HYPERLIPIDAEMIA IN THE DOG

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ABSTRACT

Hyperlipidaemia describes increased plasma concentrations of cholesterol and/or triglyceride and may arise from primary, often inherited defects in lipoprotein metabolism or appear secondary to systemic disease. The aim of this study was to characterise the abnormalities of lipoprotein metabolism responsible for hyperlipidaemia in the dog, to establish their effect upon the health of the individual, and to evaluate approaches to therapy.

A combined ultracentrifugation/precipitation technique for the measurement of very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) was adapted and validated for use with canine plasma. Alternative methods of analysis were evaluated, but none of the commercially available precipitation reagents for the measurement of human lipoproteins were found to be suitable for use in the dog. Lipoprotein electrophoresis was useful in the investigation of hyperlipidaemic patients by allowing the identification of other lipoprotein classes, namely chylomicrons, HDL_1 and β-VLDL.

The effects of age, breed, sex and health status on lipoprotein concentrations in the dog were established. The HDL concentration was greater in entire females than males and certain disease processes were found to display characteristic lipoprotein abnormalities. Dogs with diabetes mellitus and hypothyroidism presented with defects in the metabolism of LDL and HDL and triglyceride-rich lipoproteins, while hyperadrenocorticism, obstructive jaundice and protein-losing nephropathies were predominantly associated with abnormalities of cholesterol metabolism. A decreased lipoprotein lipase activity was partially responsible for the hyperlipidaemia in hypothyroid dogs. Overt hyperlipidaemia was not identified in obese dogs, but aberrations of triglyceride metabolism were identified by kinetic analysis and found to be a result of decreased lipoprotein lipase activity. Impaired triglyceride clearance in the postprandial period may therefore predispose obese dogs to the development of acute pancreatitis.

The lipoprotein abnormalities underlying idiopathic hyperlipidaemia were heterogeneous. Dogs with hypertriglyceridaemia presented with clinical signs believed to be precipitated by the hyperlipidaemia which resolved with reduction of the plasma lipid concentrations. Restriction of the dietary fat intake was successful in reducing the hyperlipidaemia in most animals, however, in some cases the hypertriglyceridaemia was found to be less responsive and required additional therapy with omega-3 fatty acids.

It is concluded that the investigation of hyperlipidaemia, including quantitative and qualitative lipoprotein analysis, measurement of the activity of lipoprotein lipase and, where necessary, the performance of an intravenous fat tolerance test will allow the identification of underlying defects of lipoprotein metabolism and a rational approach to their therapy.

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AUTHOR'S DECLARATION

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

Joan Barrie, May 1993.

DEDICATION

To my parents.

If I have seen further, it is by standing on the shoulders of giants.

Sir Isaac Newton.

CHAPTER I

INTRODUCTION

This thesis concerns the aetiology, characterisation and management of abnormalities of lipid metabolism in the dog. In this introduction an account of lipid metabolism, based on observations in man and the dog, is followed by a review of the literature regarding the clinical presentations of canine hyperlipidaemia. Lastly the aims of this study are defined.

1. LIPIDS: THE REQUIREMENT FOR LIFE

Lipids are water-insoluble biomolecules that are essential for normal physiological function. The neutral fats, or triglycerides, are the most abundant and are formed by the esterification of one molecule of glycerol with three fatty acids. Accumulation of triglycerides in cellular cytoplasm, particularly in adipocytes, provides an essential reserve of chemical energy for tissue requirements. The transfer of these stores in the form of non-esterified fatty acids (NEFA) to other tissues for oxidation provides a means of supplying energy according to cellular demand.

The polar lipids, including cholesterol and phospholipids are major components of cellular membranes. Cholesterol appears to regulate and stabilise the fluidity of the outer cell membrane, thus determining its permeability (Brown and Goldstein 1986). In addition, this sterol is an essential precursor of steroid hormones, vitamins and bile acids.

Cholesterol may exist in the free or esterified form and is formed by a complex process including the condensation of three units of acetyl Co-A to mevalonic acid, a reaction which is catalysed by the enzyme 3-hydroxy,3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34). This is the rate-limiting step in cholesterol synthesis and is subject to a number of influencing factors, including the free cellular concentration. Since the liver produces approximately 50% of the body's cholesterol pool, it follows that the delivery of dietary cholesterol to the hepatocytes has a major regulatory effect on cholesterol synthesis.

The phospholipids are derived from glycerol and contain both hydrophobic and hydrophilic regions, making them ideally suited to their role as the major components of membranes. One group of phospholipids, the lecithins, are found in both cell membranes and extracellular fluid and are important in the esterification of cholesterol catalysed by the enzyme lecithin:cholesterol acyl transferase (LCAT).

2. LIPOPROTEIN STRUCTURE AND FUNCTION

It is necessary to transfer insoluble lipid substances through the aqueous phase of the plasma to their site of utilisation or storage. This is best achieved by the formation of lipid-protein complexes which act as vehicles for the transport of triglycerides, cholesterol and cholesteryl esters. Lipoproteins are composed of a surface coat, containing phospholipid, cholesterol and apolipoproteins (apo), surrounding a hydrophobic lipid centre containing triglycerides and cholesteryl esters. The apolipoproteins are specific proteins which direct the lipoproteins to their sites of metabolism by acting as ligands for cell surface receptors and as cofactors in the enzymatic hydrolysis of triglyceride and esterification of cholesterol. There are a number of discrete populations of lipoproteins which may be classified on the basis of their size, hydrated density, lipid and apolipoprotein composition, and electrophoretic mobility. The classes recognised in the dog are chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The characteristics of these classes have been described by Mahley and Weisgraber (1974) (Fig. 1a. and 1b.).

Figure 1a. The physical characteristics of canine plasma lipoproteins.

Lipoprotein	Diameter nm	Density g/ml	Electrophoretic mobility
Chylomicron	75-200	<0.960	origin
VLDL	26-80	0.930-1.006	preβ
LDL	16-25	1.019-1.087	β
HDL_1	10-35	1.025-1.100	α_2
HDL_2	5-9	1.100-1.210	α_1

Figure 1b. The approximate lipid and protein compositions of canine plasma lipoproteins.

	Percentage of particle mass				
Lipoprotein	Trig	Chol	PL	Protein	Apolipoproteins
Chylomicron	90	3	5	2	B_{48}, A, C, E
VLDL	62	12	14	12	B_{100}, C, E
LDL	30	22	24	24	B_{100}
HDL_1	1	36	40	23	E, A, C
HDL_2	1	21	35	43	A, C, E

Each lipoprotein species has a specific function and co-ordinated interaction between lipoprotein populations and tissues ensures the efficient transport of lipid in response to physiologic demands. The chylomicrons, VLDL and LDL of the dog resemble those of man (Mahley and Weisgraber 1974), while the subpopulations of HDL identified in the dog are peculiar to that species. LDL is quantitatively the most important cholesterol carrier in human plasma but the majority of cholesteryl esters in canine plasma are transported within HDL (Solyom, Bradford and Furman 1971; Mahley and Weisgraber 1974). The following sections review the current understanding of the fate and function of the lipoprotein populations in the healthy individual, based on observations made in both man and the dog.

2.1. Exogenous Lipid Transport

As a carnivorous species, the natural diet of the dog has a high fat content, the majority of which is in the form of triglyceride with a small quantity of esterifed cholesterol and phospholipids. In the small intestine these lipids interact with bile acids to form micelles, creating an emulsion in the intestinal lumen. Pancreatic lipase is then responsible for the hydrolysis of triglycerides to monoacylglycerol and fatty acids, which are transported to the absorptive sites of the intestinal mucosa. Within the lumenal cells, the absorbed glyceride is re-esterified and packaged into chylomicrons with the cholesterol, phospholipids and apolipoproteins A-I, A-II, A-IV and B (Durrington 1989). ApoB exists in two forms: $apoB_{100}$ which is synthesised by the liver and $apoB_{48}$ which is produced by the intestine and is the major structural component of chylomicrons. Following secretion from the lumenal cells, the chylomicrons enter the intestinal lacteals and lymphatics where they transfer to the thoracic duct and finally the circulation. Chylomicron synthesis and the secretion of $apoB_{48}$ therefore accompanies fat absorption and this lipoprotein class is detected in plasma during the postprandial period.

After entering the circulation the chylomicrons deliver their core lipid to tissues by two mechanisms. On entering the plasma, apoC-II, apoC-III and apoE are transferred from HDL to the chylomicron surface. The acquisition of apoC-II, activates the enzyme lipoprotein lipase which hydrolyses the core triglycerides, liberating fatty acids for uptake by the peripheral tissues (Ben-Aviram, Ben-Zeev, Lee, Haaga, Shively, Goers, Pederson, Reeve and Schotz 1986). Lipoprotein lipase is bound to the endothelium of cardiac and skeletal muscle, and adipose tissue, where it regulates the provision of fatty acids for oxidation or storage, respectively. The activity of the enzyme is modulated by apoC-III, ensuring a regulated delivery of fatty acids to these tissues. The lipolytic process eviscerates the chylomicron particle and renders some of the surface layer (phospholipid, free cholesterol, apoA-I, apoA-II) redundant. This material is transferred back to HDL. After approximately 75% of the core triglyceride is hydrolysed, apoC-II is lost from the lipoprotein and lipolysis ceases, leaving the chylomicron remnant relatively deficient in triglyceride and enriched in cholesteryl ester (Mahley and Innerarity 1983).

Removal of the chylomicron from the circulation is a function of the apoE receptor on hepatic parenchymal cell membranes (Bergman, Havel, Wolfe and Bohmer 1971; Sherrill, Innerarity and Mahley 1980). Delipidation of the chylomicron particle appears to result in a configurational change in apoE, conferring upon the protein the ability to be recognised by that hepatic receptor. In this way, the chylomicron is responsible for the delivery of dietary triglycerides to the tissues and cholesterol to the liver (Calvert and Abbey 1985).

In the dog, a protein with a relative molecular weight of 56 kDa has been isolated from hepatic membranes and is believed to be the functional receptor protein (Hui, Brecht, Hall, Friedman, Innerarity and Mahley 1986). In addition to the uptake of chylomicron remnants the receptor is also responsible for the uptake of β-VLDL and apoE-enriched HDL called HDL_1 (also known

as HDL_C), two unique lipoproteins which may be characteristic of hypercholesterolaemia in the dog (Mahley, Weisgraber and Innerarity 1974).

Clearance of exogenous lipid from the circulation depends on a number of mechanisms, including the activity of lipoprotein lipase, the presence of apoC-II, the structural integrity of apoE and the activity of the hepatic apoE receptor.

2.2. Hepatic Lipid Synthesis: Very Low Density Lipoproteins

The liver is the central organ in the maintenance of plasma lipid levels and lipoprotein metabolism. Very low density lipoproteins, the major plasma vehicle of endogenous triglyceride are structurally similar to chylomicrons and are synthesised continuously by the liver. The major apolipoprotein of VLDL is $apoB_{100}$, which has a molecular weight approximately twice that of the protein synthesised in the intestine ($apoB_{48}$). A single molecule of $apoB_{100}$ encircles the VLDL particle and remains with the lipoprotein throughout its lifetime in the circulation (Calvert and Abbey 1985). Following its synthesis in the endoplasmic reticulum, apoB is complexed with cholesteryl esters and transferred to the junction of the smooth endoplasmic reticulum, where triglycerides are added. Endogenous triglycerides incorporated into VLDL particles arise from the *de novo* synthesis of fatty acids, from extracellular NEFA, stored hepatic triglyceride or from lipoproteins processed by hepatic mechanisms (Gibbons 1990). After secretion by the Golgi apparatus, the VLDL enter the space of Disse where they acquire apoC and apoE from HDL.

Upon entering the circulation, the core lipid of VLDL is subjected to the lipolytic action of lipoprotein lipase. This process results in partial delipidation of the lipoprotein; however, the final fate of the particle depends to a degree upon the size of the particle. Large, triglyceride-rich VLDL participate in further lipolytic reactions, followed by direct removal from the circulation via hepatic membrane receptors. Small cholesteryl ester enriched VLDL are

processed to intermediate density lipoproteins (IDL), which may be removed directly from the circulation by the hepatic apoB,E receptor, or further modified to LDL by the action of a second endothelial lipase, hepatic lipase (Brown and Goldstein 1986).

VLDL synthesis and secretion is influenced by nutritional and metabolic changes. The addition of insulin to hepatic cell cultures promotes the synthesis but prevents the secretion of triglyceride, resulting in accumulation of triglyceride within the cellular cytoplasm (Patsch, Prasad, Gotto and Bengtsson-Olivecrona 1984). Secretion occurs following removal of the insulin (Gibbons 1990). This may demonstrate a physiologic means of storing hepatic triglyceride in the postprandial period and allowing its release in the fasting period to provide an energy source for muscle.

2.3. Intermediate Density Lipoproteins

This class of lipoproteins is formed from VLDL particles through the lipolytic action of hepatic lipase. The species is therefore rich in cholesteryl esters and has been incriminated as one of the major atherogenic lipoprotein populations. The concentration of IDL in plasma is generally very low, reflecting its rapid conversion to LDL or direct removal from the circulation by the hepatic apoB,E receptor (Brown and Goldstein 1986).

2.4. Low Density Lipoproteins

LDL, the major cholesterol-carrying species in human plasma, is rich in cholesteryl esters and contains a single molecule of $apoB_{100}$ (Calvert and Abbey 1985). It is formed by the action of hepatic lipase on IDL and is considered to be the major participating lipoprotein in the process of atherosclerosis (Tyroler 1984). LDL is composed of a number of subpopulations which differ in their kinetic, chemical, physical and atherogenic properties (Babiak and Rudel 1987).

Clearance of LDL from the circulation is achieved by the apoB,E receptor which is expressed on hepatic and peripheral cell membranes (Mahley and Innerarity 1983). Once bound to the receptor glycoproteins, the LDL is rapidly internalised to form an endocytic vesicle which migrates through the cytoplasm and combines with a primary lysosome, forming a secondary lysosome. Release of cholesterol from the lysosome into the cell suppresses the HMGCoA reductase-catalysed synthesis of cholesterol and increases the activity of acylCoA:cholesterol acyl transferase activity (ACAT) which re-esterifies the cholesterol. In addition, the apoB,E receptor is down-regulated, preventing excessive accumulation of cholesterol in the cell (Brown and Goldstein 1986). After delivery of the lipoprotein constituents to the lysosome the LDL receptor proteins are recycled to the cell membrane surface (Brown and Goldstein 1986). This receptor round-trip takes approximately 10 minutes and occurs irrespective of whether the receptor has collected LDL, ensuring a rapid response to alterations in cellular cholesterol requirements (Angelin, Raviola, Innerarity and Mahley 1983). The greatest number of LDL receptors are located on the liver which accounts for approximately 70% of whole body receptor activity (Brown and Goldstein 1986). In the dog, the expression of hepatic B,E receptors decreases as an animal matures, resulting in a higher fractional catabolic rate of LDL in young dogs, perhaps reflecting the metabolic requirements for growth (Mahley, Hui, Innerarity and Weisgraber 1981). The LDL receptor is also expressed on peripheral cells, in particular those with a high cholesterol demand, *i.e.*, the adrenal glands and gonads, which require cholesterol for steroidogenesis (Mahley and Innerarity 1983). The circulating LDL which is not removed by the apoB,E receptor is cleared by non-regulated mechanisms including macrophage binding sites which display high affinity for chemically modified LDL (Mahley and Innerarity 1983). This is followed by the activation of macrophages which scavenge the lipid material. This non-

regulated uptake and accumulation of cholesteryl esters results in the formation of the typical lipid-laden "foam cells" which are thought to be the precursors of atherosclerotic lesions (Yamauchi and Hoff 1984).

2.5. High Density Lipoproteins

The HDL class shows marked heterogeneity, comprising of several subpopulations. In man two subclasses are found and are identified as HDL_3 and HDL_2. The former is the mature particle formed by transfer of cholesterol from tissues and triglyceride-rich lipoproteins, while the latter is the triglyceride-rich species formed by transfer of triglycerides from other lipoproteins in exchange for cholesteryl ester.

Nascent HDL is produced by the liver and intestine as a disc-like bilayer of phospholipid and protein, mainly apoA-I. Free cholesterol is drawn down a concentration gradient from the peripheral tissues into the disc (Nikkilä 1978; Dory, Boquet, Hamilton, Sloop and Roheim 1985). Following entry to the lipoprotein, the cholesterol is esterified by the action of LCAT on the surface of the bilayer. The acquisition of additional cholesterol leads to progressive expansion of the lipoprotein core and to the development of the mature HDL particle.

Many species, including man, possess a transfer protein which exchanges cholesteryl ester in the HDL core with triglyceride from the core of lower density lipoproteins. This exchange facilitates the formation of a triglyceride-rich HDL particle (HDL_2). HDL_2 is the preferred substrate of hepatic lipase, the action of which results in depletion of the core lipids with reformation of particles characteristic of the HDL_3 population (Nestel 1987). Thus cycles of cholesterol uptake, triglyceride transfer and delipidation progress with distribution of HDL-derived cholesterol esters through lower density lipoprotein remnants to the liver. The exact nature of the uptake of HDL by the liver has not been determined, although the small proportion of particles

which contain apoE may be directed to the apoB,E receptors. This completes the cycle of reverse cholesterol transport, *i.e.*, the delivery of cholesterol from the peripheral tissues to the liver.

In the dog, the metabolism of HDL displays important differences from that of man. In this species, HDL is the major carrier of plasma cholesterol and the individual HDL subfractions, which are classified on the basis of their density, cholesterol and apoE content, and electrophoretic mobility, show few similarities to those of man (Terpstra, Sanchez-Muniz, West and Woodward 1982). The characteristic lipoproteins in the normolipidaemic dog are HDL_2, which have physical properties that lie between those reported for human HDL_2 and HDL_3 (Edelstein, Lewis, Shainoff, Naito and Scanu 1976). This subclass possesses apoA-I and no apoE, while apoE-containing HDL are also recognised. The latter are found in the plasma of normal dogs but are recognised predominantly in hypercholesterolaemic or cholesterol fed dogs and are known as HDL_1 or HDL_c (Mahley, Weisgraber and Innerarity 1974; Mahley 1981). HDL_1 displays α_2 mobility on agarose gel electrophoresis and is isolated from the LDL density range during preparative ultracentrifugation (Mahley, Weisgraber and Innerarity 1974).

The initial stage of HDL production in the dog is similar to that of man, *i.e.*, discoidal HDL, produced by the liver and intestines interacts with peripheral tissue resulting in accumulation of cholesterol in the lipoprotein core (Dory, Boquet, Hamilton, Sloop and Roheim 1985). Progressive accumulation of cholesterol and the action of LCAT results in expansion of the particle so that it decreases in density. Further extension of the lipoprotein requires the addition of apoE, which appears to be uniquely capable of permitting expansion of the HDL core to accommodate more cholesterol (Koo, Innerarity and Mahley 1985). The cholesteryl ester (50% by mass) and apoE enriched lipoprotein produced by this mechanism is HDL_1, which may be isolated from the d1.006-1.063g/ml fraction of the plasma of

hypercholesterolaemic dogs (Sloop, Dory, Hamilton, Krause and Roheim 1983; Mahley and Innerarity 1983). The apoE of HDL_1 is responsible for the recognition and clearance of the lipoprotein by the hepatic apoB,E and E receptors (Koo, Innerarity and Mahley 1985), and acetylation of the lysine residues of this apolipoprotein retards the uptake of the particle (Sherrill, Innerarity and Mahley 1980). HDL_1 shows a higher binding affinity for the hepatic apoB,E receptor than LDL and competes with the LDL for receptor binding, internalisation and degradation (Mahley and Innerarity 1977; Koo, Innerarity and Mahley 1985).

The dog has a low plasma cholesteryl ester transfer protein activity, ensuring that cholesteryl esters in the core of HDL are not transferred to other lipoprotein species, but are directed to the liver for excretion. The containment of cholesterol esters within the HDL class and their efficient uptake by hepatic receptors may play a major role in the protection of the dog from atherosclerosis.

2.6. Miscellaneous lipoproteins

β-VLDL

Beta-VLDL are cholesteryl ester enriched lipoproteins which accumulate in the serum of hypothyroid, cholesterol fed dogs (Mahley, Weisgraber and Innerarity 1974) and human patients with type III hyperlipidaemia (DeWater, Hessels, Bakkeren and Van Berkel 1990). They are isolated from the $d < 1.006 g/ml$ range, contain both apoE and apoB and are thought to represent chylomicron and VLDL remnants (Fainaru, Funke, Boyles, Ludwig, Innerarity and Mahley 1988). Patients with type III hyperlipidaemia may display apoE2 homozygosity which results in impaired clearance of lower density lipoprotein remnants from the circulation (Davignon, Gregg and Sing 1988). The accumulated remnants have the flotation characteristics of VLDL combined with the β electrophoretic mobility of LDL and are therefore called β-VLDL. The appearance of this

unusual lipoprotein species in cholesterol fed animals may have a different aetiology to man, since the apoE complement in these individuals has a normal structure (Mahley and Innerarity 1983). Beta-VLDL in the dog are cleared from the circulation via the hepatic apoB,E receptor and by lipolytic conversion to IDL and LDL (Fainaru, Funke, Boyles Ludwig, Innerarity and Mahley 1988). In the rat, β-VLDL is rapidly cleared by the apoB,E receptor of non-macrophage cells (Goldstein, Ho, Brown, Innerarity and Mahley 1980) and the cholesteryl ester hydrolysed within lysosomes (DeWater, Hessels, Bakkeren and Van Berkel 1990). This feature, and the observation that incubation of canine β-VLDL with human peritoneal macrophages results in massive cholesterol accumulation within the cellular cytoplasm, has prompted the belief that β-VLDL is associated with a marked atherogenic potential (Goldstein, Ho, Brown, Innerarity and Mahley 1980).

Lipoprotein X (Lp-X)

This lipoprotein is characteristically identified in association with cholestatic disease in dogs (Danielsson, Ekman, Johansson and Petersson 1976) and has been isolated from the plasma of patients with familial LCAT deficiency (Miller 1990). The particle floats in the LDL density range but is rich in phospholipids and free cholesterol. The protein content of the lipoprotein is mainly albumin, which is held in the inner core (Riesen and Kloer 1989), and apoC (Danielsson, Ekman, Johansson and Petersson 1976). The physical properties of the lipoprotein result in a characteristic cathodic migration on agarose gel electrophoresis, allowing identification of the species in plasma (Durrington 1989). The mechanism of Lp-X production has not been determined, but the species may result from an interaction between plasma albumin and regurgitated biliary lipids. Neither lipoprotein lipase nor hepatic lipase appear to act upon the lipoprotein which is removed from the circulation by the cells of the reticulo-endothelial system (Riesen and Kloer 1989).

Despite this, Lp-X may interfere with the catabolism of chlyomicrons, leading to marked lipid abnormalities (Durrington 1989). The use of alterations of plasma Lp-X concentrations for the differentiation of intra and extrahepatic jaundice in man has been suggested, but has not been validated (Meredith 1986).

3. APOLIPOPROTEINS

The apolipoproteins serve a number of functions which are vital in the synthesis, modification and metabolism of lipoproteins. The most important is their ability to form lipoprotein complexes from phospholipid bilayers and insoluble lipids. In addition, the proteins are instrumental in directing the metabolic fate of the lipoproteins in their role as enzyme cofactors and receptor ligands. The apolipoproteins of the dog are similar to those of man (Mahley and Weisgraber 1974).

3.1. Apolipoprotein A

ApoA-I is the major protein constituent of canine HDL (Edelstein, Lewis, Shainoff, Naito and Scanu 1976). The mature protein (molecular weight 28kDa) is a crucial structural protein in the formation of HDL and is a cofactor in the enzymatic reaction catalysed by LCAT. It is possible that the apolipoprotein physically stabilises the enzyme with respect to its phospholipid substrate, ensuring maximal activity. The apolipoprotein is synthesised as pre-pro-apoA-I in the intestines and liver. Translational cleavage forms pro-apoA-I which is secreted from the cell and undergoes proteolytic cleavage (by a pro-apoA-I peptidase), resulting in formation of mature, plasma apoA-I (Calvert and Abbey 1985).

ApoA-II is a minor protein component of HDL while apolipoprotein A-IV is associated with the lower density lipoproteins (Weisgraber, Bersot and Mahley 1978). The exact function of apoA-IV is not certain (Nestel 1987; DeLamatre, Hoffmeier, Lacko and Roheim 1983).

3.2. Apolipoprotein B

In contrast to the other apolipoproteins, apoB is insoluble and does not transfer between lipoproteins (Calvert and Abbey 1985). Two distinct forms of the protein have been recognised in canine plasma. $ApoB_{100}$, the major protein component of VLDL, IDL and LDL, is largely of hepatic origin, although a small amount may be synthesised in the intestines. This protein is one of the largest isolated monomeric proteins, with a molecular weight of 549kDa. The second form of apoB, designated $apoB_{48}$ because its molecular weight is 48% of that of $apoB_{100}$, is synthesised in the intestines and is secreted with exogenous lipid in the form of chylomicrons. Differentiation of the two forms, on the basis of molecular weight, can be achieved using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis.

3.3. Apolipoprotein C

A number of species of apolipoprotein C have been identified using SDS polyacrylamide gel electrophoresis. Quantitatively the most important are C-II and C-III, although apoC-I, C-IV and C-V have also been identified. Each protein performs a different function in the pathways of lipoprotein metabolism. ApoC-I is identified in association with VLDL and HDL where its main role is in the stabilisation and binding of lipid for the enzymatic reaction catalysed by LCAT (Calvert and Abbey 1985). ApoC-II, also found in association with chylomicrons, VLDL and HDL is the essential activator of lipoprotein lipase. It appears to act by orientating the enzyme and substrate for maximal activity. The details of the role of apoC-III are not clear, although it does appear that the activation of lipoprotein lipase by apoC-II can be inhibited by apoC-III (Quinn, Shirai and Jackson 1982).

3.4. Apolipoprotein E

ApoE is associated with chylomicrons, VLDL, IDL and HDL. It is synthesised as a proprotein and undergoes intracellular proteolysis and glycosylation, and

extracellular desialation to form the apolipoprotein observed in plasma (Zannis, McPherson, Goldberger, Karathanasis and Breslow 1984). The protein is synthesised in peripheral tissues, including the adrenal glands and the kidneys (Blue, Williams, Zuckeer, Khan and Blum 1992), and by cholesterol-loaded mouse peritoneal macrophages and human monocytes (Basu, Ho, Brown, Bilheimer, Anderson and Goldstein 1982). ApoE is secreted with phospholipids in lamellar discs and can be recognised by apoB,E receptors for which they show greater affinity then LDL (Basu, Ho, Brown, Bilheimer, Anderson and Goldstein 1982). The rate of apoE synthesis is increased two-fold in cholesterol fed dogs when compared to control animals (Dory, Boquet, Tate and Sloop 1986). This evidence suggests that the production of apoE by peripheral tissues and its uptake by the LDL receptor may play a major role in reverse cholesterol transport, thought to be the mechanism of protection against atherosclerosis. However, the evidence for *in vivo* production of apoE by the peripheral tissues of the dog is controversial (Julien, Fong and Angel 1988).

In man, the gene specifying apoE is polymorphic. The protein products of the three common alleles are designated E2, E3 and E4. The most common allele is E3. Substitution of cysteine for the arginine at position 158 gives rise to the E4 allele, while replacement of the cysteine by arginine at position 112 results in the formation of E2 (Zannis and Breslow 1981). The variation in size and/or charge of the isoforms is due partly to genetic variation and partly to post-translational modification of the original polypeptide. These changes include sialation of the original polypeptide (Zannis and Breslow 1981), formation of apoE-apoE bonds and formation of an apoE-apoA-II complex (Weisgraber and Mahley 1978). Abnormalities of apoE binding affinity due to E2 homozygosity, rare apoE mutants or a deficiency of apoE results in type III hyperlipidaemia in man, a condition characterised by premature coronary

artery disease and peripheral vascular disease (Schaefer, Gregg, Ghiselli, Forte, Ordovas, Zech and Brewer 1986).

Unlike the human apolipoprotein, apoE in the dog has four major isoforms (Dory, Boquet, Tate and Sloop 1986) resulting from sialation of one parent polypeptide (Weisgraber, Troxler, Rall and Mahley 1980).

4. ENZYMES

4.1. Lipoprotein lipase

Lipoprotein lipase (EC 3.1.1.34) is responsible for the hydrolysis of triglyceride carried in chylomicrons and VLDL, and as such, is the rate-limiting step in the removal of these lipoproteins from the circulation. The enzyme is found on the capillary endothelium, particularly of adipose tissue, muscle and lactating mammary gland (Eckel 1989). Lipoprotein lipase allows delivery of free fatty acids to tissues, either for utilisation or for re-asssimilation to triglycerides and storage in adipose tissue. Reciprocal regulation of tissue enzyme activities ensures delivery of fatty acids to the adipose tissue during the postprandial period and channelling of fatty acids for utilisation by muscle tissue during periods of fasting (Cryer 1981). The activity of adipose tissue lipoprotein lipase correlates positively with plasma insulin concentrations (Semencovich, Chen, Wims, Luo, Li and Chan 1989) while catecholamines, ACTH, glucagon, TSH and oestrogen result in enzyme inhibition (Cryer 1981).

Lipoprotein lipase is located on the endothelium and its activity can only be detected in the plasma after the administration of heparin (LaRosa, Levy, Windmueller and Fredrickson 1972). The enzyme is attached to the endothelium via glycosaminoglycan (heparan-sulfate) chains which present a number of enzyme molecules to each lipoprotein (Eckel 1989). Intravenously administered heparin binds to the endothelium, displacing lipoprotein lipase into the plasma, in which it may be measured (Hallberg 1968). The administration of heparin is not thought to induce lipoprotein lipase activity

although heparin does stabilise the purified protein by preventing surface denaturation (Quinn, Shirai and Jackson 1982).

4.2. Hepatic lipase

An enzyme with triglyceride lipase activity, similar to human hepatic lipase has been isolated from perfused canine livers (Greten, Sniderman, Chandler, Steinberg and Brown 1974; Frost, Shore and Havel 1982). This enzyme has action against triglycerides, monoglycerides and phospholipids (Ehnholm, Shaw, Greten and Brown 1975) and is responsible for the conversion of IDL to LDL, HDL_2 to HDL_3 and the clearance of chylomicrons from the circulation. The mechanism of this latter action is unclear (Sultan, Lagrange, Jansen and Griglio 1990). The role of hepatic lipase in the lipoprotein metabolism of the dog has not been elucidated.

4.3. Lecithin:cholesterol acyl transferase

Lecithin:cholesterol acyl transferase (EC 2.3.1.43) catalyses production of plasma cholesteryl esters and is the key enzyme in the transport of cholesterol from the peripheral tissues to the liver (Grove and Pownell 1991). The enzyme is produced by the liver (Riesen and Kloer 1989) and has both acyl transferase and phospholipase A activity which are stimulated by apoA-I (Grove and Pownell 1991; Chen and Albers 1983). The substrates for action include lecithin and cholesterol on the lipoprotein surface and the reaction involves the cleavage of the fatty acid on position 2 of lecithin and its transfer to free cholesterol, forming two products: cholesteryl ester and lysolecithin. The latter is then bound to albumin, while cholesteryl ester moves into the lipoprotein core (DeLamatre, Hoffmeier, Lacko and Roheim 1983).

4.4. Cholesteryl ester transfer protein

A variety of lipid transfer proteins have been identified in human plasma (Zilversmit 1984). Cholesteryl ester transfer protein (CETP) catalyses the transfer of cholesteryl esters from HDL to the other lipoprotein species in

exchange for triglyceride (Tall 1986; Brown, Inazu, Hesler, Agellon, Mann, Whitlock, Marcel, Milne, Koizumi, Mabuchi, Takeda and Tall 1989).

There is a marked interspecies variation in CETP activity. Man is considered to have a high enzyme activity, while that in the dog is low (Ha and Barter 1982). Species lacking CETP activity are resistant to dietary atherosclerosis, while those with intermediate or high activities are susceptible (Tall 1986). In the former, HDL cholesteryl esters are removed directly from the circulation by the hepatic apoE receptor (Tall 1986).

5. HYPERLIPIDAEMIA

In clinical terms, hyperlipidaemia is defined as an elevation of plasma cholesterol and/or triglyceride concentrations. The condition may arise as the result of a primary, often inherited defect in lipoprotein metabolism or secondary to systemic disease processes. In man, the plasma lipid and lipoprotein concentrations may therefore be influenced by medical status, hormonal factors, dietary composition and genetic determinants (Tyroler 1984). The current understanding of the interactions of these factors with canine lipoprotein metabolism is based mainly on observations made during experimental procedures and a small number of clinical reports. The following is a review of these observations.

5.1. Dietary influences

A high dietary cholesterol intake over a number of months results in hypercholesterolaemia in dogs. This reflects an increase in the HDL concentration, in particular, HDL_1 (Julien, Fong and Angel 1988). Dietary cholesterol loading is also accompanied by increased plasma apoB and apoE concentrations (Melchior and Harwell 1985), which may be the result of overproduction rather than impaired catabolism, since the response to reduction in dietary cholesterol is rapid (Melchior, Dory and Roheim 1984). Expansion of the hepatic cholesterol pool may also be noted and although a

decrease in biliary excretion has been proposed as the major contributing factor, this has not been proven (Melchior and Harwell 1985). Beta-VLDL have been identified in the plasma of hypothyroid dogs fed on high cholesterol diets (Mahley, Weisgraber and Innerarity 1974; Mahley 1981).

