THE IMMUNOLOCALIZATION AND QUANTITATION OF AVIAN LIPOPROTEIN LIPASE

BY

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DECLARATION

Except where stated the work presented in this thesis, submitted for the degree of Doctor of Philosophiae in the University of Wales, is the result of my own investigations. The contents of this thesis have not previously been accepted in substance for any degree and are not being concurrently submitted in candidature for any degree.

Ian M. R. George .....

Harry D. Griffin (Director of studies) .....

Anthony Cryer (Director of studies) .....

To my parents and to Helen

ABSTRACT

The overall aim of the project was to investigate the pools of LPL activity that exist within chicken tissues *in vivo*.

Polyclonal and monoclonal anti-chicken LPL antibodies were produced using highly purified chicken adipose tissue LPL as an immunogen. An attempt was made to measure LPL at the luminal surface of the capillary endothelium of individual tissues using an iodinated anti-chicken LPL monoclonal antibody, injected intravenously, *in vivo*. However, the technique was not successful because high levels of specific antibody binding were not achieved.

Heparin-release studies using the isolated perfused heart model system found significant species differences, between rats and chickens, in the regulation of heparin-releasable LPL in response to fasting. The low percentage of heparin-releasable LPL activity observed in chicken cardiac tissue corresponded to the relatively low accumulation of triacylglycerol NEFA by the chicken muscular tissues by comparison with adipose tissue, following the intravenous injection of [^{14}C]-VLDL *in vivo*.

Using anti-chicken LPL antibodies immunocytochemical studies on a variety of chicken extrahepatic tissues showed the enzyme to be located predominantly extracellularly, at the basement membrane of the tissue parenchymal cells and in association with the interstitial capillary elements. Image-analysis was used to quantify the pools of enzyme associated with these compartments in chicken cardiac tissue. In chicken bone marrow LPL was found in association with the adipocytes and vascular elements of the marrow mass, with a lack of enzyme associated with the haematopoietic cells. Immunocytochemical techniques were also used to investigate the tissue specific developmental changes which occur in the distribution of LPL during the growth and maturation of the heart and liver of the embryonic chicken.

The present study has identified several pools of LPL within chicken tissues and proposed that the transportation of enzyme from the interstitial capillary elements to the luminal endothelial surface may play a role in the regulation of functional LPL activity in chicken tissues.

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CONTENTS

CHAPTER 1

INTRODUCTION

1.1 *Identification of lipoprotein lipase*

In 1943 Hahn observed the reversal of alimentary lipemia in dogs following a transfusion of blood containing heparin as an anticoagulant. It was established subsequently that heparin alone was responsible for the effect which Hahn (1943) described as the "clearing reaction". Anfinsen *et al.* (1952) provided evidence for the conclusion that the clearing of plasma turbidity was the result of the action of a lipolytic enzyme. As the substrate specificity became fully characterized the enzyme was defined as lipoprotein lipase, an acylglycerol hydrolase (EC 3.1.1.34).

The physiological role of the enzyme and the site of its action remained uncertain, until it was noted that rapid appearance of the enzyme in the bloodstream occurred not only after heparin injection, but also following the injection of high molecular weight dextran sulphates which could not readily escape across the vascular endothelium (Robinson *et al.*, 1959). This led to the suggestion that LPL was located on the luminal surface of vascular endothelial cells. Its physiological role at this site was proposed to be the hydrolysis of lipoprotein triacylglycerol with the non-esterified fatty acids (NEFA) that were released diffusing from the plasma into the tissues (Robinson and French, 1960). Much subsequent evidence (for reviews, see Cryer, 1981; Robinson, 1987 and Bensadoun, 1991) has supported this initial concept.

Long chain NEFA are a requirement for the structural and functional integrity of all cells and are also an important energy source. NEFA can be synthesized by the cells *de novo* or be derived from the plasma. There are two possible sources of NEFA present in the plasma. One form comprises the NEFA released during the hydrolysis of lipoprotein triacylglycerol by the action of LPL while the other form is composed of NEFA bound to albumin (Spector *et al.*, 1968).

Whilst in the circulation, plasma triacylglycerol-rich lipoproteins are the substrate for at least three enzymes, LPL, lecithin-cholesterol acyl transferase (LCAT) and hepatic lipase. Hepatic lipase which is found in

the liver of mammals and birds, but at low levels in the latter (Benson *et al.*, 1975), has been associated with the uptake of lipoprotein remnants into hepatocytes. LCAT catalyses the transfer of acyl groups from lecithin to the 3-OH position of free cholesterol and may play a role in the efflux of cholesterol from the peripheral tissues to the liver (Smith *et al.*, 1990).

LPL is certainly the most important enzyme catalysing the hydrolysis of plasma lipoprotein triacylglycerol in extrahepatic tissues to produce NEFA and glycerol. The released NEFA are then free to diffuse into the surrounding tissue to be either oxidised as an energy source or re-esterified and stored depending on the physiological requirements of the tissue.

1.2 *The interaction of LPL with the capillary endothelium*

Evidence to support the hypothesis, first proposed by Robinson and French (1960), that the "functional" site of LPL activity is the capillary endothelium, where it is involved in the hydrolysis of lipoprotein triacylglycerol, has subsequently come from many sources. The use of radiolabelled lipoprotein triacylglycerol and the precipitation of the released NEFA, using lead salts, has shown that the products of lipoprotein hydrolysis were generated only at the capillary endothelium and in the sub-endothelial space (Scow *et al.*, 1976). Pedersen *et al.* (1983) immunolocalized functional LPL at the capillary lumen in isolated rat hearts by perfusion with an anti-rat LPL antibody. A similar technique was used by Schotz *et al.* (1977) who found that the addition of anti-LPL antibodies to the perfusion medium of the isolated rat heart prevented chylomicron hydrolysis during a 15 minute period of perfusion.

The role of LPL *in vivo* has also been demonstrated by Kompiang *et al.* (1976) by injecting an anti-LPL serum intravenously into roosters in an attempt to block the catabolism of lipoproteins. Following the introduction of antibodies into the circulation there was a linear increase in the plasma triacylglycerol concentration and from this data the authors concluded that LPL mediated hydrolysis was a prerequisite for the removal of lipoprotein triacylglycerol from the circulation.

Further evidence for the role of LPL in plasma triacylglycerol clearance *in vivo* has been produced from clinical studies on patients with familial type I primary hyperlipoproteinaemia (Eckel, 1989). In these cases post-heparin plasma and/or adipose tissue LPL activities are low or absent and as a proposed consequence plasma lipoprotein triacylglycerol concentrations are extremely high. Fager *et al.* (1990) showed that in some cases this can be caused by ineffective transport of LPL from the Golgi to the cell surface of adipocytes. Alternatively, tissue LPL levels may be normal but patients may lack apolipoprotein CII (Breckenridge *et al.*, 1978), which is a requirement for maximal enzyme activity. In both cases reduced levels of lipoprotein hydrolysis leads to excessive levels of plasma lipoprotein triacylglycerol.

The existence of LPL in several distinct pools within tissues has been suggested by the results of studies on perfused hearts which distinguished two pools of enzyme activity differing in their response to heparin. The smaller heparin-releasable pool of LPL activity was thought to be present at the capillary endothelium, with the larger heparin-residual pool being present in the underlying tissue (Cryer and Jones, 1979). Removal of LPL by heparin perfusion of the isolated rat heart lead to a complete loss of the capacity to catabolize exogenous triacylglycerol (Fielding and Higgins, 1974; Rogers and Robinson, 1974). This provides evidence that the functionally active pool of the enzyme corresponds to the rapidly heparin-releasable pool of LPL activity thought to be located at the capillary endothelium.

Although the functional site of LPL action appears to be the capillary endothelium, endothelial cells appear to be incapable of synthesizing the enzyme. Cheng *et al.* (1981) demonstrated the absence of LPL on the surface of cultured endothelial cells although the cells were able to bind purified LPL with an affinity constant of 0.7×10^7 M^{-1}. These authors found that enzyme binding was inhibited by heparinase, heparin and heparan sulphate. On binding of exogenous LPL the cells, however, acquired the capacity to hydrolyse triacylglycerol. The binding capacity for LPL has been estimated at 3.75×10^6 molecules per cultured endothelial cell (Cheng *et al.*, 1981). Cryer (1987) calculated that an eight-fold molar excess of active glycosaminoglycan binding sites for LPL were present in relation to the functionally active LPL molecules bound at the endothelial cell surface.

1.3 *Molecular structure of LPL*

1.3.1 *Molecular biology*

The gene for LPL belongs to a family that includes the genes coding for hepatic lipase and pancreatic lipase. The primary sequence of human LPL is 46% homologous with rat hepatic lipase and 28% homologous with porcine pancreatic lipase (Komaromy and Schotz, 1987). The primary sequence of LPL also shows homology to the *Drosophila* yolk proteins 1,2 and 3 (Hide *et al.*, 1992) although these latter proteins do not exhibit lipase activity. The human (Kirchgessner *et al.*, 1989), guinea pig (Enerback and Bjursell, 1989) and chicken (Cooper *et al.*, 1992) LPL genes have all been isolated and characterized. Comparison of the sequences suggests that during evolution the exon-intron organization has been highly conserved. The first exon contains the 5' non-coding region of the mRNA and the LPL signal peptide. The 2nd and 8th exons contain the consensus sequences for N-linked glycosylation and the 10th exon specifies the 3' non-coding sequence. From the organization of the genes for LPL, hepatic lipase and pancreatic lipase it has been proposed that these lipases are derived from a single ancestral lipase gene which had a minimum of 14 introns (Kirchgessner *et al.*, 1989).

Based on the cDNA sequence the primary sequence of LPL from six species has been determined: chicken (Cooper *et al.*, 1989), mouse (Kirchgessner *et al.*,1987), human (Wion *et al.*, 1987), guinea pig (Enerback *et al.*, 1987), sheep (Edwards *et al.*, 1993) and bovine (Senda *et al.*, 1987). The predicted amino acid sequences for the mammalian enzymes show an 83-89% homology. However, chicken LPL, a mature protein of 465 amino acids, was found to be 73-77% homologous to the mammalian primary sequences. Conservation of the position of cysteine residues, proposed functional domains and potential N-glycosylation sites has been found in all of the species studied. Chicken LPL differs from the mammalian LPLs in the position of one N-glycosylation site and the presence of an additional 15-17 amino acids at the C-terminus (Cooper *at al.*, 1989). Based on the primary sequences of the amino-terminus region, the length of the signal peptide is 27 amino acids in mammals (Bengtsson-Olivecrona *et al.*, 1986) and 19 or 25 amino acids in chickens, depending at which of two methionine residues translation is initiated (Cooper *et al.*, 1989).

1.3.2 *Post-translational processing*

LPL is a glycoprotein containing between 3 and 10% carbohydrate by weight (Cryer, 1987) and is thought to be functionally active as a non-covalent homodimer *in vivo* (Olivecrona and Bengtsson-Olivecrona, 1987). Strieleman and Bensadoun (1987) reported the cell free translation of chicken LPL mRNA in a reticylocyte system resulted in the production of a 56 KDa non-glycosylated protein. Inclusion of pancreatic microsomal membranes resulted in the isolation of an additional 62 KDa glycoprotein and both proteins were confirmed to be LPL.

The only other post-translational modification reported to date is the sulphation of chicken LPL (Hoogewerf and Bensadoun, 1991). The sulphated moiety is associated with a complex oligosaccharide chain conjugated at Asn 45. This modification has no effect on LPL catalytic activity or on binding to cultured adipocytes and it has been proposed that it is responsible for targeting the enzyme to the plasma membrane.

1.4 *Molecular interactions of LPL*

1.4.1 *Active site and lipid binding site*

LPL shows a relatively low level of substrate specificity, hydrolysing long and short chain triacylglycerol (Egelrud and Olivecrona, 1973), tri-, di-, and mono-acylglycerides and phospholipids (Stocks and Galton, 1980). However, LPL preferentially hydrolyses primary triacylglycerol ester bonds generating sn-2 monoacylglycerides and NEFA (Nilsson-Ehle *et al.*, 1973) with a high turnover rate of about 1000 ester bonds per second (Scow and Olivecrona, 1977). The crystal structure of LPL is not presently available. However, the crystal structure of pancreatic lipase is known (Winkler *et al.*, 1990) and can serve as a model for that of LPL (Wang *et al.*, 1992). From the crystal structure of pancreatic lipase an active site triad of Ser-His-Asp has been identified with the same hydrogen bonding pattern which occurs in the active site of the serine proteases. A similar highly conserved Ser-His-Asp triad can also be identified in the primary sequences of LPL. The presence of an acyl enzyme intermediate (Rojas *et al.*, 1989) and the inhibition of LPL by inhibitors of serine proteases (eg: disopropylflurophosphate, DFP); provides further evidence for the existence of a serine based active site in LPL (Parkin *et al.*, 1982). The reaction kinetics for the inhibition of LPL activity by DFP indicates a single active site (Wang *et al.*, 1992)

while the kinetics of tri- and mono-acylglycerol hydrolysis indicates a common active site (Twu *et al.*, 1976).

It has been proposed that LPL binds to the lipid interface of the lipoprotein particles before the hydrolysis of lipoprotein triacylglycerol at the active site can occur. The lipid binding site is thought to differ from the active site because the enzyme can bind lipoprotein *in vitro* under conditions which are not accompanied by hydrolysis (Olivecrona and Bengtsson, 1984). Two lipid binding sequences have been identified from the primary LPL sequences (Cooper *et al.*, 1989), which are thought to form part of a single lipid binding site on the enzyme.

1.4.2 *Interaction with glycosaminoglycans*

LPL has a high affinity for heparin and other glycosaminoglycans. The glycosaminoglycans are thought to interact with a number of positively charged amino-acids located in a specific region of the enzyme (Enerback *et al.*, 1988). The binding of LPL to heparin has been reported to stabilise enzyme activity and bound enzyme maintains the potential for activation by apolipoprotein-CII (Bengtsson and Olivecrona, 1981). Two putative heparin binding sequences rich in basic amino acid residues have been identified at the carboxyl-terminal region which again appear to form a single three dimensional structure in the mature enzyme capable of interaction with anionic heparin-like molecules (Oka *et al.*, 1989). It has been proposed that these heparin binding sequences are located on exon 6 of the LPL gene (Deeb and Peng, 1989; Cooper *et al.*, 1992).

1.4.3 *Interaction with Apolipoprotein CII*

LPL displays only basal activity in the absence of the specific activator protein, apolipoprotein CII (apo CII). The requirement of apo CII for the maximal activity of LPL may prevent the expression of lipase activity at the intracellular site of synthesis (Wang *et al.*, 1992). The binding of apo CII to LPL is based upon a protein-protein interaction of a ß-pleated sheet region of the apo CII and a specific but unidentified region of the LPL molecule (Smith and Pownall, 1984). Apo CII appears to aid the juxtaposition of enzyme with substrate particles, allowing hydrolysis at the substrate interface and the stabilization of catalytically active enzyme (Bengtsson and Olivecrona, 1981).

The apoprotein content of chicken VLDL is different from that of mammalian VLDL (Perry *et al.*, 1985). Although at least one of the lower molecular weight avian apoproteins must be the functional equivalent of mammalian apo CII, because delipidated VLDL and portomicron preparations from immature hens activate chicken LPL strongly (Griffin and Hermier, 1988). Avian apo CII has not been positively identified, although a protein with a molecular weight of 6 KDa has been isolated from egg yolk which has the capacity to activate bovine milk LPL (Bengtsson *et al.*, 1977).

1.4.4 *Interaction of NEFA with LPL*

LPL can bind NEFA, this has been reported to reduce its affinity for lipid droplets (Bengtsson and Olivecrona, 1980) as well as for glycosaminoglycans (Olivecrona and Bengtsson-Olivecrona, 1987) and abolish activation by apo CII (Bengtsson and Olivecrona, 1979a). From these observations Bengtsson and Olivecrona (1980) proposed that product inhibition of LPL activity occurs *in vivo*. Thus, when the ability of a tissue to utilize the NEFA released by the enzyme is exceeded, the localized accumulation of NEFA causes both product inhibition and weakening of the enzyme substrate affinity. The latter effect, it has been proposed, may serve to direct plasma lipoproteins to those tissues which have the greatest requirement for NEFA (Schaefer *et al.*, 1978).

1.5 *Regulation of LPL activity*

The hydrolysis of lipoprotein triacylglycerol by LPL is a prerequisite for the uptake of triacylglycerol NEFA into the tissues. Wolfe *et al.* (1981) concluded from several studies *in vivo* on the fate of radiolabelled VLDL triacylglycerol, that oxidation of VLDL derived NEFA did not involve their prior entry into the plasma pool of NEFA. Regulation of the functional pool of LPL activity, ie: that which is thought to be present at the capillary endothelium, may therefore play a role in the partitioning of plasma lipoprotein triacylglycerol between tissues according to their physiological requirements in different nutritional and developmental states.

Regulation of the functional pool of LPL activity *in vivo* could theoretically be exercised at a number of points in the synthesis,

transport and turnover of the enzyme within the tissue. Figure 1.1 shows an overall summary of these processes.

