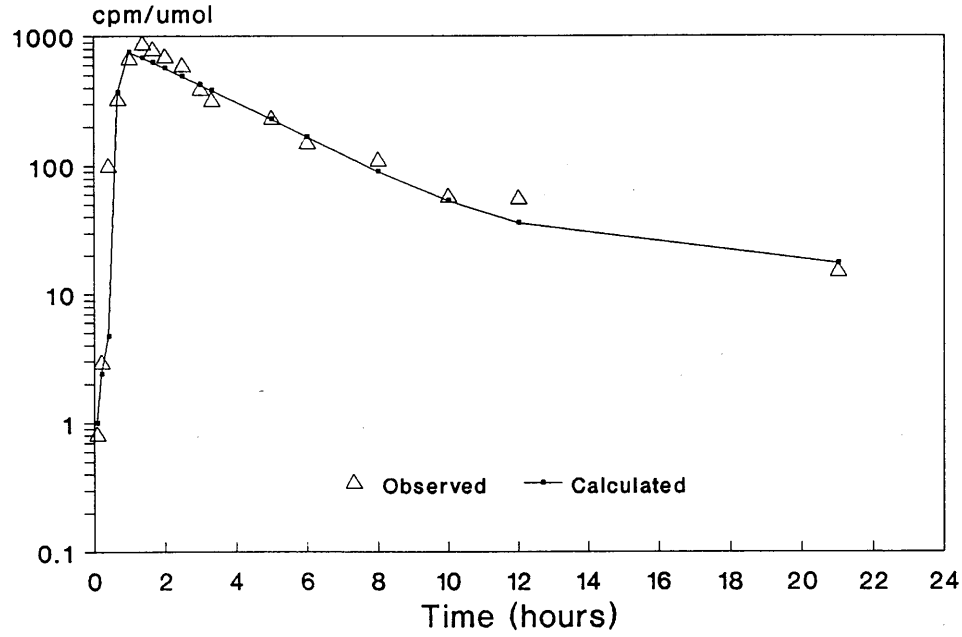
VLDL-TG

Subject 3. **Top [^{14}C]VLDL-TGFA**
Bottom [^{3}H]VLDL-TG

APPENDIX 34 (continued)



VLDL-TGFA



VLDL-TG

Subject 4. **Top [^{14}C]VLDL-TGFA**
Bottom [^{3}H]VLDL-TG

GLOSSARY

apo	apolipoprotein(s)
ACAT	acyl CoA cholesterol acyl transferase
CAD	coronary artery disease
CETP	cholesteryl ester transfer protein
Ci	curie
cm	centimetre
cpm	counts per minute
Da	Dalton
DEAE	diethyaminoethyl
dH_20	distilled water
dl	decilitre
dpm	distintegrations per minute
DTNB	5,5'-Dithio-*bis*-(2-Nitrobenzoic acid)
EDTA	ethylenediaminetetra-acetate
FDB	familial defective apoB-100
FFA	free fatty acids
g	gram
h	hour
HDL	high density lipoprotein(s)
HL	hepatic lipase
HLP	hyperlipaemia
HPLC	high performance liquid chromatography
IDDM	insulin dependent diabetes mellitus
IDL	intermediate density lipoprotein(s)
k	kilo
kb	kilobases
l	litre
LCAT	lecithin:cholesterol acyl transferase
LDL	low density lipoprotein(s)
Lp	lipoprotein
LPDP	lipoprotein deficient plasma
LPL	lipoprotein lipase
LRP	LDL receptor related protein
LTP	lipid transfer protein(s)
mg	milligram
min.	minute
mins.	minutes

GLOSSARY (continued)

ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimole
mRNA	messenger ribonucleic acid
M_r	relative molecular mass
N	normality
nd	not determined
ND	not detected
NIDDM	non-insulin dependent diabetes mellitus
nm	nanometre
nmol	nanomole
OD_{280nm}	optical density at 280nm
PBS	phosphate buffered saline
PHP	post-heparin plasma
RER	rough endoplasmic reticulum
Rf	relative band speed
rpm	revolutions per minute
sd	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SER	smooth endoplasmic reticulum
SP	Shetland pony
T	transfer
TB	Thoroughbred horse
TEMED	N,N,N',N',-tetramthylethylenediamine
TLC	thin layer chromatography
u	micro
ul	microlitre
umol	micromole
VLDL	very low density lipoprotein(s)
VLDL-TG	very low density lipoprotein triglyceride(s)
VLDL-TGFA	very low density lipoprotein triglyceride fatty acids
v/v	by volume

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