The lipid abnormalities produced when dogs are fed a high fat diet appear to depend on the degree of saturation of the fat (Grande and Schultz 1968; McCullagh, Ehrhart and Butkas 1976). Atherosclerotic lesions of the abdominal aorta, coronary and cerebral arteries have been induced in dogs maintained on a diet rich in cholesterol and hydrogenated coconut oil for one year (Mahley, Innerarity, Weisgraber and Fry 1977). Marked hypercholesterolaemia appears to be a prerequisite for the development of lesions in the dog and vascular damage alone does not induce atherosclerosis (Schaub, Keith, Bell and Hunt 1987).

Although the dog is relatively resistant to dietary induced hyperlipidaemia and atherosclerosis, it has been proposed that dietary and environmental factors may play a role in determining the fasting plasma lipid and lipoprotein cholesterol concentrations in normal dogs (Bass, Hoffman and Dorner 1976). It has been demonstrated that the total plasma cholesterol, VLDL-cholesterol and LDL-cholesterol concentrations in pet border collies were higher than those of working dogs (Crispin, Bolton and Downs 1992), but it was not possible to determine the relative effects of dietary fat intake and exercise regimes.

5.2. Diabetes mellitus

The studies of experimental diabetes mellitus in the dog have produced conflicting information, possibly as the result of differences in the methods for inducing the disease in laboratory animals. Streptozocin-alloxan induced diabetes mellitus was accompanied by increased plasma LDL, HDL_1 and apoE concentrations, but decreased concentrations of HDL_2 (Wilson, Chan, Elstad,

Peric-Golia, Hejazi, Albu and Cutfield 1986; Gleeson, Hejazi, Kwong, Chan, Le, Alberts and Wilson 1990). However, the relevance of these findings to the clinical situation is limited since the toxic methods of inducing insulin deficiency may have resulted in altered hepatic function, leading to an additional compromise of the lipoprotein metabolic pathways. Cholesterol feeding in addition to the induction of diabetes mellitus appears to be associated with increased concentrations of the triglyceride-rich lipoproteins which may arise either from increased hepatic triglyceride synthesis or decreased clearance from the circulation (Wilson, Chan, Elstad, Peric-Golia, Hejazi, Albu and Cutfield 1986). Although the activities of lipoprotein lipase and hepatic lipase have been measured in the postheparin plasma of dogs with experimental diabetes mellitus, the results are contrary. Wilson *et al.* (1986) found that the enzyme activities were the same in control and affected dogs, but pancreatectomised dogs showed a rise in the activity of hepatic lipase which was partially corrected by short term insulin therapy (Muller, Saudek and Applebaum-Bowden 1985).

The rather complex nature of lipid abnormalities associated with experimental diabetes mellitus parallels the heterogeneity of disturbances noted in dogs with naturally-occurring disease. Rogers, Donovan, and Kociba, (1975b) noted that six of nine dogs with insulin dependent diabetes mellitus had increased plasma cholesterol and triglyceride concentrations. In most of these animals the lipid elevations were accounted for by increased LDL and HDL as assessed by agarose gel electrophoresis. In five of these dogs, chylomicrons were recognised on the basis of lipoprotein electrophoresis. After the introduction of insulin therapy there was a decrease in triglyceride concentration, but the cholesterol response was erratic. Ford (1977) suggested that the development of hyperlipidaemia in diabetes mellitus was restricted to dogs with metabolic complications, *i.e.*, diabetic ketoacidosis, and that the abnormalities included hypertriglyceridaemia and hyperbetalipoproteinaemia.

These suggestions are contrary to the findings of Medaille, de La Farge, Braun, Valdiguie and Rico (1988) who identified no differences in the lipid concentrations of non-ketotic and ketotic diabetic dogs.

The information regarding the nature of hyperlipidaemia associated with canine diabetes mellitus is therefore rather confused. The comparison of results is hindered by a lack of standardisation of the methods of lipoprotein analysis, which have, to date, been limited to the use of semi-quantitative agarose gel electrophoresis.

5.3. Obesity

Obesity in both man and the rat has been associated with hypertriglyceridaemia and an increased VLDL production (Gibbons 1990). In the obese patient poor regulation of the flux of NEFA from the adipose tissue is a consequence of irregular control of the activity of hormone-sensitive lipase, the enzyme responsible for the hydrolysis of adipose triglyceride (Coppack, Evans, Fisher, Frayn, Gibbons, Humphreys, Kirk, Potts and Hockaday 1992). The increased flux of NEFA may have a number of effects including an increased hepatic triglyceride synthesis and impairment of the hepatic degradation of insulin (Anderson, Sobocinski, Freedman, Barboriak, Rimm and Gruchow 1988). The metabolic factors affecting the development of hyperlipidaemia in human obesity have not been fully defined (Durrington 1989). It has been proposed that obesity in the dog is associated with abnormalities of lipid metabolism (Zerbe 1986), but this hypothesis has not been tested.

5.4. Hypothyroidism

The induction of experimental hypothyroidism in combination with a high cholesterol diet has been used extensively to produce hypercholesterolaemia, hypertriglyceridaemia and atherosclerotic lesions in laboratory dogs (Moses 1954; Grande and Schultz 1968).

Familial hypothyroidism and hyperlipidaemia has been recognised in beagles (Manning, Corwin and Middleton 1973; Manning 1979). Affected dogs showed increased plasma cholesterol, triglyceride, VLDL and LDL concentrations and on postmortem examination had moderate to severe atherosclerotic lesions of the coronary and renal arteries. In addition to this family of beagles, there are a number of reports of lipid abnormalities secondary to spontaneous hypothyroidism in the dog, including a survey by Larsson (1988) in which two thirds of animals with thyroid dysfunction were hypercholesterolaemic. The lipid disturbances may vary in severity and nature, ranging from mild hypercholesterolaemia with a small increase in the HDL concentration, to marked hypercholesterolaemia and hypertriglyceridaemia with intense staining of the α_2, β and pre-β regions on agarose gel electrophoresis (Mahley, Weisgraber and Innerarity 1974; Rogers, Donovan and Kociba 1975b; Medaille, de La Farge, Braun, Valdiguie and Rico 1988). The introduction of thyroid hormone replacement therapy produces resolution of the lipid abnormalities in affected dogs (Manning 1979).

5.5. Hyperadrenocorticism

In man, the effect of Cushing's syndrome or the administration of exogenous corticosteroids is predominantly to increase the plasma cholesterol, LDL-cholesterol and occasionally VLDL-cholesterol concentrations (Durrington 1989).

Rogers (1977) stated that Cushing's syndrome in the dog was not associated with hyperlipidaemia. However, in a study of 115 cases of canine hyperadrenocorticism (Ling, Stabenfeldt, Comer, Gribble and Schechter 1979), 90% of dogs presented with hypercholesterolaemia. Medaille *et al.* (1988) confirmed the latter finding and suggested that increased plasma HDL and LDL concentrations were responsible for the high plasma cholesterol concentrations observed.

5.6. Cholestasis

Experimental cholestasis in the dog results in an increase in the LDL concentration, which is reflected in the total plasma cholesterol concentration (Bauer, Meyer, Goring, Beauchamp and Jones 1989). The increased LDL-cholesterol concentration is thought to be due to the presence of LpX, the lipoprotein which is characteristically identifed in cholestatic disease in man. A lipoprotein which is similar to human LpX has been quantified in the plasma of dogs with experimental cholestasis (Ritland and Bergan 1975), but other investigators have failed to identify a lipoprotein which has the high free cholesterol content of the human species (Danielsson, Ekman, Johansson and Petersson 1976 and 1977). In the study by Ritland and Bergan (1975) the LpX concentration correlated inversely with the activity of LCAT, suggesting a role for the failure of cholesterol esterification in the formation of this characteristic lipoprotein species. The information regarding the lipid and lipoprotein abnormalities associated with cholestatic disease in the dog is restricted to experimental induction of the condition.

5.7. Hepatocellular disease

Experimental hepatic necrosis in the dog is accompanied by a decrease in the plasma triglyceride and LDL concentrations (Bass, Hoffmann and Dorner 1976; Ford 1977). The triglyceride concentration may reflect a failure of hepatic synthesis and in acute hepatic disease it returned to normal before some of the parameters commonly used to assess hepatocellular insult and hepatic function, *i.e.*, the plasma alanine aminotransferase activity and the sulphobromophthalein (BSP) retention test (Bass, Hoffmann and Dorner 1976). This observation has led to the suggestion that lipid profiles may be an early prognostic indicator in acute hepatic disease in the dog (Ford 1977). The value of this approach has not been tested.

5.8. Glomerulonephritis

Nephrotic syndrome in man is characterised by an increased synthesis of triglyceride-rich lipoproteins, accompanied by a marginal reduction in lipoprotein clearance (Kaysen, Myers, Couser, Rabkin and Felts 1986). In cases of gross proteinuria, there may be urinary loss of HDL, resulting in a reduction in the source of apoC, which may in turn prevent activation of lipoprotein lipase (Chan, Persaud, Ramdial, Varghese, Sweny and Moorhead 1981). It has also been proposed that the reduction of the plasma heparan sulfate concentration detected in nephrotic humans may result in impaired lipoprotein lipase activity (Kaysen, Myers, Couser, Rabkin and Felts 1986). The plasma cholesterol and LDL cholesterol concentrations in nephrotic patients are increased and correlate inversely with the plasma albumin and plasma oncotic pressure (Appel, Blum, Chien, Kunis and Appel 1985; Joven, Villabona, Vilella, Masana, Albertí and Vallés 1990)

Glomerulonephritis in the dog has been associated with a variety of lipid abnormalities. Medaille *et al.* (1988) reported moderate hypercholesterolaemia accompanied by increased plasma VLDL and decreased HDL concentrations, findings contrary to those of Ford (1977) who identified an increased HDL concentration.

5.9. Acute pancreatitis

It has been suggested that lipaemia is commonly associated with naturally-occurring acute pancreatitis in the dog (Anderson, N.V., 1972). The identification of lipaemia, accompanied by variable increases in plasma HDL, LDL and VLDL concentrations in four dogs with pancreatitis supported this observation (Whitney, Boon, Rebar and Ford 1987). Similar increases in total plasma cholesterol and triglyceride concentrations with variable lipoprotein electrophoretic profiles have been noted by a number of investigators (Rogers, Donovan and Kociba 1975b; Ford 1977; Schaer 1979).

Experimental pancreatitis has been associated with an increase in the β peak on lipoprotein electrophoresis, but the total plasma cholesterol and triglyceride concentrations were within the reference ranges (Whitney, Boon, Rebar and Ford 1987). Other investigators have had similar difficulty in reproducing the lipaemia associated with naturally-occurring pancreatitis by experimental means (Zieve 1968; Bass, Hoffman and Dorner 1976). The association of marked lipid and lipoprotein abnormalities with spontaneous, but not experimentally-induced canine pancreatitis may suggest that hyperlipidaemia is not a consequence of pancreatic disease, but plays a role in its aetiology. This is consistent with opinion in human medicine, where patients with fasting plasma triglyceride concentrations greater than 11mmol/l are considered at risk of developing acute pancreatitis (European Atherosclerosis Society 1988).

5.10. Primary hyperlipidaemia

In man, inherited defects of lipoprotein metabolism are well characterised and occur frequently in comparison to the dog, in which familial hyperlipidaemia is considered rare. The miniature schnauzer is one of the few breeds in which hyperlipidaemia is thought to be the result of an inherited defect. The lipid abnormalities associated with this condition, including hypertriglyceridaemia, hyperchylomicronaemia and hyperbetalipoproteinaemia were first described by Rogers *et al.* (1975a). Since the first description of the entity, investigators have been unable to elucidate the underlying defect in lipoprotein metabolism, although it appears that an absolute deficiency of the enzyme lipoprotein lipase is not involved (Rogers, Donovan, Kociba 1975a; Whitney 1987). The clinical signs in affected dogs include abdominal pain, vomiting, unexplained polydipsia and generalised seizures (Rogers, Donovan and Kociba 1975a; Bodkin 1992). The introduction of a low fat diet may improve the clinical signs (Bodkin 1992)

but has little effect on the electrophoretic lipoprotein profile of individual dogs (Rogers, Donovan and Kociba 1975a).

Hypertriglyceridaemia and hyperchylomicronaemia was also described in two related Brittany spaniels (Hubert, de La Farge, Braun, Magnol 1987). Serum electrophoresis confimed the presence of chylomicrons and increased VLDL concentrations. The effect of intravenous heparin administration was abnormal, suggesting impairment of lipoprotein lipase activity. One of these dogs presented with corneal lipid deposition and alopecia while the other was clinically healthy.

Hyperlipidaemia, characterised by increased concentrations of α_2-migrating lipoproteins has been recognised in related beagle dogs (Wada, Minamisono, Ehrhart, Naito and Mise 1977). The presence of underlying endocrine disease was not excluded in these dogs and it is therefore possible that they were suffering from hyperlipidaemia secondary to familial hypothyroidism as described by Manning (1979). Increased density of the α_2 band has also been recognised in Briards in the United Kingdom (Watson, Simpson, Odedra and Bedford 1993).

The aetiology, pathogenesis and inheritance of these proposed primary hyperlipidaemias of the dog have not been elucidated.

6. AIMS OF THE STUDY

The aim of this study was to characterise the lipid abnormalities recognised in the canine population, to evaluate the effect of such aberrations on the health of individuals and to investigate therapeutic regimes for their treatment.

In order to standardise the investigation of lipid metabolism it was first necessary to identify procedures suitable for the quantitative and qualitative analysis of canine plasma lipoproteins. To this end, a combined ultracentrifugation/precipitation method was validated for the measurement of

lipoprotein cholesterol concentrations (Chapter II). Additional methods of quantitative and semi-quantitative analysis were evaluated to identify suitable alternatives for lipoprotein analysis in veterinary commercial laboratories (Chapter III) and thus expand the study of canine lipid metabolism.

The validated method of quantitative analysis was used to identify the trends in plasma lipid and lipoprotein cholesterol concentrations in dogs with secondary hyperlipidaemia (Chapter IV). The increasing incidence of obesity in pet dogs and concern about the metabolic consequences of adiposity prompted a specific investigation of triglyceride metabolism in obese dogs (Chapter V). The effects of hyperlipidaemia on the health of the canine population are addressed in Chapter VI, where the prevalence of lipoprotein abnormalities and their clinical and pathological consequences are presented.

A rational approach to the investigation of hyperlipidaemia and the management of idiopathic hyperlipidaemia is outlined in Chapter VII, followed by a discussion highlighting the significant findings of this thesis.

CHAPTER II

A METHOD FOR THE QUANTITATIVE ANALYSIS OF CANINE PLASMA LIPOPROTEINS

1. INTRODUCTION

The importance of hyperlipidaemia as a risk factor for coronary heart disease in humans has necessitated the development of methods for the quantification of plasma lipids and lipoproteins. The three methods of analysis which are commonly used are: preparative ultracentrifugation, for the isolation of individual lipoprotein classes; precipitation techniques for the selective measurement of HDL cholesterol; and electrophoresis, for the identification of specific lipoproteins and apolipoproteins. Preparative ultracentrifugation, including density gradient ultracentrifugation (Chapman, Goldstein, Lagrange, Laplaud 1981) may be time-consuming and technically difficult, often resulting in protein contamination of the HDL fraction. More commonly, ultracentrifugation is combined with a precipitation step, allowing separation of VLDL, HDL and LDL, which are then quantified on the basis of their cholesterol content (Lipids Research Clinics Program, 1982; Bachorik and Albers 1986). This method is considered an ideal compromise between expense and precision.

The four major lipoproteins of canine plasma have previously been quantified using density gradient ultracentrifugation (Terpestra, Sanchez-Muniz, West and Woodward 1982; Hollanders, Mongin, N'Diaye, Hentz, Aude and Girard 1986) and analysed in a semi-quantitative fashion using agarose gel (Rogers, Donovan and Kociba 1975b) and Geon-Pevickon block electrophoresis (Mahley and Weisgraber 1974). Although electrophoresis gives good separation of the lipoprotein classes (Noble 1968) and may be necessary for the recognition of specific lipoproteins, *e.g.*, β-VLDL, it provides only semi-quantitative data. Recently, attention has turned to the use of other methods for the analysis of canine plasma lipoproteins, including precipitation (Rhodes, Meyer, Fish and Kerns 1992) and combined ultracentrifugation/precipitation techniques (Weingand and Daggy 1992; Bolton, Downs and Crispin 1990).

While the application of these human procedures may facilitate the investigation of disorders of lipid metabolism in dogs, their continued use cannot be justified without proper validation with respect to the physical and chemical differences that exist between human and canine plasma lipoproteins. To this end, this study was designed to develop a method for the quantitative analysis of plasma lipoproteins in the dog and to fully validate the technique in healthy and hyperlipidaemic subjects.

2. MATERIALS AND METHODS

2.1. Sample collection

Blood samples were collected by jugular venepuncture from dogs which had been fasted for a minimum of 16 hours. The samples were taken into disodium ethylene-diaminotetra-acetic acid (EDTA; final concentration 1mg/ml) and the plasma separated by low speed centrifugation at 4°C for 20 minutes (Beckman TJ-6 centrifuge; Beckman Instruments Inc). Plasma was stored at 4°C for not longer than 24 hours prior to analysis and plasma for interassay (between batch) precision studies was stored for a maximum period of six days.

2.2. Quantification of lipoproteins

The concentration of cholesterol in each lipoprotein fraction was measured following separation by a combined ultracentrifugation/precipitation technique. In this, 4ml of plasma was placed in a thermoplastic ultracentrifuge tube (Ultraclear, 13x64mm; Beckman Instruments Inc.) and overlayered with 2.5ml normal saline (density 1.006g/ml). The tubes were capped and centrifuged at 164,000g, 4°C for 18 hours in a fixed angle rotor (type 50.4 Ti, Optima L-70 preparative ultracentrifuge; Beckman Instruments Inc.). The lipoproteins with density less than 1.006g/ml (VLDL) were removed in the top fraction by tube slicing and made up to a total volume of 3 ml with normal

saline. The cholesterol concentrations of the plasma and infranatant were measured on a Cobas MIRA clinical chemistry analyser (Roche) using an enzymatic colorimetric method (Ma-kit 100 Cholesterol PAP; Roche). The VLDL cholesterol (VLDL-C) was calculated as the difference between the cholesterol concentration of the plasma and that of the infranatant.

The apoB-containing lipoproteins (*i.e.*, LDL) were than precipitated from 1ml of the infranatant by the addition of 50μl of 92mM heparin-manganese chloride, followed by incubation at 4°C for 30 minutes and centrifugation at 10,800g, 4°C for 30 minutes. The cholesterol concentration in the supernatant (HDL-C) was measured and the LDL cholesterol (LDL-C) calculated as the difference between the cholesterol concentration of the infranatant and the HDL-C.

2.3. Assessment of lipoprotein separation

As lipoprotein classes exhibit different electrophoretic mobilities, the efficiency of the separation of lipoprotein fractions was assessed in normolipidaemic and hyperlipidaemic dogs using agarose gel electrophoresis. The LDL precipitate was resolubilised with 0.25ml 0.5M sodium citrate and 1ml 0.15M sodium chloride. The solution was dialysed overnight at 4°C against sodium chloride/azide (8.77g NaCl, 0.13g NaN_3, 0.372g Na_3EDTA in 1 litre, pH7.3). The lipoprotein fractions (VLDL, LDL and HDL) were then made up to 3ml with 0.15M sodium chloride and applied to 0.5% agarose gels (Paragon Lipoprotein Electrophoresis Kit, Beckman Instruments Inc) and electrophoresis was performed according to the manufacturer's instructions.

As it is possible that manipulation and storage of lipoproteins may alter their electrophoretic mobility, further confirmation of the efficiency of lipoprotein separation was sought by analysis of the apolipoprotein distribution within the fractions using SDS polyacrylamide gel electrophoresis. The density of the LDL and HDL fractions was altered to 1.225g/ml by the addition of

0.35g/ml KBr and the lipoproteins re-isolated by ultracentrifugation. The apolipoprotein content of each fraction was resolved by SDS polyacrylamide gel electrophoresis according to the method of Laemmli (1970) using a vertical slab apparatus (SE250; Hoefer Scientific Instruments). Samples were mixed in a 1:1 ratio with treatment buffer (0.125M Tris-Cl pH6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol). ApoA-I was identified on 1.5mm thick 10% polyacrylamide gels (3.33ml 30% acrylamide/2.7% bisacrylamide solution, 2.5ml 1.5M Tris-Cl pH 8.8, 0.1ml 10% SDS, 4.0ml distilled H_2O, 50μl 10% ammonium persulfate and 5μl TEMED). ApoB was identified on 4% gels (1.33ml 30% acrylamide/2.7% bisacrylamide solution, 6ml distilled H_2O). Two multiple molecular weight standards (MW-SDS-70, Sigma Chemical Company and High molecular weight-SDS calibration kit, Pharmacia) were used to determine the Rf value for each protein band. Electrophoresis was performed at a constant 20mA, for 60 minutes. The gels were stained with 1% Coomassie Blue R-250 for 20 minutes then destained with 7.5% acetic acid, 5% methanol overnight. Densitometric scanning was performed at 530nm (Chromoscan 3 Densitometer; Joyce Loebl)

2.4. Comparison of measured and calculated VLDL-C

The VLDL cholesterol concentration, taken as the cholesterol concentration in the top fraction was measured and the results compared with the calculated VLDL-C concentration (VLDL-C = plasma cholesterol - infranatant). This was done for multiple aliquots from the plasma pool with total cholesterol concentration 2.67mmol/l (n=12) and for samples from normo- and hyperlipidaemic dogs (n=28) with calculated VLDL-C concentrations ranging from 0.40 to 20.26 mmol/l.

2.5. Imprecision of the method

The intra-assay (within batch) imprecision was established for aliquots from two pools of plasma with cholesterol concentrations of 2.67 mmol/l (n=12) and

8.14 mmol/l (n=6), analysed in a single assay run. The inter-assay (between batch) variation was determined by repeated analyses performed over six consecutive days of aliquots from the lower cholesterol pool that had been stored at -20°C immediately after collection.

2.6. Effects of storage

A pool of normolipidaemic plasma was divided into 5ml aliquots and stored at -20°C for eight weeks. Samples were removed from the freezer and analysed, as above, at two and eight weeks after collection.

3. RESULTS

3.1. Separation of lipoprotein fractions

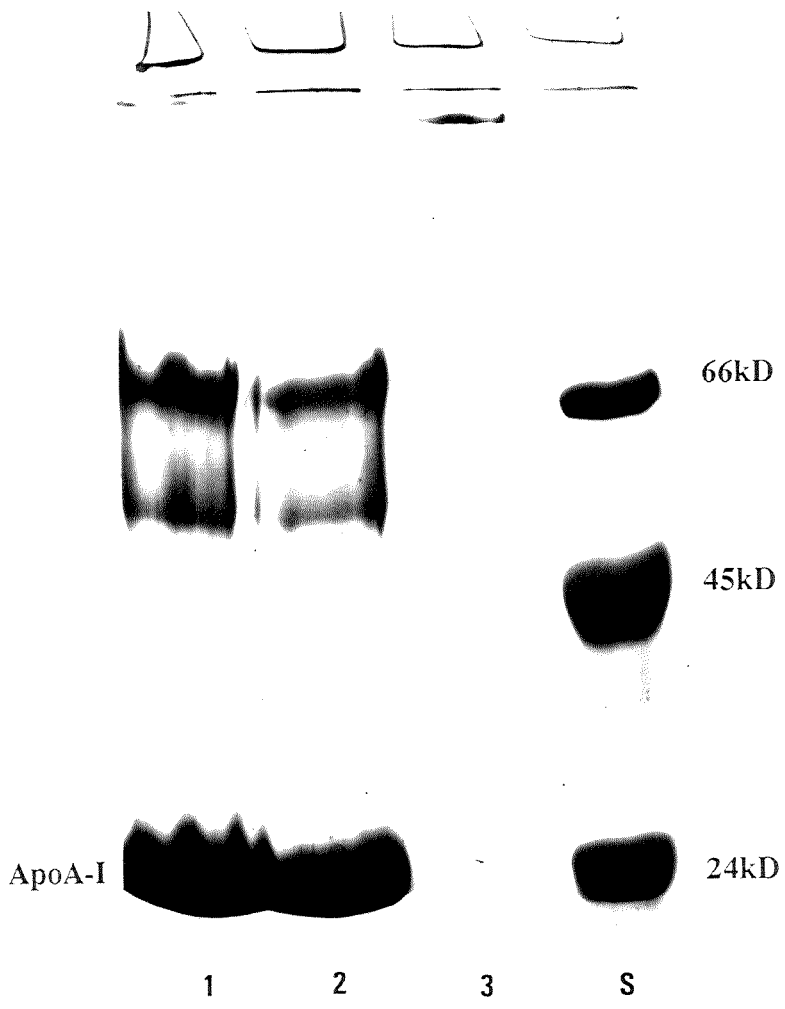
Individual lipoprotein classes were identified according to their characteristic electrophoretic mobility with the HDL in the α position, LDL in the β region and VLDL seen in the pre-β position. The VLDL band was only seen when the lipoprotein was concentrated. In normolipidaemic dogs there was no apparent contamination of the fractions by lipoproteins of other classes (Fig. 2.). Separation of the lipoprotein fractions of a dog with a plasma cholesterol concentration of 15mmol/l resulted in isolation of pure α-migrating HDL, but the LDL fractions contained lipoproteins with α and β mobility.

Four major protein bands were detected when the LDL fractions of normo- and hyperlipidaemic dogs were applied to SDS polyacrylamide gels. The molecular weights were 500-570 kDa, assumed to be apoB, and smaller bands at 340-370 kDa, 132-140 kDa and 125-128 kDa that were assumed to represent fragments of apoB. The HDL fractions of normo- and hyperlipidaemic dogs applied to the same gels showed no evidence of contamination by these proteins, indicating complete precipitation of LDL

Figure 2. Agarose gel electrophoresis of native plasma and separated lipoproteins fractions. Track 1, plasma. Track 2, concentrated VLDL showing pre-β mobility. Track 3, infranatant (HDL) before precipitation of LDL. Tracks 4 and 5, infranatant after precipitation of LDL. Tracks 6 and 7, resolubilsed precipitate.

from the infranatant. However, in samples from dogs with plasma cholesterol concentrations ranging from 4 to 15mmol/l there was some contamination of the LDL fraction by a protein of molecular weight 26.5-28.5 kDa, which was assumed to be apoA-I, the major protein of HDL (Fig. 3.). Densitometric scanning of the HDL and LDL tracks showed that in normolipidaemic dogs the mean $\pm$sd percentage of total apoA-I that was present in the LDL fraction was 3.68 $\pm$2.42 % while in hyperlipidaemic dogs this value was 8.5 $\pm$0.96 %.

Figure 3. Sodium dodecyl sulphate polyacrylamide gel (10%) electrophoresis of lipoproteins in HDL and LDL fractions. Tracks 1 and 2, infranatant (HDL) after precipitation of LDL. Track 3, resolubilsed LDL precipitate showing trace contamination with apolipoprotein A-I. S, molecular weight markers (daltons).



3.2. Comparison of measured VLDL-C and calculated VLDL-C

In samples with a total cholesterol concentration of 2.67 mmol/l, the mean ±sd measured VLDL-C (0.02 ±0.01mmol/l) was lower ($p<0.01$) than the mean calculated VLDL-C (0.13 ±0.07mmol/l). For 28 normo- and hyperlipidaemic samples, the mean ±sd measured VLDL-C concentration (2.46 ±5.70mmol/l) was also lower ($p<0.001$) than the calculated value (2.74 ±4.87mmol/l). The difference (mmol/l) between the two methods correlated negatively with the VLDL-C concentration ($r = -0.43$, $p<0.05$) (Appendix 5).

3.3. Imprecision of the method

The intra-assay (within batch) and interassay (between batch) coefficients of variation (CV) of HDL-C and LDL-C varied between 3.3% and 9.0% for plasma with cholesterol concentrations of 2.67mmo/l ($n=12$) and 8.14mmol/l ($n=6$) (Table 1.) (Appendix 3). The intra-assay CV and interassay CV for VLDL-C were higher than those of the other lipoprotein classes.

3.4. Effects of storage

Aliquots of plasma stored at -20°C for 2-8 weeks showed approximately 9 % reduction in HDL-C concentration. The LDL-C increased after storage for 8 weeks (Table 2.) (Appendix 4). During this period, there was no significant change in cholesterol concentration of the infranatant (HDL-C + LDL-C). However, the total plasma cholesterol concentration had increased at 2 weeks, which led to an increased calculated VLDL-C value. The increase in plasma cholesterol on storage was accompanied by the development of turbidity of the sample, which was cleared by low speed centrifugation and the measurements repeated. The total cholesterol concentrations after clearing the plasma were significantly lower (2.73 ±0.07mmol/l; $p<0.01$) than those in the turbid samples (2.89 ±0.05mmol/l) and were not significantly different from the concentrations in the fresh samples.

Table 1. Imprecision of the measurement of plasma lipoprotein fractions. Aliquots (5ml) of pooled plasma with cholesterol concentrations of 2.67mmol/l (n=12) and 8.00mmol/l (n=6) were analysed. The coefficients of variation (CV) are expressed.

	Cholesterol concentration (mmol/l)					
	VLDL		LDL		HDL	
Plasma	mean ±sd	CV	mean ±sd	CV	mean ±sd	CV
Within batch:						
2.67 (n=12)	0.13 ±0.07	53.8	0.72 ±0.05	6.9	1.82 ±0.06	3.3
8.14 (n=6)	0.98 ±0.18	18.4	3.63 ±0.21	5.8	3.53 ±0.16	4.5
between batch:						
2.67 (n=6)	0.40 ±0.09	22.5	0.78 ±0.07	9.0	1.66 ±0.12	7.2

4. DISCUSSION

The investigation of the metabolic abnormalities associated with hyperlipidaemia requires the recognition of both qualitative and quantitative changes in plasma lipoproteins. Qualitative changes are commonly assessed on the basis of alterations in electrophoretic mobility (Rogers, Donovan and Kociba 1975a and b; Ford 1977), but the introduction of ultracentrifugation and precipitation techniques has facilitated the quantitative analysis of the human lipoprotein classes. The application of such techniques for the quantification of canine lipoproteins may be inappropriate since it has been reported that an

Table 2. The effect of storage at -20°C on mean ±sd plasma lipoprotein cholesterol concentrations in fresh (n=12), 2 week old (n=6) and 8 week old (n=6) samples.

	Cholesterol concentration (mmol/l)				
Treatment	Total	Infranatant	VLDL	LDL	HDL
Fresh	2.67 ±0.06	2.54 ±0.06	0.13 ±0.07	0.72 ±0.05	1.82 ±0.06
Storage, 2 weeks	2.87* ±0.09	2.38 ±0.22	0.48* ±0.15	0.74 ±0.30	1.64* ±0.13
Storage, 8 weeks	2.85* ±0.03	2.45 ±0.11	0.40* ±0.09	0.79* ±0.07	1.66* ±0.12

* $p<0.05$

apolipoprotein E rich subfraction of HDL, identified in the plasma of hypercholesterolaemic dogs (Mahley, Weisgraber and Innerarity 1974), may behave like LDL under certain precipitation conditions (Rhodes, Meyer, Fish and Kerns 1992).

The combination of ultracentrifugation and precipitation described here produced an HDL fraction which was free from LDL. The purity of this fraction when applied to agarose and SDS-polyacrylamide gels suggested that 92mM heparin-manganese chloride precipitated the total LDL content of canine plasma. These findings are similar to those in man where 92mM heparin-manganese chloride results in precipitation of the total apoB content of both fresh and stored plasma (Warnick, Cheung and Albers 1979; Gibson, Rubinstein and Brown 1984).

Contamination of the LDL fraction with an α-migrating lipoprotein was evident when the lipoprotein fractions from hypercholesterolaemic dogs were

studied using agarose gel electrophoresis. It was possible that this was an artefact from the effects of manipulation and storage on the electrophoretic mobility of the lipoprotein. In addition, the precipitation of an apoE enriched, α-migrating lipoprotein by heparin-manganese chloride has previously been recognised (Mahley and Innerarity 1977). However, regardless of the electrophoretic phenomenon, resolution of the constituent apolipoproteins showed that only a small amount of the total apoA-I was present in the LDL fraction. Since apoA-I is not associated with LDL in the plasma, it followed that HDL was present as a contaminant of the precipitated LDL. This error was considered to be of low significance, representing only a 0.06mmol/l increase of the LDL-C in the normolipidaemic dog.

It is recommended by the Lipid Research Clinics Program (1982) that the VLDL-cholesterol concentration in man is calculated rather than measured. The differences found between the calculated and measured values in this study suggest that this guideline should also be applied to the dog. These differences may result from poor recovery of the top fraction after centrifugation, or from problems in measuring the cholesterol content of VLDL, particularly in the dog, where its concentration is low (Chapman 1980) and is close to the lower limit of detection of the cholesterol assay. The latter may explain the high CV reported for low VLDL-cholesterol concentrations in this study, which are similar to those achieved in established human lipid laboratories (Cathcart and Dominiczak 1990). It is unlikely that the variations will affect clinical decisions since therapeutic approaches are rarely made where the triglyceride concentration is less than 5mmol/l.