1.5.1 *Synthesis and secretion of LPL*

The amount of LPL present at the capillary endothelium may simply be related to the amount of enzyme produced and secreted by the tissue parenchymal cells. Most of the data on the synthesis and secretion of LPL has been obtained from studies on either isolated cells or cultured cell lines *in vitro*. The technique has been used to demonstrate LPL synthesis in a number of cell types including myocytes (Chohan and Cryer, 1977); adipocytes (Cryer *et al.*, 1975); preadipocytes (Plaas and Cryer, 1980); mammary acinar cells (Clegg, 1979); macrophages (Wang-Iverson *et al.*, 1982) and in tumour cells (Tajima *et al.*, 1985). For a comprehensive review see Cryer (1987).

Many studies have concluded that glycosylation is a requirement for the secretion of active LPL. For example Chajek-Shaul *et al.* (1985) have shown that, in rat preadipocytes and myocytes, inhibition of N-glycosylation with tunicamycin reduced cellular LPL activity and prevented LPL secretion. Semenkovich *et al.* (1990) used site directed mutagenesis to remove an N-linked glycosylation site by substitution of an Asn by an Ala residue at position 43 and found that intracellular and secreted LPL activity was absent.

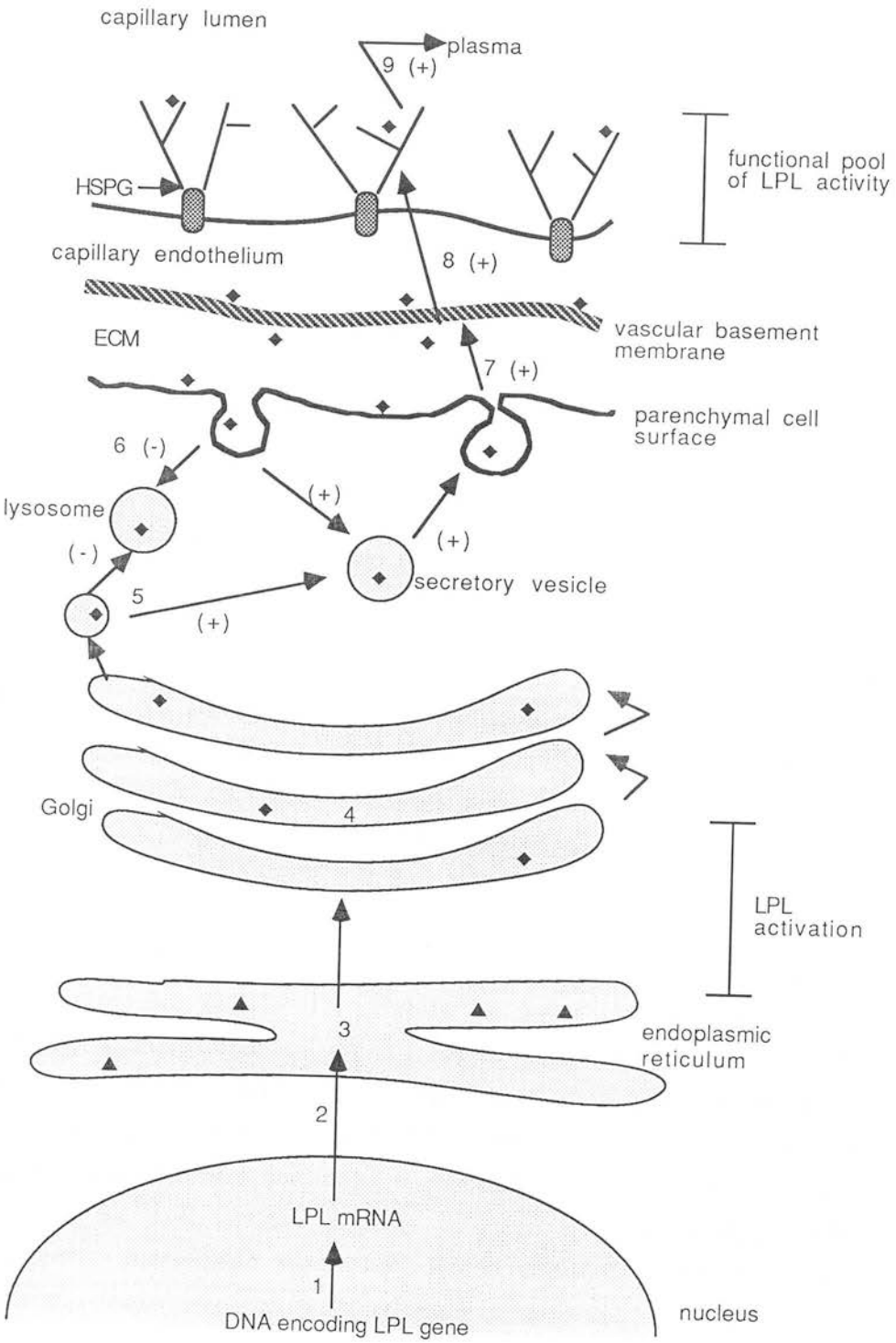
The point at which LPL acquires catalytic activity during processing has been the focus of several investigations. Active LPL is synthesized by adipocytes treated with monensin, which blocks secretion from the Golgi apparatus, but cells treated with Carbonyl cyamide m-chlorophenylhydrazone, which prevents transport between the compartments of the endoplasmic reticulum (ER), do not synthesis active LPL (Vannier and Ailhaud, 1986). This indicates that activation occurs somewhere between the ER and the Golgi apparatus, following the modification of N-linked high mannose residues. In support of this, Vannier and Ailhaud (1989) found that the fully endo H-sensitive species of LPL, bearing 3 high mannose chains, was found as an inactive monomer in mice preadipocytes and only the fully processed species bearing two complex oligosaccharide chains was present as an active homodimer. Similarly, Ben-Zeev *et al.* (1992) recently concluded that in a Chinese hamster ovary cell line, the glucose trimming of LPL

Figure 1.1 Potential regulatory processes of functional LPL.

The diagram shows a summary of the steps required for the synthesis, secretion and for the transportation of active LPL to the luminal surface of the capillary endothelium. Enzyme synthesized in the tissue parenchymal cells undergoes activation between the endoplasmic reticulum and the Golgi apparatus following the trimming of high-mannose carbohydrate units (Ben-Zev *et al.*, 1992). From the trans-Golgi the mature enzyme is then translocated to the parenchymal cell surface or to the lysosomal compartment for degradation (Ailhaud, 1990). LPL at the parenchymal cell surface is either transported through the extracellular matrix to the vascular basement membrane (Chajek-Shaul *et al.*, 1990) or undergoes internalization and subsequent degradation in the lysosomal compartment (Cisar *et al.*, 1989). Enzyme from the vascular basement membrane is moved across the endothelium to the luminal cell surface (Saxena *et al.*, 1991a) where it associates with heparan sulphate proteoglycans (Saxena *et al.*, 1991b). Only the pool of LPL at this position is functionally active ie: capable of hydrolysing plasma lipoprotein triacylglycerol (Rogers and Robinson, 1974). Control of LPL-endothelial cell surface activity could be exerted at one, or a combination of levels during the synthesis and transport of LPL. Possible regulatory processes are indicated as follows: 1) transcription; 2) RNA processing and stability; 3) translation; 4) glycosylation and trimming of high mannose units; 5) intracellular translocation; 6) endocytosis; 7) translocation to vascular basement membrane; 8) translocation across endothelium; 9) turnover at capillary endothelium.

The proposed effect of the secretatogue, heparin on these processes is denoted by (+)/(-).

Catalytically inactive LPL (▲); catalytically active LPL (◆).


capillary lumen
plasma
9 (+)
HSPG
functional pool of LPL activity
capillary endothelium
8 (+)
ECM
vascular basement membrane
7 (+)
parenchymal cell surface
6 (-)
lysosome
(+)
(+)
(-)
secretory vesicle
5
(+)
Golgi
4
LPL activation
3
endoplasmic reticulum
2
LPL mRNA
1
DNA encoding LPL gene
nucleus

oligosaccharides is essential for enzyme activation, however, further processing or translocation of the enzyme to the cis-Golgi is not a requirement for the expression of catalytic activity *in vitro*.

Several studies using cultured adipocytes and myocytes have reported that up to 80% of the synthesized LPL is degraded within the cells rather than being bound at the cell surface or secreted into the medium (Cupp *et al.*, 1987; Olivecrona *et al.*, 1987; Semb and Olivecrona, 1987). Whereas, in cultured fibroblasts and endothelial cells which do not synthesis LPL, there is a high ratio of surface binding to degradation (Friedman *et al.*, 1982; Saxena *et al.*, 1990). The LPL degradative pathway has also been recorded in the isolated rat fat pad (Speake *et al.*, 1985) and allows for the possibility that rapid increases in the rate of LPL secretion *in vivo* could occur without a change in the rate of enzyme synthesis. Pulse chase studies in isolated adipocytes led Cupp *et al.* (1987) to conclude that LPL was secreted from the cell by an apparent first order process with there being no evidence of accumulation of LPL within secretory or storage vesicles. This study also reported that 77% of synthesized LPL was degraded in control cells, whereas in heparin treated cells this figure was 22%. Also, LPL bound at the adipocyte cell surface by heparinase-sensitive high affinity binding sites has been observed to undergo internalization and degradation in the lysosomal compartment (Cisar *et al.*, 1989; Friedman *et al.*, 1982).

From these results a hypothesis was proposed to explain the mechanism of secretion of LPL and the effect of heparin on the degradative pathway (Bensadoun, 1991). Mature enzyme is transported from the trans-Golgi compartment to the adipocyte cell surface where it binds to heparan sulphate proteoglycan (HSPG) molecules. Heparin present in the medium would compete for LPL from the cell surface and soluble LPL would be released into the culture medium. In the absence of heparin a portion of the cell surface pool is released into the medium while the remainder is internalised as an LPL-HSPG complex to be degraded in the lysosomal compartment or recycled back to the cell surface (Fig 1.1).

A different hypothesis was proposed by Vannier and Ailhaud (1989) whereby LPL accumulates at the trans-side of the Golgi apparatus (the half life of the enzyme in this compartment is one hour, Olivecrona *et al.*, 1987) and is directed to compartments consisting of LPL storing vesicles

in which LPL exists in a potentially active, condensed or "polymerised" form in the presence of sulphated glycosaminoglycans (Ailhaud, 1990). Efflux from these compartments results in secretion or to lysosomal degradation. The effect of heparin is explained by increasing exocytosis to the cell-surface thereby reducing the flux of enzyme to the lysosomal compartment (Fig 1.1).

1.5.2 *LPL translocation to the capillary endothelium*

The process by which LPL is transferred from the site of synthesis in tissue parenchymal cells to its physiological site of action, the luminal surface of capillary endothelial cells has yet to be fully elucidated, although it may represent a critical regulatory step. Blanchette-Mackie *et al.* (1989) used the technique of immunogold electron microscopy to localise LPL within the myocardium of young mice. They found 78% of the LPL was present in the myocytes primarily in the sarcoplasmic reticulum, Golgi vesicles and in secretory vesicles. The extracellular matrix accounted for 3-6% of the total tissue LPL and the capillary endothelium 18%. They concluded that LPL is synthesized by the myocytes, transferred across the extracellular space travelling along cell surfaces, possibly by translocation along cell surface-associated heparan sulphates and other polyanions, and across endothelial cells within secretory vesicles or through intracellular channels to be concentrated at the surface of the endothelial luminal projections.

Several studies using the isolated perfused heart as a model system have attempted to estimate the rate of flux of LPL through the tissue compartments. Bagby (1983) estimated the $t_{1/2}$ of LPL bound at the luminal surface of the capillary endothelium to be 10 minutes in the rat. Liu and Olivecrona (1991) using pulse-chase studies in the guinea pig heart found that LPL appeared in the perfusion medium (in the presence of heparin) approximately 30 minutes after the start of the pulse. They concluded that LPL moves rapidly out of the myocytes with no obligatory delay. This supports the results of similar experiments by Cupp *et al.* (1987) in isolated adipocytes and the model of LPL secretion proposed by Bensadoun (1991) rather than that suggested by Vannier and Ailhaud (1989).

Several studies have investigated the role of the extracellular matrix and subendothelial basement membrane in the transport of LPL from its

site of synthesis to its site of action. From the results of studies using ^{125}I-labelled LPL in a cultured endothelial cell system, Chajek-Shaul *et al.* (1990) suggested that the subendothelial basement membrane contains a large number of LPL binding sites (as does the extracellular matrix) which sequester and stabilise enzyme secreted into the subendothelial space by non-endothelial cells and may therefore play a role in LPL transportation. Saxena *et al.* (1990) using a similar *in vitro* model system found evidence for a HSPG-mediated non-degradative internalization and recycling of the cell-bound endothelial pool of LPL. Saxena *et al.* (1991a) concluded that a saturatable transport system based on HSPGs exists for the transport of LPL across endothelial cells. These authors found the system to be inhibited by high concentrations of NEFA on the basal side of the cells and proposed that this mechanism may modulate the physiologically active pool of LPL *in vivo*. Saxena *et al.* (1991b) has recently identified and characterized an endothelial cell surface HSPG LPL receptor, with a molecular weight of 220 KDa, which has the properties required for the observed transport of LPL across endothelial cells and is found on both the luminal and basolateral cell surfaces. A 116 KDa LPL-binding protein has also been identified on the surface of bovine aortic endothelial cells and Sivaram *et al.* (1992) suggested that this binding protein provides highly specific, high affinity LPL binding sites, whereas the HSPG receptor provides high capacity and low specificity LPL binding sites.

To be able to partition lipoprotein triacylglycerol effectively between tissues, regulation must occur not only of the rate of synthesis and transportation of LPL to the luminal surface of the capillary endothelium but also in the turnover of enzyme at this site. The work of Bagby (1983) and of Chajek-Shaul *et al.* (1988) has shown that spontaneous release of active LPL from the capillary bed of the perfused heart into the perfusion medium occurs although it is not known if it is released in association with HSPGs. NEFA have been shown to weaken the interaction between LPL and heparin (Bengtsson and Olivecrona, 1979b) and displace LPL from cultured endothelial cells (Saxena *et al.*, 1989). It has therefore been suggested that the release of endothelial LPL by NEFA *in vivo* is another example of product regulation of functional LPL in tissues (Peterson *et al.*, 1990; Saxena *et al.*, 1989).

It has been suggested by Camps *et al.* (1990; 1991) that LPL released into the plasma can undergo several re-attachment/release events in the extrahepatic tissues before being ultimately taken up by the liver. Wallinder *et al.* (1984) found that LPL in the bloodstream of the rat had a half-life of approximately 1 minute and it has been shown that the liver operates well below saturation in its capacity for LPL removal *in vivo* (Vilaro *et al.*, 1988). Vilaro *et al.* (1988) also suggested that two different types of LPL binding sites were present in the liver. One being identical to that found in extrahepatic tissues (ie: the heparin-releasable binding of active LPL) and the other, unique to the liver, and was capable of binding inactive LPL in a non-heparin releasable fashion. Binding of LPL by the liver leads to the internalization and lysosomal degradation of the enzyme with acid soluble and proteolytic fragments rapidly appearing in the plasma (Wallinder *et al.*, 1984).

1.5.3 *Hormonal control of LPL activity*

The control of synthesis and degradation of LPL has been observed in a number of tissues and a major regulating influence on these processes was thought to be hormonal (Cryer, 1981).

Eckel (1987) proposed that insulin was the predominant hormonal regulator of adipose tissue LPL. A positive relationship between adipose tissue LPL activity and circulating insulin levels in rats has been reported in many studies (Borensztajn *et al.*, 1972; Reichl, 1972; DeGasquet *et al.*, 1981). Insulin and glucocorticoids have both been found to promote LPL activity in isolated fat pads *in vitro* (Ashby *et al.*, 1978; Ashby and Robinson, 1980).

In cultured adipocytes insulin has been shown to regulate LPL activity by several mechanisms. Chan *et al.* (1988) reported that insulin appears to bring about a rapid release of cell surface-bound LPL. The hormone was also found to increase the LPL mRNA concentration by Ong *et al.* (1989) while, Raynolds *et al.* (1990) concluded that the insulin mediated increase in LPL mRNA was due to changes in mRNA stability because the LPL gene transcription rate was not different from control cells. However, Semenkovich *et al.* (1989) concluded that insulin regulation of LPL activity in adipocytes is mediated entirely at a post-translational level. They proposed that insulin may regulate the metabolic fate of LPL within the adipocyte by controlling the fluxes of enzyme between the

degradative and secretory pathways. In perfused hearts and cultured neonatal heart cells insulin was found to have no effect on LPL activity (Stam and Hulsmann, 1984; Chajek-Shaul *et al.*, 1989).

In isolated fat pads the increased LPL activity in response to insulin and/or the glucocorticoids has been reversed by the catacholamines (Cryer *et al.*, 1973; Parkin *et al.*, 1980). These hormones appear to have an inverse regulatory effect to insulin, decreasing synthesis and increasing intracellular degradation of the enzyme in the lysosomal compartment of adipose tissue (Mooney and Lane, 1981), while increasing both the synthesis and processing of the enzyme in cultured neonatal heart cells (Friedman *et al.*, 1986a).

A new class of hormonal regulators of LPL activity was discovered following the observation that bacterial, viral and protozan infections in animals produced hyperlipidaemia (Werner, 1969) and reduced the levels of tissue LPL activity (Kawakami and Cerami, 1981). This led to the demonstration that a number of cytokines including tumour necrosis factor (TNF), interleukin 1 and the interferons exert effects on tissue LPL activity in times of infection (Beutler and Cerami, 1985). The mechanism of action is thought to be a result of the specific depression of LPL synthesis as no general reduction in protein synthesis is observed in such circumstances (Price *et al.*, 1986). Fried and Zechner (1989) found that TNF specifically decreases the levels of LPL mRNA in human adipose tissue and they concluded that TNF was a potent inhibitor of LPL gene expression. Griffin and Butterwith (1988) demonstrated that in birds, cytokines play a similar role in the depression of tissue LPL activity following endotoxin administration.