The storage of plasma samples may have deleterious effects upon the composition of lipoproteins (Bachorik, Albers, Ellefson, Kane and Wood 1982). These alterations may result from auto-oxidation, bacterial contamination and the effect of lipase and LCAT on the physical and chemical properties of the lipoproteins (Bachorik and Albers 1986). Changes in composition may alter

the precipitability of the lipoproteins. The reduction in HDL-C may therefore be a reflection of an increased precipitability of this lipoprotein class. In addition, it has been reported that prolonged storage of plasma reduces the precipitability of apoB-containing lipoproteins (Bachorik, Walker, Brownell, Stunkard and Kwiterovich 1980). This effect may become dominant in the plasma of dogs with high LDL-C concentrations. The present study showed that plasma for quantitative analysis should not be stored for periods of more than 2 weeks, and ideally, precipitation steps should be performed as soon as possible after sample collection.

The combined ultracentrifugation and precipitation method described gave effective separation of the lipoproteins of canine plasma and is presented in the remainder of this thesis as the reference method for the quantitative analysis of canine plasma lipoproteins.

CHAPTER III

METHODS FOR THE ANALYSIS OF CANINE PLASMA LIPOPROTEINS

1. INTRODUCTION

Three major procedures have been developed for the measurement of lipoproteins in clinical chemistry laboratories. These are ultracentrifugation, for the isolation of lipoprotein fractions (Terpestra, Sanchez-Muniz, West and Woodward 1982), precipitation techniques, for the measurement of HDL-C concentrations, and electrophoresis for lipoprotein and apolipoprotein characterisation. Ultracentrifugation or combined ultracentrifugation/ precipitation techniques (Lipid Research Clinics Program, 1982) are considered the reference methods for lipoprotein quantification but the techniques are more time-consuming and expensive than simple precipitation methods, limiting their availability to dedicated lipid laboratories and research establishments. As a result of these constraints, the precipitation methods, in conjunction with the estimation of LDL-C concentration by the Friedewald formula have gained popularity and are used in many routine chemical pathology laboratories.

A number of classes of precipitation reagent have been employed, the most successful of which are high molecular weight polysaccharides (dextran sulphate), lower molecular weight polysaccharides with divalent metals (heparin-manganese; phosphotungstate-magnesium) and neutral polymers (polyethylene glycol; polyvinylsulphate (PVS)) (Warnick, Cheung and Albers 1979; Rifai, Warnick, McNamara, Belcher, Grinstead and Frantz 1992). The measurement of HDL-C concentrations by these precipitation techniques is often combined with the calculation of LDL-C using the formula derived by Friedewald *et al.* (1972). The use of this formula is restricted to fasted samples with no evidence of chylomicronaemia and a plasma triglyceride concentration less than 4.5mmol/l. The formula is as follows:

LDL-C = (total cholesterol) - (HDL-C) - (total triglyceride x 0.45)

The application of this formula only requires the measurement of plasma triglyceride, total cholesterol and HDL-C concentrations, and therefore provides an inexpensive alternative to ultracentrifugation methods for the analysis of many human samples.

In veterinary medicine, investigators have concentrated on the use of electrophoresis for the semi-quantitative analysis of canine plama lipoproteins (Rogers, Donovan and Kociba 1975a and b; Ford 1977). More recently, attention has focused on the use of precipitation techniques for the quantification of canine plasma lipoproteins. The selective measurement of HDL-C is achieved by the use of precipitation reagents which induce precipitation of all the lipoprotein classes except HDL, leaving this class in the supernatant. However, Rhodes, Meyer, Fish and Kerns (1992) determined that phosphotungstate in the presence of magnesium ions, a reagent commonly used for the selective measurement of human HDL-C, causes precipitation of canine HDL in addition to the apoB-containing lipoproteins which results in an appreciable underestimate of plasma HDL-C concentrations. Weingand and Daggy (1992) combined this precipitation reaction with a preceding ultracentrifugation step, a method which resulted in minimal contamination of the precipitate with HDL and is thus considered an appropriate method for the quantification of canine plasma lipoproteins. Downs, Bolton, Crispin and Wills (1993) also utilised a combined ultracentrifugation/precipitation technique to quantify the plasma lipoproteins of normal dogs but did not explain their validation techniques or quantify the analytical error incurred by their selected method.

The aim of this study was to evaluate the use of commercially available precipitation reagents with canine plasma in order to identify techniques which might allow for the convenient and economical quantification of canine plasma lipoproteins. In addition, the application of the Friedewald formula for the

estimation of LDL-C and the use of electrophoresis for semi-quantitative analysis of canine lipoproteins was assessed.

2. MATERIALS AND METHODS

2.1. Blood collection

Blood samples were collected from fasted dogs by jugular venepuncture. Serum was harvested for the evaluation of LDL precipitation reagents as recommended by the manufacturers, and disodium ethylene-diaminetetra-acetic acid (EDTA; final concentration 1mg/ml) plasma collected for the assessment of HDL precipitation reagents and electrophoresis of plasma lipoproteins. All analytical procedures were performed on fresh plasma or serum.

2.2. Evaluation of precipitation methods

Five commercial reagents were selected for evaluation. Those for the measurement of HDL-C were phosphotungstic acid (0.55mmol/l)/magnesium chloride (25mmol/l) solution (HDL-cholesterol precipitant; Randox Laboratories) and polyethylene glycol in 0.1M phosphate buffer (Quantolip HDL-cholesterol precipitation reagent; Immuno Ltd). The reagents for the quantification of LDL were heparin (50, 000IU/l)/sodium citrate (0.064mol/l, pH 5.04) solution (LDL-cholesterol; Randox Laboratories Ltd), PVS solution (LDL-cholesterol; Boehringer Mannheim GmbH Diagnostics) and dextran sulphate in a tris HCl buffer (pH 7.4) (Quantolip LDL-cholesterol precipitation reagent; Immuno Ltd).

Each sample was aliquoted and analysed by the reference ultracentrifugation/precipitation method (Chapter II 2.2.) and by one or more of the selected precipitation methods. The agreement between each technique and the reference method was assessed as described (Chapter III 2.6.).

The efficiency of separation of the lipoprotein classes was determined by electrophoresis. The precipitates formed in both the HDL and LDL precipitation reactions were re-solubilised by the drop-wise addition of 0.5M sodium citrate. The supernatants and dissolved precipitates were applied to 0.5% agarose gels (Paragon Lipoprotein Electrophoresis Kit, Beckman, U.K.) and electrophoresis performed according to the manufacturer's instructions.

2.3. Imprecision of the precipitation reagents

The intra-assay coefficients of variation (CV) of HDL-C and LDL-C obtained by the precipitation reagents were measured on a pool of plasma with HDL-C concentration 5.22mmol/l and LDL-C concentration 1.09mmol/l (derived using the validated ultracentrifugation/precipitation method).

2.4. The Friedewald formula and its modification

The LDL-C concentration was measured in normo- (n=37) and hyperlipidaemic (n=44) dogs by the ultracentrifugation/precipitation technique. The estimated LDL-C was calculated according to the standard Friedewald formula described above, using the plasma cholesterol and triglyceride concentrations and the HDL-C concentration measured using the reference method (Chapter II 2.2.).

Since the VLDL-C:plasma triglyceride ratio on which the Friedewald formula is based may differ slightly between selected populations and between species, the relationship between these two variables in 101 dogs (46 normolipidaemic and 55 hyperlipidaemic) was determined by linear regression. The estimated LDL was then calculated using two modifications of the Friedewald formula and the results compared to the reference method.

2.5. Electrophoresis

Native plasma from 14 normo- and hyperlipidaemic cases was aliquoted and analysed by the reference method and by agarose gel electrophoresis. The native plasma was applied to 0.5% agarose gels (Paragon Lipoprotein

Electrophoresis Kit; Beckman Inc) and electrophoresis performed according to the manufacturer's instructions. Following electrophoresis, the gels were stained for lipid with Sudan Black B (7.0% w/w) and the lipid content of each band quantified by densitometric scanning (Appraise Junior Densitometer; Beckman Instruments Inc). The relative distribution of the lipoprotein fractions were compared to the percentage of the total plasma cholesterol measured in each lipoprotein class.

2.6. Statistical methods

The product moment correlation coefficient was used to describe the relationship between the lipoprotein concentrations measured by the reference method and those derived from the precipitation reactions and the Friedewald formula. Spearman's rank correlation coefficient was used in the assessment of measured lipoprotein cholesterol concentrations (HDL-C and LDL-C) and those determined by electrophoretic means. In addition, the agreements between lipoprotein cholesterol concentrations measured by ultracentrifugation/precipitation and those obtained using the techniques under evaluation were assessed using the method of Altman and Bland (1983). In this, a plot of the difference between the two methods against their mean illustrates the measurement error and reveals any relationship between the magnitude of the error and that of the mean. The difference in measurements can therefore be summarized by standard statistical descriptions, *i.e.*, by the mean difference (d) and the standard deviation of the differences (s). Assuming the differences are normally distributed, 95% of the differences lie within d-2s and d+2s. If the differences within these limits are not clinically significant then the test method may be used to replace the reference method.

Since there is little information on the clinical significance of lipoprotein concentrations in the dog, the proposal of acceptable agreement limits between

methods was not undertaken and the data are presented in their original graphic form.

The agreement between the Friedewald formula and the estimated LDL-C concentration has been assessed in man by classifying the percentage of "acceptable" results in each study. An "acceptable" result was defined as one which fell within 10% of the LDL-C concentration measured by an ultracentrifugation/precipitation technique (Warnick, Knopp, Fitzpatrick and Branson 1990). The agreement between measured and estimated LDL-C in canine plasma was assessed by this method in addition to the manipulations of Altman and Bland (1983).

3. RESULTS

3.1. HDL-C concentrations: reference method vs precipitation reagents

The mean ±sd HDL-C concentrations derived from the reference method for the sample pools (Table 3.) were greater than, although significantly correlated with, those obtained using the Randox ($r=0.81$, $p<0.01$) and the Immuno reagents ($r=0.83$, $p<0.001$) (Appendix 5). The mean ±sd differences between the HDL-C concentration derived from the reference method and from the test precipitation methods were 0.59 ±0.94mmol/l and 0.19 ±1.64mmol/l, for the Randox and Immuno reagents respectively (Fig. 4.). In the case of the Randox reagent the HDL-C derived from the kit may be between 1.29mmol/l less than and 2.47mmol/l greater than the concentration measured by the standard technique, while for the Immuno reagent the value may fall between 1.49mmol/l less than, and 1.87mmol/l greater than the reference HDL-C concentration.

The intra-assay CVs for the Randox and Immuno reactions were 1.5% and 2.7% respectively (Table 4., Appendix 8).

The resolubilised precipitates from both the Randox and Immuno reactions contained lipoproteins of α_2 mobility suggesting contamination of the precipitate with HDL_1.

3.2. LDL-C concentrations: reference method vs precipitation reagents

The mean ±sd LDL-C concentrations measured by the Randox ($r=0.91$, $p<0.001$), Immuno ($r=0.67$, $p<0.01$)) and the Boehringer ($r=-0.02$, p=n.s.) reagents were less than the mean of the same samples analysed by the standard technique (Table 3., Appendix 7). The mean differences between these methods and the reference values were -0.18 ±5.70mmol/l for the Randox reagent, 2.34±4.92mmol/l for the Immuno reagent and 1.99 ±2.08 mmol/l for the Boehringer reagent (Fig. 5.). The LDL-C concentration obtained using the Randox kit may be between 11.58mmol/l less than, and 11.22mmol/l greater than the LDL-C of the same sample using the reference method. These limits were similar for the Immuno and Boehringer reagents (Table 3.)

Assessment of the precision of the precipitation techniques revealed intra-assy CV of 14.7%, 11.9% and 66.6% for the Randox, Immuno and Boehringer reagents, respectively (Table 4., Appendix 8).

3.3. Comparison of the measured LDL-C concentration and the estimated LDL-C derived from the Friedewald formula

The mean ±sd measured LDL-C in 81 samples was 4.16 ±4.24mmol/l while that calculated by the Friedewald formula was 4.39 ±4.39mmol/l ($r=0.994$, $p<0.001$) (Table 5., Appendix 9). The mean ±sd difference between the two methods was 0.23 ±0.50mmol/l. When samples with plasma triglyceride concentrations greater then 4.5mmol/l were excluded ($n=76$) the mean ±sd measured LDL-C concentration was 3.56 ±2.66mmol/l compared to an estimation of 3.75 ±2.71mmol/l ($r=0.991$, $p<0.001$). The mean ±sd difference between the two methods was 0.20 ±0.35mmol/l.

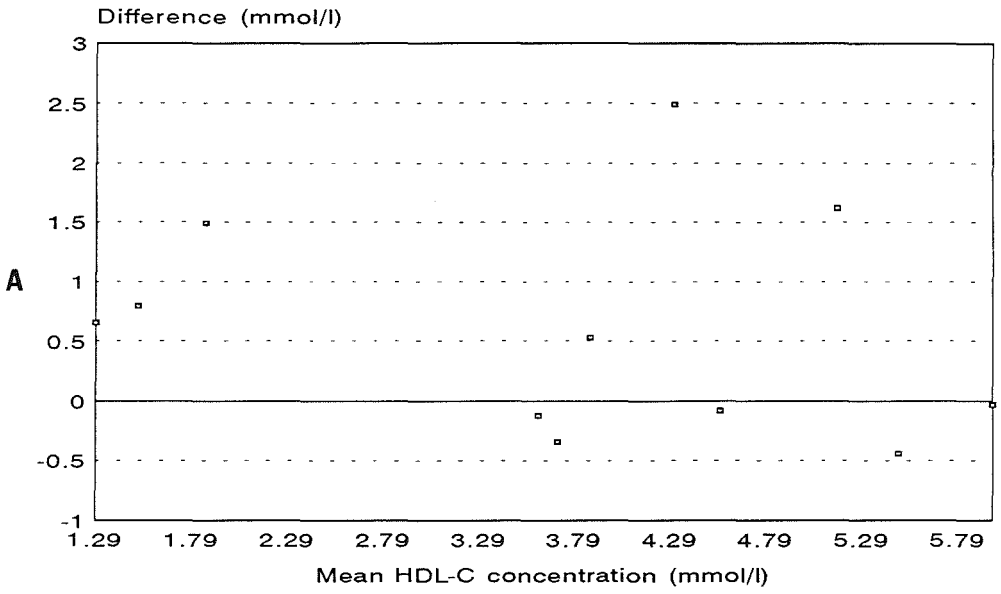
Table 3. Lipoprotein cholesterol concentrations obtained using the reference method (method A) and 5 precipitation reagents.

	mean (±sd) cholesterol (mmol/l)			mean difference ±2sd
	method A	reagent	difference	
HDL-cholesterol reagents				
Randox (n=13)	3.78 ±1.46	3.18 ±1.55	0.59 ±0.94	-1.29 - 2.47
Immuno (n=12)	3.90 ±1.44	3.70 ±1.41	0.19 ±1.68	-1.49 - 1.87
LDL-cholesterol reagents				
Randox n=13)	6.37 ±2.82	6.55 ±2.21	-0.18 ±5.70	-11.58 - 11.22
Immuno (n=13)	5.34 ±6.11	2.99 ±2.19	2.34 ±4.92	-7.50 - 12.18
Boehringer (n=12)	2.87 ±2.02	0.88 ±0.49	1.99 ±2.08	-2.17 - 6.15

Table 4. The intrassay precision of precipitation reagents (n=8).

	mean (±sd) lipoprotein cholesterol concentration (mmol/l)	CV %
HDL reagents		
Randox	5.16 ± 0.08	1.5
Immuno	5.26 ± 0.14	2.7
LDL reagents		
Randox	1.83 ± 0.27	14.7
Boehringer	0.15 ± 0.10	66.6
Immuno	1.85 ± 0.22	11.9

Figure 4. The difference between the HDL-C concentration derived from the reference method and a precipitation reagent plotted against the mean HDL-C derived from the two methods. A, Randox HDL-C precipitation reagent (n=13). B, Immuno HDL-C precipitation reagent (n=12).



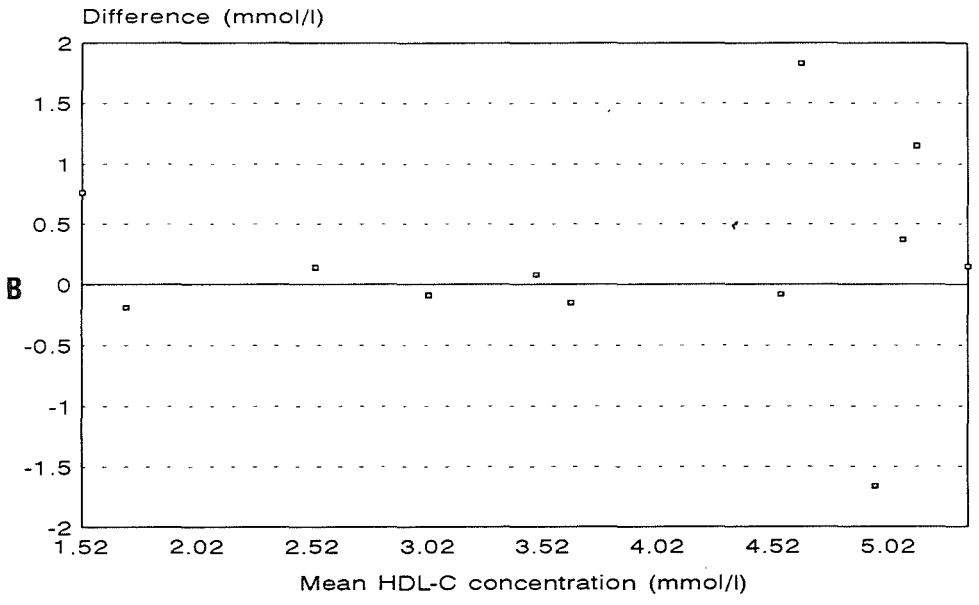
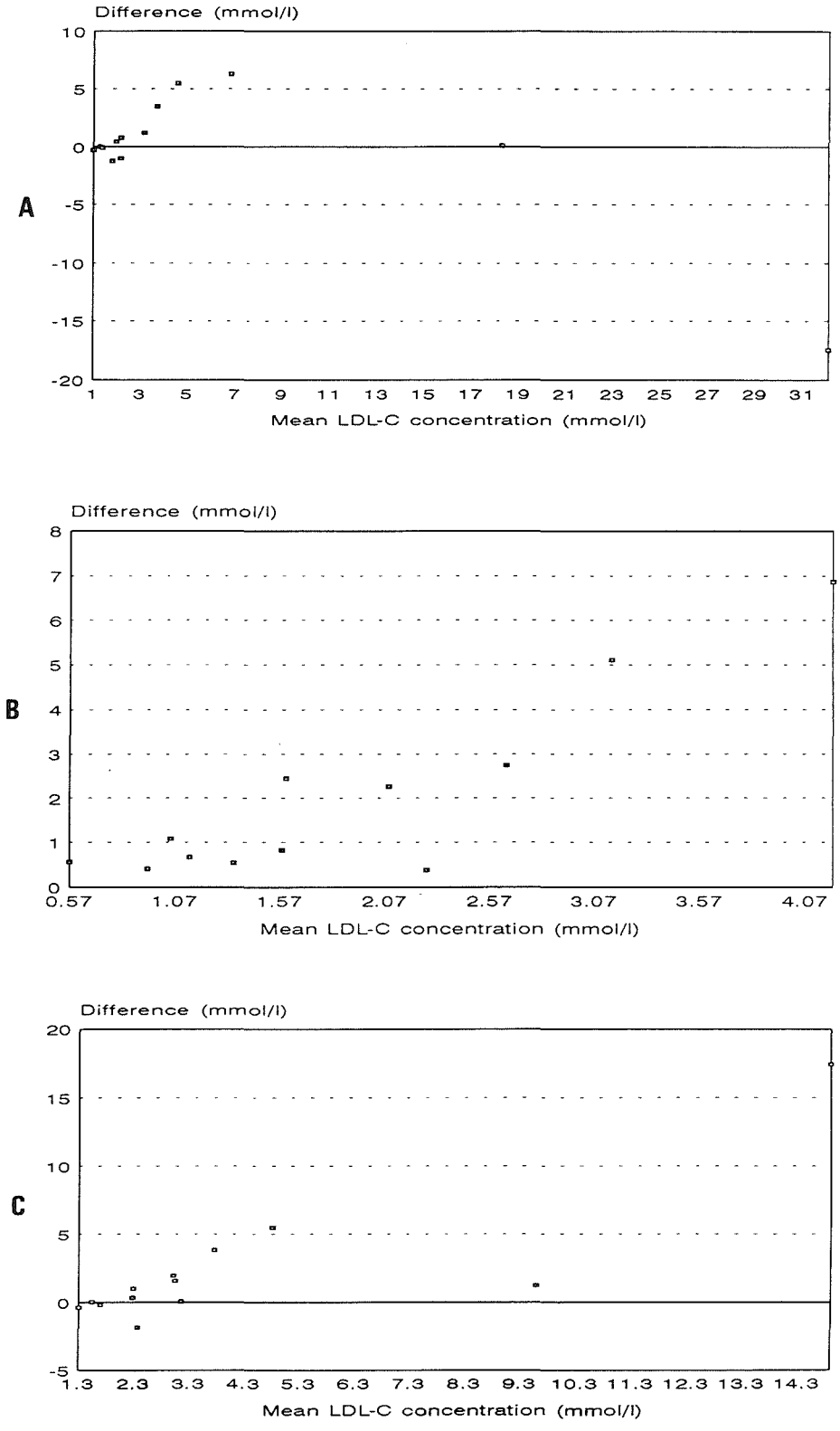


Figure 5. The difference between the LDL-C concentration derived from the reference method and a precipitation reagent plotted against the mean LDL-C derived from the two methods. A, Randox LDL-C precipitation reagent (n=13). B, Boehringer LDL-C precipitation reagent (n=12). C, Immuno HDL-C precipitation reagent (n=13).



When the raw data in this study are subjected to the criteria of Warnick, Knopp, Fitzpatrick, and Brarson (1990), the results may be classified as follows: for dogs with plasma triglyceride concentrations less than 1.75mmol/l, 49/71 (69%) of results were acceptable, when the plasma triglyceride was 1.75-4.5mmol/l only 2/5 (40%) of results were acceptable and in cases with plasma triglyceride concentrations greater than 4.5mmol/l, 2/5 (40%) values were within acceptable limits. When classified according to the LDL-cholesterol concentration, 4/8 (50%) samples with LDL-C less than 1.0mmol/l were acceptable while the values for samples with plasma LDL-C concentrations in the ranges 1-2mmol/l, 2-3mmol/l, 3-4mmol/l and greater than 4mmo/l were 9/19(47%), 7/15 (47%), 5/11 (45%) and 23/28 (82%), respectively (Fig. 6.).

3.4. Comparison of the measured LDL-C concentration and the estimated LDL-C derived from a modified Friedewald formula

The equation of the linear regression between plasma VLDL-C concentration and plasma triglyceride concentration in 101 dogs was as follows:

$$\text{VLDL-C} = 0.23 + 0.461 \times \text{triglyceride}$$

Two modifications of the Friedewald formula were therefore tested. The first (modified formula A) was as follows:

$$\text{LDL-C} = \text{plasma cholesterol} - \text{HDL-C} - (\text{plasma triglyceride} \times 0.46)$$

For all 81 cases where the Friedewald formula was calculated, the mean $\pm$sd estimated LDL-C concentration was 4.39 $\pm$4.40mmol/l ($r=0.993$, $p<0.001$) and the mean $\pm$sd difference between this and the measured LDL-C concentration was 0.20 $\pm$0.35mmol/l (Table 5.). After exclusion of the five cases with plasma triglyceride concentrations greater than 4.5mmol/l, the mean $\pm$sd estimated LDL-C and mean $\pm$sd difference were 3.56 $\pm$2.66mmol/l ($r=0.991$, $p<0.001$) and 0.20 $\pm$0.35mmol/l, respectively. The second modification of the Friedewald formula (modified formula B) was:

$$\text{LDL-C} = \text{plasma cholesterol} - \text{HDL-C} - \{(\text{plasma triglyceride} \times 0.46) + 0.23\}$$

For the total population the mean estimated LDL-C concentration using this formula was 4.14 ±4.39mmol/l (r=0.993, p<0.001), with a mean difference between the estimated and measured values of -0.02 ±0.52mmol/l. Removal of samples with high triglyceride concentrations altered these to 3.51 ± 2.72mmol/l (r=0.990, p<0.001) and -0.05 ±0.38mmol/l for the mean ±sd estimated LDL-C and difference, respectively (Table 5.).

Table 5. Mean (±sd) estimated LDL-C concentration (mmol/l) for the Friedewald formula, modified formula A and modified formula B. The mean measured LDL-C in 81 cases was 4.16±4.24mmol/l and for 76 cases with plasma triglyceride concentrations less than 4.5mmol/l, the mean concentration was 3.56±2.66mmol/l.

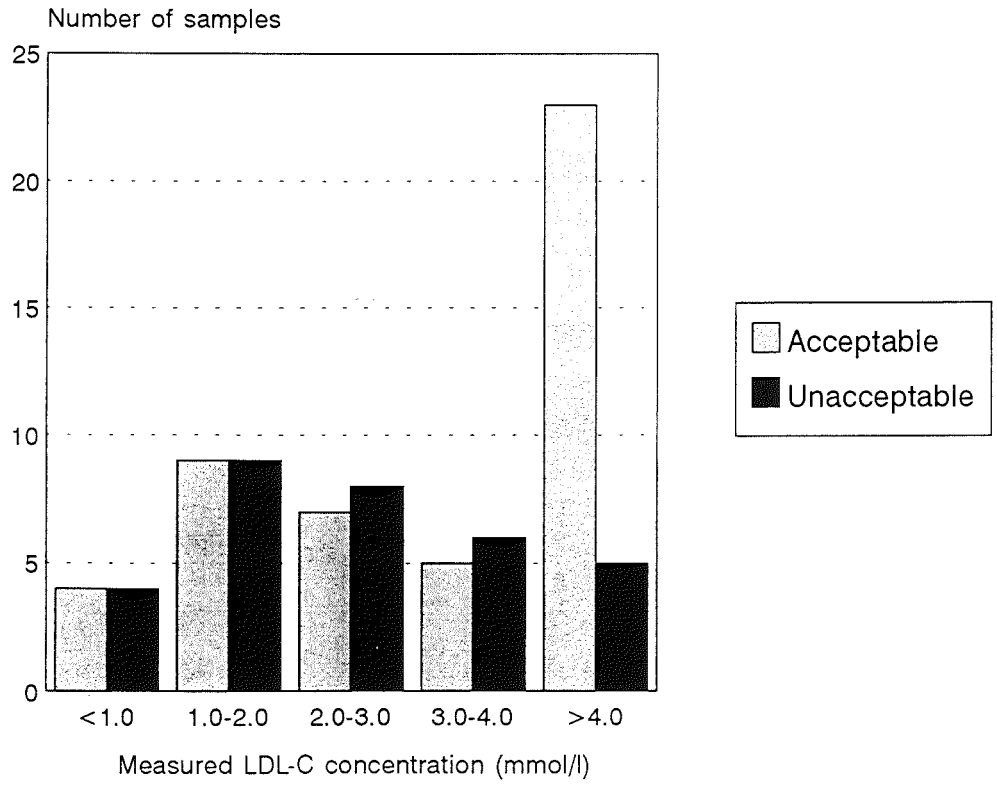
	n=81		n=76	
	LDL-C	difference	LDL-C	difference
Friedewald Formula	4.39 ±4.39	0.23 ±0.50	3.75 ±2.71	0.20 ±0.35
Formula A	4.39 ±4.40	0.23 ±0.53	3.75 ±2.70	0.20 ±0.35
Formula B	4.14 ±4.39	-0.02 ±0.52	3.51 ±2.72	-0.05 ±0.38

3.5. Comparison of semiquantitative electrophoresis and a quantitative method of lipoprotein analysis

The mean ±sd measured HDL-C and LDL-C were 3.78 ±1.40mmol/l and 1.31 ±0.94mmol/l, respectively. When converted to percentages of the total plasma cholesterol these represented 66.5 ±12.9% and 19.6 ±7.9%, respectively. The electrophoretic estimations of relative distributions of HDL and LDL were 74.9

±16.0% (r=0.77, p<0.01) and 17.84 ±7.46% (r=0.56, p<0.05), respectively. In only 2/14 cases was VLDL clearly identified as a separate band in the pre-β region (Appendix 10).

Figure 6. The measured LDL-C concentration versus the number of samples classified as acceptable and unacceptable.



4. DISCUSSION

The precipitation of apoB-containing lipoproteins by polyanions (dextran, heparin) and divalent cations (Mn^{2+}, Mg^{2+}) is frequently used as a means of measurement of human HDL-C, since the HDL class remains in the supernatant of the incubation and may be measured directly. It is recognised that each precipitation technique may give rise to considerable differences in HDL-C quantification (Warnick, Cheung and Albers 1979), *e.g.*, phosphotungstic acid precipitation yields a lower HDL-C concentration than

heparin manganese methods (Warnick, Mayfield and Albers 1981). This is confirmed in the present study of canine plasma, where the HDL-C concentration obtained with the Randox method was lower than that of the combined ultracentrifugation/precipitation method. This was caused by a greater precipitation of HDL by phosphotungstic acid than by heparin manganese solutions. The co-precipitation of HDL_1 with the former precipitation reagent was also noted by Rhodes, Meyer, Fish and Kerns (1992). In the human medicine, this is not considered of quantitative importance (Gibson, Rubinstein and Brown 1984), but in the hyperlipidaemic dog, in which HDL_1 is one of the major cholesterol carriers, this source of error may be of considerable importance.

Despite a significant correlation between the Randox precipitation reagent and the reference method, the study of the mean difference and the standard deviation of the differences between the two methods indicates that 95% of the HDL-C concentrations derived by the phosphotungstic acid method would lie between 1.29mmol/l less than and 2.47mmol/l greater than the HDL-C value achieved using the heparin manganese method. This lack of agreement may suggest that the reagent is not suitable for use with canine plasma, but considering the ease of performance of this method and the random nature of the differences between it and the reference procedure it is worth considering means of improvement. These may include alteration of the concentration of the reagent or the analysis of multiple aliquots from each sample and recording the mean HDL-C concentration.

The use of polyethylene glycol reagents for the quantification of human HDL has been associated with concentrations lower than those achieved with heparin manganese (Warnick, Mayfield and Albers 1981), a feature also confirmed in the analysis of canine plasma HDL-C. This reagent showed favourable correlation with the reference method, but the correlation coefficient failed to reveal the lack of agreement between the two methods

demonstrated by the distribution of the mean differences. This presentation of polyethylene glycol is therefore not suitable for the quantification of HDL-C in canine plasma; however modification of the concentration of the reagent may alter the precipitation properties favourably (Chira, Akizawa, Fujisawa, Osaka-Nakamori, Iwasaki, Suzuki, Intoh, Matsuno, Mitamura and Kobayashi 1992).

The phosphotungstate and the polyethylene glycol reagents displayed favourable CV but both methods produced mean HDL-C concentrations lower than that of the reference method. The lack of agreement between these methods appeared to be a result of excessive precipitation of HDL. This has also been recognised in human lipid chemistry, where precipitates from such reactions have a higher apoA-I content (an index of HDL) in samples from female patients than samples from males (Warnick, Cheung and Albers 1979). The implications of this source of error are obviously of importance in the dog, in which HDL is the major cholesterol carrier (Solyom, Bradford and Furman 1971; Mahley and Weisgraber 1974).

The quantification of plasma LDL by precipitation methods produces lower concentrations than obtained by ultracentrifugation/precipitation techniques (Rifai, Warnick, McNamara, Belcher, Grinstead and Frantz 1992) and show poor precision at clinically significant LDL concentrations (Cathcart and Dominiczak 1990). The same trends were identified in this study, where the mean LDL-C concentrations using precipitation reagents were less than those achieved using the reference method. Precipitation of human LDL by PVS has been reported to give good agreement with ultracentrifugation techniques when the total plasma triglyceride concentration is low (Rifai, Warnick, McNamara, Belcher, Grinstead and Frantz 1992). However, in this study there was a marked negative bias in LDL-C concentration derived from the PVS reaction and examination of the distribution of the differences between the two methods indicates that the method is not acceptable for use with canine plasma.

Study of the three reagents revealed large, unacceptable discrepancies in the measurement of LDL-C in canine plasma. Despite data sheet indications that LDL precipitation reagents may be used with samples with high triglyceride concentrations, two samples with markedly elevated triglyceride concentrations (18.3mmol/l and 39mmol/l) displayed large differences between precipitation methods and the reference method. Using the heparin reagent, the differences were 17.44mmol/l and 6.29mmol/l, respectively, while the dextran sulphate reagent produced differences of 17.43mmol/l and 1.23mmol/l, respectively.

The CVs of the LDL precipitation reagents were much poorer than those of the HDL methods suggesting that in addition to poor agreement with the reference method the kits do not display adequate repeatability characteristics when used with canine plasma.

Agarose gel electrophoresis gives good separation of the plasma lipoproteins (Noble 1968) and is the most commonly used means of lipoprotein analysis in the dog (Rogers, Donovan and Kociba 1975a and b; Ford 1977). The lipoprotein classes are separated on the basis of their size and charge, resulting in migration to a characteristic position , *i.e.*, LDL in the β position, VLDL in a pre-β position and HDL displaying α mobility. The lipid content of the lipoprotein is then stained with a fat soluble dye, *e.g.*, Sudan Black. The differential affinity of Sudan Black for triglyceride versus cholesterol has not been determined and therefore the relative distribution assessed by densitometric scanning may not reflect the absolute cholesterol content of the lipoprotein classes. If the staining characteristics are not equal it may explain the poor visualisation of VLDL (a triglyceride-rich and cholesterol poor lipoprotein) using this electrophoretic protocol. Despite this unknown variable there was a significant correlation between electrophoretic HDL concentrations and the measured HDL-C value and a moderate correlation between the LDL concentrations derived by the two methods.

Electrophoresis remains essential for the evaluation of lipoprotein mobility and the recognition of individual lipoproteins, *e.g.*, β-VLDL and LpX (Danielsson, Ekman, Johansson and Petersson 1976). In addition, it may be used to give an estimate of relative plasma concentrations of HDL and LDL but the poor visualisation of VLDL limits its use in the investigation of lipid abnormalities. Nevertheless, electrophoresis, in combination with the measurement of plasma cholesterol and triglyceride concentrations, is integral to the investigation of hyperlipidaemia and the relative inexpense of the procedure is obviously attractive to clinical pathology laboratories. The clinical applications of electrophoresis may improve on the introduction of agarose gel electrophoresis combined with enzymatic cholesterol assays, allowing the derivation of the absolute HDL-C and LDL-C concentrations from the total plasma cholesterol concentration and the relative distribution of cholesterol (Warwick, Packard and Shepherd 1990).

A common approach to lipoprotein analysis in human medicine is the direct measurement of plasma triglyceride, cholesterol and HDL-C concentrations. The LDL-C concentration is then calculated by the Friedewald formula. In the analysis of human plasma this allows the estimation of LDL-C without the use of preparative ultracentrifugation. The formula is used on the premiss that VLDL is the major carrier of plasma triglyceride. However, in the case of type III hyperlipidaemia the VLDL cholesterol content is very high and is underestimated by the use of the Friedewald formula. The converse is true of samples with low VLDL cholesterol concentrations, resulting in an underestimation of the plasma LDL-cholesterol concentration. The formula is not recommended for use in cases of hypertriglyceridaemia where the plasma triglyceride concentration is greater than 4.5mmol/l, since the ratio of VLDL cholesterol to triglyceride is lower than 0.45 in these samples.

In the canine population studied here there was a good correlation between the LDL-C measured by the reference technique and that estimated

by the Friedewald formula. However the agreement, either by the statistical method of Altman and Bland (1983) or by the 10% limits used by Warnick, Knopp, Fitzpatrick and Branson (1990), is not sufficient to allow replacement of measurement of LDL by estimation using this formula. Examination of the number of acceptable samples versus the LDL-C concentration indicated that the agreement between the measured and calculated values are improved at higher LDL-C concentrations. This may simply be a result of the number of cases in each group but may also reflect poorer precision in the quantitative method at lower LDL concentrations. The Friedewald formula may therefore be an acceptable alternative to ultracentrifugation/precipitation methods in the evaluation of hypercholesterolaemic cases which display only marginal elevations of plasma triglyceride concentrations.

It may be predicted that alteration of the factors used in the Friedewald formula to account for the VLDL-C/triglyceride ratio in the dog may allow derivation of a more appropriate formula for the estimation of canine plasma LDL-C concentrations. However, modification of the formula did not correct for the heterogeneity of VLDL in the population and therefore gave no improvement on the original factor of 0.45.

In this evaluation of the Friedewald formula, the HDL-C concentration was measured using the combined ultracentrifugation/precipitation technique, but the estimation of LDL-C will not provide an additional benefit in labour and equipment costs until a suitable reagent is identified for the direct measurement of HDL in plasma.

In conclusion, it has been demonstrated that the precipitation methods currently available for the quantification of human plasma lipoprotein concentrations are not suitable replacements for the quantification of canine plasma lipoproteins by validated ultracentrifugation/precipitation techniques.

The use of electrophoresis for the detection of β-VLDL and HDL_1 remains an essential part of the investigation of hyperlipidaemia in the dog.

CHAPTER IV

PLASMA LIPID AND LIPOPROTEIN CHOLESTEROL CONCENTRATIONS IN THE DOG: THE EFFECTS OF AGE, BREED, SEX AND SYSTEMIC DISEASE

1. INTRODUCTION

The lipoprotein abnormalities associated with spontaneous disease in the dog have been characterised by measuring plasma lipid concentrations and electrophoretic profiles (Rogers, Donovan and Kociba 1975b; Ford 1977). In addition to the effects of disease, a small number of investigators have attempted to define the influence of breed (Downs, Bolton, Crispin and Wills 1993) and environment (Schiller, Berglund, Terry, Reichlin, Trueheart and Cox 1964; Crispin, Bolton and Downs 1992) upon plasma lipoprotein concentrations in the dog. To date, the lack of standardised methods for the quantification of canine plasma lipoproteins has limited our understanding of lipoprotein metabolism in the healthy and hyperlipidaemic dogs. In addition, the paucity of information regarding the plasma activities of lipoprotein lipase and hepatic lipase in dogs has contributed to the lack of progress in this area of research. In this study the influences of breed, age and gender on the plasma lipid and lipoprotein concentrations of the dog have been evaluated, and the lipoprotein abnormalities associated with spontaneous systemic disease and obesity quantified. Lastly, the activities of lipoprotein lipase and hepatic lipase were measured in dogs with endocrine dysfunction.

2. MATERIALS AND METHODS

2.1. Case selection

Dogs suffering from diabetes mellitus, hyperadrenocorticism, hypothyroidism, protein-losing nephropathy, hepatic disease and obesity were selected from cases referred to Glasgow University Veterinary School (G.U.V.S.). The health status of each individual was established by routine screening, which included urinalysis and haematology/biochemistry profiles. The diagnoses were confirmed as follows: diabetes mellitus by glucosuria and fasting blood glucose

>12mmol/l; hyperadrenocorticism by Synacthen (Ciba Laboratories) stimulation test (60 minute cortisol > 660nmol/l) and followed, where necessary by a low dose dexamethasone suppression test (0.01mg/kg; 8 hour cortisol >40nmol/l); hypothyroidism by TSH (thyroid stimulating hormone; Sigma T-3538) stimulation test (5 I.U.; 6 hour thyroxine increased to a concentration less than 1.5 times the basal concentration). The assay of serum cortisol and thyroxine were performed by Serono Clinical Laboratory Services, Cambridge. The diagnosis of protein-losing nephropathy was made on the basis of persistent proteinuria and hypoalbuminaemia. Hepatic disease was defined biochemically as a condition which resulted in impaired hepatic function with or without hepatocellular enzyme leakage. Obese animals were selected on the basis that their body weight was greater than 115% of their breed and sex ideal weights (Lewis, Morris and Hand 1987; Glover 1977). The control group of dogs was comprised of clinically healthy animals screened as part of a routine health or pre-anaesthetic examination at G.U.V.S.

The total postheparin lipolytic, lipoprotein lipase and hepatic lipase activities were measured in six clinically normal, adult beagles, 10 dogs with diabetes mellitus and five dogs with hypothyroidism. All the diabetic dogs were receiving insulin at the time of sampling, but had been referred to G.U.V.S. because of apparent poor glycaemic control. The postheparin lipolytic activity was also measured in two of the hypothyroid dogs after six weeks of thyroid replacement therapy.

2.2. Routine blood biochemistry

Measurement of biochemical parameters was performed using commercial reagent kits on a Cobas MIRA clinical chemistry analyser (Roche). Alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by standard methods (Unimate 3 ALP DGKC, Uni-kit II ASAT, Ma-kit 10 ALAT; Roche). Glucose concentrations

were determined by enzymatic, colorimetric means (Unimate 7 Gluc-PAP). Total protein was measured by a modified biuret reaction with blank correction and albumin by a bromocresol green automated method on a Technicon Auto Analyser AAII (Technicon Instruments Corporation). Quality assurance was guaranteed using control sera (Precipath U and Precilip EL; Boehringer Mannheim GmbH). Quality control was monitored by the Scottish Quality Assessment Scheme and UKEQAS for general clinical chemistry (Appendix 1).

2.3. Patient preparation, blood collection and lipoprotein analysis

Blood samples were collected for lipoprotein quantification as previously described (Chapter III 2.1.) and all analyses were performed within 24 hours. Blood was collected into fluoride oxalate for glucose estimation and lithium heparin for the remaining biochemical analyses. Quantification of the plasma lipoproteins was performed using the ultracentrifugation/precipitation technique previously validated for use with canine plasma (Chapter III 2.2.). Qualitative analysis of the plasma lipoproteins was performed by agarose gel electrophoresis (Paragon Lipoprotein Electrophoresis Kit; Beckman Inc.) according to the manufacturer's instructions. Since the VLDL band of canine plasma is rarely recognised in native plasma, the concentrated VLDL fraction isolated by ultracentrifugation was also applied to the gels.

2.4. Postheparin lipolytic and lipoprotein lipase activity

Postheparin plasma lipase activities were measured in heparinised plasma from fasted dogs. Blood was collected before, and 10 minutes after the intravenous administration (70 IU/kg) of sodium heparin (Heparin (Mucous) Injection BP; Leo Laboratories Ltd.). Samples were collected into lithium heparin and placed on ice before separation by low speed centrifugation at 4°C for 15 minutes. The plasma was divided into 1ml aliquots and stored at -20°C for no longer than 12 weeks before analysis.

Radioactive triolein was formed by the addition of 50μCi glycerol tri (1-^{14}C) oleate (CFA 258; Amersham International plc) to 24.5ml toluene, then divided into 3.5ml aliquots and placed in round bottom glass flasks. To each flask was added 3.5ml cold triolein (500mg triolein (T7140; Sigma) in 25ml toluene). The substrate was then dried down under nitrogen in a water bath at 55°C, washed three times with 3ml heptane and stored under nitrogen at -20°C. The substrate emulsion was prepared 30 minutes prior to use by the addition of 5.5ml 5% gum arabic in 0.2M Tris-HCl pH 8.4, followed by sonication at 18 microns for 4 minutes. Then 5.5ml 10% bovine serum albumin (Fraction V; Sigma A-4503) in 0.2M Tris-HCl, pH8.4 was added.

For the measurement of total postheparin plasma (PHP) lipase activity, 10μl of post heparin plasma was diluted in 30μl 0.15M NaCl and incubated in duplicate with 200μl substrate, 250μl low salt buffer (0.2M Tris, 0.2M NaCl, pH8.4) and 50μl pooled, heat-inactivated serum at 28°C for 60 minutes. Hepatic lipase (HL) activity was assayed by the dilution of 10μl postheparin plasma in 30μl 0.15M NaCl and incubated in duplicate with 200μl substrate, 250 μl high salt buffer (0.2M Tris, 2.0M NaCl, pH8.4) under the same conditions. Fatty acids were extracted by adding 3.25ml methanol:chloroform:heptane (1.41:1.25:1 parts) and 0.75ml 0.14M K_2CO_3, 0.14M H_3BO_3, pH10.5. The tubes were vortexed and centrifuged at 3,000 rpm, 4°C for 30 minutes. One ml of the upper fraction was removed and counted in 10ml liquid scintillant (Ultima Gold; Packard Instrument Co.) and 200μl acetic acid. The radioactivity in 1ml of the upper fraction of blank incubations (containing 10μl 0.15M sodium chloride instead of postheparin plasma) was subtracted from the sample counts and the total radioactivity taken as the counts in 1ml of the lower fraction of the blank incubations. The lipolytic activity was calculated according to the following equation (see Appendix 2 for derivation):

$$\text{Activity } (\mu\text{molFA/ml/hr}) = \frac{(\text{Sample cpm} - \text{Blank cpm}) \times 755.1}{\text{Total cpm} - \text{background count}}$$

Lipoprotein lipase (LPL) activity was calculated as the difference between the total postheparin lipolytic activity and hepatic lipase activity.

2.5. Statistical Analyses

The independent effects of breed, age, gender and health status (control, diabetes mellitus, hyperadrenocorticism, hypothyroidism, protein-losing nephropathy, hepatic disease or obesity) on plasma lipid and lipoprotein concentrations of the dogs were determined by analysis of covariance; breed, age, gender and health status were fitted as fixed effects while age was fitted as a covariate. The mean values for each group, *i.e.*, control, diseased, obese were then estimated after correcting for the effects of breed, age and gender by the least squares method. When the main effect was statistically significant, means were then compared by multiple t-tests. Analyses were performed on the SAS software package (SAS Institute Inc., U.S.A.). The lipid and lipoprotein concentrations of two dogs with obstructive jaundice were noted, but no statistical analysis was performed. The analysis of paired plasma enzyme activites from the same individual was performed using the Sign test. The level of significance, unless stated otherwise, was taken as $p < 0.05$.

3. RESULTS

3.1. Age, breed and gender distribution in the control and disease populations

The disease populations in this study showed a gender bias with a preponderance of neutered females in both the obese (10/20) and hyperadrenocorticism (6/14) groups and a preponderance of entire males in the other populations (Table 6.). The mean $\pm$sd age was significantly greater in dogs suffering from diabetes mellitus (9.46 $\pm$3.1 years),

hyperadrenocorticism (11.36 ±1.5 years) and hepatic disease (11.00 ±2.45 years) than in the controls (6.36 ±3.7 years).

Table 6. Breed, gender and age distribution of control, obese and systemic disease groups.

	Number of cases								
	Breed				Sex				Age
	S	M	L	G	M	F	MN	FN	(mean ±sd)
Control (n=33)	5	12	16	0	18	11	0	4	6.4(±3.7)
Obesity (n=20)	5	2	12	1	4	4	2	10	7.1(±1.9)
Diabetes mellitus (n=11)	6	1	4	0	4	5	0	2	9.5(±3.1)*
Hyperadrenocorticism (n=14)	4	9	1	0	3	5	0	6	11.5(±1.5)*
Hypothyroidism (n=10)	1	2	7	0	6	2	1	1	7.6(±2.9)
Protein-losing nephropathy (n=4)	1	0	3	0	4	0	0	0	6.3(±3.2)
Hepatic disease (n=6)	2	1	3	0	5	0	0	1	11.0(±2.5)*

* $p < 0.05$

S = small, M = medium, L = large, G = giant, M = male, F = female, MN = neutered male, FN = neutered female.

3.2. The effect of age, breed and sex on plasma lipid and lipoprotein concentrations

The plasma concentrations of cholesterol, VLDL-C, LDL-C and HDL-C were unaffected by breed or age. The same was largely true of gender, with the notable exception that mean ±sem HDL-C concentration was significantly lower in entire males (least squares mean ±sem; 3.19 ±0.26mmol/l) than intact females (3.99 ±0.31mmol/l; $p<0.01$) and neutered females (3.75 ± 0.29mmol/l; $p<0.05$). The effect of gender on the plasma triglyceride concentration approached statistical significance ($p=0.07$), with highest mean concentration in neutered females and the lowest in entire males.

3.3. The effect of disease on plasma lipid and lipoprotein concentrations

Plasma cholesterol concentrations were significantly greater than those of the control group in animals with diabetes mellitus ($p<0.01$), hyperadrenocorticism ($p<0.01$) hypothyroidism ($p<0.001$), protein-losing nephropathy ($p<0.01$) and obstructive jaundice (Table 7.; Appendix 11). In dogs with diabetes mellitus this was due to significantly increased concentrations of VLDL-C ($p<0.01$) and HDL-C. The concentration of LDL-C was significantly increased in the dogs with hyperadrenocorticism and obstructive jaundice, while in the hypothyroid dogs, plasma triglyceride, VLDL-C, LDL-C ($p<0.001$) and HDL-C were significantly higher than the control group. Protein-losing nephropathy was characterised by significant increases in LDL-C and HDL-C. The plasma lipid and lipoprotein concentrations in obese dogs and dogs with hepatic disease were not significantly different from the control dogs. The plasma cholesterol ($p<0.001$) and LDL-C ($p<0.001$) were greater in dogs with hypothyroidism than those with either diabetes mellitus or hyperadrenocorticism. The plasma lipid and lipoprotein concentrations in dog with hyperadrenocorticism and those with diabetes mellitus were not significantly different.

Table 7. The mean ±sd plasma lipid and lipoprotein cholesterol concentrations in dogs with systemic diseases and in clinically healthy dogs.

	Plasma concentration (mmol/l)				
	Triglyceride	Cholesterol	VLDL-C	LDL-C	HDL-C
Control (n=33)	0.61 ±0.20	4.51 ±1.49	0.47 ±0.70	1.72 ±1.88	2.34 ±0.70
Diabetes mellitus (n=11)	1.78 ±1.33	9.31** ±3.76	1.79** ±2.23	3.58 ±2.60	3.92* ±1.04
Hyperadrenocorticism (n=14)	1.59 ±1.41	8.86** ±2.32	0.87 ±0.70	4.14** ±2.05	3.82 ±1.25
Hypothyroidism (n=10)	3.49*** ±3.96	16.17*** ±9.93	2.05* ±2.34	10.65*** ±8.23	3.48* ±1.02
Obesity (n=20)	1.04 ±0.53	6.03 ±1.79	0.70 ±0.31	1.88 ±1.19	3.47 ±1.22
Nephropathy (n=4)	0.73 ±0.39	10.69** ±1.21	0.55 ±0.40	5.86* ±1.85	4.28* ±0.84
Hepatic disease (n=6)	1.06 ±0.84	6.46 ±2.26	0.49 ±0.34	3.11 ±1.09	2.89 ±1.57
EHBDO (n=2)	1.96 ±1.62	18.22 ±4.53	0.84 ±0.07	5.06 ±5.04	2.32 ±0.59

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

EHBDO = Extrahepatic bile duct obstruction

3.4. Agarose gel electrophoresis

The individual lipoproteins were identified according to their characteristic electrophoretic mobility, *i.e.,* bands of α-migrating lipoproteins (HDL), β-migrating lipoproteins (LDL) and pre-β migrating species (VLDL), isolated from the fraction containing lipoproteins of density less than 1.006g/ml. Electrophoresis of the plasma of one diabetic dog showed evidence of hyperchylomicronaemia, indicated by the presence of chylomicrons at the origin of the gel. Beta-VLDL were identified in the plasma of one dog with spontaneous hypothyroidism.

3.5. Plasma lipolytic activity associated with secondary and idiopathic hyperlipidaemia in the dog

The total plasma lipolytic activity and the lipoprotein lipase activity in dogs with diabetes mellitus were similar to those of control beagles, but the hepatic lipase activity was significantly higher (Table 8., Appendix 18). On initial presentation, the total lipolytic, lipoprotein lipase and hepatic lipase activities were lower in dogs with hypothyroidism ($p < 0.01$), but the activities increased after appropriate drug therapy (n.s.).

4. DISCUSSION

The effect of disease upon the lipoprotein phenotype of the dog has been studied previously using semi-quantitative electrophoretic and densitometric scanning methods (Rogers, Donovan and Kociba 1975a and b; Rogers 1977). These studies did not allow for the effects of breed, age and gender on plasma lipid and lipoprotein concentrations. Here, a quantitative method of lipoprotein analysis has been used to estimate and correct for the effect of these individual factors.

Table 8. The mean ±sd total plasma lipolytic, LPL and HL activities in dogs with diabetes mellitus and hypothyroidism (before and after replacement therapy) and a control group of six beagles.

Group	Lipolytic activity (μmolFA/ml/hr) Total	LPL	HL
Control (n=6)	17.65 ±1.45	10.73 ±2.66	6.92 ±1.44
Diabetes mellitus (n=5)	22.39# ±10.31	10.31 ±5.37	10.78* ±2.04
Hypothryoidism (before) (n=5)	5.16** ±1.18	3.20** ±1.50	1.96** ±1.27
Hypothyroidism (after) (n=2)	13.40 ±3.11	5.16 ±1.05	10.24 ±4.89

* $p<0.05$, ** $p<0.01$, # n = 10

In man, total plasma cholesterol concentrations are dependent upon age and gender. The plasma cholesterol concentrations of both men and women rise from childhood to mid-life. This trend is more marked in males than in females although in the latter, plasma cholesterol concentrations increase again in the post-menopausal period (Kissebah and Schectman 1987). The present study failed to reveal any change in canine plasma cholesterol and lipoprotein concentrations with age. It may be that no such trends exist but it is also possible that the number of animals included in this study was insufficient to identify any relationships. The mean HDL-C concentration in women is higher than that of men (Crook and Seed 1990), a feature which may be explained by the lower hepatic lipase activity in females. This study showed a similar pattern in the canine population, where the mean HDL-C concentration in entire

bitches was greater than that of entire males. The effect of gender upon the activity of hepatic lipase in the dog has not been determined.

Genetic mutations or variations, resulting in abnormalities of lipoprotein metabolism, may give rise to hyperlipidaemia in man, *e.g.*, familial hypercholesterolaemia. In this way, the prevalence of hyperlipidaemia in a population depends to a degree upon the gene pool. In the dog, such genetic predisposition to lipoprotein disorders is rare, but recently, investigators have suggested that clinically normal dogs of different breeds may display variation in plasma cholesterol concentrations (Armstrong and Ford 1989). No such breed variation in lipid concentrations was noted in this study, although the limited number of cases in each population dictated that only breed type (small, medium, large and giant) was tested in the analysis of covariance. A more comprehensive study may therefore be necessary before final conclusions are drawn regarding breed variation in plasma lipid and lipoprotein concentrations.

The plasma lipid and lipoprotein concentrations associated with experimentally-induced canine hypothyroidism, in combination with cholesterol-loading have been studied previously (Mahley, Weisgraber and Innerarity 1974). Under these circumstances there was a heterogeneous response with some dogs classed as hypo-responders, in which a moderate increase in plasma cholesterol was accompanied by an increase in HDL and LDL concentrations. The present study has corroborated these results; dogs with spontaneous hypothyroidism displayed an increase in plasma cholesterol, LDL-C and HDL-C concentrations. In the original experimental study (Mahley, Weisgraber and Innerarity 1974), a second group of animals, classed as hyper-responders, developed very high cholesterol levels with an increase in HDL concentrations and the appearance of a novel lipoprotein with a density less than 1.006g/ml, but displaying β-migration on electrophoresis, called β-VLDL. In the present study, β-VLDL was evident on electrophoresis of the plasma of one dog with spontaneous hypothyroidism (Chapter VII 3.2.). The

hypercholesterolaemia in this case was particularly marked (47.80mmol/l), but the clinical and pathological factors which dictate the accumulation of the lipoprotein remnants in this case and not in others have not been determined.

The lipoprotein abnormalities noted in the dogs with hypothyroidism may be explained as follows. First, it has been demonstrated that hypothyroidism results in a down-regulation of the hepatic LDL receptor (Scarabottolo, Trezzi, Roma and Catapano 1986) secondary to deficiency of tri-iodoythyronine (T3) (Chait, Bierman and Albers 1979). Since T3 plays a role in the regulation of biliary excretion, a deficiency of this hormone results in an increased hepatic cholesterol pool, followed by down-regulation of LDL receptor activity which prevents excess sterol accumulation in the liver. Consistent with this, impaired clearance of LDL from the circulation appeared to be the predominant abnormality of lipoprotein metabolism associated with hypothyroidism in the dog.

However, hypothyroidism in humans is accompanied by reduced activities of lipoprotein lipase and hepatic lipase (Valdemarsson, Hansson, Hedner and Nilsson-Ehle 1983). Lipoprotein lipase is responsible for the hydrolysis of the core lipid of triglyceride-rich lipoproteins and their modification to remnant particles, thus facilitating their removal from the circulation. The impaired lipoprotein lipase activity recorded in this study may account for the raised plasma triglyceride and VLDL-C concentrations found in the dogs with hypothyroidism. Hepatic lipase is responsible for the conversion of VLDL to IDL, IDL to LDL, and the interconversion of HDL subfractions. It is possible that the decreased hepatic lipase activity contributed to the changes in HDL-C concentration noted with hypothyroidism. Although the number of cases included in this study is small and the changes are not significant, it does appear that thyroid relacement therapy results in an increase in the plasma activities of these enzymes. It has been proposed that the increased lipoprotein lipase activity is mediated via increased protein synthesis, while hepatic lipase is

under direct control by thyroid hormones (Valdermarsson, Hedner and Nilsson-Ehle 1982).

Insulin-dependent diabetes mellitus in man is associated with a number of lipoprotein abnormalities. Diabetic ketoacidosis is commonly accompanied by raised plasma triglyceride and VLDL concentrations secondary to an increased flux of NEFA from the adipose tissue. In the dog, however, experimental evidence suggests that the hypertriglyceridaemia associated with diabetes mellitus is the result of impaired clearance of triglyceride from the circulation (Basso and Havel 1970), possibly as a consequence of decreased lipoprotein lipase activity. The reduced catabolism of VLDL, with or without an increase in its synthesis would explain the raised VLDL-C concentrations associated with canine diabetes mellitus in this study.

It has been reported previously that dogs with diabetes mellitus have an intensely staining β-band on lipoprotein electrophoresis consistent with increased LDL (Rogers 1977). Individual animals examined in this study had raised LDL-C, but the group was heterogeneous in this respect and the mean value was not significantly different from the control population. The increase in plasma LDL-C concentrations in individual dogs may be the result of over-production of the lipoprotein class, secondary to raised VLDL concentrations. The raised HDL-C concentration found in dogs with spontaneous disease has previously been recognised in the hyperlipidaemia of experimental canine diabetes mellitus combined with dietary cholesterol loading (Wilson, Chan, Elstad, Peric-Golia, Hejazi, Albu and Cutfield 1986). The increase in this lipoprotein class may reflect an increase in cholesterol synthesis, or be the consequence of impaired removal of the lipoprotein from the circulation secondary to alterations in hepatic receptor activity (Gleeson, Hejazi, Kwong, Chan, Le, Alberts and Wilson 1990).

In humans, diabetes mellitus is accompanied by a reduction in lipoprotein lipase activity (Abbate and Brunzell 1990), which corrects rapidly

on insulin therapy (Nikkilä, Huttunen and Ehnholm 1977). It is not surprising therefore, that in diabetic dogs receiving insulin therapy the lipoprotein lipase activities are not significantly different from controls. The plasma lipolytic activities in two dogs (29.40 μmolFA/ml/hr and 40.46 μmolFA/ml/hr) were considerably greater than in control dogs (upper limit, 19.10μmolFA/ml/hr). In one of these the lipoprotein lipase activity was also increased (18.50 μ molFA/ml/hr). This may reflect the effect of hyperinsulinaemia secondary to the systemic administration of insulin. The activity of hepatic lipase is increased both in diabetic humans (Nikkilä, Huttunen and Ehnholm 1977) and dogs with experimental diabetes mellitus which are under poor glycaemic control (Muller, Saudek and Applebaum-Bowden 1985). The present study confirmed these findings in naturally-occurring diabetes mellitus in the dog although the exact mechanism of the increase in plasma hepatic lipase activity is not clear.

Hyperadrenocorticism in man is not commonly associated with plasma lipoprotein abnormalities, although the administration of prednisolone results in increased plasma cholesterol and HDL-C concentrations in both sexes, and plasma triglyceride concentrations in females (Zimmerman, Fainaru, Eisenberg 1984). In one study of canine Cushing's syndrome, 90% of all cases showed increased plasma cholesterol concentrations (Ling, Stabenfeldt, Comer, Gribble and Schechter 1979). In the present study, dogs with hyperadrenocorticism had raised plasma cholesterol and LDL-C concentrations. If the underlying defects in lipoprotein metabolism were the consequence of glucocorticoid antagonism of insulin alone, then elevations of plasma triglyceride and triglyceride-rich lipoproteins would be expected. These features were, in fact, present in only three of the 14 cases of canine Cushing's syndrome. The most consistent abnormality was raised LDL-C, resulting in increased plasma cholesterol concentrations. One effect of the administration of synthetic steroids is an alteration in the bile acid composition, resulting in a

reduction of the rate of biliary excretion (Berk and Javitt 1978). Such an effect may increase the hepatic sterol pool with a consequent down-regulation of the hepatic LDL receptor and an increase in circulating LDL concentrations. Lipoprotein abnormalities similar to those described in this study have also been recognised in dogs with experimentally-induced obstructive jaundice (Bauer, Meyer, Goring, Beauchamp and Jones 1989). The changes seen in these dogs and in dogs with hyperadrenocorticism may therefore be the result of down-regulation of the hepatic LDL receptor secondary to reduced biliary excretion, or as a consequence of hepatocellular pathology.

Dogs which presented with hepatic disease *per se* showed a heterogeneity of abnormalities including hypercholesterolaemia, hypocholesterolaemia and hypertriglyceridaemia. Under these circumstances the underlying hepatic pathology and, in particular, the degree of biliary stasis versus hepatocellular damage may play an important role in determining the character and severity of lipoprotein abnormalities. Extra-hepatic bile duct obstruction secondary to chronic pancreatic disease was found to induce increases in plasma cholesterol and LDL-C concentrations similar to previous reports of experimentally-produced lesions (Danielsson, Ekman, Johansson and Petersson 1977). Hepatic parenchymal disease, however, was associated with a wide variety of lipoprotein disturbances which may have been the result of a combination of factors including down-regulation of the LDL receptors, decreased activities of LCAT and hepatic lipase and increased hepatic cholesterol synthesis. Lipoprotein-X, an unusual lipoprotein identified specifically in cholestasis and LCAT deficiency in man (Miller 1990) was not identified from the plasma of dogs with naturally-occurring hepatic disease in this study.

Obesity in man may be associated with lipoprotein abnormalities which do not necessarily result in overt hyperlipidaemia (Kissebah and Schectman 1978). Increased adipose tissue mass, particularly abdominal adiposity

(Després 1991) results in increased flux of NEFA via the portal circulation to the liver, stimulating over-production of VLDL. Hormone sensitive lipase regulates the release of NEFA from the adipose tissue. In healthy individuals the activity of this enzyme decreases in the postprandial period but in the obese patient this response is less marked (Coppack, Evans, Fisher, Frayn, Gibbons, Humphreys, Kirk, Potts and Hockaday 1992), resulting in poor regulation of the flux of NEFA from adipose tissue. In our population, the mean plasma VLDL-C concentration was higher than that of control dogs, but this difference was not significant. It is possible, therefore, that kinetic disorders, similar to those recognised in man may exist in the canine population and that studies including a greater number of cases may identify such abnormalities.

In conclusion, this study has identified and quantified a range of abnormalities of lipid metabolism associated with systemic disease in the dog. Such lipoprotein phenotyping may help in the differential diagnosis of hyperlipidaemia in the dog.

CHAPTER V

ABNORMALITIES IN LIPID AND LIPOPROTEIN METABOLISM ASSOCIATED WITH CANINE OBESITY

1. INTRODUCTION

Obesity is defined as the excessive accumulation of fat (triglyceride) in the adipose tissue of the body, leading to physiological dysfunction and a deterioration of health (Anderson, J., 1972). In man, the condition is associated with a high incidence of metabolic abnormalities, including insulin resistance, hypertriglyceridaemia, hypercholesterolaemia and cardiovascular disease (Grundy 1987). The development of such metabolic disorders is related not only to the presence of excessive adipose tissue but also to the distribution of the adipose tissue in the body. In particular, several indicators of poor health, including hypertension, hypercholesterolaemia, hypertriglyceridaemia and illness in terms of sick leave, were accompanied by an accumulation of fat in the deep and subcutaneous abdominal tissues, rather than the gluteal-femoral region (Després 1991).

The disturbances of lipoprotein metabolism include an overproduction of VLDL, which may be compensated for by an increase in the removal rate of triglyceride from the circulation. Plasma triglyceride concentrations in many obese individuals are therefore maintained within normal limits, and exception to this suggests that there is concurrent impairment of the mechanisms of triglyceride clearance (Kissebah and Schectman 1987). Since adipose tissue is an important cholesterol storage organ and is capable of making a major contribution to lipid production, its excessive accumulation may also be associated with hypercholesterolaemia (Krause and Hartman 1984). Adiposity is considered an independent risk factor for coronary heart disease, an observation which suggests that even without overt hyperlipidaemia there may be impairments of lipid metabolism which contribute to the development of atherosclerosis (Kissebah and Schectman 1987). All of the disturbances of lipid metabolism associated with obesity are reversible by calorie restriction and weight reduction (Grundy 1987).

In the dog, the excessive accumulation of adipose tissue has been associated with glucose intolerance and hyperinsulinaemia (Mattheeuws, Rottiers, Baeyens and Vermeulen 1984) and is related to the development of other abnormalities, including respiratory, neoplastic and locomotor disorders (Clutton 1988). It has been proposed that obesity in the dog may contribute to the development of hyperlipidaemia (Hand 1988; Whitney 1992), but this relationship has not been investigated. The aim of this study was to identify if there are abnormalities of lipoprotein metabolism associated with obesity in the dog and to evaluate the role of calorie restriction and weight loss in the correction of any such disturbances.