Other hormones that have been implicated in the control of LPL activity include thyroid hormones, glucagon, growth hormones, prolactin, sex steroids (androgens, estrogens, progesterons) and fibroblast like-growth factor (see reviews by: Eckel, 1987; Cryer, 1987; Butterwith, 1988).

1.5.4 *Tissue-specific regulation*

LPL activity has been detected in a wide range of tissues and the differing roles played by the enzyme in the lipid and energy metabolism of a variety of tissues is summarised in Table 1.1. The tissue-specific regulation of LPL activity has been observed in a variety of different

Table 1.1 Suggested role of LPL in various tissues.

Tissue	Role	Reference
Muscle (cardiac, skeletal)	Energy provision	Borensztajn (1987)
White adipose tissue	Triacylglycerol storage	Eckel (1987)
Brown adipose tissue	Thermogenesis	Carneheim *et al.* (1984)
Lactating mammary gland	Milk triacylglycerol synthesis	Scow & Chernick (1987)
Lung	Surfactant synthesis	Coonrod *et al.* (1989)
Brain	Phospholipid/glycolipid synthesis	Eckel & Robbins (1984)
Foetal liver	Energy provision/triacylglycerol storage	Gimenez-Llort *et al.* (1989)

Table modified from Eckel (1989).

nutritional or developmental states, with the level of enzyme activity responding to the physiological requirements of the individual tissues.

Tissue-specific changes in tissue LPL activities occur during the feeding-starvation and refeeding cycle. These changes may be caused by a complex interaction of many factors, including: insulin, glucagon and glucocorticoid levels; sympathetic innervation and the components of the diet. During a period of fasting, plasma insulin levels decrease, levels of counter-regulatory hormones increase and the lipolysis of triacylglycerol stored in adipose tissue accelerates. Fasting has been found to reduce the level of adipose tissue LPL activity in mammals and the concomitant uptake of NEFA derived from lipoprotein triacylglycerol (Cryer, 1987). Reciprocal changes in total tissue LPL activity occur in mammalian adipose and muscle tissues during fasting and exercise (Cryer, 1981) indicating that LPL in mammals does play a role in the partitioning of lipoprotein triacylglycerol between storage and oxidation (NEFA being an important energy source in muscle tissues). Farese *et al.* (1991) suggested that such changes are due to the divergent effects of insulin on LPL activities in adipose tissue and muscle tissue.

The mechanism by which LPL activity is regulated appears to vary depending on the species and the exact point in the feeding/fasting cycle studied. For example, after a prolonged fast of 48 hours, guinea pig (Enerback *et al.*, 1988) and chicken (Cooper *et al.*, 1989) adipose tissue LPL mRNA levels and rates of synthesis respond in parallel to changes in LPL activity upon refeeding. Semb and Olivecrona (1989) concluded that the nutritional regulation of LPL in guinea pig adipose tissue occurs by relatively slow changes in mRNA levels and more rapid changes in the transportation and turnover of the enzyme. Whereas, in rat adipose tissue Doolittle *et al.* (1990) found that, after fasting, LPL activity was decreased by 50% while LPL mRNA levels and rates of synthesis increased nearly 2 fold. These authors concluded that nutritional regulation of LPL activity occurred post-translationally with the redistribution of LPL within adipose tissue. After fasting, the high mannose form is the predominant species while the intermediate/complex species predominates after feeding. This suggests a shift of LPL out of the ER and into the post-Golgi secretory pathway. Doolittle *et al.* (1990) also proposed that control occurs over the flux of

newly synthesized enzyme between the secretory and degradative pathways, the latter increasing during fasting.

The regulation of LPL during lactation is also tissue-specific. The role played by LPL during lactation has been fully reviewed by Scow and Chernick (1987). In late pregnancy and after parturition, prolactin levels rise and LPL activity is greatly increased in the mammary gland and markedly decreased in adipose tissue. The net result of this is the incorporation of plasma lipoprotein triacylglycerol into milk production rather than storage in adipose tissue (Scow *et al.*, 1977). In lactating rats hypophysectomy was found to reverse these changes, emphasizing the possible role of prolactin in this process (Scow and Chernick, 1987).

Most studies on the tissue-specific developmental expression of tissue LPL activities have used the rat as a model. In the heart, skeletal muscle and adipose tissue, LPL mRNA levels and activity rise during suckling and after weaning (Semenkovich *et al.*, 1989; Tavangar et al., 1992). This can be related to the metabolic transition which occurs at birth from a mainly carbohydrate and NEFA calorie supply derived from the maternal circulation to primarily lipid during suckling. At this stage 70% of all metabolic energy in the rat is derived from milk lipids (Cryer, 1987) of which 97% is triacylglycerol (Fernando-Warnakulasuriya *et al.*, 1981), the neonate is therefore dependent on the action of LPL to release NEFA as an energy source. In the muscular tissues the increase in the LPL activity after birth parallels the increase in the use of NEFA as an energy source (Schiebler and Wolff, 1966). LPL activity is also found in foetal and newborn rat liver (Llobera *et al.*, 1979; Grinberg *et al.*, 1985), turning the neonatal liver into a net importer of triacylglycerol. However, in this tissue enzyme activity declines during suckling and is absent at weaning. These changes are coincident with the changes in the nutritional intake at weaning and the onset of hepatic lipogenesis, turning the liver into a net exporter of triacylglycerol.

1.6 *Plasma lipoprotein metabolism*

1.6.1 *Differences between birds and mammals*

It has been shown by Saadoun and Leclercq (1983) using [^{3}H] water that chicken adipose tissue has a very low capacity for lipogenesis *de novo*. Therefore, the triacylglycerol that accumulates in avian adipose tissue is derived mainly from the liver, an important site of lipogenesis

in birds, or from the diet. Griffin *et al.* (1992) calculated that approximately 80-85% of the fatty acids that accumulate in the adipose tissue of broiler chickens are derived from plasma lipoproteins.

Triacylglycerol rich lipoproteins are synthesized by the intestine to transport exogenous dietary lipid to the rest of the body and by the liver to supply the extrahepatic tissues with endogenous triacylglycerol. These lipoproteins have a core of apolar lipids (Shen *et al.*, 1977) principally triacylglycerol (>70%) surrounded by a monolayer of surface-active lipid, predominantly phospholipid and some unesterified cholesterol (Zilversmit, 1965). The apoprotein polypeptides characteristic of the lipoprotein also reside in this outer layer and play a significant role in their catabolism and metabolic fate (Mahley *et al.*, 1984). Although the apoprotein pattern of chicken lipoproteins was found to be different from that of mammals (Chapman *et al.*, 1977; Perry *et al.*, 1985) chicken apoproteins must perform functions equivalent to those of their mammalian counterparts (Griffin and Hermier, 1988).

In both birds and mammals, dietary lipid is hydrolysed in the intestine by the action of pancreatic lipase and the products, NEFA and monoglycerides, are absorbed by the mucosal cells lining the intestine. However, differences exist in the transport of exogenous dietary lipid from the intestine of birds and mammals. In mammals, the absorbed NEFA are re-esterified and incorporated into chylomicron lipoproteins (150-300nm in diameter) which are secreted by the epithelial cells of the small intestine into the lymphatic system and enter the bloodstream via the thoracic duct. Because the intestinal lymphatic system is poorly developed in birds, those absorbed NEFA which are re-esterifed and incorporated into lipoproteins are secreted directly into the venous portal system (Noyan *et al.*, 1964). These triacylglycerol rich lipoproteins (150 nm diameter) are termed portomicrons due to their route of entry into the circulation (Bensadoun and Rothfield, 1972) and have a similar lipid composition to mammalian chylomicrons.

The absorbed NEFA which are not re-esterifed are also released into the portal vein to be complexed with albumin in the plasma (Sklan *et al.*, 1984). In birds up to 50% of the absorbed NEFA may enter the

bloodstream in this fashion while in rats the figure is not more than 10% (Griffin and Hermier, 1988).

Due to the short half life of portomicrons in the circulation, 3-4 minutes (Bensadoun and Kompiang, 1979) and the low level of lipid in standard poultry diets, most of the triacylglycerol present in the plasma of birds is found in very low density lipoproteins, VLDL, (Griffin and Hermier, 1988). In birds VLDL are synthesized solely in the liver and have a similar lipid and apoprotein composition to portomicrons but are of smaller size.

The hydrolysis of triacylglycerol from the core of VLDL or portomicron particles by the action of LPL is accompanied by the transfer of apolipoproteins, phospholipid and cholesterol to high density lipoproteins (Behr *et al.*, 1981). The density of the lipoproteins increases as more triacylglycerol is hydrolysed than protein transferred (Griffin and Hermier, 1988). VLDL appears to be the precursor of intermediate and low density lipoproteins in immature chickens, with the latter disappearing entirely from the plasma when lipoprotein hydrolysis is blocked with an anti-LPL antiserum *in vivo* (Behr *et al.*, 1981).

1.6.2 *LPL and fat deposition in birds*

LPL activity in avian adipose tissue is limited by substrate concentration at the VLDL range normally present in immature hen plasma (Griffin *et al.*, 1987). The rate of synthesis and secretion of portomicrons and VLDL into the circulation is therefore of primary importance in controlling the rate of incorporation of lipoprotein triacylglycerol into avian adipose tissue (Griffin and Hermier, 1988). Evidence of this relationship is provided by the observation that plasma VLDL concentrations in fully-fed broilers correlates closely (r=0.7) with abdominal and total body fat content (Griffin *et al.*, 1982; Whitehead and Griffin, 1982). Divergent selection for high and low plasma lipoprotein concentration has produced fat and lean broiler lines (Whitehead, 1988). Following 10 generations of selection, a 20 fold difference in plasma VLDL concentration between the lines was produced which was due mainly to differences in the rate of VLDL synthesis and secretion from the liver (Griffin *et al.*, 1989; Griffin *et al.*, 1991a). Studies on the fate of VLDL triacylglycerol in these lines found a 2.5-fold greater uptake into the

abdominal fat pad of the fat line by comparison to the lean line (Griffin *et al.*, 1989). However, this was found not relate to LPL levels as a lower total LPL activity per pad was measured in the abdominal fat of the fat line birds. Similarly, although a higher LPL activity was found in the muscle tissues of the lean line birds this did not result in a higher percentage of the VLDL triacylglycerol being oxidized to CO_2 *in vivo* (Griffin *et al.*, 1989).

The role of LPL in plasma lipoprotein metabolism has also been studied in lean and fat broiler lines produced by divergent selection for abdominal fat pad weight (Leclercq *et al.*, 1980). In these lines a 2-fold greater uptake of VLDL triacylglycerol into the abdominal fat pad of the fat line birds, correlated with a 2-fold increase in the total LPL activity of the abdominal fat pad of these birds (Hermier *et al.*, 1989). Lemarchal *et al.* (1988) concluded that the differences in fatness in these lines were not due to differences in the turnover of adipose tissue triacylglycerol because the half life of the triacylglycerol was found to be similar in the adipose tissue of the 2 lines.

Studies have also been made on the plasma lipoprotein kinetics of rapidly growing broiler chickens which have more than twice the proportional body fat content of layer chickens. In a recent study, Griffin *et al.* (1991b) found that the total LPL activity present in the abdominal fat pad was 16-fold higher in broiler than in layer chickens. However, the percentage of radiolabelled VLDL taken up by the abdominal fat pad was similar in each case. In relation to the total tissue LPL activity, the uptake of NEFA derived from VLDL triacylglycerol into the abdominal fat pad of layers was high and occurred at 4 times the rate of deposition, while in broilers it accounted for only 65-85% of fat accumulation. In contrast to the conclusions of Lemarchal *et al.* (1988) following studies on selected lines of broilers, Griffin *et al.* (1991b) concluded that the relative fatness of broilers was due to the turnover of adipose tissue triacylglycerol in layers with a half-life of 2-3 days, while in broilers there was no evidence of significant turnover of adipose tissue triacylglycerol. Griffin *et al.* (1991b) also found that only about 20-30% of triacylglycerol from intravenously injected radiolabelled-VLDL was oxidized to CO_2 within 8 hours in either broiler or layer chickens. From this Griffin (1993) postulated that changes in tissue LPL activities resulting from selection would have a greater

impact on the partitioning of lipoprotein triacylglycerol between different fat depots rather than between oxidation and storage.

The LPL activity measured in post-heparin plasma is an indication of the total functional LPL available for lipoprotein hydrolysis in the whole animal. Despite the correlation, observed by Griffin *et al.* (1982) and Whitehead and Griffin (1982), between plasma VLDL concentration and abdominal and whole body fat in broiler chickens. No such correlation between measurements of post-heparin plasma LPL activity and either abdominal or total body fat has been reported (Guo *et al.*, 1988; Hermier *et al.*, 1989). Similarly, although a significant correlation was found between the total LPL activity per fat depot and fat pad weight, little or no correlation between specific activity of the enzyme and fat pad weight has been observed in broilers (Guo *et al.*, 1988; Pinchasov and Cahaner, 1991). Such studies concluded that measurement of LPL activity in adipose tissue biopsy samples or in post-heparin plasma cannot be used as a predictor of fatness in broilers.

In general, from these studies (with different conclusions being drawn from different lines of birds) it would appear that there is a lack of direct quantitative evidence that, in chickens, LPL plays a significant role in the partitioning of lipoprotein triacylglycerol between oxidation and storage.

1.6.3 *LPL and fat deposition in mammals*

Vernon and Clegg (1985) concluded from the results of several studies on the fate of radiolabelled lipoprotein triacylglycerol that in the fed state, mammalian adipose tissue LPL is limited by substrate concentration and does not work at maximal velocity. Despite this, early studies found a relationship between the uptake of NEFA derived from lipoprotein triacylglycerol into mammalian adipose tissue *in vivo* and total tissue LPL activity. Cryer *et al.* (1976) positively correlated nutritional changes in total tissue LPL activity with the accumulation of [^{14}C]-chylomicron triacylglycerol by the epididymal fat pad. Using radiolabelled triacylglycerol, Ramirez (1981) positively correlated the uptake of NEFA with eastradiol-induced changes in the LPL activity of rat adipose tissues. Similarly, Maggio and Greenwood (1982) correlated the elevated total tissue LPL levels of the fat depots of obese Zucker rats with an increased accumulation of radiolabelled triacylglycerol

NEFA. However, recent studies have found no such correlation between lipoprotein triacylglycerol uptake into rat muscle tissues (Brown and Layman, 1988) or human adipose tissues (Marin *et al.*, 1990) and total tissue LPL activities. These authors proposed that factors such as the blood flow to a given depot were equally as important as LPL levels. Eckel (1987) reviewing the evidence for a correlation between LPL activity and triacylglycerol NEFA uptake *in vivo* concluded that,: adipose tissue LPL activity has been associated with, but is insufficient to predict, serum triacylglycerol levels and/or alterations in triacylglyceride-rich lipoprotein triacylglyceride kinetics.

A loose association between adipose tissue LPL activity and the origin and/or maintenance of the obese state in both rats and man has been noted by Eckel, (1987; 1989). In the obese Zucker (*fa/fa*) rat, throughout development and in adult life, LPL when expressed per adipocyte or per fat pad has been found to be elevated (Gruen and Greenwood, 1981; Quig *et al.*, 1983). However, when LPL activity was expressed per g or per mg of protein increases (Wang *et al.*, 1984), decreases (Bertin *et al.*, 1985) or a lack of change (McNamara *et al.*, 1982) by comparison with the enzyme activity of non-obese controls was found. Similar inconclusive results have been found for the heparin-release of LPL activity from adipose tissue segments from obese humans which has been shown to be increased or unchanged when compared to controls, depending on the manner in which enzyme activity was expressed (Arner *et al.*, 1982; Bosello *et al.*, 1984).

A critical, initiating role for LPL in the accumulation of lipid by adipose tissue has however been found following lipectomy (Roth *et al.*, 1981) or refeeding after a prolonged fast (Fried *et al.*, 1983). In both cases enzyme activity increased before lipid loading occurred and in the latter case, enzyme activity rose to twice their prefasting values before returning to the initial levels on restoration of adipocyte cell size. From this it was suggested that LPL may play a role in regulating the uptake of lipoprotein triacylglycerol to reach a predetermined adipocyte cell size. However, no increase in LPL activity has been reported to precede the increase in adipocyte cell size or adipose tissue hypertrophy, associated with obesity. Eckel (1989) suggested that apparent increases in the levels of LPL activity in adipose tissue of obese animals preserves rather than causes the condition.

In conclusion, although the hydrolysis of lipoprotein triacylglycerol by LPL is a prerequisite for the uptake of triacylglycerol NEFA by tissues, there appears to be little direct quantitative evidence for a significant correlation between LPL levels and the deposition of triacylglycerol in the fat depots of either birds or mammals. It is therefore probable that several factors other than the level of functional LPL are operating in the partitioning of lipoprotein triacylglycerol between tissues. Because adipose tissue LPL does not work at maximal velocity in the fed state due to limiting substrate concentrations (Vernon and Clegg, 1985; Griffin *et al.*, 1987), this may include any processes which increase the time spent by individual lipoproteins at the capillary bed of a given tissue or which regulate the concentration of lipoproteins delivered to a tissue or fat depot. For example, blood flow has been found to vary between fat depots (Herd *et al.*, 1968) and depending on the species studied, shows nutritional- (West *et al.*, 1989), diurnal- (Herd *et al.*, 1968) or exercise- (Larsen *et al.*, 1981) related changes.