2. MATERIALS AND METHODS

2.1. Inclusion criteria and experimental design

Thirteen obese dogs were recruited from veterinary practices in the Glasgow area for inclusion in this study. All the dogs were greater than 115% of the ideal weight for their breed, age and sex (Lewis, Morris and Hand 1987; Glover 1977). The animals were considered clinically healthy as judged by examination, routine haematology and biochemistry profiles, and urinalysis. Where necessary, endocrine disorders were excluded by the methods previously described (Chapter IV 2.1.). The dogs included in this study formed a subgroup of the obese dogs described in Chapter IV 2.1.

After confirmation of the satisfactory health status of each individual, samples for plasma lipoprotein analysis and postheparin plasma lipase activity were collected. Three days elapsed between these manipulations and the performance of an intravenous fat tolerance test. Plasma lipid and lipoprotein cholesterol concentrations were quantified at two weekly intervals and intravenous fat tolerance tests and postheparin plasma lipase assays repeated at the end of the study (6-8 weeks).

After diagnostic testing and collection of baseline samples, each dog was gradually introduced to a reduced calorie, high fibre diet with a nutritional analysis (% dry matter) of 22% protein and 8% fat, metabolizable energy 3.30kcal/g (Perform Lite; The Center for Animal Nutrition, Glendale, California). The daily ration was calculated for 70% maintenance requirement (Hand 1988). The weight, and dimensions of the neck, girth and the thigh above the stifle were recorded at weekly intervals for each dog. In individuals displaying no weight loss for two consecutive visits, the dietary intake was reduced by a further 20%. In addition to dietary manipulation, owners were encouraged to exercise their animals for increasing periods of time, tailored to the individual client and animal.

The control group consisted of six, non-obese, healthy, normolipidaemic adult beagles (4 entire males, 2 entire females) maintained on a commercial dry diet, on which plasma lipid and lipoprotein analysis, postheparin plasma lipase activities and intravenous fat tolerance tests were performed.

2.2. Plasma lipids and lipoproteins

Plasma lipid and lipoprotein concentrations were quantified by the ultracentrifugation/precipitation method validated for use in the dog (Chapter II 2.2.). Agarose gel electrophoresis was performed on plasma and VLDL fractions in order to confirm the presence of normal lipoprotein electrophoretic mobility and exclude the presence of β-VLDL particles (Chapter II 2.3.). Non-esterified fatty acids were measured using an enzymatic colorimetric method (NEFA- C; Wako) modified for use on the Cobas MIRA (Roche Diagnostics).

2.3. Postheparin plasma lipase activities

Total plasma postheparin lipase activity, lipoprotein lipase and hepatic lipase activities were assayed according to the methods described in Chapter IV 2.4. Samples were collected before introduction to the reducing diet and at the end of a six week period, irrespective of the degree of weight loss.

2.4. Intravenous fat tolerance tests

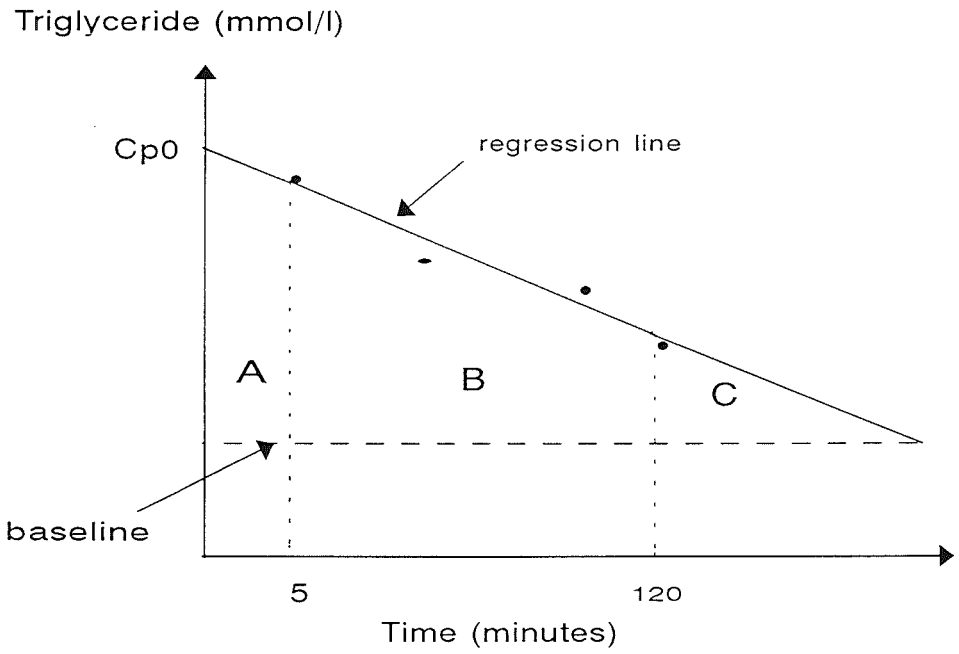
Dogs were fasted for a minimum of 16 hours and a baseline blood sample taken into EDTA (final concentration 1mg/ml). A soya bean triglyceride/egg yolk phosphatidyl choline emulsion (Intralipid* 10%; Kabi Pharmacia Ltd.) was administered by intravenous injection (0.1g/ml) at a rate of one to three millilitres per second. Serial blood samples were collected at 5, 10, 20, 30, 40, 90, and 120 minutes after Intralipid* administration and immediately placed onto ice. The triglyceride concentration of the samples was measured and corrected for free glycerol (GY105; Randox Laboratories).

The triglyceride clearance characteristics for each intravenous fat tolerance test were calculated by analysing the changes in plasma triglyceride concentration versus time. All the kinetic analyses were performed using a nonlinear regression Fortran IV curve stripping computer program, CSTRIP. All samples were standardised by subtracting the fasting plasma triglyceride concentration from each time point.

The initial plasma concentration (Cp0) in mmol/l was the calulated triglyceride concentration at time 0 (extrapolated from the regression line) (Fig. 7.) and the Cmax was taken as the peak observed plasma triglyceride concentration. The area under the plasma concentration versus time curve for observed values (AUC_{obs}), in mmol.min/l, from time 0 to time infinity, was determined for observed concentrations using the trapezoidal rule (Matthews, Altman, Campbell and Royston 1990). The area under the regression line between time 0 and the first sample and the area between the last sample and baseline were calculated (Appendix 17). The area under the first moment curve for observed values ($AUMC_{obs}$) for time 0 to time infinity, *i.e.* the plasma concentration time versus time curve in $mmol.min^2/l$, was calculated in a similar fashion. The ratio of AUMC to AUC for any substance is a measure of its mean residence time (MRT), which is a quantitative estimate of the

persistence of the substance in the body and the time taken for 63.2% of the dose to be eliminated.

Figure 7. A diagramatic representation of the parameters used in the description of the plasma triglyceride clearance curves.



Baseline: baseline triglyceride concentration.

regression line: regression line through observed points.

Cp0: Initial triglyceride concentration in plasma following Intralipd* administration.

A: area of the triangle between time 0 and the first sample.

B: area under the plasma concentration versus time curve for observed values.

C: area of the triangle between the last sample and 0 concentration.

2.5. Statistical methods

Plasma lipid and lipoprotein cholesterol concentrations, enzyme activities and triglyceride clearance curve characteristics were compared using the Mann Whitney test for non-parametric data. In addition, the independent effect of obesity on the plasma lipid and lipoprotein concentrations were determined by analysis of covariance, correcting for the effects of breed, age and gender (Chapter IV 2.5.). Paired data derived from the same animals were analysed by the Sign test.

The relationship between the plasma lipid and lipoprotein concentrations and the following parameters were described by the product-moment correlation coefficient and tested by linear regression: body weight, neck, girth and thigh circumference, and the ratios of the neck circumference to weight, the girth circumference to weight and the stifle circumference to weight.

3. RESULTS

3.1. Population distribution

The study group consisted of nine neutered females, two entire females, one entire male and one neutered male (Table 9.). The mean $\pm$sd age was 7.10 $\pm$ 2.36 years. One neutered female was withdrawn from the trial soon after the start and two were lost to follow up at 4 weeks. The mean $\pm$sd weight loss over the trial period for the 10 remaining dogs was 9.2 $\pm$5.7% of the initial body weight, which represented 41 $\pm$27.3% of the target weight loss. In four dogs the weight reduction was minimal (<25% target weight loss). A failure of owner compliance with the feeding regime was suspected as the underlying cause for poor weight reduction in 2 cases.

Table 9. Details of obese dogs.

Number	Breed	Age (yrs)	Sex
1	Labrador retriever	6.0	MN
2	Labrador retriever X	10.0	FN
3	Springer spaniel	10.0	FN
4	Rottweiller	7.5	M
5	Labrador retriever X	7.0	FN
6	Bernese mountain dog	10.0	FN
7	Golden retriever	10.1	FN
8	Rough collie	7.0	FN
9	Jack Russell terrier	5.0	FN
10	Shetland sheepdog	3.2	F
11	Shetland sheepdog	6.1	F
12	Beagle	4.0	FN
13	Terrier X	6.0	FN

3.2. Plasma lipid and lipoprotein concentrations before and after dietary intervention

On initial presentation, four of the obese dogs were hypercholesterolaemic, one of which was also hypertriglyceridaemic. The mean plasma triglyceride, VLDL-C and LDL-C concentrations in obese dogs were significantly ($p<0.05$) greater than those of control dogs (Table 10., Appendix 12). The plasma cholesterol, HDL-C and concentrations of the obese dogs were not significantly different from control animals (Table 10.). The NEFA concentration in obese animals was similar to that of the controls (0.98 $\pm$0.25mmol/l and 0.92 $\pm$0.47mmol/l, respectively). When determined by analysis of covariance, obesity had no significant effect on the plasma lipid or any of the lipoprotein concentrations.

The plasma cholesterol, triglyceride, VLDL-C and LDL-C concentrations in obese dogs after calorie restriction for 6 weeks, were lower than those on initial presentation (Table 10.) and the HDL-C concentration was higher after introduction of the reducing diet. None of the differences in lipid and lipoprotein concentrations before and after caloric restriction were statistically significant at $p < 0.05$.

There were moderate correlations between the VLDL-C concentration and the stifle circumference ($r=0.60$; $p<0.05$), VLDL-C and the stifle:weight ratio ($r=-0.64$; $p<0.05$) and LDL-C and the percentage weight over the ideal weight ($r=0.58$, $p<0.05$) on initial presentation (Appendix 13). The relationships which were close to significance at the 0.05 level were plasma triglyceride and stifle:weight ratio ($r=-0.51$; $p=0.09$), VLDL-C and neck circumference ($r=0.49$; $p=0.09$), VLDL-C and girth circumference ($r=0.50$; $p=0.08$), and VLDL-C and neck:weight ratio ($r=-0.52$; $p=0.07$).

Table 10. Mean ±sd plasma lipid and lipoprotein cholesterol concentrations (mmol/l) in obese dogs before and after maintainance on a restricted calorie intake.

	Plasma Triglyceride	Cholesterol concentration (mmol/l)			
		Plasma	VLDL	LDL	HDL
Before (n=12)	0.97* ±0.44	5.97 ±2.01	0.61* ±0.30	1.75* ±1.28	3.61 ±0.99
After (n=12)	0.82* ±0.31	5.35 ±1.24	0.51 ±0.21	1.21 ±0.53	3.78 ±0.70
Control (n=6)	0.47 ±0.19	4.43 ±1.76	0.32 ±0.13	0.69 ±0.46	3.41 ±1.36

* $p<0.05$

3.3. Postheparin plasma lipase activities

For 13 obese dogs, on initial presentation, the total plasma lipase and lipoprotein lipase activities were significantly lower than those of the control population (Table 11., Appendix 14). The plasma hepatic lipase activities were not significantly different. In eight dogs, calorie restriction resulted in no significant alteration in these enzyme activities (Table 12., Appendix 15).

Table 11. Mean (±sd) PHP, LPL and HL activities in obese and control dogs.

	Lipolytic activity (μmolFA/ml/hr)		
	PHP	HL	LPL
Obese (n=13)	10.00** ±6.59	4.89 ±2.73	5.11** ±4.70
Control (n=6)	17.65 ±1.45	6.92 ±1.44	10.73 ±2.66

**p<0.01

Table 12. Mean (±sd) PHP, LPL and HL activities in eight obese dogs before and after dietary intervention.

	Lipolytic activity (μmolFA/ml/hr)		
	PHP	HL	LPL
Before	11.19 ±7.92	5.83 ±2.80	5.37 ±5.65
After	10.84 ±5.21	6.71 ±3.33	4.12 ±2.50

3.4. Intravenous fat tolerance tests

The plasma triglyceride concentrations (mmol/l) at 0, 5, 10, 20, 30, 40, 90 and 120 minutes after the intravenous administration of Intralipid*, for the obese population before and after dietary intervention are shown in Table 13 (Appendix 16).

The triglyceride concentrations in samples taken at 0, 5, 10, 15, 30, 45, 60, 90 and 120 minutes from the control group are shown in Table 14.

In the analysis of the intravenous fat tolerance tests the curves from the obese and control populations fitted a single exponential ($r^2 > 0.80$), but two curves fitted with poorer r^2 values ($r = 0.68$). These clearance curves also had the highest AUC values and were obtained from two obese dogs prior to weight loss. The triglyceride clearance curve after administration of an Intralipid* bolus to Case 4 on initial presentation did not fit a single exponential model ($r^2 = 0.02$). When the test was repeated after dietary intervention, the clearance data fitted a monoexponential equation with a poor r^2 value ($r^2 = 0.62$). While the other triglyceride concentration versus time curves showed a maximum concentration at five minutes, the first clearance curve from Case 4 reached a peak at 20 minutes. The owner did not consider it possible that the dog had gained access to food in the 16 hours prior to the performance of the intravenous fat tolerance test and therefore the abnormalities displayed by this case were considered to be genuine rather than an effect of intestinal fat absorption. The data sets from two obese dogs could be fitted by both monoexponential and bi-exponential equations, but to facilitate standardisation, the characteristics quoted are calculated from the former.

Table 13. Mean ±sd plasma triglyceride concentration at serial time points after the intravenous administration of 0.1mg/kg Intralipid* to obese dogs (n=8) before and after dietary manipulation.

	Time (minutes)							
	0	5	10	20	30	40	90	120
Before	0.56 ±0.60	1.74 ±0.52	1.55 ±0.48	1.52 ±0.48	1.22 ±0.65	1.05 ±0.73	0.51 ±0.41	0.62 ±0.63
After	0.42 ±0.24	1.39 ±0.39	1.23 ±0.49	1.09 ±0.45	0.96 ±0.48	0.87 ±0.48	0.52 ±0.36	0.47 ±0.34

Table 14. Mean ±sd plasma triglyceride concentration at serial time points after the intravenous administration of 0.1mg/kg Intralipid* to control dogs (n=6).

	Time (minutes)								
	0	5	10	15	30	45	60	90	120
Control	0.20 ±0.06	1.54 ±0.46	0.92 ±0.39	0.56 ±0.23	0.22 ±0.07	0.20 ±0.06	0.18 ±0.07	0.17 ±0.07	0.18 ±0.07

The AUC_{obs}, $AUMC_{obs}$ and MRT were significantly ($p<0.05$) greater in the obese population than in the control dogs (Table 15.). After calorie restriction for 6 weeks the AUC_{obs}, $AUMC_{obs}$ and MRT were not significantly different from the values on initial presentation (Table 15.). When examined according to weight loss, the mean ±sd AUC_{obs} values before and after dietary intervention for four cases with a mean weight loss of 3.2 ±3.2% of body weight (12.0 ±10.6% target weight loss) were 76.3 ±25.1mmol.min/l and 68.0 ± 27.8mmol.min/l compared to 68.4 ±48.6mmol.min/l and 33.7 ±

15.3mmol.min/l for four cases with mean weight loss of 11.9 ±1.13% of bodyweight (62.3 ±11.8% target weight loss).

Since some clearance curves did not fit a single exponential, the AUC from time 5 minutes to time 120 minutes was calculated in order to test the relationship between the AUC and the other curve characteristics and was found to correlate negatively with the lipoprotein lipase activity ($r=-0.63$, $p<0.05$). The peak plasma triglyceride concentration (Cmax) correlated positively with the fasting triglyceride concentration ($r=0.74$, $p<0.01$) and there were weak correlations between the fasting triglyceride and LPL activity ($r=-0.41$; n.s.), Cmax and LPL activity ($r=-0.45$; n.s.), and Cmax and the AUC ($r=0.40$; n.s.).

Table 15. The mean ±sd AUC_{obs} (mmol.min/l) and $AUMC_{obs}$ (mmol.min^2/l) from time 0 to time infinity and MRT (mins) for the triglyceride clearance curves of eight obese dogs before and after calorie restriction and for six control dogs.

	AUC (mmol.min/l)	AUMC (mmol.min^2/l)	MRT (mins)
Obese (before)	76.8 ±36.5	3288 ±2533	37.9 ±17.5
Obese (after)	50.9 ±27.7	2179 ±2151	35.1 ±17.7
Control	21.9 ±8.4	179 ±87	7.9 ±1.4

4. DISCUSSION

In man, hypertriglyceridaemia is often recognised in association with obesity. This is the result of an increase in hepatic VLDL production which arises secondary to an increased flux of NEFA from the adipose tissue (Durrington 1989). In the obese dogs, fasting plasma triglyceride, VLDL-C and LDL-C concentrations were significantly greater than those of the control group of adult, non-obese beagles. However, these findings are contrary to the findings of Chapter IV which suggest that the lipid and lipoprotein concentrations in obese dogs were not significantly different from a control group of healthy pet dogs. There are two main contributing factors to the differences in these findings. The first important consideration is the selection of the control dogs. In the study of Chapter IV, 20 obese dogs were compared with a control population of 33 healthy pet dogs. It is recognised that breed differences (Downs, Bolton, Crispin and Wills 1993) and environmental factors may influence plasma LDL-C and HDL-C concentrations (Crispin, Bolton and Downs 1992). When the control dogs of Chapter IV were compared with the beagles used as control animals in this study, it was evident that the experimental dogs have lower triglyceride, VLDL-C and LDL-C concentrations. No significant differences in plasma lipid and lipoprotein concentrations were observed when this group of thirteen obese dogs were compared to the control population of pet dogs.

Different methods of statistical analysis were used to identify population variations in this data set and that described in Chapter IV. In the previous chapter, the effect of breed, age, gender, and health status on plasma lipid and lipoprotein concentrations were determined by analysis of covariance. This is particularly important since the gender effect on plasma triglyceride concentrations was close to statistical significance. Since the small subset of obese dogs presented in this chapter was composed predominantly of spayed

bitches, it is possible that the gender effect resulted in a population bias which was not accounted for by the Mann Whitney test. This data highlights the necessity for the formation of lipid and lipoprotein reference ranges for the population to be studied, *i.e.*, pet dogs versus laboratory animals. However, irrespective of the control population selected, the obese dogs do show similar lipoprotein trends to the obese human, *i.*e. hypertriglyceridaemia and raised VLDL-C concentrations.

The abnormalities of lipoprotein metabolism associated with obesity in man are corrected by prolonged fasting (Yeshurun, Aviram, Barak, Baruch and Brook 1986), calorie restriction and weight reduction (Grundy 1987). In the obese dogs which presented with hyperlipidaemia, there was partial correction of these abnormalities after calorie restriction. However, since only a minority of the obese animals did, in fact, present with raised lipid concentrations, the effect of calorie restriction on the whole group was minimal. There was a decrease in the LDL-C concentration which was reflected in a small decrease in the plasma cholesterol concentration. The minimal elevation of the mean HDL-C after dietary intervention masked the marked increase in some individuals. Calorie restriction results in mobilisation of adipose tissue cholesterol stores (Krause and Hartman 1984), which, in turn, may cause an elevation of the plasma concentration of HDL, the lipoprotein which serves as an acceptor of lipid from peripheral tissues.

Gender-specific patterns of obesity are recognised in people. The presence of abdominal adiposity (android obesity), commonly described in men, is associated with an increased risk of the development of coronary heart disease, non-insulin dependent diabetes mellitus and hyperlipidaemia, when compared to the relatively benign gluteal-femoral adiposity (gynoid obesity) recognised in women (Després 1991). An estimation of these patterns of obesity may be achieved by applying a number of simple computations, including the waist girth, hip girth, the waist:hip circumference ratio (WHR)

and the Quetelet index (kg/height^2). These anthropometric estimates correlate positively with plasma cholesterol and triglyceride concentrations, and negatively with HDL-C concentrations. The lipoprotein profile associated with human obesity is therefore also associated with an increase in atherogenic risk (Anderson, Sobocinski, Freedman, Barboriak, Rimm and Gruchow 1988).

Gender-specific patterns of obesity have not been described in the dog, although reports indicate that bitches have, on average, 16% more body fat than males (Meyer and Stadtfeld 1980) and that the prevalence of obesity is greater in the female (Mason 1970). Excess adipose tissue tends to accumulate over the thorax and tailhead, cranial to the tuber coxae and intra-abdominally. A number of methods have been proposed for the assessment of obesity in the dog (Wilkinson and Mooney 1991) but for all practical purposes this remains a subjective evaluation (Hand 1988). The use of bioelectrical impedence (Hand, Armstrong and Allen 1989) and A mode ultrasound (Wilkinson and McEwan 1991) may, in the future, allow improved estimation of the degree of obesity of individual dogs. In the meantime, the measurement of obesity is dependent upon manual and visual appraisal (Edney and Smith 1986), peripheral body measurements and the percentage over "ideal" body weight. This practical difficulty undoubtedly creates an obstacle when attempting to assess the effect of obesity on canine metabolism. The anthropometric indices evaluated in this study do show some correlation with the plasma triglyceride and triglyceride-rich lipoprotein concentrations, suggesting that in the dog, obesity may be associated with abnormalities of chylomicron and VLDL lipoprotein metabolism. Since the dietary fat intake may play a role in the determination of obesity and the plasma lipid concentrations it would be of interest to determine the independent effects of both factors. However, this was not possible within the scope of this study due to the small number of cases included and the difficulty in assessing the fat content of the diet, considering

that most of the animals, including those on commercial reducing diets were fed varying quantities of home-cooked food each day.

The hypertriglyceridaemia identified in obese humans has been attributed to an increased VLDL production (Després 1991). In patients with normal triglyceride concentrations, the increased VLDL synthesis is balanced by an increased removal of the lipoprotein from the circulation (Kissebah and Schectman 1978). In support of this, increased lipoprotein lipase activities per single fat cell and per total body fat mass have been described in association with obesity in man (Taskinen and Nikkilä 1981). However, in this study there was a decreased lipoprotein lipase activity in the postheparin plasma of obese dogs, compared with control animals. It is noted in man that caloric restriction is associated with a decrease in adipose tissue lipoprotein lipase activity (Taskinen and Nikkilä 1981). Three of the dogs included in this study were already receiving a reduced calorie diet before evaluation. In the remaining nine dogs, the owners reported unsuccessful attempts at weight reduction. It may be that partial caloric restriction in the six weeks between owners agreeing to participate in this trial and the beginning of the trial was sufficient to suppress lipoprotein lipase activity when compared to a normal population. There was a further reduction in lipoprotein lipase activity throughout the period of calorie restriction, but this change was not significant. Since the lipoprotein lipase of postheparin plasma is derived from both skeletal muscle and adipose tissue it is possible that the alterations in activity reflect changes in the muscle rather than the adipose tissue enzyme.

The Intralipid* intravenous fat tolerance test for the assessment of chylomicron clearance has been employed in both man (Cohen 1989) and the dog (Carlson and Hallberg 1963). In both species the clearance of intravenously-administered Intralipid* follows the same kinetics as chylomicron clearance. It has been proposed that the clearance curve in man may be fitted by a single exponential in all subjects (Carlson and Rössner 1972; Cohen 1989).

This confirmed the earlier findings of Carlson and Hallberg (1963), who found that the elimination of Intralipid* (0.1g/kg administered intravenously) in clinically healthy dogs could be fitted to a single exponential. The same was true for the control dogs and most of the obese dogs in this study. However, four data sets displayed a very poor fit to a monoexponential equation. When these plasma triglyceride curves were examined it was evident that the concentrations in these cases increased at some point during the 120 minutes sample time, *i.e.*, the clearance curve had a second "peak" or "shoulder". A number of hypotheses may explain the complex nature of these curves. It is possible, that in the obese dog, the Intralipid* triglyceride was rapidly cleared to a body compartment from which it was later released, *e.g.*, rapid hepatic uptake, followed by secretion in the form of VLDL triglyceride. In the normal scenario, insulin results in an increased synthesis of VLDL triglyceride accompanied by a reduction in secretion, resulting in an intracellular accumulation of triglyceride which is released on decline of the circulating insulin concentrations (Gibbons 1990). Prolonged exposure of hepatocytes to insulin results in down-regulation of the insulin receptors and an increase in hepatic VLDL secretion. It is possible that in obese dogs, in which insulin resistance is a metabolic complication, the inhibitory effect of insulin upon hepatic triglyceride secretion is lost and such animals may produce VLDL particles in a poorly regulated fashion. The increase in plasma triglyceride concentrations during the intravenous fat tolerance tests in the obese dogs of this study may, therefore, reflect abnormal regulation of hepatic triglyceride synthesis.

The delayed clearance in obese patients was consistent with the identification of reduced lipoprotein lipase activity in obese dogs when compared to the non-obese control population. It is evident from this data that obese individuals display a prolonged plasma triglyceride clearance which may exacerbate the postprandial hyperlipidaemia noted in normal individuals

(Watson, MacKenzie, Packard and Shepherd 1993). In cases where the lipid metabolism of an individual is further compromised, *e.g.*, in dogs with concurrent familial or secondary hyperlipidaemia, the compound effect may result in marked hypertriglyceridaemia. The delayed clearance of triglyceride from the circulation of the obese dog in the postprandial period may explain the higher incidence of acute pancreatitis in obese animals fed a high fat diet (Anderson, N.V., 1972).

With the exception of two cases which lost minimal quantities of weight (Cases 4 and 9), and in one case (Case 12) which may have gained access to food on the morning of the second test, the values for the areas under the triglyceride clearance curves were reduced after caloric restriction. This was irrespective of the degree of weight reduction. The owner's compliance with the feeding regime was questioned in one case in which the AUC did not decrease, but in the other case, no such problem was suspected. It appears, therefore, that caloric restriction alone may improve plasma triglyceride clearance in obese dogs. This may be related to a decreased plasma triglyceride pool, *i.e.*, a reduction in endogenous triglyceride synthesis (Taskinen and Nikkilä 1981). However, the reduction in AUC values in the group which lost weight does suggest that there is an effect of weight reduction *per se* on the triglyceride clearance curves, in addition to any effect secondary to caloric restriction. This may reflect changes in hepatic triglyceride synthesis, since there was no significant change in lipoprotein lipase activity.

These findings confirm the benefits of weight reduction in the correction of the kinetic lipoprotein abnormalities associated with obesity and may reduce the risk of the development of pancreatitis.

CHAPTER VI

THE CLINICAL PRESENTATIONS AND METABOLIC CONSEQUENCES OF HYPERLIPIDAEMIA IN THE DOG

1. INTRODUCTION

The literature regarding idiopathic and secondary hyperlipidaemia in the dog is limited to the description of small numbers of cases (Rogers, Donovan and Kociba 1975a and b; Ford 1977) and review articles (DeBowes 1987; Armstrong and Ford 1989). The incidence and prevalence of hyperlipidaemia in the canine population have not been determined.

The pathological consequences of hyperlipidaemia have been alluded to by some investigators (Zerbe 1986) and may be classified according to the role of hypertriglyceridaemia or hypercholesterolaemia in the induction of certain lesions. Hypertriglyceridaemia may be associated with the development of ocular lesions, pancreatitis, eruptive xanthomas and more uncommonly, disturbances of the central nervous system (Shepherd and Packard 1987). The ocular abnormalities include lipid-laden aqueous humor and lipaemia retinalis. In man, these clinical and pathological aberrations are generally detected in association with a marked increase in the plasma triglyceride concentration (>11mmol/l) and are collectively referred to as the chylomicronaemia syndrome (Brunzell and Bierman 1982). Hypertriglyceridaemia has also been implicated in the pathogenesis of hepatic lipidosis in both the dog and the cat (Hardy 1989). Hypercholesterolaemia is associated with atherosclerosis and corneal arcus, an arc-like lipid deposit of the cornea (Crispin and Barnett 1983). The effect of increased plasma cholesterol concentrations upon the cholesterol content of cell membranes, in particular red blood cells and platelets, may result in haematological abnormalities, which may predispose the individual to thrombosis (Gasser and Betteridge 1990) and the blood to *in vitro* haemolysis.

The aim of this study was to determine the incidence of idiopathic and secondary hyperlipidaemia in a referral population and to catalogue the associated clinical signs and the pathological findings.

2. MATERIALS AND METHODS

2.1. The incidence of hyperlipidaemia

The incidence of hyperlipidaemia in a referral population at G.U.V.S. was assessed during the six month period from October 1990 to March 1991. For the purposes of the study, hyperlipidaemia was defined by raised fasting plasma concentrations of cholesterol (>6.5mmol/l) and/or triglyceride (>1.75mmol/l). Plasma lipid concentrations were measured by the routine chemical methods described in Chapter II 2.2.

Affected dogs were allocated to a group depending on their health status: secondary hyperlipidaemia associated with underlying disease process previously recognised as a cause of lipid disorders, *i.e.,* hypothyroidism, hyperadrenocorticism, diabetes mellitus, nephrotic syndrome and obstructive jaundice. If no underlying disease process was detected, the hyperlipidaemia was defined as idiopathic (or primary).

2.2. Classification of hyperlipidaemia

Samples for lipid analysis were collected from 128 dogs referred to G.U.V.S. with hyperlipidaemia or diseases commonly associated with the development of lipoprotein abnormalities. Fasting plasma cholesterol and triglyceride concentrations were measured and the animals classified on the basis of the presence of no lipid abnormalities, hypercholesterolaemia, hypertriglyceridaemia, or combined hyperlipidaemia (increased plasma cholesterol and triglyceride).

2.3. The clinical signs and alterations in haematological and biochemical parameters associated with hyperlipidaemia

Hyperlipidaemia was identified in 92 dogs referred to G.U.V.S. for the investigation of medical or surgical disorders. In 57 cases the hyperlipidaemia was associated with systemic disease: diabetes mellitus (10),

hyperadrenocorticism (13), hypothyroidism (15), hepatic disease (5), nephrotic syndrome (4), septicaemia (3) and obesity (7). No underlying pathological process was identified in 35 dogs with hyperlipidaemia (idiopathic hyperlipidaemia). In addition to the cases examined at G.U.V.S., case histories and plasma for lipoprotein quantification were submitted from nine dogs with corneal lipid deposition. The clinical signs and pathological abnormalities putatively associated with hyperlipidaemia were recorded. The biochemical indicators of hepatocellular insult and renal disease, in addition to haematological abnormalities were also noted.

3. RESULTS AND DISCUSSION

3.1. The incidence of hyperlipidaemia

During the period of the study, 362 new, consecutive canine referrals were examined at G.U.V.S. Hyperlipidaemia was identified in 53 dogs (15%), of which 43 (81%) had secondary hyperlipidaemia. No underlying disease was found in 10 dogs (19%), in which the hyperlipidaemia was considered idiopathic (Table 16.). The secondary hyperlipidaemia group consisted of dogs with diabetes mellitus, hyperadrenocorticism, hypothyroidism, nephrotic syndrome, obstructive jaundice, hepatic disease other than obstructive jaundice, renal disease other than protein-losing nephropathy and septicaemia. Concurrent hypothyroidism or hyperadrenocorticism was identified in a number of dogs with diabetes mellitus.

The pattern of disease distribution in this survey reflects both the incidence of diseases known to be associated with hyperlipidaemia in the dog and the type of medical cases referred to Glasgow University Veterinary School. Hyperlipidaemia has previously been reported in association with

Table 16. The classification of hyperlipidaemia in 53 dogs according to disease association.

Classification	Number
Secondary hyperlipidaemia	43
Diabetes mellitus*	17
Hyperadrenocorticism	13
Hypothyroidism	3
Nephrotic syndrome	2
Obstructive jaundice	1
Hepatic disease	4
Renal disease	1
Septicaemia	2
Idiopathic hyperlipidaemia	10

* Five cases with concurrent hyperadrenocorticism and two with concurrent hypothyroidism.

endocrine disease in the dog (Rogers 1977; Medaille, de La Farge, Braun, Valdiguie and Rico 1988). The identification in this study of increased plasma lipid concentrations in dogs with diabetes mellitus, hypothyroidism and hyperadrenocorticism therefore confirms previous reports. The incidence of secondary hyperlipidaemia in a referral clinic is dictated by a number of factors, not entirely related to the incidence of the disease in the general population. At the time of this study, a clinical trial designed to evaluate a new insulin product was also in progress and certainly resulted in a higher number of diabetic dogs referred to the hospital. Dogs with diabetes mellitus and hyperadrenocorticism are generally under poor glycaemic control, and are therefore referred for further investigative or therapeutic measures, explaining the high incidence of complicated diabetes mellitus in this hospital population. Hypothyroidism, although considered a relatively common endocrine disease in

the dog is not well represented in this study, possibly due to ease of diagnosis and therapy of the condition in general practice. Conversely, the toxicity of op'DDD therapy for hyperadrenocorticism results in a high referral rate for the diagnosis and management of the disease.