1.7 *Aims of the project*

Excessive fat deposition in broiler chickens has increased over the last 30 years due to selection on the basis of faster growth rate, a characteristic which is associated with an increase in fat deposition (Cahaner and Nitsan, 1985; McCarthy and Siegel, 1983). It is not unusual for fat to constitute between 15 and 20% of the total body weight of modern broiler chicken strains (Leenstra, 1986). Much of this body fat is lost during processing, reducing the meat yield and resulting in a loss to the producer. Fat deposition in chickens is influenced by genetic, dietary and environmental factors. The role played by LPL in the genetic susceptibility of broiler chickens to excessive fat deposition is unclear. Broiler chickens are a particularly useful model system for the study of the role of LPL in plasma lipoprotein kinetics and fat deposition. This is due in part, to their rapid growth and rate of fat deposition in early life (Scanes, 1991) and also because lipid accumulating in chicken adipose tissue is derived, via plasma lipoproteins, predominantly from lipogenesis in the liver or from the diet rather than as a result of *de novo* fatty acid synthesis within the adipocytes themselves (Griffin and Hermier, 1988). Comparative studies are also possible due to the availability of selected lines which differ considerably in body fat composition (Leclercq *et al.*, 1980; Griffin *et al.*, 1982).

From this review it can be seen that although the molecular structure and interactions of LPL have been well characterised, many unanswered questions on the transportation and regulation of LPL activity (both functional and non-functional) in the whole animal remain. Little information is available on the physiologically active pool of LPL activity, present at the capillary endothelium, in chickens (or other species) and its role in the partitioning of lipoprotein triacylglycerol between storage and oxidation or its role in the growth and maturation of tissues during development. The effect of enzyme fluxes through the tissue compartments on functional enzyme activity and the factors regulating enzyme translocation are also largely uncharacterised.

The lack of information on the functional pool of LPL activity *in vivo* results from the absence of a reliable method for its assessment. Enzyme activity measured in acetone diethyl-ether powders or after extraction of tissue homogenates in the presence of detergents represents the level of total tissue LPL activity, consisting of both intracellular and extracellular pools. Incubation of tissue segments in heparin-containing medium (Iverius and Brunzell, 1985) may cause the release of enzyme from several different compartments within the tissue. Using this method, different incubation conditions can lead to the release of varying amounts of LPL and losses of enzyme activity released into the medium over the incubation period cannot be excluded.

The overall aim of the present project was to develop techniques to quantify the pools of LPL activity (especially the functional pool) that exist within chicken tissues *in vivo*. Nutritional and developmental changes in the level and distribution of LPL within chicken tissues were studied using the techniques of: heparin-release of enzyme activity from the isolated perfused heart; the use of an iodinated anti-LPL monoclonal antibody *in vivo* to measure LPL protein available at the capillary bed of individual tissues; the fate of [^{14}C] labelled VLDL and semi-quantitative immunohistochemistry.

CHAPTER 2

THE PURIFICATION OF CHICKEN LIPOPROTEIN LIPASE

2.1 INTRODUCTION

The overall aim of the present project was the the immunoquantitation and immunolocalization of functional chicken LPL at the capillary endothelium. Samples of pure LPL were therefore required as an immunogen for the production of both polyclonal and monoclonal antibodies and for their subsequent detection and assessment by ELISA. Because the antibodies were required for studies in the perfused heart model system and also for use *in vivo*, the preservation of the enzyme in its native state during the purification procedure was essential for its potential use as an immunogen. In common with many other proteins, the reaction of LPL with specific antibodies has been found to be conformation dependent (Friguet *et al.*, 1989). A number of previous studies have however, reported the production of polyclonal antibodies which are more reactive towards the denatured conformation of LPL than that of the native species (Olivecrona and Bengtsson, 1983). Indeed, Peterson, *et al.*, (1992) characterized a monoclonal antibody which reacted only with the denatured species of human and bovine LPL and suggested that the antibody was specific for an epitope previously hidden but exposed by a conformational change in the enzyme used as an immunogen.

The first stage in the purification of LPL from tissue sources in most published protocols has been the production of acetone diethyl-ether dried powders of the tissue. This is due to the stability of LPL activity during and following acetone diethyl-ether treatment whereas other lipases present (eg: hormone sensitive lipase) are inactivated by the procedure (Palmer and Kane, 1983). It has been proposed that the high level of LPL activity measured in such powders was due to the release of membrane enclosed (cryptic) enzyme by the delipidation procedure (Chohan and Cryer, 1978).

The original observation that bovine milk LPL interacted strongly with immobilized heparin (Olivecrona *et al.*, 1971) has led to the use of affinity chromatography, using heparin as the ligand, for the production

of high specific activity preparations of bovine milk LPL. Since its introduction, this procedure has also been used widely to purify LPL from many tissue sources. However, although the heparin affinity chromatography step produced significant levels of purification, it has been followed by other sequential chromatography stages in order to purify the enzyme to homogeneity. For example, LPL was purified to homogeneity from pig adipose tissue by Bensadoun *et al.* (1974) using two sequential affinity chromatography steps. In this case, the first step used heparin covalently linked to Sepharose and the second concanavalin-A also covalently bound to Sepharose, the latter being employed because of the strong affinity between the heavily glycosylated LPL and the affinity ligand. To achieve the purification of LPL from human milk however, Zechner (1990) used hydrophobic interaction chromatography on phenyl-Sepharose as the second chromatography step. By contrast with such protocols, for the purification of LPL to homogeneity from post-heparin plasma, a hydroxylapatite column was introduced between an initial heparin-Sepharose affinity step and either a concanavalin-A (Hayashi *et al.*, 1986) or phenyl-Sepharose affinity column (Cheng *et al.*, 1985). The hydroxylapatite column was useful in this context because of the co-purification of large quantities of plasma-derived antithrombin III with LPL during the heparin-Sepharose affinity chromatography stage.

There have been four previously reported purifications of LPL from chicken adipose tissue and the characteristics of the enzyme produced in each case are shown in Table 2.1. In all of these cases, LPL has been purified from adipose tissue using essentially the procedure first described by Bensadoun *et al.* (1974) ie: chromatography on heparin-Sepharose followed by chromatography on concanavalin A-Sepharose. In this Chapter, the procedures available for the purification of chicken LPL were evaluated to ensure that enzyme of high specific activity and native conformation could be purified routinely in the quantities required for immunization and the screening of antibodies. An initial objective was to determine the most appropriate tissue source of LPL, with chicken heart and abdominal fat being investigated, both having high levels of enzyme activity (Guo, *et al.*, 1988; Griffin, *et al.*, 1991b). A second objective was to compare the use of concanavalin-A affinity chromatography and phenyl-Sepharose hydrophobic interaction chromatography with respect to optimizing the overall yield and specific

activity of the LPL that was recovered.

Table 2.1 Characteristics of chicken LPL preparations reported previously.

Tissue source	Molecular Weight (KDa)	Specific Activity (units*/mg protein)	Reference
Adipose	60	10,815	Cheung *et al.* (1979)
Adipose	60	19,600	Gershenwald *et al.* (1985)
Adipose	60	7,385	Kompiang *et al.* (1976)
Adipose	73	13,312	Bensadoun & Kompiang (1979)

* µmoles NEFA released per hour.

2.2 MATERIALS AND METHODS

2.2.1 *Preparation of acetone diethyl-ether powders*

Chicken hearts were obtained following the evisceration process on a commercial slaughter house line and on collection they were immediately placed into liquid nitrogen. Abdominal fat pads were removed from 6-8 week old broiler chickens that had been killed by cervical dislocation. This tissue was also frozen in liquid nitrogen immediately following collection. Both tissues were stored at -70°C for up to three months before use without any loss of activity.

The method adopted for the preparation of acetone diethyl-ether powders from cardiac tissue was essentially that described by Borensztajn *et al.* (1970). For this, frozen hearts were crushed and homogenized in 50 mM NH_3/NH_4Cl buffer, pH 8.1, (0.5 g/ml) at 4°C using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The resultant homogenate was then poured into ice cold acetone (10 ml/g wet weight of tissue) and the precipitate that formed allowed to flocculate for 10 min. The material was then filtered through Whatman filter paper No1 (Whatman Int Plc, Maidstone, Kent) using a Buchner filter funnel. The protein-containing precipitate was then washed on the filter paper using acetone (10 ml/g original wet weight of tissue) and diethyl-ether (10 ml/g original wet weight of tissue) at 20°C.

For the production of acetone diethyl-ether powders from fat pads, the frozen tissue was crushed and homogenized using a Polytron homogenizer directly into ice cold acetone (20 ml/g wet weight of tissue) and stirred on a magnetic stirrer for 30 min at 0°C. The protein precipitate which formed was filtered, resuspended in ice cold diethyl-ether (10 ml/g wet weight of tissue) and stirred on a magnetic stirrer for a further 30 min at 0°C. The precipitate was finally collected by filtration through Whatman filter paper No1. and residual diethyl-ether present in the tissue powders was rapidly removed under vacuum.

The tissue powders were stored for up to a week at -70°C without loss of activity.

2.2.2 *Measurement of LPL activity*

LPL activity was measured using Intralipid (Kabi-Vitrum Ltd, Uxbridge, UK.), a commercially available phospholipid-stabilised triacylglycerol emulsion, as substrate (Guo *et al.*, 1988). Any non-esterified fatty acids (NEFA) present in the Intralipid were removed before use by incubation with NEFA depleted BSA (produced by the method described in Appendix 1). This was achieved by mixing 7 ml of Intralipid with 21 ml of 50 mM Tris/HCl buffer, pH 8.0, containing 2% (w/v) NEFA depleted BSA , 2 mM $CaCl_2$ and incubating the mixture at 37°C for 1 hour. The mixture was then centrifuged at 20,000 *g* av for 20 min with the treated Intralipid being recovered by aspiration of the floating layer and resuspended in 0.9% (w/v) saline to give the initial volume of 7 ml. The treated Intralipid was activated by incubation for 1 hour at 37°C with 3 volumes of immature broiler serum, which serves as a donor of the LPL-activator protein, apo CII (Olivecrona and Bengtsson-Olivecrona, 1987). The enzyme activity assay mixture was prepared by adding an equal volume of 250 mM Tris/HCl buffer, pH 8.0, containing 10% (w/v) NEFA depleted BSA and 10 mM $CaCl_2$ to the activated Intralipid. For the determination of enzyme activity, duplicate 20, 40 and 60 µl aliquots of varying dilutions of enzyme source ie: homogenate, supernatant or column eluant were incubated with 100 µl of enzyme activity assay mixture together with 50 µl of 20 mM Tris/HCl buffer, pH 8.0, containing 20 µg of heparin/ml. The enzyme reaction was allowed to proceed for 30 min at 37°C with continuous shaking. Reactions were then stopped by the addition of 2.4 ml of 100 mM glycine buffer, pH 2.7. The NEFA released were extracted using 3.2 ml of di-n-butyl-

ether/ethanol/heptane (225:150:25) and measured as described by Bowyer *et al.* (1978) using a continuous flow Autoanalyser (Technicon Corp., Tarrytown, New York) The assay reagents were freshly prepared as described in Appendix 2. Briefly, the NEFA present in the upper organic phase, obtained from the extraction of assay samples, were converted to a copper-soap complex. The amount of copper present in this complex was determined using a colorimetric reaction produced by the addition of 1,5-diphenylcardazide to the samples. The absorbance of the samples at 550 nm was then determined using a tubular flow cell colorimeter (Technicon Corp.).

From a stock soln of palmitic acid in chloroform (1 µmole/ml), standards containing 0.5, 0.4, 0.3, 0.2, 0.1, 0.0 µmoles of NEFA were prepared and treated in the same manner as assay tubes. NEFA release in the assays was found to be linear over the incubation period and one unit of LPL activity was defined as the release of 1 µmole of NEFA per hour. With each batch of assays, control tubes were included in which the enzyme source was omitted or inhibited by the addition of 1.0 M NaCl. In these cases no significant levels of NEFA release were detected.

2.2.3 *Solubilization of LPL activity from chicken heart acetone diethyl-ether powders*

Soluble extracts of acetone diethyl-ether powders of chicken cardiac tissue were produced by homogenizing the powders (30 mg/ml) in either 10 mM phosphate buffer, pH 6.5, containing 1.2 M NaCl and 30% (v/v) glycerol or 5 mM sodium barbitone buffer, pH 7.5, containing 0.1% Triton X-100 and 30% (v/v) glycerol using a Polytron homogenizer. Each homogenate was then stirred on a magnetic stirrer continuously at 4°C for 30 min and then centrifuged at 30,000 *g* av at 4°C for 15 min. The resultant supernatant was removed by aspiration and the pellet was resuspended in an equal amount of the original buffer. LPL activity was then determined in duplicate in all three samples (homogenate, supernatant and pellet) in order to ascertain the efficiency of LPL extraction from the powder.

2.2.4 *Purification of LPL*

The initial step in the purification of LPL was accomplished using the method of Bensadoun *et al.* (1974) which employs affinity chromatography on heparin-Sepharose. The heparin-Sepharose matrix

was prepared by the method of Iverius (1971) using cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co.) and porcine mucosal heparin as described in Appendix 3. Extraction and solubilization of LPL was achieved by homogenising the acetone diethyl-ether powders (50mg/ml) in 1.2 M NaCl in 10 mM phosphate buffer, pH 6.5, containing 30% glycerol (Buffer A), as described in section 2.2.3. The homogenate was then centrifuged at 30,000 *g* av at 4°C for 15 min. The resultant supernatant was removed by aspiration and then incubated, with occasional mixing, for 30 min together with heparin-Sepharose (40 ml of packed gel per 150 ml of extract) previously equilibrated with Buffer A. The gel was then packed under gravity into a glass column (2.4 cm by 20 cm) and washed with Buffer A containing 0.9 M NaCl until the measured absorbance of the eluant at 280 nm, approached zero. At this point Buffer A containing 2.0 M NaCl was applied to the column at a flow rate of one column volume per hour in order to elute the bound LPL.

Fractions eluted from the heparin-Sepharose column were assayed for LPL activity and those which were found to be rich in enzyme activity were pooled and adjusted to 1.0 M NaCl prior to application (at a flow rate of 10 ml/hour) onto a 5 ml column of concanavalin A-Sepharose (Sigma Chemical Co.), previously equilibrated with Buffer A containing 1.0 M NaCl, 1.0 mM $MnCl_2$ and 1.0 mM $CaCl_2$. The column was then washed with 10 column volumes of Buffer A containing 1.0 M NaCl and subsequently eluted, at a flow rate of 20 ml/hour with Buffer A containing 0.6 M methyl α-D-mannopyranoside and 0.6 M methyl α-D-glucopyranoside (Parkin *et al.*, 1982).

Chicken cardiac LPL, partially purified by either heparin-Sepharose affinity chromatography or sequential heparin-Sepharose and concanavalin A-Sepharose affinity chromatography, was loaded at a flow rate of 10 ml/hour directly on to a 10 ml column of phenyl-Sepharose CL-4B (Sigma Chemical Co.), the column having previously been equilibrated with the buffer used to elute the enzyme from the previous column (heparin-Sepharose or concanavalin A-Sepharose). The phenyl-Sepharose column was then washed with 10 column volumes of 5 mM phosphate buffer, containing 20% (v/v) glycerol, before elution, at a flow rate of 20 ml/hour, with buffer containing 4 mM SDS, 50 mM octyl-ß-D-glucopyranoside (Cheng *et al.*, 1985) or deoxycholate, 5 mg/ml, (Zechner, 1990).

Fractions containing LPL activity were concentrated under nitrogen in an Amicon ultrafiltration cell (Amicon Corp, Lexington, Mass, USA) using a PM30 membrane, diluted with Buffer A to reduce the NaCl concentration to below 0.1 M and then reconcentrated before being stored at -70°C. The protein content of samples at each stage of the purification procedure was determined by the method of Bradford (1976) using a Coomassie (blue) protein assay reagent (Pierce, Rockford, Il, USA). Samples were then subject to polyacrylamide gel electrophoresis (section 2.2.5) to check the purity of the preparation before its use as an immunogen (section 3.2.1/2).

2.2.5 *Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate* (SDS-PAGE)

Vertical polyacrylamide gel electrophoresis was performed using the buffer system described by Laemmli (1970). In brief, gels were prepared using dilutions of a stock acrylamide/bis solution containing 40% (w/v) acrylamide and 1% (w/v) N,'N'-methylenebisacrylamide. Slab gels, of 12% (w/v) acrylamide were run on a Protean II system (Biorad, Richmond, California, USA). Gels containing linear gradients of 8-20% (w/v) acrylamide, were run on a dual mini slab gel apparatus (Atto Corporation, Tokyo, Japan). A 5% (w/v) acrylamide stacking gel was used in both systems and the final buffer concentrations were as follows: 0.125 M Tris/HCl, pH 6.5, containing 0.1% (w/v) SDS in the stacking gel and 0.375 M Tris/HCl, pH 8.6, containing 0.1% (w/v) SDS in the resolving gel. The gels were polymerized by the addition of 10% (v/v) N,N,N,'N'-tetramethylethylenediamine and 7.5% (w/v) ammonium persulphate solution. The composition of the solutions used in the preparation of the gels are given in Table 2.2.

Samples for electrophoresis (10-20 µl) were mixed with an equal volume of sample buffer which consisted of 1.0 ml stacking gel buffer, 0.2 ml of 2-mercaptoethanol, 0.4 ml glycerol, 80 mg SDS, 0.3 ml distilled water and 0.1 ml 0.05%w/v bromophenol blue (Sigma Chemical Co.). The mixture was heated to 100°C for 5 min and transferred to a well in the stacking gel.

Electrophoresis was carried out at a constant current of 30 mA with an electrode buffer of 0.25 M Tris/HCl, pH 8.3, containing 0.192 M glycine

and 0.1% (w/v) SDS and was terminated when the bromophenol blue tracking dye had travelled to within 0.5 to 1.0 cm of the lower edge of the gel. The gels were then stained at 20°C in a solution of 0.125% (w/v) Coomassie Brilliant Blue (Sigma Chemical Co.) in 10% (v/v) acetic acid, 25% (v/v) isopropanol. The gels were subsequently destained using repeated changes of 8% (v/v) methanol, 10% (v/v) acetic acid in distilled water.

Table 2.2 Table of solutions used to prepare SDS-PAGE gels.

Acryl-amide (ml)	Gel buffer (ml)	Stacking gel buffer (ml)	H_2O (ml)	TEMED (µl)	APS (µl)	Final vol (ml)	Final % acrylamide
1.0	2.5	--	1.5	20	20	5.04	8
2.5	2.5	--	--	10	10	5.02	20
7.5	12.5	--	5.0	75	75	25.15	12
0.625	--	2.5	1.725	50	50	4.95	5 (SG)

Composition of stock solutions used to prepare SDS-polyacrylamide gels.
Acrylamide: 40% (w/v) acrylamide, 1% (w/v) N 'N' methylenebisacrylamide.
Resolving gel buffer: 0.75 M Tris/HCl, pH 8.6, containing 0.2% (w/v) SDS.
Stacking gel buffer: 0.25 M Tris/HCl, pH 6.5, containing 0.2% (w/v) SDS.
TEMED: NNN'N' Tetramethylethylenediamine (TEMED), 10% (w/v) H_2O.
APS: Ammonium persulphate, 75 mg/ml in H_2O. SG: stacking gel

The molecular weight of unknown proteins was estimated by comparison of their migration to that of protein standards of known molecular weight. This comparison was done graphically by plotting the relative mobility of each standard (ie: distance moved in relation to the tracking dye) against the $\log_{10}$ of their respective molecular weight. Two commercially available mixtures of standard protein markers of known molecular weight were used in the work described in this Chapter. The molecular weights of the components of Electran molecular weight markers (Sigma Chemical Co.) were: bovine serum albumin, 66.0 KDa; ovalbumin, 45.0 KDa; glyceraldehyde-3-phosphate dehydrogenase, 36.0 KDa; carbonic anhydrase, 29.0 KDa; trypsinogen, 24.0 KDa; trypsin inhibitor, 20.1 KDa and lactalbumin, 14.2 KDa. A linear relationship was obtained by plotting the $\log_{10}$ of the molecular weight of the standards against their electrophoretic mobility on slab gels made up of 12% (w/v) acrylamide. The molecular weights of the components of the second standard mixture Rainbow molecular weight markers (Amersham Int

plc, Amersham, Bucks, UK) were: myosin, 200 KDa; phosphorylase b, 97.4 KDa; bovine serum albumin, 69 KDa; ovalbumin, 46 KDa; carbonic anhydrase, 30 KDa; trypsin inhibitor, 21.5 KDa and lysosyme, 14.3 KDa. Plots of the $\log_{10}$ of the molecular weight of the standards against their electrophoretic mobility, on linear gradient polyacrylamide gels (8-20% w/v), typically gave a curve identical to that given by the manufacturers. The molecular weights of sample proteins were found to be similar when determined using the two standard mixtures.

2.3 RESULTS

2.3.1 *The solubilization of chicken LPL from acetone diethyl-ether powders*

All previous reports of the isolation of chicken LPL have used abdominal fat as the initial tissue source, probably because of the high level of LPL activity present in the tissue (Guo *et al.*, 1988; Griffin *et al.*, 1991b). However, because chicken hearts also readily produced high activity powders and were easily collected in large quantities from a commercial slaughterhouse line, it was this tissue that was initially investigated as a suitable alternate source of LPL.

Several buffers have been described for the solubilization of LPL from acetone diethyl-ether powders. The effectiveness of a high salt containing buffer (as described by Bensadoun *et al.*, 1974) and a detergent containing buffer (as described by Parkin *et al.*, 1982) for the solubilization of chicken cardiac LPL was investigated. Soluble extracts of chicken cardiac powders were produced using either 1.2 M NaCl in 10 mM phosphate buffer, pH 6.5, or 0.1% Triton X-100 in 5 mM sodium barbitone buffer, pH 7.5, as described in section 2.2.3. Both buffers contained 30% (v/v) glycerol in order to help stabilize enzyme activity. The results are summarised in Table 2.3.

Table 2.3 The effectiveness of high salt- and detergent- containing buffers in the solubilization of LPL activity from acetone diethyl-ether powders of chicken cardiac tissue.

Buffer additive	Activity in homogenate (units*/ml)	Activity in supernatant (units*/ml)	Activity in pellet (units*/ml)	Percentage extraction
1.2 M NaCl	12.30	10.29	2.94	84
0.1% Triton	9.84	1.95	6.24	20

The results represent mean data derived from two extractions of 30 mg/ml of chicken cardiac tissue acetone diethyl-ether powders, using the method described in section 2.2.3. The tissue pellet was resuspended in an equal volume of buffer to assay for remaining LPL activity (*μmoles NEFA release per hour).

Extraction in the presence of buffer containing 1.2 M NaCl was found to be approximately 4 times as effective as 0.1% Triton X-100 in the extraction of chicken cardiac LPL activity from acetone diethyl-ether powders and was therefore used in all subsequent purifications.

2.3.2 *The purification of LPL from cardiac tissue*

At each stage of the purification procedure described in section 2.2.4, samples were taken for determination of LPL specific activity and assessment of the protein constituents by SDS-PAGE.

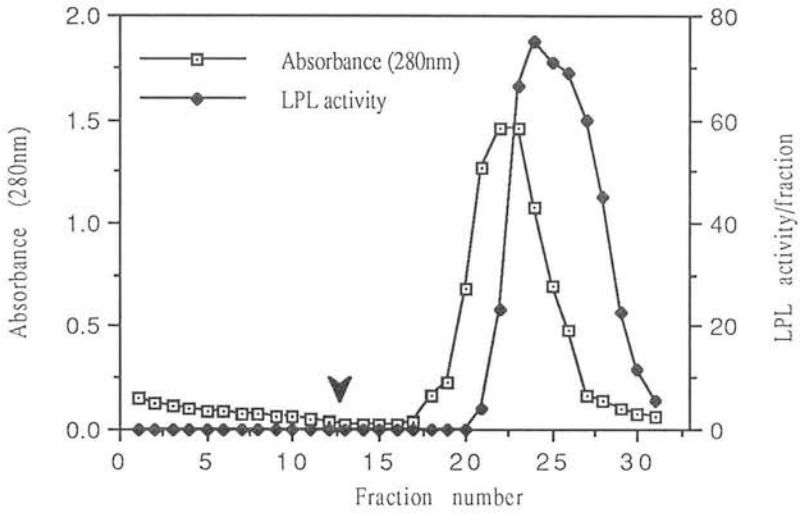
During the first stage of the purification using heparin-Sepharose affinity chromatography, the LPL activity peak was consistently eluted slightly later than the main protein peak following the addition of buffer containing 2.0 M NaCl to the heparin-Sepharose column. A representative elution profile is shown in Fig 2.1. The mean specific activity of the LPL following this stage in the purification procedure was 896 units/mg (Table 2.4, mean of 2 typical purifications) which represented a 2240-fold purification when compared to the starting material (cardiac tissue acetone diethyl-ether dried powder).

Table 2.4 Summary of the purification of LPL from chicken cardiac tissue

Stage	Total Protein (mg)	Total LPL Activity (units)	LPL Specific Activity (units/mg)	Yield of Step (%)	Overall Yield (%)	Fold Purification
Acetone/ether powder	14670	5868	0.4	--	100	1.00
Extract of acetone/ether powder	3210	4790	1.5	82	82	3.75
Eluate from heparin-affinity column	2.70	2420	896	51	41	2240
Eluate from concanavalin-A affinity column	0.12	1364	11366	56	23	28415
Eluate from phenyl-Sepharose column	0.05	984	19680	72	17	49200

LPL was purified from chicken cardiac tissue by the procedure described in section 2.2.4. LPL activity (μmoles NEFA released/hr) and protein levels were determined in the samples as described in sections 2.2.2 and 2.2.4 respectively. The data shown is the mean of two typical purifications and the average wet weight of cardiac tissue used was 117g.

Figure 2.1 Chromatography of the soluble extract chicken cardiac tissue acetone diethyl-ether dried powder on a heparin-Sepharose affinity column.



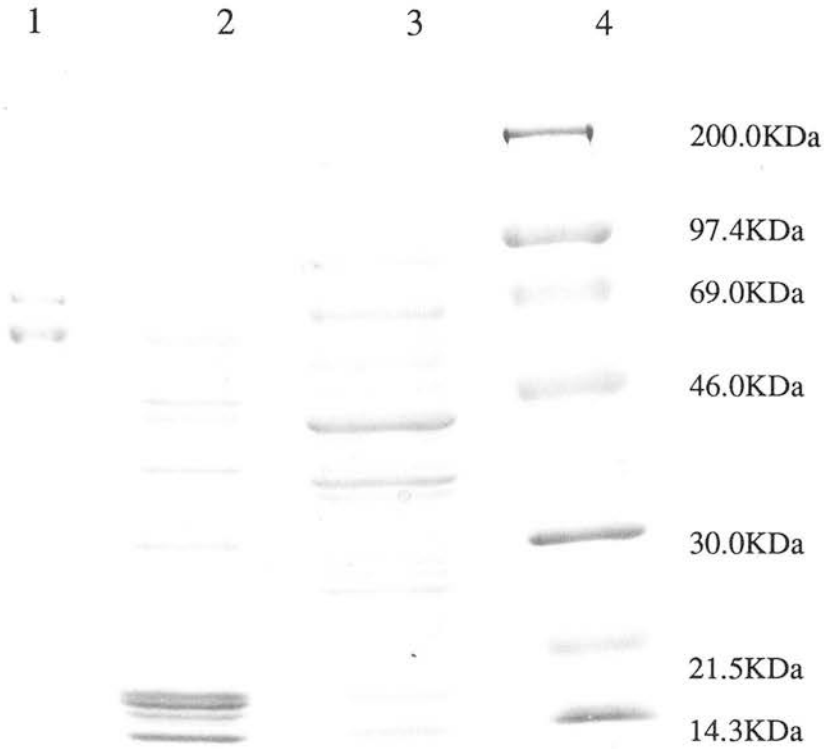
The measured absorbance at 280nm of the fractions eluted from a 80 ml heparin-Sepharose affinity column to which the soluble extract of chicken cardiac tissue acetone diethyl-ether dried powder had been applied, as described in section 2.2.4. The point at which buffer containing 0.9 M NaCl was replaced with buffer containing 2.0 M NaCl is indicated by an arrow. Those fractions (numbers 23-30, each of 7.5 ml) found to be rich in enzyme activity were pooled and retained for further chromatography steps.

Analysis of the protein components of heparin-Sepharose eluate by SDS-PAGE revealed that LPL was not a major component with the majority of protein constituents being found in the 14-20 KDa molecular weight range (Fig. 2.2).

Those fractions from the heparin-Sepharose column identified as being rich in LPL activity were loaded onto a concanavalin A-Sepharose column from which bound LPL was eluted as a broad peak of activity within 10 column volumes after the addition of 0.6 M methyl α-D-mannopyranoside and 0.6 M methyl α-D-glucopyranoside. The mean specific activity of the pooled eluate fractions from the concanavalin A-column was 11,366 units/mg (Table 2.4, mean of 2 typical purifications). Analysis of the protein constituents of the preparation by SDS-PAGE revealed the presence of equal quantities of 2 major component proteins with molecular weights of 61 KDa and 66 KDa (Fig. 2.2).

Experiments were also carried out to investigate the use of phenyl-

Figure 2.2 Polyacrylamide gel electrophoresis of protein samples obtained during the purification of chicken cardiac LPL by chromatography on sequential heparin-Sepharose and concanavalin A-Sepharose columns.



Samples of chicken cardiac LPL were obtained from various stages in the purification procedure described in section 2.2.4. The samples were analysed by SDS-PAGE under reducing conditions on an 8-20% linear gradient polyacrylamide gel, as described in section 2.2.5, with amounts of protein indicated per lane.
Lane 1 material eluted from the concanavalin A-Sepharose column (5 µg).
Lane 2 material eluted from a heparin-Sepharose column (15 µg)
Lane 3 extract of chicken cardiac tissue acetone/diethyl-ether dried powder (25 µg).
Lane 4 Rainbow molecular weight markers (Amersham Plc.)

Sepharose hydrophobic interaction chromatography as a possible alternative to the concanavalin-A affinity column.

In preliminary experiments, the method of Zechner (1990) using deoxycholate to elute the phenyl-Sepharose was found to give a low yield of recovered LPL. By contrast, the method of Cheng *et al.* (1985), using octyl ß-D-glucopyranoside and SDS to elute the bound LPL from the phenyl-Sepharose column, consistently gave a recovery of over 50% of the enzyme activity loaded onto the column (eg: Table 2.4). However, the material eluted from the phenyl-Sepharose column, using the method of Cheng *et al.* (1985), contained several contaminants of low molecular weight (10-14 KDa) which were evident on analysis by SDS-PAGE (Fig. 2.3). The mean specific activity of the latter preparation (5,033 units/mg, mean of 2 typical purifications) was also considerably lower than that obtained using concanavalin A-Sepharose as the column matrix.

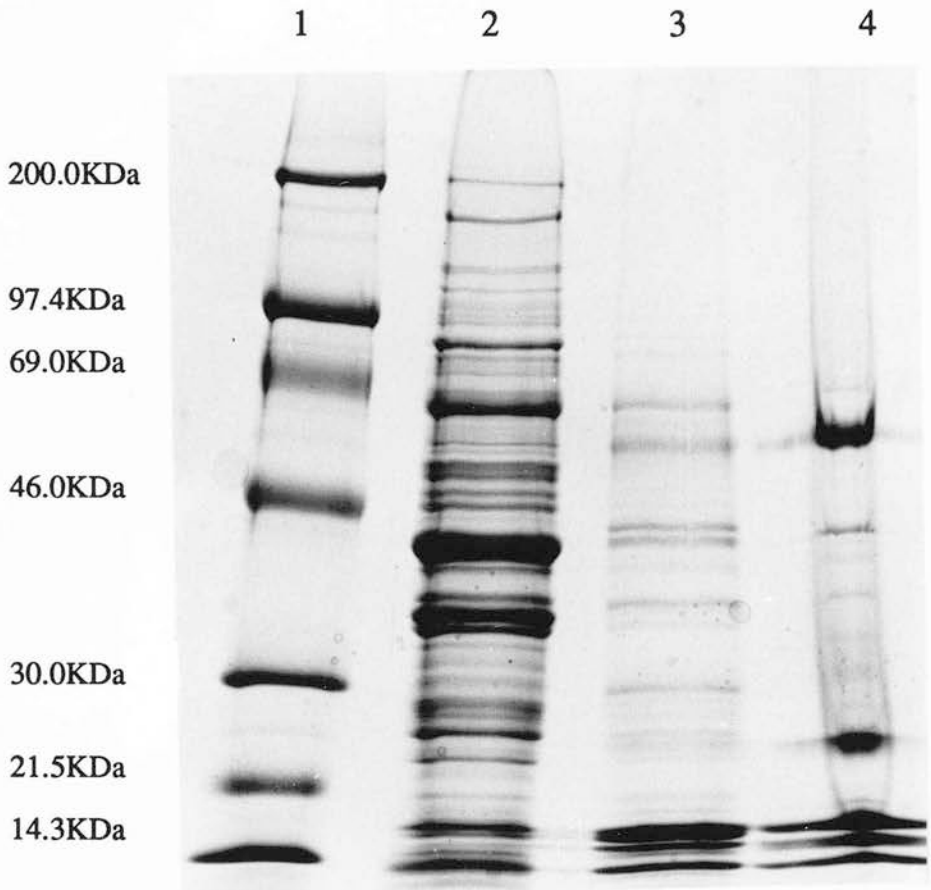
Neither purification procedure (ie: heparin-Sepharose chromatography followed by sequential chromatography on either concanavalin A-Sepharose or phenyl-Sepharose) resulted in the production of an homogenous preparation of chicken cardiac LPL. Because only a single common protein with a molecular weight of 61 KDa (the approximate molecular weight of chicken LPL, Table 2.1) was identified by SDS-PAGE in the LPL preparations produced by the two procedures, a purification procedure which included all 3 chromatography steps sequentially (ie: heparin-Sepharose, concanavalin A-Sepharose and phenyl-Sepharose) was adopted. LPL purified in this manner had a molecular weight of 61 KDa and a mean specific activity of 19680 units/mg. The enzyme had not been purified to homogeneity, because of the presence of minor protein components with apparent molecular weights of 35 KDa and 28 KDa, the former being in the highest concentration (Fig 2.4).