Disorders of lipid metabolism have been noted in both experimental (Bass, Hoffman and Dorner 1976) and naturally-occurring liver disease (McCullagh 1978) in the dog. The appearance of hyperlipidaemia in association with both obstructive jaundice and hepatic dysfunction is therefore consistent with the limited information available for the dog, and with extrapolation from human lipid metabolism (Miller 1990). The relationship between hyperlipidaemia and renal disease (impaired glomerular filtration rate or protein-losing nephropathy) has been established in man (Brøns, Christensen and Hørder 1972; Short and Durrington 1990). The results of the present study and previous case reports (Ford 1977) suggest that similar associations may exist in the dog.

Hypercholesterolaemia and hypertriglyceridaemia have been observed in dogs naturally infected with *Leishmania infantum* (Nieto, Barrera, Habela, Navarrete, Molina, Jiménez and Serrera 1992) and with experimental gram negative sepsis (Griffiths, Groves and Leung 1973). The increase in plasma lipids and lipoproteins in these processes may serve to modulate the immune or host reaction to the pathogen and may be related to an increase in the circulating concentrations of tumour-necrosis factor (Feingold and Grunfeld 1987). In the light of these experimental findings and the association between septicaemic processes and hyperlipidaemia demonstrated in this study, it would seem that further lines of investigation in this direction may yield information regarding the role of lipids in the regulation of the immune system.

Primary, inherited hyperlipidaemia is considered a rare condition in the dog, although a familial disturbance of lipoprotein metabolism is recognised in the miniature schnauzer in North America (Rogers, Donovan and Kociba

1975a; Whitney 1987) and is suspected in the Briard in the United Kingdom (Watson, Simpson, Odedra and Bedford 1993). On examination of the results of this study, however, it is apparent that the prevalence of idiopathic hyperlipidaemia is much higher than suggested by the prevalence of recorded inherited lipoprotein disorders. Since investigations into hyperlipidaemia in the dog are in their infancy, it is possible that additional inherited lipoprotein disorders may be recognised and characterised in the future, or that diet, age and other undetermined environmental factors may be important in the aetiology of such abnormalities.

3.2. Classification of hyperlipidaemia

Blood samples were collected from dogs with diabetes mellitus (n=12), hyperadrenocorticism (n=17), hypothyroidism (n=15), renal disease (n=4), hepatic disease (n=6), obesity (n=20) and idiopathic hyperlipidaemia (n=35). Normolipidaemic dogs were present in the dogs with diabetes mellitus (2/12), hyperadrenocorticism (3/17), hepatic disease (2/6) and obesity (13/20) (Table 17.). None of the cases presented with hypertriglyceridaemia alone. Hypercholesterolaemia was the predominant lipoprotein abnormality in all groups, with the exception of the hypothyroid dogs, where increases in both plasma cholesterol and triglyceride concentrations were more common.

Increased plasma cholesterol, with or without concurrent hypertriglyceridaemia, has previously been recognised in association with diabetes mellitus and hypothyroidism in the dog (Zerbe 1986). Ford (1977) suggested that dogs with uncomplicated diabetes mellitus have no lipoprotein abnormalities, while those with ketoacidosis are hypertriglyceridaemic and possess increased plasma LDL concentrations (detected by lipoprotein electrophoresis). This observation agrees in part with the findings of our study,

Table 17. Characterisation of the lipid abnormalities associated with secondary and idiopathic hyperlipidaemia. The number of cases with hypercholesterolaemia (HC), combined hyperlipidaemia (CH) and normal lipid concentrations (N) are expressed. No dogs had hypertriglyceridaemia alone.

	Number of cases		
	N	HC	CH
Obesity	13	5	2
Diabetes mellitus	2	6	4
Hyperadrenocorticism	3	10	4
Hypothyroidism	0	6	9
Renal	0	4	0
Hepatic disease	2	3	1
Idiopathic hyperlipidaemia	0	25	10

where the four dogs with combined hyperlipidaemia were also suffering from diabetic ketoacidosis (characterised by glucose and ketones in the urine, hyperglycaemia and dehydration). However, six dogs with uncomplicated diabetes mellitus were hypercholesterolaemic. The two normolipidaemic dogs had received insulin therapy, but were under poor glycaemic control. The plasma lipid concentrations prior to therapy were not available and therefore the effect of therapy in these two cases could not be determined.

Rogers (1977) stated that hyperadrenocorticism is not associated with the development of hyperlipidaemia, and that the finding of lipid abnormalities in dogs with Cushing's syndrome should prompt further investigation to identify the aetiology. This suggestion is, however, contrary to the findings of other authors and to the data presented in this study. A study of 117 cases of canine

hyperadrenocorticism (Ling, Stabenfeldt, Comer, Gribble and Schechter 1979) confirmed hypercholesterolaemia in 90% of individuals, while the present study identified increased cholesterol concentrations in 82% of dogs. Feldman and Nelson (1987) suggested that lipaemia is at least as frequent as hypercholesterolaemia in canine Cushing's syndrome. This statement was not confirmed in the present study where hypertriglyceridaemia was present in only 31% of dogs.

Hypercholesterolaemia is present in approximately two thirds of dogs with hypothyroidism (Rogers, Donovan and Kociba 1975b; Larsson 1988). Of these, approximately 50% have combined hyperlipidaemia (Rogers, Donovan and Kociba 1975b) which is similar to the present study. The cases of canine hypothyroidism sampled at G.U.V.S. were all hyperlipidaemic. Since hypothyroidism is commonly recognised, diagnosed and treated by veterinary surgeons in general practice, cases referred to Glasgow University may reflect the positive selection of cases with hyperlipidaemia, rather than suspected hypothyroidism. This would certainly explain the difference between the prevalence of hyperlipidaemia in dogs with hypothyroidism reported by Rogers *et al.* (1975b) and that described here.

3.3. Gastrointestinal disease, abdominal pain and pancreatitis

In this study, anterior abdominal pain, characterised by restlessness, a praying stance and pain on palpation, was detected in four dogs. Plasma amylase and lipase activities were raised, indicative of acute pancreatitis, in only two of these cases. The first of these, a 5.5 year-old, neutered, female Tibetan terrier (Case 112714, Appendix 19) was suffering from concurrent diabetes mellitus and hypothyroidism with marked hyperlipidaemia (plasma cholesterol 17.51 mmol/l and triglyceride 14.15 mmol/l). The second, a 12 year-old golden retriever bitch (Case 122074, Appendix 19) presented with anorexia, vomiting and depression. Biochemical analyses revealed increases in alkaline phosphatase

activity (1155 IU/l), plasma cholesterol (14.88mmol/l) and triglyceride concentrations (4.37mmol/l). Plasma from both dogs was visibly lipaemic. The clinical signs of both animals were consistent with acute pancreatic disease and the pain was localised to the anterior abdomen by the examining veterinary surgeon.

In two other dogs hyperlipidaemia was accompanied by intermittent episodes of anorexia, vomiting and abdominal pain. In neither case was the pain convincingly localised to the anterior abdomen, nor were plasma amylase and lipase activities increased. The first case was a seven year-old crossbred neutered dog (Case 119993, Appendix 19) which presented to the referring veterinary surgeon with recurrent vague signs of abdominal discomfort and vomiting. The plasma was lipaemic and a routine biochemical profile revealed a triglyceride concentration of 39.00mmol/l and cholesterol of 16.48mmol/l. The second dog was an eight year-old neutered bitch (Case 120360, Appendix 19) with a history of weekly episodes of vomiting and anorexia, which responded to dietary restriction and antibiotic therapy. The plasma cholesterol concentration in this bitch was within the reference range (6.23mmol/l) but the triglyceride concentration was markedly raised (11.30mmol/l).

In three of the four animals which presented with abdominal pain and intestinal signs, no underlying disease process was detected to which the hyperlipidaemia may have been attributable. The question remains as to whether the lipid disturbances were responsible for the clinical signs or whether both the hyperlipidaemia and the clinical manifestations were the result of a common pathological process. The introduction of a low fat diet (Canine low fat diet; Waltham) resulted in partial resolution of the hyperlipidaemia and complete remission of the clinical signs in all three cases, with no recurrence to date. It is likely that hyperlipidaemia, and in particular hypertriglyceridaemia, plays a role in the development of the symptoms described. The clinical signs in the latter two cases were similar to those of classical recurrent pancreatitis

with the major exception of the generalised, rather than localised abdominal pain. This has also been noted in the chylomicronaemia syndrome of man (Brunzell and Bierman 1982) and may indicate pathological processes, as yet undetermined. It is possible, however, that these two dogs were indeed suffering from classical pancreatitis, but that interference in the analysis of amylase by the lipaemia of the plasma samples resulted in spuriously low results.

Investigators have previously attempted to clarify the relationship between hyperlipidaemia and pancreatitis by studying the lipid changes in experimentally-induced canine pancreatitis. The infusion of bile into the pancreatic duct of dogs resulted in slight increases in the plasma triglyceride concentrations and decreases in the rate of triglyceride clearance from the circulation (Zieve 1968), while bile duct ligation (Whitney, Boon, Rebar and Ford 1977) induced hypercholesterolaemia secondary to an increase in LDL concentrations. Plasma from three of the four dogs presented in this study was submitted for lipoprotein quantification. All the samples contained increased concentrations of VLDL-cholesterol and LDL-cholesterol (Appendix 19). In the one dog with concurrent diabetes mellitus and hypothyroidism, these changes are likely to be secondary to the underlying disease processes, but the fact that the other three dogs presented with similar clinical and lipoprotein disturbances suggests an association between this lipoprotein profile and the clinical syndrome. These data confirm that hypertriglyceridaemia plays a role in the development of pancreatitis, rather than arising as a consequence of pancreatic disease. The aetiology may involve the formation of lipid emboli and ischaemia of pancreatic tissue.

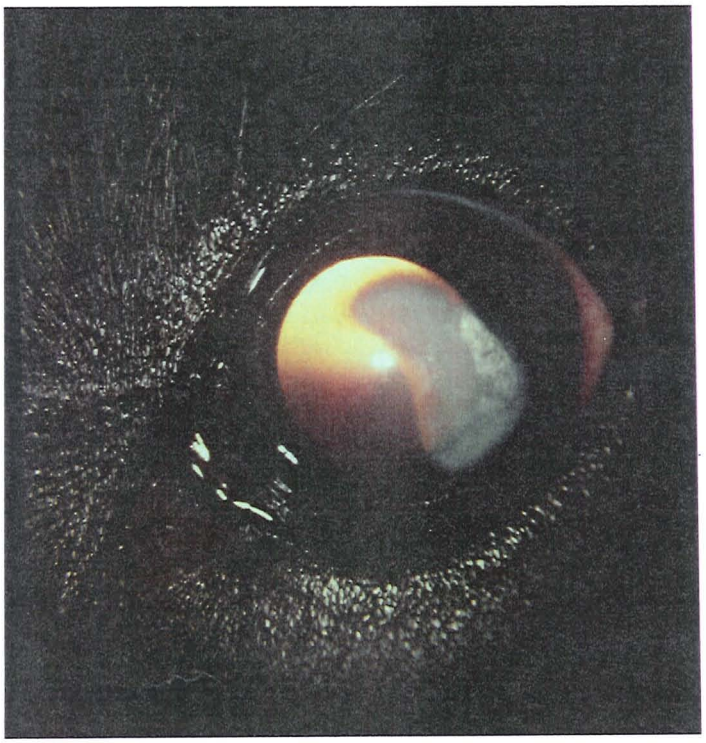
3.4. Ocular abnormalities

One case, a 3 year old, female cocker spaniel (Case 116313, Appendix 19), was referred for the investigation of bilateral corneal opacities (Figs 8. and 9.). Lipid accumulation was noted in both corneas, with denser areas laterally and fainter deposits medially. In the right eye the lesion was horse-shoe shaped and early vascularisation was identified on both sides. The plasma cholesterol concentration at this time was above the upper limit of the reference range (8.4mmol/l), but the triglyceride concentration was normal (0.59mmol/l). The dog was considered moderately obese and a low fat diet (Hills r/d diet, Hills Pet Products Ltd) was introduced to induce weight reduction and control the hyperlipidaemia. Within three weeks of the initial presentation the plasma cholesterol concentration had fallen to 5.7mmol/l and by six weeks to 4.3mmol/l. The lipid concentrations remained within normal limits and the corneal lesions remained unchanged for the following six months. The dog then presented with bilateral epiphora and anterior segment inflammation which persisted for approximately one year, despite topical therapy, at which point she developed an episcleral mass on the lateral aspect of the left globe. Biopsy of the lesion revealed an inflammatory reaction consistent with scleritis/episcleritis. Systemic and topical corticosteroid therapy relieved the scleral inflammation and epiphora but an increase in the extent and density of the corneal lipid deposition was noted. The latter appeared to worsen after each inflammatory episode, suggesting that anterior segment inflammatory disease and corneal oedema may have played a role in the development of corneal lipid deposition in this dog.

A small, dense lipid deposit was also noted in the ventrolateral quadrant of right eye of an adult male Dobermann (Case 112876, Appendix 19) suffering from hypothyroidism. On presentation the dog was both hypercholesterolaemic

Figure 8. Corneal lipid deposition in the right eye of Case 116313.

Figure 9. Corneal lipid deposition in the left eye of Case 116313.



(17.67mmol/l) and hypertriglyceridaemic (2.61mmol/l). After the introduction of thyroid replacement therapy the lesion became fainter but was still present six months later.

In addition to the cases examined at Glasgow Veterinary School, plasma samples from nine dogs (Cases 1-9, Appendix 19) with corneal lipidosis were submitted for plasma lipoprotein quantification. Of these, one dog with unilateral facial nerve paralysis and corneal lipid deposition was also hypothyroid. This animal was hypercholesterolaemic (14.41mmol/l) and hypertriglyceridaemic (2.04mmol/l). Of the remaining dogs, four (50%) were hypercholesterolaemic (range 6.93mmol/l - 11.35mmol/l) and one dog was hypertriglyceridaemic (1.84mmol/l). The hyperlipidaemia in the latter case may have reflected insufficient fasting before sampling.

Arcus lipoides corneae is an annular lipid infiltration of the peripheral cornea and perilimbal zone of the sclera by lipid. There is usually a clear ring at the limbus but the lesion may extend towards the central cornea. The condition has been recognised as an infiltration of cholesterol, fatty acids and phospholipids in German shepherd dogs with hypothyroidism and hyperlipidaemia (Crispin and Barnett 1978). In the present study three dogs with corneal lipid deposition were German shepherd dogs, but none of these animals was hyperlipidaemic or had clinical signs of hypothyroidism. In these three cases, and in that of the cocker spaniel with anterior segment disease, the corneal lipid deposition may have been secondary to local changes in blood vessel permeability or inflammatory reactions, resulting in an increased transfer of lipids into the corneal stroma (Crispin 1989), rather than systemic factors.

Lipaemia retinalis is the alteration in colour of the retinal vessels due to hyperlipidaemia and has been recognised in cats with inherited chylomicronaemia (Jones, Wallace, Harding, Hancock and Campbell 1983), but appears to be rarely identified in dogs (Wyman and McKissick 1973). The condition is only of diagnostic significance since the excess of blood lipids has

been demonstrated to have no detrimental effect on the retina (Gelatt 1991). Crispin and Barnett (1978) described a single case of lipaemia retinalis in a dog with a plasma cholesterol concentration of 22.1mmol/l. The plasma triglyceride concentration was not recorded. Lipaemia retinalis was not among the ocular abnormalities detected in the present series of cases, which included one dog with a plasma triglyceride concentration of 39mmol/l at the time of ophthalmic examination. The failure to recognise lipaemia retinalis in any of the cases studied at Glasgow Veterinary School may, however, reflect ophthalmic inexperience, rather than absence of the condition. This must be considered the most likely scenario, since the appearance of blood samples from a number of cases resembled "tomato-soup" or "strawberry milk shake" and such abnormalities might well be associated with visual distortion of the retinal vessels.

3.5. Atherosclerosis

Experimentally, canine atherosclerosis may be induced by oral vitamin D poisoning (McAllister and Waters 1950), the chemical or surgical induction of hypothroidism combined with high cholesterol diets (Moses 1954) and by feeding semisynthetic diets for prolonged periods (McCullagh, Ehrhart and Butkus 1976; Mahley, Innerarity, Weisgraber and Fry 1977). Naturally-occurring atherosclerosis is rarely reported in the dog, but has been recognised in association with thyroid dysfunction (Robinson 1976; Patterson, Rusley and Zachary 1985; Liu, Tilley, Tappe and Fox 1986). The development of atherosclerosis in the dog usually begins in small vessels and later in the aorta and coronary arteries (McGill 1965).

Atherosclerosis was recognised in a 9 year-old, male whippet (Case 114522, Appendix 19). The dog presented with hind limb paresis. On clinical examination the hind limbs were cold and the nailbeds cyanosed. No femoral pulse was detected on the right side but a thickened cord, thought to be the

femoral artery was identified. A provisional diagnosis of aortic saddle thrombosis was made. Biochemical analyses revealed hypercholesterolaemia (47.80mmol/l) and hypertriglyceridaemia (5.60mmol/l). A TSH stimulation test confirmed the presence of thyroid dysfunction. The dog was euthanased on humane grounds and post mortem examination revealed the presence of atherosclerotic plaques of the coronary vessels, descending aorta and peripheral vessels (Fig. 10.). A large thrombus was present in approximately one third of the right femoral artery and the right testicle was completely infarcted secondary to vessel occlusion.

Histologically the lesions were characterised by an accumulation of lipid deposits in the media of the vessels (Fig. 11.). The peripheral vascular disease observed in this case was similar to that found in human type III hyperlipidaemia (European Atherosclerosis Society 1988). It is interesting to note that type III hyperlipidaemia is associated with the accumulation of β-VLDL, an unusual lipoprotein which represents the accumulation of remnants of triglyceride-rich lipoproteins. Beta-VLDL was also isolated from the plasma of this case of canine hypothyroidism (Chapter VII 3.2.), suggesting a possible role for this lipoprotein in the pathogenesis of peripheral vascular disease in the dog.

3.6. Neurological disturbances

In man, dementia, characterised by fluctuations in memory performance which correlated inversely with plasma lipid concentrations, has infrequently been recognised in association with hyperlipidaemia (Mathew, Meyer, Achari and Dodson 1976). Disorientation, coma, circling and visual impairment have been noted in dogs with cerebral atherosclerosis secondary to primary hypothyroidism (Liu, Tilley, Tappe and Fox 1986; Patterson, Rusley and Zachary 1985). Similar signs were noted in the hypothyroid whippet previously

Figure 10. Atherosclerosis of a branch of the left coronary artery. The epicardial arteries appear as prominent cord-like structures due to narrow irregular white plaques within their walls.

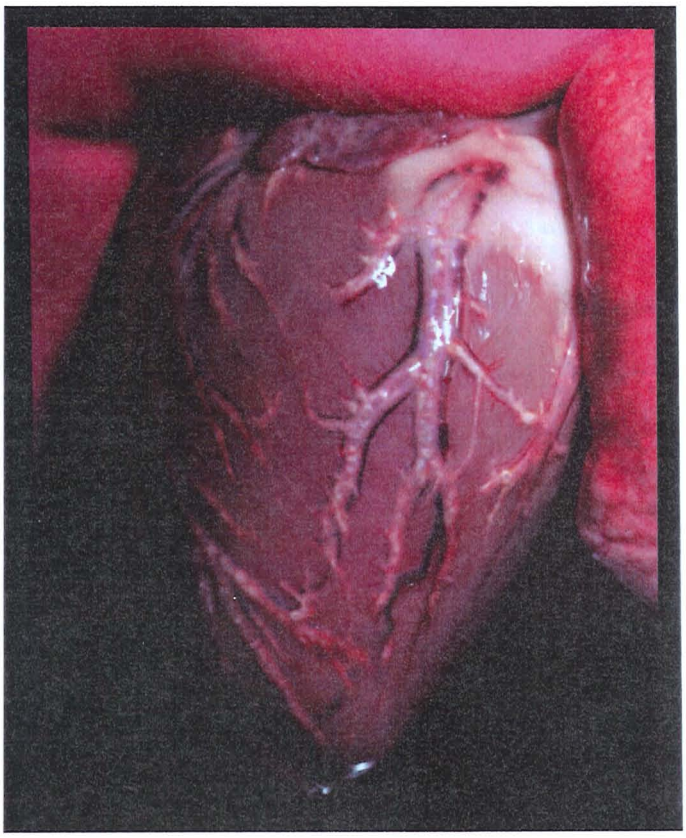


Figure 11. Cross section of the carotid artery showing clear spaces where lipid filled macrophages have replaced the smooth muscle cells of the media. Cholesterol clefts are also present (c). (Sirius Red)

mentioned. This dog had sporadic episodes of aimless wandering and apparent confusion during the three months prior to referral to G.U.V.S.

Generalised seizures have been reported in miniature schnauzers with variable plasma triglyceride concentrations (Rogers, Donovan and Kociba 1975a; Bodkin 1992). The relationship between hyperlipidaemia and disorders of the central nervous system have not been determined. None of the dogs with hyperlipidaemia identified in the presented study suffered from seizures.

3.7. Peripheral neuropathies

Peripheral neuropathies in man have been associated with diabetes mellitus and hyperlipidaemia, but have also been identified in a small number of non-diabetic hyperlipidaemic cases suggesting that hyperlipidaemia, *per se* has a role in the aetiology of such neurological deficits (Sandbank, Bechar and Bornstein 1971; Mathew, Meyer, Achari and Dodson 1976). In the present study, peripheral neuropathies were detected in a number of dogs with endocrine disease. Two dogs with diabetes mellitus had hind limb polyneuropathies and one had paralysis of the parasympathetic component of the third cranial nerve. One dog with hypothyroidism had a polyneuropathy and one dog with hyperadrenocorticism showed signs of generalised weakness and paralysis of the seventh cranial nerve.

Only one dog with idiopathic hyperlipidaemia (Case 119993, plasma cholesterol 16.48mmol/l and triglyceride 39.00mmol/l) had evidence of peripheral nerve dysfunction. In this case, the dog presented to the referring veterinary surgeon with a unilateral facial nerve paralysis and hyperlipidaemia. The dog had previously suffered recurrent episodes of bilateral otitis externa which may have progressed to otitis media, resulting in neurological signs. At the time of this presentation, however, auriscopic examination revealed only minor erythematous changes in the ipsilateral ear.

In man, the pathogenesis of hyperlipidaemic neuropathy is associated with demyelination, myelin disorganisation and perineural connective tissue proliferation (Mathew, Meyer, Achari and Dodson 1976; Sandbank, Becher and Bornstein 1971), while in cats with idiopathic hyperlipidaemia peripheral neuropathy is believed to be the result of compression by subcutaneous xanthomata (Jones, Johnstone, Hancock and Wallace 1986). Due to the impracticalities of nerve biopsy, it was not possible to study the pathology associated with the endocrine and hyperlipidaemia related neuropathies identified in this study.

3.8. Cutaneous xanthomatosis

Xanthoma are dermatological lesions which result from the accumulation of lipids in foam cells of the dermis. The lipids contained within them are thought to originate from circulating plasma lipids, a theory which is supported by the appearance and regression of xanthomas in concert with the changes in plasma lipoproteins. In their eruptive phase, the lesions are particularly triglyceride-rich, while the resolving lesions are cholesterol enriched (Parker, Bagdade, Odland and Bierman 1970). Cutaneous xanthomas have been described rarely in animals but are recognised in association with feline idiopathic hyperchylomicronaemia (Jones, Johnstone, Hancock and Wallace 1986) and diabetes mellitus in both the dog (Chastain and Graham 1978) and the cat (Jones, Wallace, Hancock, Harding and Johnstone 1985). The paucity of case reports was reflected in the present study, where skin lesions were detected in only one case, *i.e.*, the adult, male Dobermann with hypothyroidism and corneal lipid deposition (Case 112876; Appendix 19, see section 3.4.). Raised, thickened plaques with a grey surface and hyperkeratotic edges were present on the pinnae, hocks and elbows of this dog. The lesions regressed within one month of thyroid hormone replacement, but their exact nature was not classified by histological examination. If these lesions were classical xanthomas,

it is surprising that this individual animal, with a plasma triglyceride concentration of 2.63mmol/l (plasma cholesterol 19.13mmol/l) should be affected and others with more severe lipid abnormalities did not show dermatological lesions. However, the presence of xanthomas with a major fibrohistiocytic proliferation have been reported in normolipidaemic dogs, although the origin of the lipid component has not been determined (Gross and Ihrke 1992).

3.9. Pseudopregnancy

The female cocker spaniel previously described (case 116313) has been maintained on a low fat diet (Hills r/d diet, Hills Pet Products Ltd.) from the time of her initial consultation at G.U.V.S. (January 1991) to the present date. During this period her plasma cholesterol concentrations, which were monitored at four to six weekly intervals, remained between 3.0mmol/l and 4.0mmol/l. The plasma triglyceride concentration was stabilised at 0.50-0.65mmol/l. On 21 August 1991, the bitch's plasma cholesterol concentration was 3.55mmol/l. This rose to 5.59mmol/l on 1 October and 7.13mmol/l on 24 October 1991. This increased plasma cholesterol concentration reflected an increase in both HDL-C and LDL-C concentrations (3.69mmol/l and 2.71mmol/l, respectively). From 4-10 October the dog displayed behavioural changes and milk production consistent with pseudopregnancy. The clinical signs regressed within 8 days without the use of medical therapy. Pseudopregnancy in the bitch is believed to be a an exaggeration of the normal non-pregnant state. The onset is dependent on falling progesterone and rising oestrogen concentrations, in addition to an increased prolactin concentration (Feldman and Nelson 1987). Hyperlipidaemia is commonly recognised in pregnant women and in those prescribed certain oral contraceptives (Crook and Seed 1990). Under these circumstances, lipoprotein metabolism appears to be influenced by changes in progesterone, oestrogen and insulin. The effect of

lactation *per se* appears to be limited to an increase in the cholesterol content of HDL particles (Knopp, Bergelin, Wahl and Walden 1985). It is possible, therefore, that the hormone changes responsible for the development of pseudopregnancy in the cocker spaniel bitch presented here may also have resulted in alterations of lipid metabolism.

3.10. Hepatic enzymes

The alkaline phosphatase, ALT and AST activities in obese dogs and dogs with idiopathic and secondary hyperlipidaemia (Table 18., Appendix 20) were compared with the plasma lipid and lipoprotein concentrations. In each group, the correlations were very weak, with the exception of those dogs with idiopathic hyperlipidaemia, in which the following relationships were noted: plasma cholesterol and alkaline phosphatase activity ($r=0.46$, $p<0.05$), AST activity ($r=0.64$, $p<0.01$) and ALT activity ($r=0.52$, $p<0.05$); LDL-C and AST activity ($r=0.70$, $p<0.01$) and ALT activity ($r=0.54$, $p<0.05$); plasma triglyceride and alkaline phosphatase ($r=0.56$, $p<0.05$) and AST activity ($r=0.79$, $p<0.001$), and plasma VLDL-C and AST activity ($r=0.71$, $p<0.01$) and ALT activity ($r=0.58$, $p<0.01$). In addition, a negative relationship ($r=-0.49$, $p<0.05$) was observed between plasma HDL-C and AST activity.

The increases in hepatic enzyme activities in diabetes mellitus, hyperadrenocorticism and hepatic disease reflect the underlying pathology in these cases. There were only two cases of obstructive jaundice, a disease process in which one might expect the plasma cholesterol concentration to be related to the degree of cholestasis (judged by the alkaline phosphatase activity) and these were not included in this study. When considering hyperadrenocorticism it is important to note that the increase in liver enzyme activities is related to the induction of a specific isoenzyme of alkaline phosphatase and hepatocyte swelling secondary to glycogen accumulation. Under these circumstances a relationship between plasma lipid concentrations

and hepatic enzyme activities might not be expected. However, in diabetes mellitus, where the underlying pathology includes hepatic lipidosis, *i.e.*, hepatocellular triglyceride accumulation, it would seem likely that a relationship between plasma lipids and hepatocellular insult might exist. Such a relationship has not been demonstrated by this study, perhaps because of insufficient data. It is, for example possible that the effect of hepatocyte triglyceride accumulation on the activities of hepatic enzymes is disproportionate to the effect of insulin deficiency on the circulating plasma lipoproteins. It is perhaps more likely that the activities of alkaline phosphatase, ALT and AST in the plasma do not reflect the degree of lipid accumulation in the hepatocytes, particularly in dogs with other metabolic disturbances, *e.g.*, cardiovascular hypoxia.

The relationships described between plasma lipid and lipoprotein concentrations and hepatic enzyme activities may be artefactual, resulting from the small number of cases, or as a consequence of leakage of red cell constituents secondary to *in vitro* haemolysis, which is often noted in lipaemic samples. This latter hypothesis may explain the positive correlation between the plasma triglyceride concentration and VLDL-C concentration and the AST and ALT activities. If, however, these relationships are a consequence of physiological rather than technical influences, it is possible that increases in plasma triglyceride and VLDL are accompanied by hepatic triglyceride accumulation, resulting in swelling of the hepatocytes and release of AST and ALT.

3.11. Renal parameters and plasma lipoprotein concentrations in protein-losing nephropathy

Hyperlipidaemia is recognised as a component of the nephrotic syndrome in man (Keane and Kasiske 1990). A significant negative correlation has been identified between the total plasma cholesterol and both the plasma albumin

and the plasma oncotic pressure (Appel, Blum, Chien, Kunis and Appel 1985). In the present study, very weak negative correlations, which were not significant, were identified between the plasma triglyceride and cholesterol concentrations and and plasma albumin ($r=-0.26$ and $r=-0.16$, respectively) and between the plasma triglyceride and total protein concentrations ($r=-0.55$) in dogs with protein-losing nephropathy ($n=4$). The small number of cases in this study make it impossible to form any meaningful conclusions regarding the relationships between plasma lipoprotein and protein concentrations in the dog.

3.12. Haematological abnormalities

The haemoglobin, mean cell haemoglobin and mean cell haemoglobin concentrations were above the upper limit of the reference range in three cases with lipaemic plasma. Non-regenerative anaemia was noted in one obese dog, one dog with hypothyroidism and one case with hepatic dysfunction. Target cells were identified in blood smears from one dog with hyperadrenocorticism and one with hepatic disease.

Lipaemia of a sample may interfere with the determination of haemoglobin by spectrophotometric means, in a similar fashion to the interference with biochemical parameters (DeBowes 1987). The erroneously elevated haemoglobin concentration in lipaemic samples subsequently affects the mean corpuscular haemoglobin and the mean corpuscular haemoglobin concentration, as reported here.

Non-regenerative anaemia is a common haematological abnormality associated with hypothyroidism (Feldman and Nelson 1987) and hepatic disease in the dog (Center 1989) and the occurrence of this complication in dogs included in this study is therefore not surprising. Target cells (leptocytes)

Table 18. The mean ±sd alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in obese dogs and dogs with idiopathic and secondary hyperlipidaemia.

Disease Status	ALP IU/l	ALT IU/l	AST IU/l
Obesity (n=17)	246 ±324	42 ±57	23 ±10
Diabetes mellitus (n=7)	1649 ±2399	155 ±215	38 ±23
Hyperadrenocorticism (n=14)	1840 ±1792	165 ±162	34 ±17
Hypothyroidism (n=9)	142 ±154	37 ±8	52 ±81
Nephropathy (n=4)	240 ±180	40 ±10	65 ±35
Hepatic disease (n=6)	4143 ±3015	177 ±670	61 ±57
Idiopathic hyperlipidaemia (n=20)	404 ±405	52 ±31	32 ±26
Reference range	<230	<35	<35

have been recognised in portosytemic shunts and obstructive jaundice in the dog. Their occurrence in association with a deficiency of plasma LCAT activity in man (Barter, Hopkins and Rajaram 1987) suggests a role for excess accumulation of cell membrane cholesterol and lecithin in their formation. It is possible that the hepatic pathology present in the two cases in which target cells

were identified may have resulted in impaired LCAT function and abnormalities of the red cell membrane.

CHAPTER VII

THE INVESTIGATION AND MANAGEMENT OF HYPERLIPIDAEMIA IN THE DOG

1. INTRODUCTION

Secondary hyperlipidaemia is more common in dogs than idiopathic or primary hyperlipidaemia (Chapter VI 3.1.). Despite this, idiopathic hyperlipidaemia was identified in a significant number of animals in the survey described in Chapter VI. In an attempt to identify the underlying lipid disturbances in these cases, the quantification of plasma lipids and lipoproteins, the measurement of postheparin plasma lipase activities, the performance of intravenous fat tolerance tests and the assessment of lipoprotein mobility by electrophoresis has been undertaken.

Following the identification of hyperlipidaemia, it is then necessary to consider whether, and what therapeutic strategies should be implemented. In the dog, where atherosclerosis is rare, the need for such lipid-lowering therapy is not immediately obvious. However, the clinical findings associated with hyperlipidaemia (Chapter VI 3.3-3.12), including classical pancreatitis, abdominal and gastrointestinal signs, neurological defects and ocular abnormalities, suggest that lipid-lowering could be important in the management of idiopathic hyperlipidaemia. The first line of approach in any hyperlipidaemic case is to exclude the possibility of underlying disease processes, particularly, endocrine disease. In cases of secondary hyperlipidaemia the lipoprotein abnormalities resolve partially or completely after therapy for the underlying condition (Ford 1977), but in the case of idiopathic hyperlipidaemia specific therapy may be required. In man, initial therapy involves the introduction of a low fat diet, that is followed, where necessary, by additional lipid-lowering drugs.

The aim of this study was to characterise the lipoprotein abnormalities associated with idiopathic hyperlipidaemia in the dog and to evaluate the efficacy of therapeutic regimes.

2. MATERIALS AND METHODS

Idiopathic hyperlipidaemia was diagnosed in dogs with increased plasma cholesterol and/or triglyceride concentrations with no identifiable systemic disease process. Endocrine disease and organ dysfunction were excluded by the investigations desribed in Chapter IV 2.1.

The quantification of the plasma lipoproteins was performed by an ultacentrifugation/precipitation method (Chapter II 2.2.) and the effect of breed, age and gender on the lipoprotein concentrations were corrected for by the methods described in Chapter IV 2.5. Lipoprotein electrophoresis was performed to identify the presence of β-VLDL as described in Chapter II 2.3.

3. RESULTS AND DISCUSSION

3.1. The characterisation of idiopathic hyperlipidaemia

The mean $\pm$sd age of this group was 7.34 $\pm$ 3.2 years. These were 16 entire males, seven entire females and 12 neutered females. Nine dogs had increased plasma cholesterol and triglyceride concentrations while 26 had hypercholesterolaemia alone. The distributions of the cholesterol and triglyceride concentrations are shown in Figures 12 and 13. The plasma cholesterol and LDL-C concentrations (Table 19., Appendix 10) in the group were significantly greater ($p < 0.001$ and $p \leq 0.01$, respectively) than those of the control population.

Idiopathic hyperlipidaemia in the dog was accompanied by increased plasma concentrations of cholesterol, triglyceride, VLDL-C, LDL-C and HDL-C. Due to the marked heterogeneity of the group, only the increases in total cholesterol and LDL-cholesterol were significant. The plasma triglyceride concentration associated with hyperlipidaemia was biased by three dogs with hypertriglyceridaemia (cases 119993, 122074 and 120360). In these cases, the

plasma triglyceride, VLDL-C and plasma LDL-C concentrations were increased. These abnormalities may be the consequence of increased hepatic triglyceride synthesis or delayed clearance of both triglyceride-rich lipoproteins and LDL from the circulation.

Table 19. The plasma lipid and lipoprotein concentrations (mmol/l) in dogs with idiopathic hyperlipidaemia (n=35) and control dogs (n=33).

	Plasma concentration (mmol/)				
	Triglyceride	Cholesterol	VLDL-C	LDL-C	HDL-C
Control (n=33)	0.60 ±0.20	4.51 ±1.49	0.47 ±0.70	1.72 ±1.88	2.34 ±0.70
Idiopathic (n=35)	2.44 ±6.72	8.86** ±2.93	0.98 ±1.03	3.95* ±2.31	3.84 ±1.39

* p=0.01, **p<0.001.

3.2. β-VLDL and apolipoprotein E

Beta-VLDL was identified on the basis of its density and migration to the β position on agarose gel electrophoresis. This lipoprotein was detected in the plasma of one dog referred to G.U.V.S., a 10 year old male Whippet (Case 114522, Appendix 16) suffering from bilateral hind limb paresis and iliac thrombosis (Chapter VI 3.5.). On examination, the blood collected for routine haematological and biochemical evaluation was lipaemic and the dog was diagnosed as suffering from thyroid dysfunction on the basis of a thyroid hormone stimulation test, although the animal showed no clinical signs of hypothyroidism. The plasma cholesterol, triglyceride and VLDL-C and LDL-C

Figure 12. The distribution of plasma cholesterol concentrations of dogs with idiopathic hyperlipidaemia (n=35).

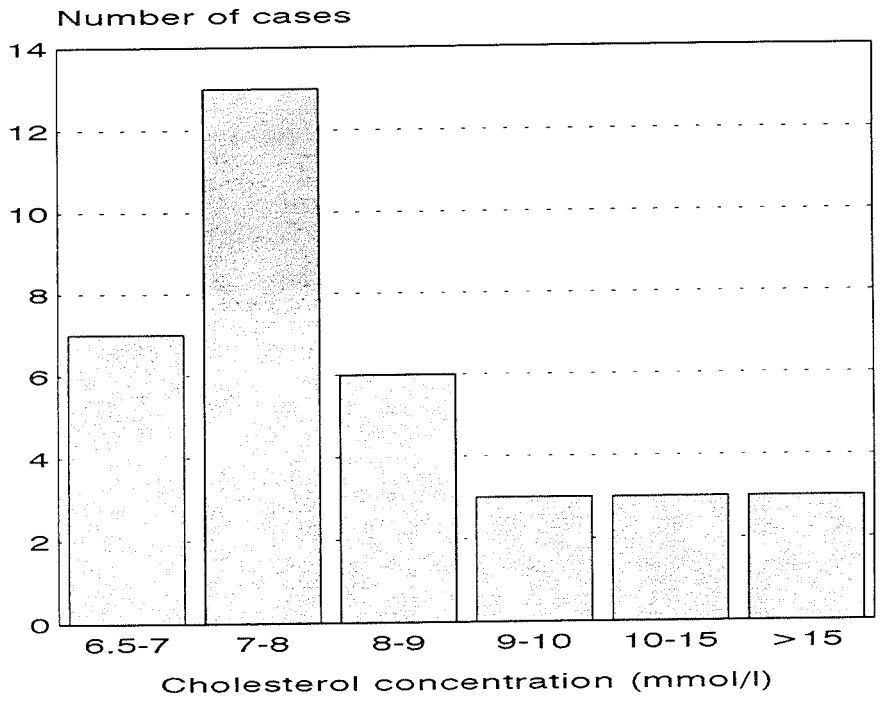
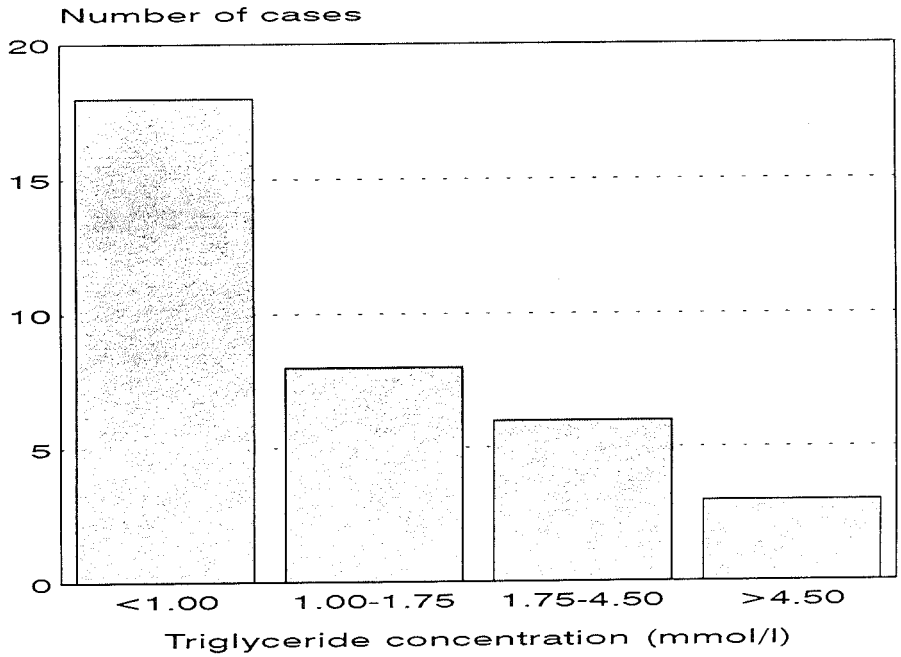


Figure 13. The distribution of the plasma triglyceride concentrations of dogs with idiopathic hyperlipidaemia (n=35).



concentrations were 47.80 mmol/l, 5.60 mmol/l, 22.31 mmol/l and 23.74mmol/l, respectively. The extremely high cholesterol content of the lipoproteins with density less than 1.006 g/ml is characteristic of β-VLDL. On agarose gel electrophoresis the lipoproteins of this density also displayed β-migration. The β -VLDL previously recognised in plasma from dogs fed high cholesterol diets possessed a normal apoE complement. However, an abnormality of apoE function in addition to endocrine disease may have resulted in the formation of β -VLDL in this pet dog which was not receiving a high fat diet and this is still under investigation.

3.3. The management of hyperlipidaemia in the dog

Ten cases of idiopathic hyperlipidaemia were selected for the evaluation of the efficacy of dietary management. Nine of the dogs presented with hypercholesterolaemia and six were hypertriglyceridaemic (Appendix 21). The mean ±sd plasma cholesterol and triglyceride concentrations after dietary intervention (8.74 ±4.18 mmol/l and 4.17 ± 7.15 mmol/l, respectively) were lower then those before introduction of the low fat diet (11.93 ±4.04 mmol/l and 5.89 ±5.59 mmol/l) (Appendix 18). The alterations in plasma cholesterol were significant ($p<0.01$), but those of triglyceride were not. Taken on an individual basis, the hyperlipidaemia resolved in three dogs. In six cases hypercholesterolaemia persisted and in four dogs the hypertriglyceridaemia did not resolve. On initial presentation three of these cases displayed gastrointestinal signs, while two additional dogs were considered at risk of developing pancreatitis secondary to hypertriglyceridaemia. Of these five cases, only two dogs retained the marked elevations of plasma triglyceride after dietary manipulation.

One dog (Case 119993) with marked increases of plasma cholesterol (16.48 mmol/l) and triglyceride (39.00 mmol/l) concentrations did not respond to dietary fat restriction and therapy with gemfibrizol (Lopid*, Parke-Davis; 300

mg daily) was introduced. This fibric acid derivative was selected because its main actions include the reduction of triglyceride concentrations by decreasing hepatic VLDL synthesis and increasing the activity of lipoprotein lipase (Tikkanen 1990). After six weeks of therapy there was no reduction of the cholesterol and triglyceride concentrations. At this point repeat biochemical analyses and urinalysis revealed a moderate hypoalbuminaemia secondary to proteinuria. Gemfibrizol therapy was discontinued. The toxicological studies on this drug indicate that it was well tolerated by dogs in the short term and in the long term it was tolerated at doses of 15 times the normal human dosage (15mg/kg) (Kurtz, Fitzgerald, Fisken, Schardein, Reutner and Lucas 1976). It is unlikely, therefore, that the protein-losing nephropathy was a consequence of drug therapy and more likely that the dog was suffering from a previously unrecognised renal disorder. At this point a home-formulated low fat, low protein diet was introduced and the plasma triglyceride and cholesterol concentration steadily declined over the following three weeks. Since then, the plasma cholesterol has remained moderately increased (7-9 mmol/l), but the triglyceride concentration has been consistently within normal limits (<1.75 mmol/l). At no time did the dog show signs of weight loss and the hypoalbuminaemia and proteinuria are steadily resolving. Long term remission and spontaneous recovery of canine nephropathies have been noted and the findings in this case are believed to be consistent with this observation, although the possibility that renal disease was drug-induced cannot be completely excluded.

In two other dogs it was necessary to consider means of reducing the plasma triglyceride concentration. One dog (Case 116313, Appendix 19) did not display fasting hypertriglyceridaemia, but suffered persistent corneal lipid deposition secondary to anterior segment disease. The owner was keen to maintain the fasting triglyceride concentrations at a minimum. The second dog, a 9 year-old male Cavalier King Charles spaniel (Case 120848, Appendix 19) was

suffering from idiopathic hyperlipidaemia with lethargy and intermittent anorexia. The hyperlipidaemia in this case not respond completely to dietary fat restriction.

Therapy with fish oils (omega-3 pc'yunsaturated fatty acids) has been advocated in human hypertriglyceridaemia (Phillipson, Rothrock, Connor, Harris and Illingworth 1985) where it is particularly effective in moderating postprandial lipaemia (Williams, Moore, Morgan and Wright 1992). The supplementation of fish oils is also associated with few side effects and may be used for the treatment of hypertriglyceridaemia in patients who cannot tolerate other therapies (Schaefer 1988). For these reasons an omega-3 polyunsaturated fatty acid supplement was administered to the two cases described above in an attempt to limit the plasma triglyceride concentrations. The fasting and postprandial triglyceride concentrations of both dogs were measured before and after the inclusion of an omega-3 polyunsaturated fatty acid supplement (Maxepa liquid, 15mg/kg/day; Duncan, Flockhart) in the diet. The results (Table 20.) indicate that fish oil supplementation in the dog is effective in the reduction of hypertriglyceridaemia, particularly postprandial hypertriglyceridaemia. It is possible that this therapy may play a role in modulating the hypertriglyceridaemia associated with some cases of idiopathic hyperlipidaemia and with familial hyperlipidaemia in the miniature schnauzer, thus reducing the predisposition to clinical manifestations, including pancreatitis.

Table 20. The fasting and postprandial triglyceride concentrations (mmol/l) in two dogs before and 3 weeks after dietary supplementation with omega-3 fatty acids.

	120848		116313	
	before	after	before	after
Fasting	2.13	1.47	0.62	0.38
Postprandial				
0.5 hr	2.09	1.40	0.58	0.44
1 hr	2.12	1.48	0.73	0.70
2 hr	2.96	1.78	0.71	0.77
3 hr	3.28	n.d.	n.d.	0.10

CHAPTER VIII

CONCLUSIONS AND FUTURE STRATEGIES

The aim of this thesis was to characterise the abnormalities of lipid metabolism recognised in dogs, to establish the effect of such aberrations on the health of individuals and to evaluate the efficacy of therapeutic regimes. The purpose of this chapter is to collate the significant findings of the study and to outline future avenues of investigation in the field of canine lipid metabolism.

1. THE ANALYSIS OF CANINE PLASMA LIPOPROTEINS

Methods of quantitative lipoprotein analysis have not previously been applied to the investigation of canine lipid abnormalities associated with naturally-occurring disease. Differences in the physical and chemical properties of the lipoproteins between humans and dogs necessitated techniques specific for canine plasma. The validation of an ultracentrifugation/precipitation method for the quantification of lipoprotein cholesterol concentrations was therefore central to the execution of this study. Attempts to identify an accurate, reproducible and inexpensive technique for use in veterinary laboratories without ultracentrifugation facilities were unsuccessful. However, the combination of the measurement of HDL-C by a precipitation procedure, followed by the estimation of the LDL cholesterol using the Friedewald formula may have potential. The development of a simple method for the measurement of HDL-C will allow both researchers and clinicians access to information which will help in understanding hyperlipidaemia in the canine population. Electrophoresis remains a useful adjunct in the investigation of lipid disorders, facilitating the recognition of chylomicrons, HDL_1 and β-VLDL which are not isolated by the combined precipitation/ultracentrifugation technique.

This thesis has demonstrated that canine plasma lipoprotein concentrations are influenced by gender and perhaps by dietary and/or other

environmental factors. This highlights the need for specific lipoprotein reference ranges that take these effects into account.

2. THE LIPID ABNORMALITIES ASSOCIATED WITH SYSTEMIC DISEASE

Quantitative lipoprotein analysis was used for the first time to characterise the disturbances of lipid metabolism associated with systemic disease. The results indicate that diabetes mellitus and hypothyroidism are associated with defects in the metabolism of both cholesterol-carrying and triglyceride-rich lipoproteins, while hyperadrenocorticism, extrahepatic bile duct obstruction and protein-losing nephropathy are accompanied solely by increases in plasma cholesterol and LDL concentrations. These observations provided clues to the origin of the underlying defects in lipoprotein metabolism. The identity of these defects was investigated as far as possible, given that the cases were client owned, by the measurement of lipoprotein and hepatic lipase activities, and the use of the intravenous fat tolerance test to assess plasma triglyceride clearance.

These methods demonstrated impaired triglyceride clearance associated with a decreased lipoprotein lipase activity in obese dogs and dogs with thyroid dysfunction. Furthermore, the plasma triglyceride clearance curves in obese dogs suggested that the hyperlipidaemia may be complicated by the poor regulation of hepatic triglyceride synthesis. These abnormalities of triglyceride metabolism may predispose obese dogs to acute pancreatitis. The restriction of caloric intake and subsequent weight reduction was shown to improve the abnormalities in lipid kinetics associated with obesity and have an important role in the prevention of acute pancreatic disease.

Further understanding of lipid abnormalities could only have been gained from measurement of other intravascular enzymes, namely, LCAT and the investigation of apoE and receptor function.

3. IDIOPATHIC HYPERLIPIDAEMIA

This study identified a number of dogs with defects of lipoprotein metabolism which cannot be attributed to underlying disease processes. Gastrointestinal clinical signs were associated with hypertriglyceridaemia and were observed to regress in parallel with the lipid abnormalities. Hyperlipidaemia *per se*, should therefore, be considered detrimental to the health of individual dogs.

The nature of the underlying defects in lipoprotein metabolism could not be established. It was unlikely that genetic variations/defects in the regulatory elements of the plasma lipoproteins e.g., lipoprotein lipase or receptor deficiencies, were solely responsible as the dogs were generally mid- to old-aged rather than puppies. However, it is possible that acquired or environmental factors such as age or diet, may have further compromised a lipoprotein system impaired by an inherited defect.

Dogs related to those presented with hyperlipidaemia were not available for study and therefore the investigation of familial trends in lipoprotein disturbances could not be pursued. However, the techniques validated in this study for the investigation of idiopathic and secondary hyperlipidaemia will allow a rational approach to the investigation of the proposed familial hyperlipidaemias of the miniature schnauzer and the Briard.

The measurement of the plasma lipoprotein cholesterol concentrations and lipoprotein lipase activity allows a rational approach to the therapy of idiopathic hyperlipidaemia which should include dietary fat restriction as the first measure. In cases that respond poorly to this action, additional therapy with omega-3 fatty acids may be considered. The data regarding supplementation with marine oils is still sparse but the results show potential and future work should be aimed at targeting suitable cases for therapy and closely monitoring the response of both fasting and postprandial lipaemia, as well as any side effects.

LIST OF APPENDICES

LIST OF APPENDICES

APPENDIX 1

Suppliers of reagents and equipment.

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

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

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

Ciba Laboratories,
Horsham, England.

Hoefer Scientific Instruments,
Scottish Biotechnology Instrumentation,
Blairgowrie Business Centre, 60 High Street, Blairgowrie, PH10 6AF.

Idexx Laboratories Inc.
1 Idexx Drive, Westbrook, Portland, Maine, U.S.A.

Immuno AG,
Immuno Ltd.,
Arctic House, Rye Lane, Dunton Green, Nr. Sevenoaks, Kent.

Joyce-Loebl,
Dukesway, Team Valley, Gateshead, Tyne & Wear, NE11 0PZ.

Kabi Pharmacia Ltd.,
Davy Avenue, Knowlhill, Milton Keynes, MK5 8PH, Bucks.,

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.

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.

APPENDIX 1

Suppliers of reagents and equipment (continued).

Randox Laboratories Ltd.,
Ardmore, Diamond Road, Crumlin, Co. Antrim BT29 4QY.

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

Scottish Quality Assessment Scheme
Biochemistry Department, Victoria Infirmary, Glasgow.

SCL Bioscience Services Ltd.,
211 Cambridge Science Park, Milton Road, Cambridge CB4 4ZA.

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

Technicon Instruments Corporation
Tarrytown, New York 10591, U.S.A.

U.K. External Quality Assessment Scheme,
Department of Clinical Chemistry,
Queen Elizabeth Medical Centre, Edgbaston, Birmingham.

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

APPENDIX 2

The derivation of the equation for the calculation of post-heparin plasma lipolytic activity.

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

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

[a] is μmol triolein/assay tube
[b] three fatty acids are released from one molecule of triolein
[c] to correct 10μl sample to 1ml
[d] volume of upper phase after extraction
[e] volume of lower phase after extraction
[f] incubation time in hours
[g] % oleic acid extracted into upper phase from Belfrage and Vaughan (1969)

APPENDIX 3

Performance characteristics of a method for the quantitative analysis of canine plasma lipoproteins.

Intra-assay (Within batch) performance

	Cholesterol concentration (mmol/l)			
Aliquot	Plasma	VLDL	LDL	HDL
Low cholesterol pool				
A	2.58	0.02	0.77	1.79
B	2.63	0.03	0.75	1.85
C	2.71	0.18	0.73	1.80
D	2.72	0.19	0.67	1.86
E	2.71	0.21	0.74	1.76
F	2.58	0.04	0.65	1.89
G	2.75	0.12	0.79	1.84
H	2.61	0.11	0.62	1.88
I	2.64	0.23	0.71	1.70
J	2.70	0.12	0.72	1.86
K	2.71	0.20	0.71	1.80
L	2.64	0.11	0.72	1.81
High cholesterol pool				
A	8.20	0.28	3.64	3.54
B	8.20	0.29	3.47	3.61
C	8.19	0.26	3.39	3.70
D	8.32	0.29	3.53	3.67
F	7.85	0.32	3.76	3.39
H	8.06	0.30	3.97	3.29

APPENDIX 4

The plasma and lipoprotein cholesterol concentrations after storage for two and eight weeks.

	Cholesterol concentration (mmol/l)				
Aliquot	Plasma	Infranatant	VLDL	LDL	HDL
Two weeks					
A	2.82	2.42	0.39	0.65	1.76
B	2.88	2.63	0.25	0.80	1.83
C	2.87	2.50	0.37	0.84	1.67
D	2.82	2.31	0.51	0.83	1.48
E	2.90	2.48	0.42	0.87	1.61
F	2.94	2.03	0.91	0.36	1.67
Eight weeks					
A	2.81	2.41	0.39	0.65	1.76
B	2.88	2.63	0.25	0.80	1.83
C	2.87	2.50	0.37	0.83	1.67
D	2.82	2.31	0.51	0.83	1.48
F	2.90	2.43	0.47	0.84	1.59
H	2.83	2.41	0.42	0.77	1.64

appendix 5

The calculated and measured VLDL-C concentration in 28 samples of canine plasma.

Calculated VLDL-C (mmol/l)	Measured VLDL-C (mmol/l)
3.28	2.15
0.63	0.04
0.47	0.02
1.54	0.93
18.64	20.56
20.26	23.58
1.02	0.28
3.27	2.37
0.76	0.04
0.99	0.05
0.64	0.10
1.85	0.68
0.79	0.02
2.71	2.26
0.40	0.17
0.54	0.10
1.28	0.91
0.65	0.13
2.02	0.93
1.33	0.75
0.62	0.03
0.52	0.13
1.22	0.97
0.54	1.22
1.10	0.94
5.89	6.20
2.17	2.61

APPENDIX 6

HDL-C concentrations measured by a combined ultracentrifugation/precipitation technique and commercial precipitation reagents.

HDL-C (mmol/l)	
Method A	Method B
3.54	3.66
2.97	3.00
4.51	4.59
4.13	3.60
1.90	1.11
4.20*	1.26
3.53	3.87
5.97	4.35
5.55	3.06
2.29*	6.06
1.61	0.96
5.26	5.70
2.60	1.11

HDL-C (mmol/l)	
Method A	Method C
3.54	3.69
2.97	3.06
4.51	4.59
4.13	5.79
1.90	1.14
4.20*	0.81
3.53	3.45
5.43	5.28
5.55	3.72
1.61	1.80
5.26	4.89
2.60	2.46
5.71	4.56

Method A: Ultracentrifugation/precipitation technique.
Method B: HDL-cholesterol precipitant (Randox Laboratories).
Method C: Quantolip HDL-cholesterol precipitation reagent (Immuno Ltd.).

* plasma triglyceride > 5.00mmol/l.

APPENDIX 7

LDL-C concentrations measured by a combined ultracentrifugation/precipitation technique and commercial precipitation reagents.

LDL-C (mmol/l)		LDL-C (mmol/l)	
Method A	Method B	Method A	Method C
2.82	2.06	2.82	1.84
4.02	2.84	4.02	2.07
18.63	18.51	23.65*	6.22
23.65*	41.09	1.47	3.33
1.47	2.72	1.14	1.54
1.14	1.43	5.71	1.88
5.71	2.21	1.59	1.57
1.59	1.57	3.86	2.29
10.23*	3.94	10.23*	9.00
1.44	2.96	1.62	1.83
1.62	1.72	7.59	2.10
7.59	2.10	2.46	2.15
2.46	2.04	3.22	3.17

Method A: Ultracentrifugation/precipitation technique.
Method B: LDL-cholesterol precipitant (Randox Laboratories).
Method C: Quantolip LDL-cholesterol precipitation reagent (Immuno Ltd.).

* plasma triglyceride > 5.00mmol/l.

APPENDIX 7

LDL-C concentrations measured by a combined ultracentrifugation/precipitation technique and commercial precipitation reagents (continued).

LDL-C (mmol/l)	
Method A	Method D
2.82	0.38
4.02	1.28
1.47	0.80
1.14	0.73
5.71	0.59
1.59	0.51
1.99	1.16
1.62	1.08
7.59	0.72
2.46	2.08
3.22	0.96
0.85	0.29

Method A: Ultracentrifugation/precipitation technique.

Method D: LDL-cholesterol precipitation reagent (Boehringer Mannheim GmbH Diagnositics).

APPENDIX 8

The performance characteristics of the precipitation reagents for the measurement of HDL and LDL cholesterol concentrations.

HDL-C (mmol/l)		LDL-C (mmol/l)		
Method A	Method B	Method C	Method D	Method E
5.10	5.28	2.09	0.30	1.54
5.25	5.52	1.65	0.09	1.76
5.13	5.31	1.65	0.18	2.09
5.25	5.31	1.43	0.12	1.76
5.13	5.07	1.98	1.40	1.54
5.07	5.25	1.32	1.29	1.54
5.28	5.22	1.54	1.16	1.76
5.10	5.13	1.54	2.06	1.43

Method A: HDL-cholesterol precipitant (Randox Laboratories).
Method B: Quantolip HDL-cholesterol precipitation reagent (Immuno Ltd.).
Method C: LDL-cholesterol precipitant (Randox Laboratories).
Method D: LDL-cholesterol precipitation reagent (Boehringer Mannheim GmbH).
Method E: Quantolip LDL-cholesterol precipitation reagent (Immuno Ltd.).

APPENDIX 9

The LDL-cholesterol concentration measured by a combined ultracentrifugation/precipitation technique and calculated by the Friedewald formula in 81 canine plasma samples.

Plasma triglyceride (mmol/l)	Plasma cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	Friedewald LDL-C (mmol/l)
0.95	11.36	2.77	8.13	8.16
0.66	6.36	2.68	3.03	3.38
0.81	10.28	4.39	5.05	5.52
2.34	9.35	4.91	4.24	3.38
1.44	7.93	4.46	2.92	2.82
1.66	8.25	3.79	3.36	3.71
0.82	9.23	2.58	5.95	6.28
2.16	11.73	2.43	7.37	8.32
0.96	7.19	4.43	2.06	2.32
0.49	6.17	2.40	2.85	3.55
1.15	9.08	6.36	1.44	2.20
5.92	13.76	5.19	5.86	5.88
0.98	9.10	3.31	5.27	5.34
0.55	3.27	1.89	0.61	1.13
0.95	4.47	1.92	2.08	2.12
0.43	6.32	2.71	3.52	3.41
1.73	7.58	3.85	2.96	2.99
0.57	5.86	1.03	3.99	4.57
1.42	6.65	4.36	1.45	1.64
0.40	5.82	1.60	3.59	4.04
0.46	7.82	1.32	5.76	6.29
1.35	7.59	1.02	6.11	5.96
0.65	7.57	2.13	4.55	8.14
0.90	6.55	3.46	2.55	2.68
0.41	6.01	3.25	2.59	2.57

APPENDIX 9

The LDL-cholesterol concentration measured by a combined ultracentrifugation/precipitation technique and calculated by the Friedewald formula in 81 canine plasma samples (continued).

Plasma triglyceride (mmol/l)	Plasma cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	Friedewald LDL-C (mmol/l)
0.34	5.34	3.69	1.00	1.50
1.66	5.89	3.66	1.62	1.48
0.83	6.67	4.41	1.72	1.88
0.90	7.29	2.43	4.76	4.48
0.56	5.60	3.27	1.56	1.59
2.46	11.59	5.73	4.84	4.74
0.52	7.00	4.18	2.03	2.58
5.57	25.57	2.73	16.95	20.31
3.70	37.85	2.15	29.43	29.47
4.84	18.04	3.75	11.84	12.09
1.41	11.53	2.30	8.60	8.59
1.06	11.90	3.88	6.86	7.54
2.81	17.67	1.65	13.85	14.74
0.69	8.72	3.70	4.65	4.71
0.47	6.89	2.46	3.66	4.22
1.59	3.87	0.78	2.81	1.50
0.42	6.97	2.86	3.62	3.43
0.70	6.61	3.40	2.18	2.89
0.82	15.02	2.73	11.50	11.92
0.28	12.43	4.20	7.85	8.10
0.72	10.55	4.78	5.30	5.44
1.42	12.70	5.36	6.76	6.69
0.68	9.69	5.31	3.43	4.07
1.25	10.10	3.26	6.44	6.28
1.57	8.76	4.73	3.63	3.32
1.13	6.74	5.14	1.06	1.09
1.97	6.30	3.02	1.59	2.40

APPENDIX 9

Plasma triglyceride (mmol/l)	Plasma cholesterol (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	Friedewald LDL-C (mmol/l)
0.53	8.12	4.46	3.27	3.42
0.60	7.91	3.73	2.97	3.91
1.08	8.98	1.37	6.31	6.72
0.40	4.20	1.83	2.08	2.19
0.59	5.35	3.44	1.45	1.64
0.85	5.83	3.69	1.57	1.74
1.01	5.60	3.14	1.80	2.00
0.56	3.99	2.47	1.10	1.27
1.30	6.48	2.46	3.55	3.43
0.50	3.20	2.19	0.42	0.78
0.74	4.91	1.77	2.38	2.81
0.63	4.34	1.95	2.34	2.10
0.93	6.05	2.71	2.83	2.92
0.68	3.51	1.24	1.82	1.96
0.57	4.81	2.63	1.97	1.92
0.54	3.99	1.87	1.55	1.87
0.56	4.24	1.90	1.55	2.09
0.44	2.95	1.98	0.72	0.77
0.49	0.49	1.98	0.62	0.77
0.49	0.49	2.12	0.73	0.63
0.46	0.46	2.13	1.33	1.22
0.45	0.45	1.99	0.94	0.98
0.44	0.44	1.88	0.62	0.57
0.47	0.47	2.16	0.48	0.55
1.69	14.62	3.90	10.08	9.95
4.86	9.80	3.62	4.33	3.26
1.40	5.37	2.49	1.69	2.24
1.59	8.51	2.78	5.57	5.01
1.30	9.01	2.95	5.63	5.47

APPENDIX 10

The lipoprotein cholesterol concentration measured by an ultracentrifugation/precipitation technique and the relative distribution of plasma lipoproteins on agarose gel electrophoresis for 14 samples of canine plasma.

Cholesterol concentration (mmol/l)				electrophoretic distribution (%)		
Plasma	VLDL	LDL	HDL	pre-beta	beta	alpha
3.52	0.34	0.53	2.65	3.32	17.79	72.79
8.32	0.96	1.43	5.93	-	14.53	83.53
7.21	0.25	1.51	5.45	-	25.01	66.25
3.65	0.48	0.50	2.67	-	21.92	75.61
2.95	0.45	0.24	2.26	-	14.96	81.80
6.03	0.25	0.95	4.83	-	8.05	90.44
7.50	0.85	1.74	4.91	-	20.39	78.40
15.00	7.72	2.31	4.97	35.44	23.72	39.14
4.73	0.15	1.14	3.28	-	40.76	36.54
2.72	0.41	0.56	1.75	-	11.54	78.73
4.20	0.47	0.87	2.86	-	12.58	86.64
9.92	1.34	3.75	4.83	-	36.30	41.35
4.00	0.60	0.43	2.97	-	29.85	70.15
4.83	0.63	1.51	2.67	-	20.86	84.52

APPENDIX 11

Plasma cholesterol and lipoprotein cholesterol concentrations in control, obese and diseased dogs.

	Cholesterol concentration (mmol/l)		
Plasma	VLDL	LDL	HDL
Control			
4.20	0.29	2.08	1.83
5.35	0.46	1.45	3.44
5.83	0.57	1.57	3.69
5.60	0.60	1.80	3.14
3.99	0.42	1.10	2.47
6.48	0.47	3.55	2.46
3.10	1.00	0.69	1.31
3.20	0.60	0.42	2.19
4.91	0.76	2.38	1.77
4.34	0.06	2.34	1.95
6.05	0.51	2.83	2.71
3.51	0.45	1.82	1.24
4.81	0.21	1.97	2.63
6.72	1.59	3.43	1.70
7.00	1.17	3.78	2.05
3.99	0.57	1.55	1.87
4.24	0.79	1.55	1.90
4.93	0.38	1.21	3.34
3.77	0.18	1.25	2.34
4.92	0.12	2.60	2.20
2.95	0.25	0.72	1.98
2.97	0.39	0.62	1.98
2.97	0.05	0.73	2.12
2.93	0.14	0.60	2.18
3.56	0.08	1.33	2.13

APPENDIX 11

Plasma cholesterol and lipoprotein cholesterol concentrations in control, obese and diseased dogs (continued).