This purification procedure is summarized in Table 2.4 which gives the mean values of 2 typical purifications.

2.3.3 *The purification of LPL from adipose tissue*

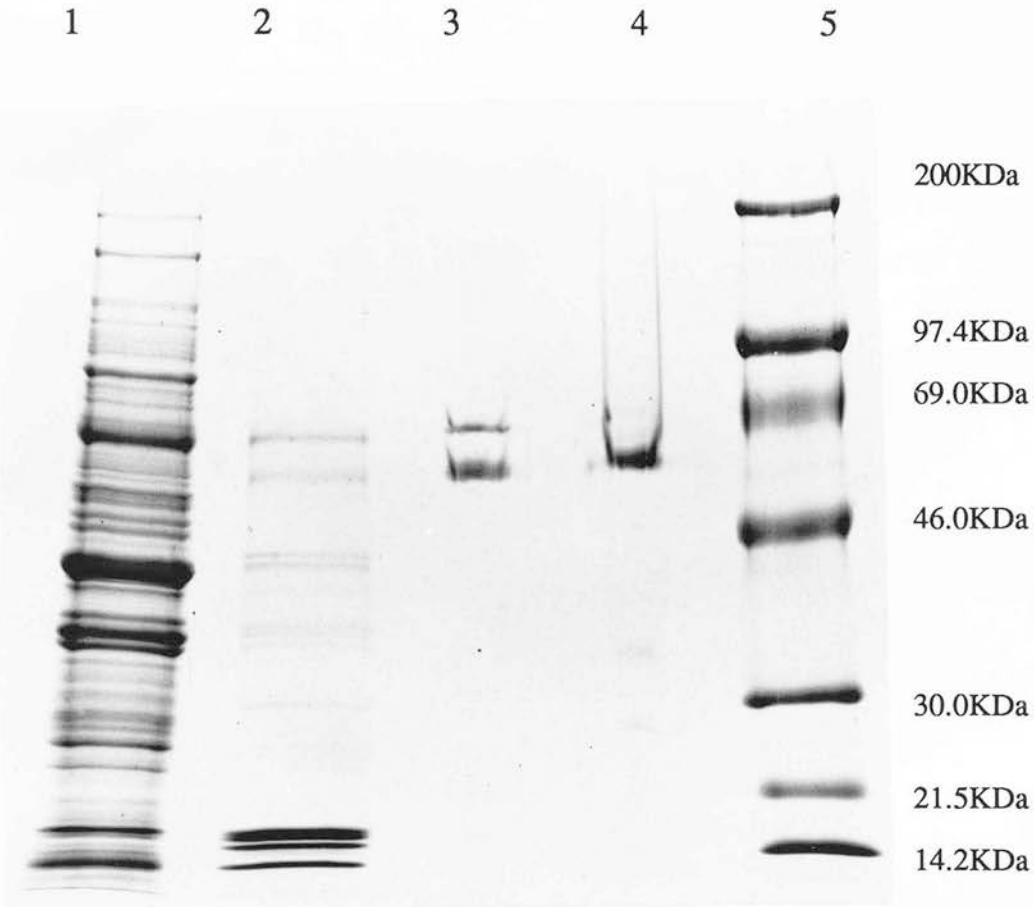
Much of the work described in section 2.3.2 for the purification of chicken heart LPL was repeated using chicken abdominal fat pads as the tissue source of the enzyme.

Figure 2.3 Polyacrylamide gel electrophoresis ofprotein samples obtained from the purification of chicken cardiac LPL by chromatography on sequential heparin-Sepharose and phenyl-Sepharose columns.



Samples of chicken cardiac LPL were obtained from various stages in the purification procedure described in section 2.2.4. The samples were analysed by SDS-PAGE under reducing conditions on an 8-20% linear gradient polyacrylamide gel, as described in section 2.2.5, with amounts of protein indicated per lane.
Lane 1. Rainbow molecular weight markers (Amersham Plc.).
Lane 2. extract of chicken cardiac tissue acetone/diethyl-ether dried powders (25 µg).
Lane 3. material eluted from a heparin-Sepharose affinity column (10 µg).
Lane 4. material eluted from a phenyl-Sepharose column (10 µg).

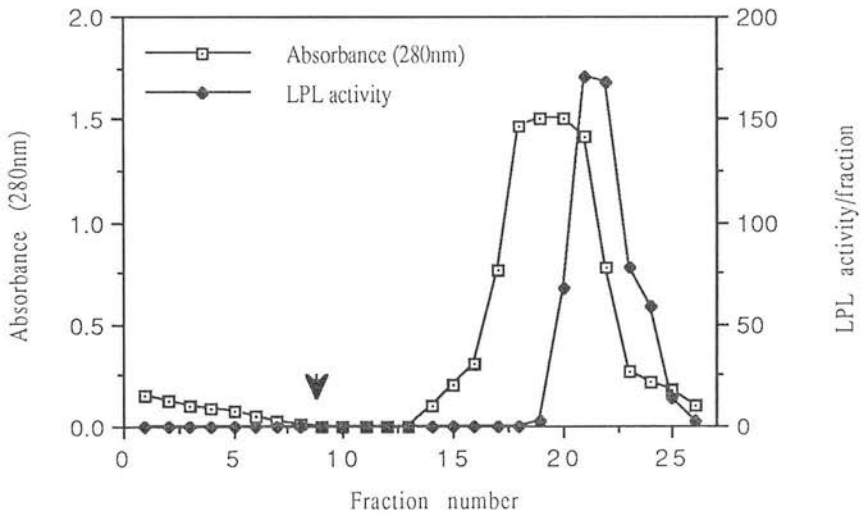
Figure 2.4 Polyacrylamide gel electrophoresis of samples obtained during the purification of chicken cardiac LPL using sequential heparin-Sepharose, concanavalin A-Sepharose and phenyl-Sepharose chromatography steps.



Samples of cardiac tissue LPL were obtained from each stage in the purification procedure described in section 2.2.4. The samples were analysed by SDS-PAGE under reducing conditions on an 8-20% linear gradient polyacrylamide gel, as described in section 2.2.5, with amounts of protein indicated per lane.
Lane 1. soluble extract of cardiac tissue acetone/diethyl-ether dried powder (25 µg).
Lane 2. material eluted from a heparin-Sepharose affinity column (10 µg).
Lane 3. material eluted from a concanavalin A-Sepharose affinity column (5µg)
Lane 4. LPL eluted from a phenyl-Sepharose column (5 µg).
Lane 5. Rainbow molecular weight markers (Amersham Plc.).

Adipose tissue was found to produce acetone diethyl-ether powders with specific activities twice those of cardiac tissue, ie: 0.97 units/mg and 0.40 units/mg respectively (Tables 2.4 and 2.5). As for chicken cardiac LPL, chicken adipose tissue LPL activity was consistently eluted slightly later than the main protein peak from the heparin-Sepharose column following the addition of 2.0 M NaCl. A representative elution profile is shown in Fig 2.5. The mean specific activity of 2 typical preparations at this stage in the purification procedure was 1,241 units/mg (Table 2.5) which represent a 1283-fold purification when compared to adipose tissue acetone diethyl-ether dried powder.

Figure 2.5 Chromatography of the soluble extract chicken adipose tissue acetone diethyl-ether dried powder on a heparin-Sepharose affinity column.



The measured absorbance at 280nm of the fractions eluted from a 40 ml heparin-Sepharose affinity column to which the soluble extract of chicken adipose tissue acetone diethyl-ether dried powder had been applied, as described in section 2.2.4. The point at which buffer containing 0.9 M NaCl was replaced by buffer containing 2.0 M NaCl is indicated by an arrow. Those fractions (numbers 21-26, each of 4.5 ml) found to be rich in enzyme activity were pooled and retained for further chromatography steps.

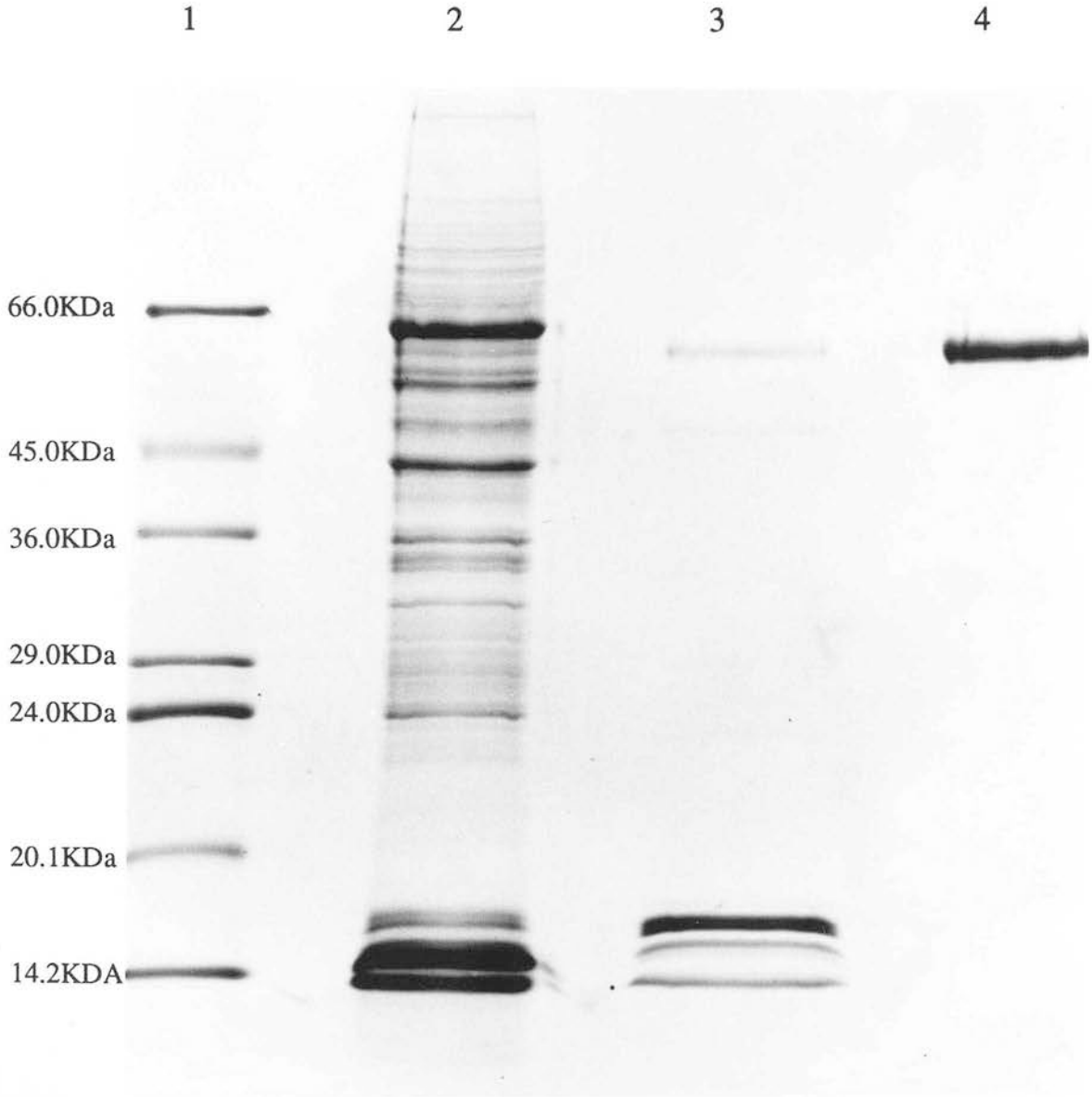
As for the purification of LPL from cardiac tissue when the heparin-Sepharose eluate was analysed by SDS-PAGE, proteins of low molecular weight 14-20 KDa were found to be the major components (Fig 2.6). Subsequent, chromatography on a sequential concanavalin A-column produced material with a mean specific activity of 17,582 units/mg (mean of 2 typical purifications) which represented a 18,182-fold purification for the overall scheme. When analysed by SDS-PAGE, the

Table 2.5 Summary of the purification of LPL from chicken adipose tissue

Stage	Total Protein (mg)	Total LPL Activity (units)	LPL Specific Activity (units/mg)	Yield of Step (%)	Overall Yield (%)	Fold Purification
Acetone/ether powder	6150	6980	0.97	--	100	1.00
Extract of acetone/ether powder	750	5950	7.90	85	85	8.17
Eluate from heparin-affinity column	2.20	2731	1241	46	39	1283
Eluate from concanavalin-A affinity column	0.14	2444	17582	89	35	18182

LPL was purified from chicken adipose tissue by the procedure described in section 2.2.4. LPL activity (μmoles NEFA released/hr) and protein levels were determined in the samples as described in sections 2.2.2 and 2.2.4 respectively.The data shown was the average of two representative purifications and the average wet weight of adipose tissue used was 199g.

Figure 2.6 Polyacrylamide gel electrophoresis of samples obtained during the purification of LPL from chicken adipose tissue.



Samples of chicken adipose tissue LPL were obtained from each stage in the purification procedure described in section 2.2.4. The samples were analysed by SDS-Page under reducing conditions on a 12% polyacrylamide gel, as described in section 2.2.5, with amounts of protein indicated per lane.
Lane 1. Electran molecular weight markers (Sigma Chemical Co).
Lane 2. extract of chicken adipose tissue acetone/diethyl-ether dried powder (40 µg).
Lane 3. material eluted from a heparin-Sepharose affinity column (15 µg).
Lane 4. material eluted from a concanavalin A-Sepharose affinity column (5 µg)

final preparation was found to comprise a single major constituent protein with a molecular weight of 61 KDa (Fig 2.6). The final preparation could be classified as being close to homogenous, with the presence of only a minor protein impurity with a molecular weight of 66 KDa (Fig 2.6). The purification procedure is summarized in Table 2.5, which gives the mean values of 2 typical purifications.

2.4 DISCUSSION

All the previously reported purifications of chicken LPL (Table 2.1) have used the high salt containing buffer described by Bensadoun *et al.* (1974) for the solubilization of LPL from acetone diethyl-ether powders. This buffer was found to be considerably more efficient in the solubilization of chicken LPL (Table 2.3) than the detergent containing buffer described by Parkin *et al.* (1982), for the solubilization and purification of rat cardiac LPL and was therefore used thereafter.

In the purification of both heart and adipose tissue LPL, heparin-Sepharose chromatography was highly successful in removing the high molecular weight components present in the soluble extracts of acetone diethyl-ether powders and the fold purification at this stage was the greatest in the overall schemes.

The final mean specific activity of preparations purified by heparin-Sepharose and concanavalin A-Sepharose was 17,582 units/mg for adipose tissue, which is in close agreement with the highest published value for chicken adipose tissue LPL of 19,600 units/mg (Gershenwald *et al.* (1985), whereas for heart tissue a lower value of 11,366 units/mg was obtained.

In both heart and adipose tissue, an impurity with an apparent molecular weight of 66 KDa was present in material eluted from the concanavalin-A column (Figs 2.2 and 2.6). It is possible that this protein is antithrombin III which also binds to both heparin (Danishefsky *et al.*, 1978; Bengtsson and Olivecrona, 1979b) and concanavalin-A (Denton *et al.*, 1981) and in the chicken has a reported molecular weight of 62 KDa (Amrani *et al.*, 1985). This possibility is strengthened by the fact that

the protein was found to be a major constituent of LPL preparations from cardiac tissue but a minor constituent of LPL preparations from adipose tissue, the former tissue being highly vascularised and contained large quantities of clotted blood on collection from a commercial slaughter house line. This protein was, however, effectively separated from chicken cardiac LPL by hydrophobic interaction chromatography on phenyl-Sepharose. In the purification of LPL from post-heparin plasma, Cheng *et al.* (1985) showed that LPL binds strongly to phenyl-Sepharose whereas antithrombin III shows no such interaction.

The final mean specific activity of chicken heart LPL following three sequential chromatography steps on heparin-Sepharose, concanavalin A-Sepharose and phenyl-Sepharose was 19,680 units/mg and this was similar to that of adipose tissue LPL preparations following heparin-Sepharose and concanavalin-A Sepharose columns. The minor protein constituents of 35 KDa and 28 KDa molecular weight present in the final preparation (Fig 2.4) are similar in molecular mass to immunoreactive components identified in previous studies by immunoblotting with anti-LPL antibodies. Kern *et al.* (1988); Socorro and Jackso, (1985) and Friedman *et al.* (1986b), all found that such minor LPL immunoreactive components increased at the expense of the level of mature LPL protein present in samples on prolonged incubation at 37°C. The components were therefore classified by these authors as degradative or proteolytic breakdown products of LPL rather than physiological derivatives of the enzyme. In the present study, the predominant 35 KDa protein was also demonstrated in chicken cardiac tissue homogenates (shown in section 6.3.2) by immunoblotting using an anti-chicken adipose tissue LPL polyclonal antibody (the production of which is described in Chapter 3). It seems probable therefore that this species was derived from the mature enzyme by the action of degradative processes occurring during the purification procedure.

The LPL purified from chicken adipose and heart tissue by the two procedures had identical molecular weights of 61 KDa and this value is in close agreement with previously reported values for chicken adipose LPL (Table 2.1).

The existence of tissue specific variants of LPL has been suggested by several early studies which found heart and adipose tissue LPL to have different physical or kinetic properties. For example, Fielding (1976) reported that rat muscle LPL had a higher affinity for lipoprotein substrates and a different Km value than the LPL from rat adipose tissue. However, later studies have found no tissue specific variations in the molecular mass or immunological reactivity of LPL. This has been demonstrated in the chicken (Guo, 1988), the rat (Soteriou, 1991) and in the guinea pig (Semb and Olivecrona, 1986) by the purification, immunoprecipitation or the immunoblotting of LPL from a variety of tissue sources for each species.

The overall yield for the two procedures was on average 17% for heart preparations and 35% for adipose preparations. Elution of the phenyl-Sepharose column required the use of detergent concentrations which Cheng *et al.* (1985) showed to increase the rate of loss of LPL activity and possibly cause the loss of the native configuration.

In conclusion, it was decided to use adipose tissue LPL preparations purified by the method of Bensadoun *et al.* (1974) for subsequent studies, as the high overall yield of the procedure allowed large quantities of LPL to be purified for the immunization of animals and screening of antibodies, while maintaining catalytic activity and the native conformation of LPL essential for the production of antibodies for *in vivo* and whole organ studies.

CHAPTER 3

THE PRODUCTION OF ANTI-CHICKEN LPL ANTIBODIES

3.1 INTRODUCTION

The ability to purify LPL to homogeneity, has led to the production of both polyclonal and monoclonal antibodies against the enzyme from a number of different species. Such anti-LPL antibodies have, for example, been used for the immunocytochemical localization of LPL (eg: Blanchette-Mackie *et al.*, 1989; Camps *et al.*, 1990), to quantify LPL protein by immunoassay (eg: Ikeda *et al.*, 1990; Kern *et al.*, 1988) and to block functional LPL activity both *in vitro* (Schotz *et al.*, 1977) and *in vivo* (eg: Kompiang *et al.*, 1976).

Two basic protocols have been used for the detection of anti-LPL antibodies. ELISA systems have been used successfully in, for example, the detection of hybridomas secreting anti-LPL antibodies (Goldberg *et al.*, 1986; Socorro and Jackson, 1985; Gershenwald *et al.*, 1985). In other contexts, the production of anti-LPL antibodies has also been assessed by their measured ability to inhibit LPL activity. In this latter case, Kompiang *et al.* (1976) and Schotz *et al.* (1977) used enzyme inhibition to assess the level of anti-LPL polyclonal antibodies present in serum samples following immunization, while Goldberg *et al.* (1986) used the method to screen hybridomas for the secretion of inhibitory anti-LPL monoclonal antibodies.

Using purified chicken adipose LPL as an immunogen, Kompaing *et al.* (1976) raised an inhibitory antiserum in rabbits, whereas Gershenwald, *et al.* (1985) produced several murine anti-chicken LPL monoclonal antibodies, two of which were inhibitory. The work in this Chapter describes the production and characterization of a sheep anti-chicken LPL polyclonal antibody and murine monoclonal antibodies raised against the same immunogen. To screen the sera of immunized animals and hybridoma cell supernatants, a non-competitive ELISA system was developed and optimized for the detection of anti-chicken LPL antibodies. For the further development of the current project, the identification of a monoclonal antibody capable of binding functional LPL in the perfused heart and *in vivo* was a requirement. For this the

antibody would have to recognise LPL in its native state and bind to the enzyme when bound to endothelial heparan sulphate proteoglycans at the glycocalyx. A second requirement was for an antibody which was capable of localizing LPL in fixed tissue sections. This antibody (polyclonal or monoclonal) would need to recognise denaturation-resistant epitope(s) on the enzyme.

The work described in this Chapter details the production of anti-chicken LPL monoclonal and polyclonal antibodies and the subsequent investigation of potentially useful antibody characteristics.

3.2 METHODS

3.2.1 *Production of anti-LPL polyclonal serum in sheep*

An antiserum was raised in a female Greyface sheep (Blue Headed Leicester sire, crossed with a Scottish Blackface dam) by the use of multiple-site subcutaneous injections at the flexure of the upper thigh and lower abdomen. The immunogen for this was 100-150 µg of chicken adipose LPL purified by heparin-Sepharose and concanavalin A-Sepharose chromatography (section 2.3.3) administered at 3/4 week intervals. For the initial immunization, LPL was emulsified in complete Freund's adjuvant (CFA) by sonication at 4°C for 30 seconds. Incomplete Freund's adjuvant (IFA) was used for the 6 subsequent immunizations and test-bleeds were taken 7-14 days after each of these injections. The blood was collected from the external jugular vein on to EDTA to give a final concentration of 4 mM and the plasma was obtained by centrifugation at 1000 *g* av for 10 min at 4°C. The plasma was recovered by aspiration and $CaCl_2$ added to give a final concentration of 5 mM $CaCl_2$. The mixture was allowed to clot overnight at 4°C and then homogenized using a Polytron homogeniser. After centrifugation at 10,000 *g* av for 15 min at 4°C to remove the insoluble debris, the recalcified plasma was stored in aliquots at -70°C.

3.2.2 *Immunization of mice for the production of specific immune spleen donors*

Chicken adipose LPL purified by heparin-Sepharose and concanavalin A-Sepharose chromatography (section 2.3.3) was emulsified in CFA by sonication and injected intraperitoneally (i.p.) and subcutaneously (s.c.), at the flexure of the dorsal thigh and abdomen of 6 female Balb/c mice.

The mice were each given a subsequent immunizations, i.p. and s.c., using IFA, 28 days after the primary injection. Both immunizations were of 30 μg of LPL in 0.2 ml of adjuvant and were divided equally between the two sites of immunization in each mouse. A week after the second injection, test bleeds were taken from the tail vein. Those mice with test bleeds showing a high titre of anti-chicken LPL antibodies (assessed by ELISA) were given a final i.p. injection of 30 μg of LPL in IFA. Three days later the mice were killed and the spleens removed as a source of immune-splenocytes for fusion.

3.2.3 *Production of hybridomas*

The immunized mice were killed by ether anaesthesia with the spleen being removed under sterile conditions and placed into serum-free Dulbecco's modified Eagle's medium (DMEM). A single cell suspension was prepared by gentle disruption of the spleen in serum-free medium using a sterile loose-fitting Teflon/glass hand-held homogenizer. The cell suspension was then centrifuged at 800 *g* av and the resultant pellet resuspended in 10 ml of serum-free medium with the number of splenocytes obtained determined using a haemocytometer. The yield of cells from a single immune spleen was typically between $5x10^7$ to $20x10^7$ cells. Spleen cells were fused using the method of Galfre and Milstein (1981) with the mouse plasmacytoma cell line NS-O as the fusion partner. For this, spleen cells and plasmacytoma cells were mixed at a ratio of 1:1 and fused by incubating in 1 ml of polyethylene glycol 1500 (BDH Ltd, Poole, UK.) for 90 seconds at 37°C. The fusion was halted by the gradual addition of 20 ml of DMEM, prewarmed to 37°C. The cell suspension was then centrifuged for 10 min at 200 *g* av with the supernatant being discarded and the pellet being resuspended in 160 ml of complete DMEM containing 25% (v/v) rat mixed thymocyte culture-conditioned medium (MTM), 10^{-4} M hypoxanthine, $4x10^{-5}$ M aminopterin and $1.6x10^{-5}$ M thymidine (stock HAT soln from Sigma Chemicals). The suspension of fused cells was then distributed into ten 96 well tissue culture plates (200 μl/well) with the medium being replaced after 7 days and every 3 days thereafter. Initial screening of the hybridoma cell media, for the production of anti-LPL IgG, was carried out by ELISA 10 days after fusion.

The MTM was produced using the method of Micklem *et al.* (1987). Briefly, the thymus was removed from a 6 week old female Sprague-

Dawley rat under sterile conditions and gently disrupted by homogenization using a sterile Teflon/glass hand-held homogenizer in serum-free DMEM. The cells were washed three times in DMEM and cultured in complete DMEM ($5x10^6$ cells/ml) for 48 hours. The cell medium obtained after centrifugation at 800 *g* av was filtered through a 0.2 μm membrane (Corning Laboratory Science Co, New York, USA), to ensure sterility and stored at -70°C in aliquots.

Table 3.1 Composition of complete Dublecco's modified Eagle's medium (DMEM).

Serum-free DMEM	
Foetal Calf serum	10% or 20% (v/v)
Glutamine	2 nM
Penicillin	200 units/ml
Streptomycin	200 μg/ml

The composition of the medium used for the maintenance of myeloma/hybridoma and thymocyte feeder cells in culture. The foetal calf serum was pre-tested for its ability to support clonal myeloma cell growth. All tissue culture media and supplements were obtained from Sigma Chemical Co.

3.2.4 *ELISA for the detection of specific anti-LPL antibodies*

Chicken adipose LPL purified by heparin-Sepharose and concanavalin A-Sepharose chromatography (section 2.3.3) was absorbed (50 ng/well) on to 96 well polystyrene microtitre plates (Immobilon II, Dynatech Laboratories Ltd, Billingshurst, Sussex) in 0.1 M carbonate/bicarbonate buffer, pH 9.6, 100 μl per well for 16 hours at 4°C. Between each stage of the ELISA (to remove unbound LPL or antibodies), the wells were washed 3 times with 200 μl/well of PBS (composition given in Appendix 4) containing 0.05% (v/v) Tween-20 (PBS-T). After washing with PBS-T, any residual binding sites were blocked by the addition of 200 μl of 3% (w/v) BSA in PBS for 2 hours at 20°C. Following this procedure and a wash stage, primary antibody, in the form of either diluted sera or cell supernatant, was added to the plates (100 μl/well) and incubated for 2 hours at 20°C. After washing with PBS-T, the presence of bound primary antibodies was detected by incubation with an appropriately diluted secondary species-specific anti-IgG horseradish peroxidase-conjugated antibody (Sigma Chemical Co.) for 1 hour at 20°C. For the dilution of antibodies, PBS-T containing 0.1% (w/v) BSA was used. The presence of bound enzyme conjugate was detected after a wash stage by the addition of 100 μl per well of the horseradish peroxidase substrate,

0.04% (w/v) σ – phenylenediamine dihydrochloride (Sigma Chemical Co.) and 0.012% (v/v) H_2O_2 in phosphate-citrate buffer, pH 5.0 (25.7 ml 0.2 M dibasic sodium phosphate, 24.3 ml 0.1 M citric acid and 50 ml deionized H_2O) The colour was allowed to develop in a linear fashion for 15 min in the dark and the enzyme reaction was stopped by the addition of 50 µl of 2.5 M sulphuric acid to each well. The absorption was determined at 492 nm using an automatic spectrophotometric plate reader (Titerteck Twinreader Plus, Flow Laboratories Ltd, Rickmansworth, Herts). The titre of specific polyclonal anti-LPL antibodies was defined as the dilution giving half maximal binding to the antigen, under the conditions of the assay.

3.2.5 *Cross-reactivity with rat LPL*

The cross-reactivity of the anti-chicken LPL antibodies with rat LPL was determined using a modification of the ELISA system described in section 3.2.4. This required the production of partially purified rat LPL which was accomplished using the method of Parkin *et al.* (1982). The solubilization, purification on heparin-Sepharose and concentration of LPL in an ultrafiltration cell have been described in detail for the purification of chicken LPL in section 2.2.4. Briefly, LPL was solubilized from rat adipose acetone diethyl-ether powders (produced as described in section 2.2.1) using 5 mM sodium barbitone buffer, pH 7.5, containing 0.1% Triton X-100 and was batch absorbed on to heparin-Sepharose, as described in section 2.2.4. The matrix was then packed into a column under gravity and the column was then washed with barbitone buffer containing 0.7 M NaCl until the absorbance measured at 280 nm approached zero. The LPL activity was eluted from the heparin-Sepharose column by 2.0 M NaCl and concentrated under nitrogen in an ultrafiltration cell using a PM 30 membrane and stored at -70°C.

The partially purified rat LPL (10 µg/ml) was absorbed on to polystyrene microtitre plates (Imobillon II, Dynatech) in 0.1 M carbonate/bicarbonate buffer, pH 9.6, (100 µl/well) for 16 hours at 4°C. The ELISA was then continued as described in section 3.2.4

3.2.6 *Immunoreactivity of anti-chicken LPL antibodies towards acetone fixed cryostat sections of chicken and rat cardiac tissue*

Small cubes of cardiac tissue (chicken or rat) were frozen in isopentane cooled with liquid nitrogen and stored at -70°C. Tissue sections of 10 µm

thickness were then cut using a cryostat (Bright Ltd, UK.) at -26°C. The sections were mounted on to slides precoated with 0.1% poly-L-lysine (Sigma Chemical Co.) and fixed by immersion for 5 min in acetone at -20°C. The mounted sections were then incubated for 15 min with 20% (v/v) normal serum diluted in PBS. In all cases, normal serum from the species which was the donor of the second antibody preparation was used to block the non-specific IgG binding. The sections were then incubated with primary antibody ie: sheep anti-chicken LPL IgG (4 µg/ml) or Cal-11 monoclonal IgG (1 µg/ml), diluted in PBS containing 10% (v/v) normal serum or hybridoma cell medium respectively. For detection by immunofluorescence, the sections were then washed with three changes of PBS and incubated for 1 hour at 20°C with either anti-sheep- or anti-mouse- IgG-FITC antibody conjugate (Sigma Chemical Co.) diluted 1/100 (v/v) in PBS containing 10% (v/v) normal serum. The secondary anti-mouse IgG-FITC conjugate preparation used for the detection of mouse IgG binding in rat tissues was pre-absorbed against rat IgG by the manufactures (Sigma Chemical Co) to reduce non-specific interactions. The sections were then washed with PBS (three incubations each of 10 minutes) and mounted in 90% (v/v) glycerol and 2.5% (w/v) 1,4 diazabicyclo-[2.2.2]octane in PBS for viewing. Immunofluorescent staining was visualised using a fluorescent photomicroscope (Olympus BH2, Olympus Optical Co., Tokyo, Japan) and photographed using Kodak Ektachrome P800/1600 film (Eastman Kodak, Rochester NY).

3.2.7 *Inhibition of enzyme activity*

The inhibitory effect of the sheep anti-chicken LPL polyclonal serum was determined by adding increasing amounts of enzyme, partially purified by heparin-Sepharose affinity chromatography, to a fixed amount of antibody in a total assay volume of 250 µl. LPL activity was measured immediately following the addition of enzyme to the antibody, as described in section 2.2.2. A final dilution of 1/100 of the sheep anti-chicken LPL serum in the assay volume was used (ie: 2.5 µl of serum was added to the assay mixture). LPL activity was also determined in control tubes containing a 1/100 dilution of non-immune sheep serum.

3.2.8 *Determination of antibodies specific for carbohydrate epitopes*

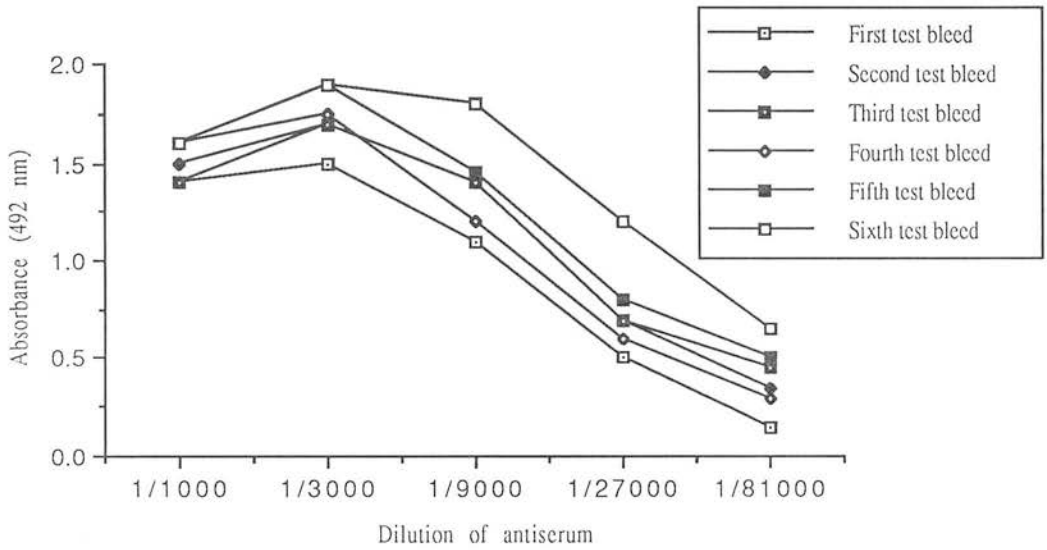
To determine whether any of the monoclonal antibodies were specific for any putative carbohydrate epitopes on the enzyme, LPL was bound to the wells of ELISA plates as described in section 3.2.4 and then treated with periodic acid, by the method of Woodward *et al.* (1985), before the addition of primary antibodies. The residual binding sites on the plate were blocked as described in section 3.2.4 and the absorbed LPL was incubated in 50 mM sodium acetate buffer, pH 4.5, for 30 min at 20°C. Half of the sample wells were then subjected to 20 mM periodic acid in acetate buffer, pH 4.5, for 1 hour at 20°C, while the other half were left in acetate buffer alone. At the end of the treatment, all the samples were incubated for 30 min at 20°C with 50 mM sodium borohydride in PBS to stop the reaction. After washing, the primary antibody at varying dilutions was added in duplicate to the treated and untreated wells. The ELISA was then completed as described in section 3.2.4.