Plasma	Cholesterol concentration (mmol/l) VLDL	LDL	HDL
Control			
2.65	0.15	0.62	1.88
2.92	0.27	0.48	2.16
4.73	0.31	1.14	3.28
7.00	0.79	2.03	4.18
4.83	0.63	1.51	2.69
4.24	0.79	1.55	1.90
7.57	0.89	4.55	2.13
6.01	0.96	2.44	2.61
Obesity			
3.27	0.77	0.61	1.89
4.47	0.47	2.08	1.92
6.32	0.09	3.52	2.71
7.58	0.82	2.03	4.70
5.86	0.84	3.99	1.03
6.65	0.84	1.45	4.36
7.72	1.44	1.62	4.66
5.74	0.50	1.41	3.83
5.44	0.52	1.24	3.68
7.23	1.10	1.94	4.17
4.20	0.47	0.87	2.86
3.35	0.41	0.78	2.16
5.83	0.69	2.02	3.12
7.50	0.85	1.74	4.91
4.50	0.76	0.52	3.23

APPENDIX 11

Plasma cholesterol and lipoprotein cholesterol concentrations in control, obese and diseased dogs (continued).

Plasma	Cholesterol concentration (mmol/l) VLDL	LDL	HDL
Obesity			
9.96	1.20	5.02	3.74
9.49	0.36	2.91	6.22
5.84	0.66	2.10	3.08
4.81	0.50	0.98	3.33
5.28	0.68	0.73	3.87
Diabetes mellitus			
8.76	0.40	3.63	4.73
6.74	0.54	1.06	5.14
6.30	1.68	1.59	3.02
14.62	0.64	10.08	3.90
9.80	1.85	3.62	4.33
17.77	8.30	7.32	2.08
5.37	1.19	1.69	2.49
9.15	0.50	4.52	4.13
9.61	1.01	3.83	4.77
7.01	2.01	1.47	3.53
7.23	0.64	1.55	5.04
Hyperadrenocorticism			
5.70	0.44	2.66	2.60
11.36	0.46	8.13	2.77
6.36	0.29	3.03	2.68
10.28	0.64	5.25	4.39
9.35	0.20	4.24	4.91
7.93	0.55	2.92	4.46
8.25	1.00	3.36	3.79
9.23	0.70	5.95	2.58

APPENDIX 11

Plasma cholesterol and lipoprotein cholesterol concentrations in control, obese and diseased dogs (continued).

Cholesterol concentration (mmol/l)			
Plasma	VLDL	LDL	HDL
Hyperadrenocorticism			
11.73	1.93	7.37	2.43
7.19	0.70	2.06	4.43
6.17	0.92	2.85	2.40
9.18	1.28	1.44	6.36
13.76	2.71	5.86	5.19
7.62	0.33	2.82	4.47
Hypothyroidism			
25.57	5.89	16.95	2.73
6.71	0.43	3.07	3.21
37.85	6.30	29.43	2.15
18.04	1.82	11.84	3.75
7.86	0.18	4.24	3.44
10.93	0.63	8.60	2.03
11.90	1.16	6.86	3.88
14.41	0.92	9.60	3.89
15.23	0.89	9.69	4.65
11.47	1.16	5.13	4.18
Protein-losing nephropathy			
9.69	0.95	3.43	5.31
12.43	0.38	7.85	4.20
10.5	0.47	1.45	4.36
10.10	0.40	6.44	3.26

APPENDIX 11

Plasma cholesterol and lipoprotein cholesterol concentrations in control, obese and diseased dogs (continued).

	Cholesterol concentration (mmol/l)		
Plasma	VLDL	LDL	HDL
Hepatic disease			
8.71	1.16	2.30	5.25
8.72	0.37	4.65	3.70
6.89	0.28	3.66	3.14
3.87	0.28	2.81	0.78
6.97	0.49	3.62	2.86
3.59	0.36	1.62	1.61
Obstructive Jaundice			
15.02	0.79	11.50	2.73
21.42	0.89	18.63	1.90
Idiopathic Hyperlipidaemia			
7.82	0.74	5.76	1.32
7.59	0.46	6.11	1.02
7.57	1.59	4.55	2.13
6.55	0.54	2.55	3.46
6.67	0.54	1.72	4.41
7.29	0.10	7.37	2.43
11.59	1.02	4.84	5.73
7.00	0.79	2.03	4.18
10.82	1.70	3.20	5.92
9.96	1.20	5.02	3.74
9.10	0.52	5.27	3.31
7.71	1.61	3.61	2.82
7.23	1.10	1.93	4.17

APPENDIX 11

Plasma cholesterol and lipoprotein cholesterol concentrations in control, obese and diseased dogs (continued).

Plasma	Cholesterol concentration (mmol/l) VLDL	LDL	HDL
Idiopathic hyperlipidaemia			
7.00	0.09	3.78	2.05
7.22	0.17	2.97	3.76
6.70	0.82	2.20	3.68
6.93	0.70	3.52	2.71
7.72	1.44	1.62	4.66
6.67	0.54	1.72	4.41
18.02	5.32	10.38	1.62
6.72	0.02	2.10	3.20
6.65	0.84	1.45	4.36
7.58	0.14	2.96	3.80
8.81	0.86	3.79	4.16
8.81	1.31	4.51	2.99
7.50	0.85	1.74	4.91
8.56	0.64	4.58	3.34
9.49	0.36	2.90	6.22
8.15	0.62	2.24	5.29
8.49	0.99	2.11	5.39
14.88	1.18	7.11	6.59
16.48	3.96	10.23	2.29
7.58	0.68	2.50	4.40
8.08	0.42	2.66	5.00
15.11	7.07	7.07	5.04

APPENDIX 12

The plasma lipid and lipoprotein cholesterol concentrations of 13 obese dogs on initial presentation.

Case	Plasma triglyceride (mmol/l)	Cholesterol concentration (mmol/l)			
		Plasma	VLDL	LDL	HDL
1	0.81	5.44	0.52	1.24	3.68
2	1.15	4.50	0.76	0.52	3.23
3	1.93	7.23	1.10	1.96	4.17
4	1.52	9.96	1.20	5.02	3.74
5	0.54	4.20	0.47	0.87	2.86
6	0.74	5.74	0.50	1.41	3.83
7	1.10	5.83	0.69	2.02	3.12
8	1.20	5.28	0.68	0.73	3.87
9	1.00	3.35	0.41	0.78	2.16
10	0.41	5.84	0.18	2.58	3.08
11	0.56	9.49	0.36	2.91	6.22
12	0.73	4.81	0.50	0.98	3.33
13	1.16	7.50	0.85	1.74	4.91

APPENDIX 12

The plasma lipid and lipoprotein cholesterol concentrations of 12 obese dogs after calorie restriction for six weeks.

Case	Plasma triglyceride (mmol/l)	Cholesterol concentration (mmol/l)			
		Plasma	VLDL	LDL	HDL
1	0.72	5.32	0.38	1.40	3.54
2	0.77	4.92	0.62	0.40	3.90
3	0.60	5.26	0.56	0.66	4.04
4	0.88	7.55	0.71	1.87	4.97
5	0.59	5.49	0.73	1.14	3.62
6	0.69	5.24	0.39	1.24	3.41
7	1.15	3.24	0.87	1.20	3.17
8	0.63	5.57	0.28	1.60	3.69
9	1.18	3.94	0.66	0.56	2.72
10	0.52	5.76	0.23	1.43	4.10
11	0.55	7.46	0.25	2.12	5.09
12	1.50	4.46	0.45	0.85	3.16

The plasma lipid and lipoprotein cholesterol concentrations of six adult beagle dogs.

Case	Plasma triglyceride (mmol/l)	Cholesterol concentration (mmol/l)			
		Plasma	VLDL	LDL	HDL
1915	0.44	7.21	0.25	1.51	5.45
2108	0.42	3.19	0.16	0.43	2.60
2132	0.68	3.52	0.34	0.53	2.65
9022	n.d.	6.03	0.25	0.95	4.83
3004	0.60	3.65	0.48	0.50	2.67
2519	0.51	2.95	0.45	0.24	2.26

APPENDIX 13

The body measurements and weight reduction performance of 13 obese dogs.

Case	Weight (kg)	% over ideal	% weight reduction*	Circumference (cm)		
				Neck	Girth	Stifle
1	42	24	63	49	90	37
2	35	28	50	42	85	36
3	23	18	-	40	73	-
4	71	42	24	69	105	52
5	38	23	7	49	88	44
6	76	15	-	63	99	49
7	38	27	0	50	87	38
8	28	16	78	56	82	34
9	13	33	17	36	64	26
10	11	33	67	31	57	28
11	12	46	46	31	63	24
12	17	31	58	38	64	28
13	22	22	-	40	71	34

* weight loss expressed as a percentage of the target weight loss.

APPENDIX 14

The postheparin plasma lipolytic, lipoprotein lipase and hepatic lipase activities in 13 obese dogs and six beagle dogs.

Case	Lipolytic activity (μmolFA/ml/hr) PHP	LPL	HL
1	6.83	1.41	5.42
2	8.50	1.80	6.70
3	12.39	9.11	3.28
4	7.50	1.12	6.38
5	8.64	4.84	3.80
6	3.05	2.09	0.96
7	7.05	4.93	2.12
8	2.86	0.97	1.89
9	7.27	1.21	6.06
10	17.10	9.30	7.80
11	28.14	17.24	10.90
12	9.96	6.25	3.71
13	10.74	6.21	4.53
1915	16.20	7.00	9.20
2108	15.80	8.10	7.70
9022	19.10	12.30	6.80
2132	19.00	12.50	6.50
2519	17.20	10.80	6.40
2809	18.60	13.70	4.90

PHP: Postheparin plasma.
LPL: Lipoprotein lipase.
HL: Hepatic lipase.

APPENDIX 15

The postheparin plasma lipolytic, lipoprotein lipase and hepatic lipase activities in 8 obese dogs after calorie restriction for six weeks.

Case	Lipolytic activity (μmolFA/ml/hr) PHP	LPL	HL
1	13.80	5.70	8.10
2	6.35	1.22	5.13
4	6.92	2.17	4.75
5	7.46	2.84	4.62
8	5.72	1.34	4.38
10	19.60	5.90	13.70
11	10.43	6.21	4.22
12	16.40	7.60	8.80

PHP: Postheparin plasma.
LPL: Lipoprotein lipase.
HL: Hepatic lipase.

APPENDIX 16

Plasma triglyceride concentration at serial time points after the intravenous administration of Intralipid* to 8 obese dogs before and after calorie restriction.

	Time (minutes)							
Case	0	5	10	20	30	40	90	120
Before								
1	0.33	1.42	1.43	1.35	0.98	1.15	n.d.	0.14
2	0.56	2.48	1.71	1.38	1.49	n.d.	n.d.	0.88
4	1.93	2.26	2.31	2.80	2.46	2.34	n.d.	1.96
5	0.11	1.57	1.64	1.55	1.08	1.04	0.36	0.34
7	0.83	2.17	1.71	1.77	1.53	n.d.	1.23	1.00
8	0.17	1.08	0.84	0.84	0.49	0.41	0.28	0.18
9	0.26	1.80	1.80	1.74	1.26	1.06	0.38	0.24
12	0.27	1.16	0.93	0.71	0.44	0.31	0.28	0.23
After								
1	0.24	1.34	1.25	1.08	0.94	0.69	0.23	0.23
2	0.18	0.92	0.82	n.d.	0.64	0.61	0.33	0.24
4	0.69	1.83	1.73	1.45	1.43	1.24	0.72	0.74
5	0.43	1.51	1.39	1.34	1.41	1.18	0.72	0.56
7	0.31	1.41	1.11	0.90	0.74	0.64	0.41	0.45
8	0.23	0.94	0.59	0.28	0.13	0.16	0.23	0.20
9	0.85	2.01	2.07	1.64	1.52	1.70	1.24	1.14
12	0.41	1.17	0.91	0.94	0.85	0.72	0.25	0.18

APPENDIX 16

Plasma triglyceride concentration at serial time points after the intravenous administration of Intralipid* to six beagle dogs.

	Time (minutes)							
Case	0	5	10	15	30	45	90	120
2519	0.16	1.98	1.21	0.78	0.20	0.21	0.16	0.18
1915	0.25	2.03	1.44	0.82	0.29	0.23	0.22	0.23
2108	0.14	1.11	0.55	0.29	0.15	0.11	0.11	0.10
3004	0.19	1.49	0.97	0.60	0.27	0.21	0.13	0.15
2132	0.15	0.91	0.41	0.27	0.14	0.13	0.12	0.11
9023	0.29	1.74	0.94	0.58	0.29	0.28	0.29	0.29

The r^2 value, AUC, AUMC and MRT for the triglyceride clearance curves of six beagle dogs.

Case	r^2	AUC (mmol.min/l)	AUMC (mmol.min^2/l)	MRT (min)
2519	0.91	27.63	281.7	10.19
1915	0.85	33.44	267.6	8.00
2108	1.00	14.68	101.9	6.94
3004	0.74	24.07	207.8	8.63
2132	0.99	10.60	69.3	6.53
9023	1.00	21.14	147.4	6.97

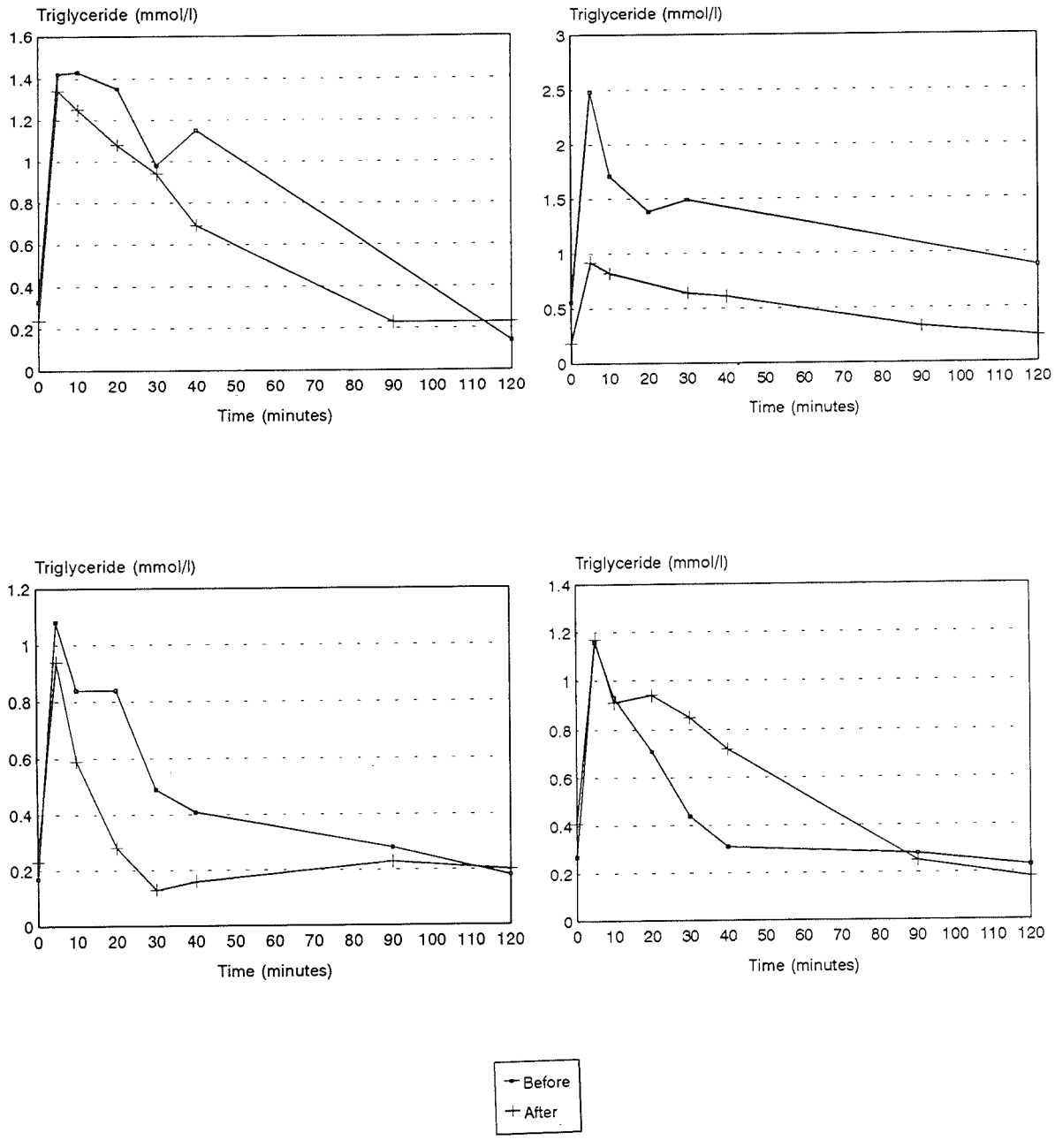
APPENDIX 16

The r^2 value, AUC, AUMC and MRT for the triglyceride clearance curves of eight obese dogs before and after calorie restriction.

Case	r^2	AUC (mmol.min/l)	AUMC (mmol.min^2/l)	MRT (min)
Before				
1	0.68	104.10	2376.1	22.83
2	0.68	115.80	7279.0	62.86
4	0.02	41.26	1441.1	34.93
5	0.95	101.09	5902.0	58.38
7	0.89	82.04	3728.5	45.45
8	0.95	34.24	1048.6	30.63
9	0.81	80.74	2369.8	29.35
12	0.91	19.52	311.13	15.94
After				
1	0.96	44.98	1487.5	33.07
2	0.91	42.70	1838.4	43.05
4	0.62	51.24	1299.5	25.36
5	0.88	78.07	3549.9	45.47
7	0.95	40.45	1268.8	31.37
8	0.97	11.55	79.2	6.86
9	0.88	102.25	6920.3	67.68
12	0.80	35.64	985.9	27.66

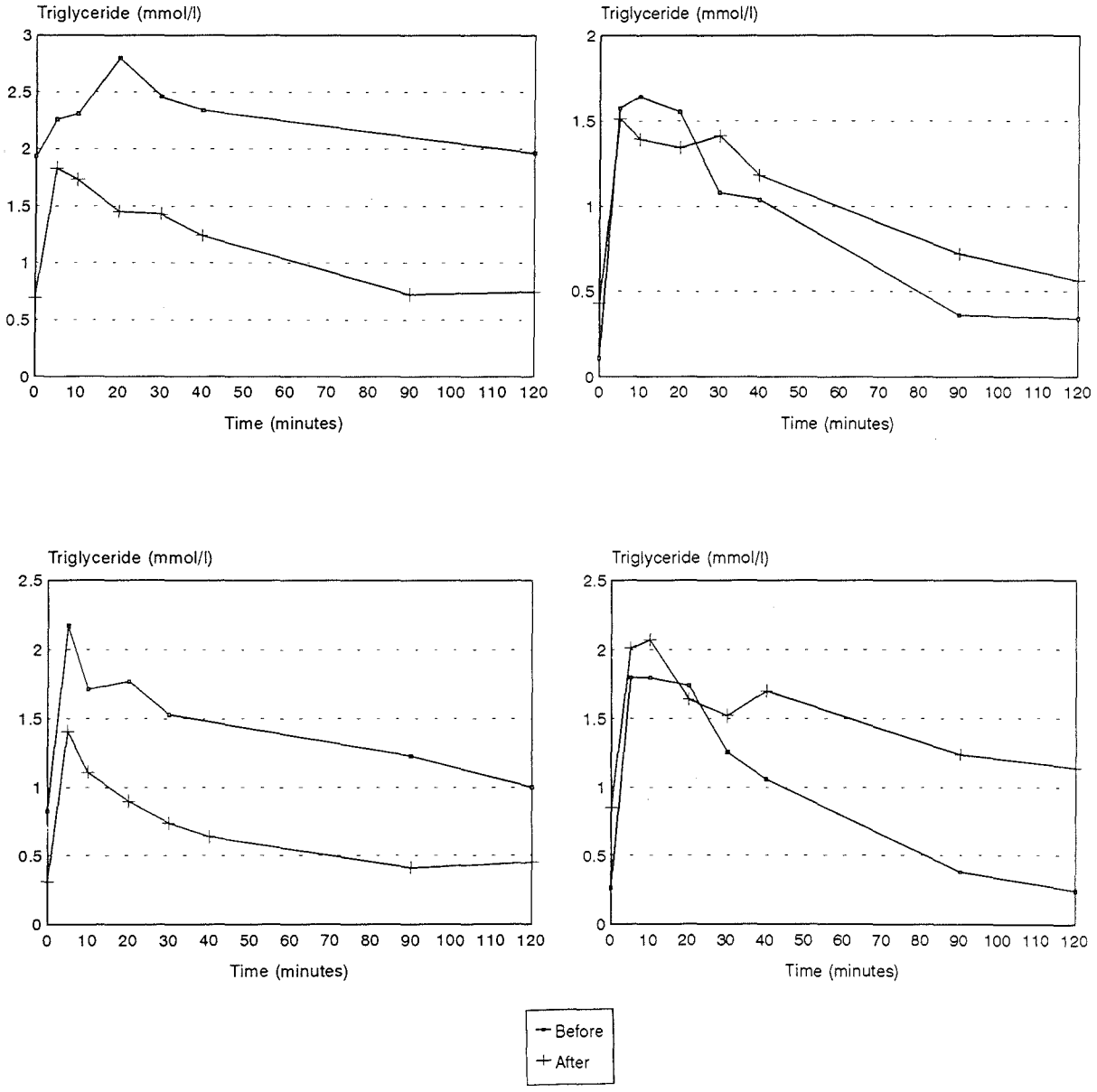
APPENDIX 16

The intravenous fat tolerance test curves before and after calorie restriction in four obese dogs which lost weight.



APPENDIX 16

The intravenous fat tolerance test curves before and after calorie restriction in four obese dogs which lost minimal quantities of weight.



APPENDIX 17

Pharmacokinetic analysis and terms.

The Fortran IV curve stripping programme generates up to 3 y-axis intercepts (A1, A2 and A3) and exponents (B1, B2 and B3) (first to last) for the equations best describing the data. The area under the plasma concentration versus time curve for observed values (AUC_{obs}), in mmol.min/l, from time 0 to infinity was determined for observed concentrations using the trapezoidal rule. The area of the triangle between time 0 and the first sample was calculated by taking the average of the Cp0 and the first observed concentration multiplied by the first time point, and the area of the triangle between the last sample and 0 concentration was calculated by dividing the final concentration with the terminal slope, B1. The area under the first moment curve for observed values ($AUMC_{obs}$) from time 0 to infinity, *i.e.* the plasma concentration time versus time curve in $mmol.min^2/l$, and the area between time 0 and the first sample were calculated in a similar fashion. The area (AUMC) of the triangle between the last sample and concentration 0 was calculated using the product of time and the last plasma concentration divided by B1.

AUC	Total area under the triglyceride concentration versus time or zero moment curve (mmol.min/l) from time 0 to time infinity calculated using the trapezoidal rule.
AUMC	Total area under the triglyceride concentration time versus time or first moment curve ($mmol.min^2/l$) from time 0 to time infinity calculated using the trapezoidal rule.
Cmax	Maximum plasma triglyceride concentration following Intralipid* administration.
Cp0	Initial concentration of triglyceride in plasma following administration of an intravenous bolus of Intralipid*.

APPENDIX 18

Postheparin plasma lipolytic, lipoprotein lipase and hepatic lipase activity in dogs with diabetes mellitus and hypothyroidism.

Case	Lipolytic activity (μmolFA/ml/hr) PHP	LPL	HL
Hypothyroidism			
1	6.49	2.47	4.02
2	4.59	2.92	1.67
3	4.64	3.09	1.55
4	3.78	1.78	2.00
5	6.30	5.73	0.57
Hyothyroidisim (after therapy)			
1	15.60	5.90	13.70
4	11.20	4.42	6.78
Diabetes mellitus			
6	29.40	18.50	10.90
7	16.43	8.47	7.96
8	22.96	12.75	10.21
9	17.60	6.41	11.19
10	19.08	5.43	13.65
11	17.37	n.d.	n.d.
12	13.34	n.d.	n.d.
13	26.86	n.d.	n.d.
14	20.44	n.d.	n.d.
15	40.46	n.d.	n.d.

PHP: Postheparin plasma.
LPL: Lipoprotein lipase.
HL: Hepatic lipase.

APPENDIX 19

Plasma lipid and lipoprotein concentrations in 16 dogs.

Case 112714

Tibetan terrier, 5.5 years, female (N), diabetes mellitus and hypothyroidism.

Biochemical parameters:

Cholesterol	17.51	mmo/l
Trigyceride	14.15	"
VLDL-C	8.41	"
LDL-C	6.97	"
HDL-C	2.13	"
Amylase	5294	IU/l

Case 122074

Golden retriever, 12 years, female (N).

Biochemical parameters:

Cholesterol	14.88	mmo/l
Triglyceride	4.37	"
VLDL-C	1.18	"
LDL-C	7.11	"
HDL-C	6.59	"
Amylase	> 3000	IU/l
Lipase	> 1000	IU/l

Case 119993

X-bred, 7 years, male(N).

Biochemical parameters:

Cholesterol	16.48	mmo/l
Triglyceride	39.00	"
VLDL-C	3.96	"
LDL-C	10.23	"
HDL-C	2.29	"
Amylase	2763	IU/l
Lipase	308	IU/l

APPENDIX 19

Plasma lipid and lipoprotein concentrations in 16 dogs (continued).

Case 120360

Yorkshire terrier, 8 years, female (N).

Biochemical parameters:

Cholesterol	6.23 mmo/l
Triglyceride	11.30 "
Amylase	1860 IU/l
Lipase	480 IU/l

Case 116313

Cocker spaniel, 3 years, female.

Biochemical parameters (24.10.91):

Cholesterol	7.13 mmo/l
Triglyceride	0.74 "
VLDL-C	0.08 "
LDL-C	2.71 "
HDL-C	3.69 "

Case 112876

Dobermann, 8 years, male, hypothyroidism.

Biochemical parameters:

Cholesterol	17.67 mmo/l
Triglyceride	2.61 "
VLDL-C	2.17 "
LDL-C	13.85 "
HDL-C	1.65 "

APPENDIX 19

Plasma lipid and lipoprotein concentrations in 16 dogs (continued).

Case 114522

Whippet, 9 years, male, hypothyroidism.

Biochemical parameters:

Cholesterol	47.80	mmo/l
Triglyceride	5.60	"
VLDL-C	22.31	"
LDL-C	23.74	"
HDL-C	1.75	"

Case 1

Golden retriever, 10 years, female.

Biochemical parameters:

Cholesterol	14.41	mmo/l
Triglyceride	2.04	"
VLDL-C	0.92	"
LDL-C	9.60	"
HDL-C	3.89	"

Case 2

German shepherd dog, 3 years, female (N).

Biochemical parameters:

Cholesterol	5.13	mmo/l
Triglyceride	0.60	"
VLDL-C	0.51	"
LDL-C	1.99	"
HDL-C	2.63	"

APPENDIX 19

Plasma lipid and lipoprotein concentrations in 16 dogs (continued).

Case 3

German shepherd dog, 7 years, female.

Biochemical parameters:

Cholesterol	5.88	mmo/l
Triglyceride	0.90	"
VLDL-C	0.85	"
LDL-C	2.20	"
HDL-C	2.83	"

Case 4

German shepherd dog, 3 years, female.

Biochemical parameters:

Cholesterol	4.37	mmo/l
Triglyceride	0.99	"
VLDL-C	0.59	"
LDL-C	1.42	"
HDL-C	2.36	"

Case 5

Rottweiller, 3 years, female.

Biochemical parameters:

Cholesterol	11.35	mmo/l
Triglyceride	1.20	"
VLDL-C	1.05	"
LDL-C	4.81	"
HDL-C	5.49	"

APPENDIX 19

Plasma lipid and lipoprotein concentrations in 16 dogs (continued).

Case 6

X-bred, 3 years, female.

Biochemical parameters:

Cholesterol	6.93	mmo/l
Triglyceride	0.83	"
VLDL-C	0.73	"
LDL-C	1.99	"
HDL-C	4.21	"

Case 7

X collie, 7 years, female.

Biochemical parameters:

Cholesterol	8.15	mmo/l
Triglyceride	0.76	"
VLDL-C	0.65	"
LDL-C	2.84	"
HDL-C	4.66	"

Case 8

Labrador retriever, 3 years, female.

Biochemical parameters:

Cholesterol	6.16	mmo/l
Triglyceride	1.84	"
VLDL-C	1.51	"
LDL-C	1.20	"
HDL-C	3.45	"

APPENDIX 19

Plasma lipid and lipoprotein concentrations in 16 dogs (continued).

Case 9

Alaskan Malamute, 5 years, female.

Biochemical parameters:

Cholesterol	6.16	mmo/l
Triglyceride	1.84	"
VLDL-C	1.51	"
LDL-C	1.20	"
HDL-C	3.45	"

Case 120848

Cavalier King Charles spaniel, 9 years, male.

Biochemical parameters:

Cholesterol	15.11	mmo/l
Triglyceride	2.46	"
VLDL-C	0.58	"
LDL-C	9.49	"
HDL-C	5.04	"

APPENDIX 20

The plasma lipid and lipoprotein concentrations and hepatic enzyme activities of 20 dogs with idiopathic hyperlipidaemia.

Case	Plasma triglyceride (mmol/l)	Cholesterol concentration (mmol/l)			
		Plasma	VLDL	LDL	HDL
1	1.52	9.96	1.20	5.02	3.74
2	0.88	7.71	1.61	3.61	2.82
3	1.93	7.23	1.10	1.93	4.17
4	0.68	7.00	0.09	3.78	2.05
5	0.50	6.70	0.82	2.20	3.68
6	0.43	6.93	0.09	3.52	2.71
7	2.34	7.72	1.44	1.62	4.66
8	0.83	6.67	0.54	1.72	4.41
9	n.d.	18.02	5.32	10.38	1.62
10	n.d.	6.72	0.02	2.10	3.20
11	1.42	6.65	0.84	1.45	4.36
12	1.73	7.58	0.14	2.96	3.80
13	0.34	8.81	0.86	3.79	4.16
14	n.d.	8.81	1.31	4.51	2.99
15	0.56	9.49	0.36	2.91	6.22
16	0.78	8.15	0.62	2.24	5.29
17	1.55	8.49	0.99	2.11	5.39
18	39.00	16.48	3.96	10.23	2.29
19	2.46	15.11	0.58	7.07	5.04
20	4.37	14.88	1.18	7.11	6.59

APPENDIX 20

The plasma lipid and lipoprotein concentrations and hepatic enzyme activities of 20 dogs with idiopathic hyperlipidaemia.

Case No.	ALP	AST	ALT
1	966	23	33
2	78	19	24
3	64	24	37
4	154	31	72
5	49	29	68
6	168	29	37
7	277	18	21
8	109	14	76
9	409	77	135
10	132	81	50
11	448	18	23
12	1130	11	40
13	55	28	23
14	67	20	30
15	60	31	22
16	480	12	104
17	892	21	48
18	1289	16	98
19	356	106	53
20	901	28	52

APPENDIX 21

The plasma lipid concentrations before and after dietary fat restriction in 10 dogs.

Cholesterol (mmol/l)		Triglyceride (mmol/l)	
Before	After	Before	After
8.41	3.99	0.59	0.75
11.44	5.78	10.83	1.96
17.51	15.14	17.20	23.57
11.35	9.35	1.20	1.15
14.88	11.67	4.37	1.16
6.23	4.49	11.30	6.77
16.13	15.38	2.62	2.13
7.23	5.26	1.93	0.60
9.96	7.55	1.52	0.88

GLOSSARY

α	alpha
apo	apolipoprotein
ACAT	acyl coA cholesterol acyl transferase
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
β	beta
CETP	cholesteryl ester transfer protein
Ci	curie
cm	centimetre
cpm	counts per minute
Da	Dalton
EDTA	ethylenediaminetetra-acetate
FA	fatty acids
g	gram
h	hour
HDL	high density lipoprotein(s)
HDL-C	high density lipoprotein cholesterol
HL	hepatic lipase
IDL	intermediate density lipoprotein(s)
kg	kilogram
l	litre
LCAT	lecithin:cholesterol acyl transferase
LDL	low density lipoprotein(s)
LDL-C	low density lipoprotein cholesterol
LPL	lipoprotein lipase
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mmol	millimole
Mr	relative molecular mass
N	normality
n.d.	not determined
NEFA	non-esterified fatty acids
n.s.	not significant

GLOSSARY

PVS	polyvinylsulphate
PHP	postheparin plasma
Rf	relative band speed
rpm	revolutions per minute
sd	standard deviation
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N,N,N',N',-tetramethylethylenediamine
μl	microlitre
VLDL	very low density lipoprotein(s)
VLDL-C	very low density lipoprotein cholesterol

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