3.3 RESULTS

3.3.1 *Production of a sheep anti-chicken LPL antiserum and assessment of titre by ELISA*

The ELISA system described in section 3.2.4 was optimised initially by titrating the concentrations of absorbed LPL and secondary anti-sheep IgG peroxidase conjugated antibody. The combination of reagent dilutions chosen was that giving the maximal specific binding measurements (data not shown). Using this system, the presence of anti-chicken LPL antibodies could be readily detected in serum samples following the initial immunizations of the animal, as described in section 3.2.1. As shown in Fig. 3.1, the polyclonal titre of specific anti-chicken LPL antibodies increased progressively with successive immunizations (6 in all). The titre assessed at half maximal binding rose from 1/27,000 after the initial immunization reaching a peak of 1/81,000 following the sixth immunization. Large samples of blood were taken after the fifth and sixth immunizations producing 300 and 150 ml of recalcified plasma, respectively.

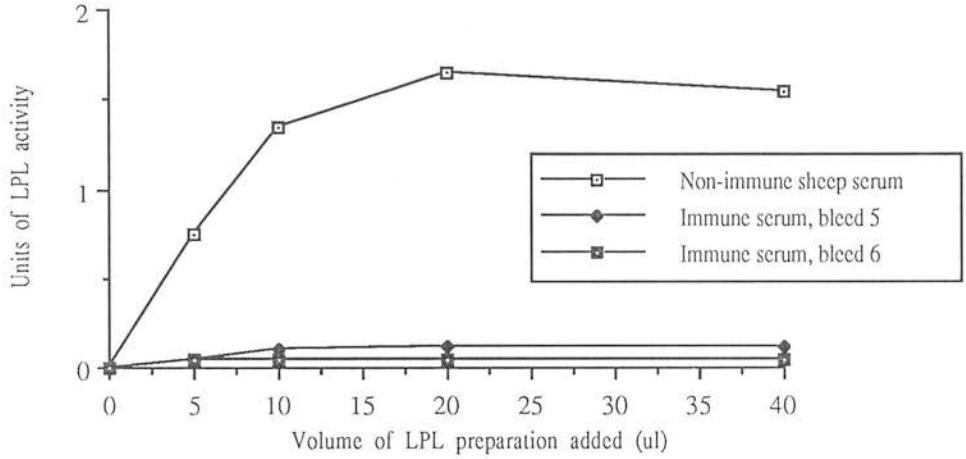
Figure 3.1 Assessment of the levels of anti-chicken LPL antibodies present in the serum of a sheep following successive immunizations with purified chicken LPL.



Test bleeds were taken from the sheep following each successive immunization with purified chicken LPL, as described in section 3.2.1. The titre of anti-chicken LPL antibodies was determined in the serum using the ELISA system described in section 3.2.4. The absorbances shown describe the levels of specific antibody binding from which the absorbances of control wells incubated with non-immune sheep serum have been deducted.

The titre assessed by ELISA closely correlates with the ability of test bleeds to inhibit LPL activity, with blood samples 5 and 6 in particular showing substantial inhibitory activity, as assessed by the method described in section 3.2.7. Recalcified sheep plasma from bleeds 5 and 6, at a 1/100 final dilution, was found to inhibit LPL activity by a maximal 92.8% and 96.8% respectively, Fig 3.2.

Figure 3.2 The inhibition of LPL activity by sheep anti-chicken LPL serum.

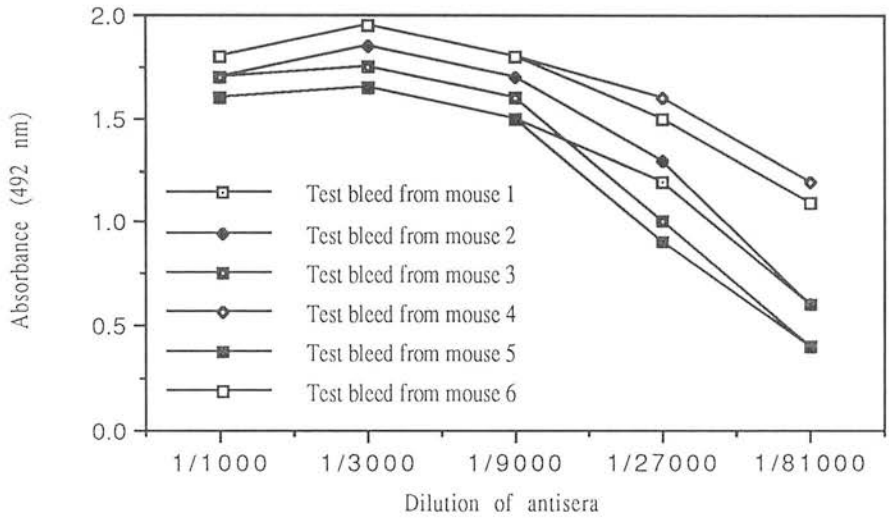


Immune sheep serum, following the 5th and 6th successive immunizations of chicken LPL, was obtained as described in section 3.2.1. Sheep serum, immune and non-immune, was added to the LPL assay mixture to give a final dilution of 1/100 and incubated with increasing amounts of partially purified chicken LPL, as described in section 3.2.7.

3.3.2 *Immunization of mice for the production of potential immune spleen donors*

The ELISA system described in section 3.2.4 was initially optimised by titrating the amount of absorbed LPL and the concentration of secondary anti-mouse IgG peroxidase conjugated antibody. The combination of reagent dilutions chosen was that giving the maximal specific binding measurements (data not shown). Following the immunization schedule described in section 3.2.2, the serum of all six immunized mice was found to contain anti-chicken LPL IgG after the secondary immunizations. As shown in Fig 3.3, mice 1,2,3 and 5 had half-maximal binding titres of 1/81,000, while mice 4 and 6 had titres in excess of 1/81,000. From these titres, the immunization schedule described in section 3.2.2 was judged successful in producing potentially suitable donors of immune splenocytes for fusion *in vitro* and the production of monoclonal antibodies.

Figure 3.3 Assessment of the titre of anti-chicken LPL antibodies present in the sera of mice immunized with purified chicken LPL.



Test bleeds were taken from mice immunized with purified chicken LPL, as described in section 3.2.2. The titre of anti-chicken LPL antibodies was determined in the sera using the ELISA system described in section 3.2.4. The absorbances shown describe the levels of specific antibody binding from which the absorbances of control wells incubated with non-immune mice serum have been subtracted.

3.3.3 *Production of hybridoma cell lines*

Two separate splenocyte/myeloma cell fusions were carried out, as described in section 3.2.3, using splenocytes from mice 2 and 4. The initial screening of the first fusion (mouse 2) after the hybridomas had been cultured for 10 days, (using the ELISA system described in section 3.2.4) identified 10 wells containing anti-LPL antibodies in the cell supernatant (out of 816 wells plated down with cells). A well was considered to contain a hybridoma capable of secreting anti-LPL antibodies if the cell medium produced an absorbance of five times the mean background level in the ELISA.

The initial screening of the second fusion (mouse 4) after 10 days of culture resulted in the identification of 11 positive wells out of 640 wells plated down with cells, Table 3.2. These wells, containing hybridomas secreting anti-LPL antibodies, were expanded into 24 well plates and re-tested for the presence of specific antibodies. Seven of these cell lines remained positive, Table 3.2, and were continually expanded to produce cell supernatant (>100 ml) for characterization and cells (3 vials of 15 x 10^6 cells) to freeze and store in liquid nitrogen.

Table 3.2 Summary of hybridoma cells produced by second fusion.

Well	Initial Screen	Expanded Hybridoma Cells	
Source of LPL absorbed	Chicken LPL 20 ng/well	Chicken LPL 50 ng/well	Rat LPL 1 µg/well
1E7	0.7	1.2	0.0
1G9	0.7	1.6	0.0
2A8	0.8	1.5	0.0
3H3	0.7	0.1	0.0
3A8	0.7	0.0	0.0
4F6	0.6	1.2	0.0
5F2	0.8	1.3	0.0
5G3	0.8	1.5	0.0
5G4	0.6	0.2	0.0
6A1	0.8	1.3	0.0
6B3	0.7	0.1	0.0
No of +ve wells	11/640	7/640	0/640
Cal-11	----	1.22	0.0
Sheep polyclonal	----	1.58	0.1

Figures represent the OD_{492} of wells in the ELISA system described in section 3.2.4 & 3.2.5. The initial screen of the fusion plates was carried out 10 days after the fusion of immune-splenocytes from mouse 4 and the values shown describe the level of specific antibody binding after the subtraction of the mean background level of absorbance. At this stage 11 out of the 640 wells plated down with cells were considered to be positive ie: possessed OD_{492} readings of at least 5 x background. The second screen was carried out after the hybridomas, from wells determined to be positive in the initial screening, had been expanded into 24 well plates and the values represent the mean of duplicate wells after the absorbance of myeloma culture conditioned medium has been subtracted. Cal-11 and the sheep anti-chicken LPL polyclonal antibody were both used at 1/3000 dilution with non-immune mouse or sheep IgG being used as the control.

3.3.4 *Antibody characterization*

The anti-chicken LPL antibodies described in this Chapter and the monoclonal antibody, Cal-11 (a kind gift of Professor A. Bensadoun of Cornell University, New York), were characterized for their ability to immunolocalize LPL in acetone fixed cryostat sections and crossreact with rat LPL.

The ability of the antibodies to immunolocalize LPL in acetone fixed cryostat sections was studied as this characteristic would be required for future quantitative immunocytochemical studies (Chapter 6). Four of the monoclonal antibodies, Cal-11 and those secreted by hybridomas

1G9, 2A8 and 6A1 were able to immunolocalize LPL on acetone-fixed cryostat sections of chicken cardiac tissue, Fig 3.5. The sheep polyclonal antibody was also found to have this property and the pattern of immunostaining obtained with all 5 antibodies was found in chicken cardiac tissue to be identical, Figs 3.5 and 6.3. The antibodies secreted by the remaining hybridomas gave no detectable immunofluorescence, Fig 3.5. None of the antibodies capable of immunolocalization of LPL in chicken cardiac tissue were able to detect LPL in similarly prepared sections of rat cardiac tissue, Fig 3.5. Similarly none of the anti-chicken LPL antibodies, polyclonal or monoclonal, cross-reacted with partially purified rat LPL (specific activity 320 units of enzyme activity/mg of protein) in the ELISA system described in section 3.2.4, Table 3.2.

Treatment of absorbed LPL by periodic acid, in the ELISA system described in section 3.2.8 was not found to affect the binding of any of the anti-chicken LPL monoclonal antibodies to chicken LPL, Table 3.3, at any of the antibody dilutions tested.

Table 3.3 The lack of effect of periodic acid treatment of absorbed chicken LPL on the subsequent binding of anti-chicken LPL monoclonal antibodies as measured in an ELISA system.

Antibody	OD_{492} Treated wells	OD_{492} Control wells
1E7	0.68±0.08	0.71±0.01
1G9	0.71±0.07	0.77±0.05
2A8	0.58±0.07	0.65±0.08
4F6	0.66±0.01	0.67±0.03
5F2	0.88±0.03	0.93±0.05
5G3	0.70±0.02	0.72±0.01
6A1	0.63±0.02	0.66±0.02
Cal-11	0.50±0.01	0.50±0.03

Purified chicken LPL was bound to the wells of ELISA plates and treated with periodic acid, before the addition of anti-chicken LPL antibodies, as described in section 3.2.8. The values represent the means ± SE (n=4) for 1/4 dilutions of the hybridoma cell supernatants or in the case of Cal-11 a 1/6000 dilution. No significant difference (student's test) between treated and untreated (control) wells was found.

Figure 3.5 Immunolocalization of LPL in sections of cardiac tissue using anti-chicken LPL monoclonal antibodies.

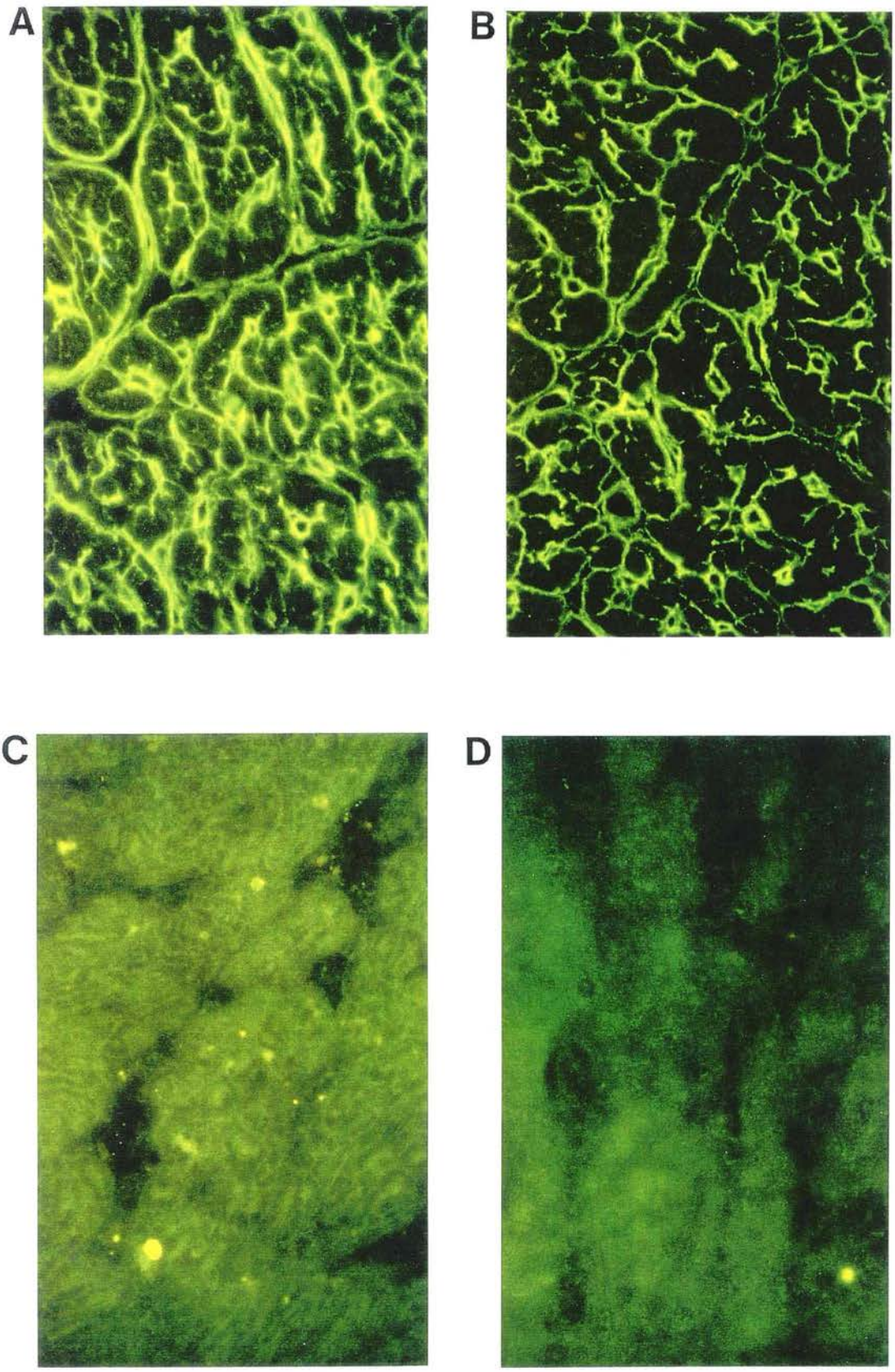
LPL was localized in cryostat sections of the chicken or rat left ventricular myocardium using anti-chicken LPL monoclonal antibodies in the immunofluorescent staining procedure described in section 3.2.6.

A) Immunofluorescent staining of sections of chicken cardiac tissue, obtained by using cell medium from hybridoma 2A8 as the source of primary anti-LPL antibodies. An identical pattern of immunoreactivity was also obtained using cell medium from hybridomas 1G9 or 6A1 (data not shown). Print x 476.

B) Immunofluorescent staining of sections of chicken cardiac tissue, obtained by using purified Cal-11 IgG as the primary anti-LPL antibody. The pattern of immunoreactivity was identical to that obtained above (Fig 3.5a), using cell medium from hybridoma 2A8 as the source of anti-LPL antibodies. Print x 476.

C) Lack of specific immunofluorescent staining of sections of chicken cardiac tissue, obtained when using cell medium from hybridoma 5F2 as the source of primary anti-LPL antibodies. This is representative of results obtained using cell medium from hybridomas 4F6, 5G3 and 1E7 (data not shown). Print x 476.

D) Lack of specific immunofluorescent staining of cryostat sections of rat cardiac tissue, obtained when using cell medium from hybridoma 2A8 as the source of primary anti-LPL antibodies. This was representative of the lack of reaction shown by all the anti-chicken LPL antibodies, polyclonal and monoclonal, to rat LPL. Print x 476.


A
B
C
D

3.4 DISCUSSION

The work in this Chapter describes the production of an inhibitory anti-chicken LPL sheep antiserum. Several groups have previously produced inhibitory antisera against LPL. Etienne *et al.* (1985) and Goldberg *et al*, (1986) raised an inhibitory antiserum against bovine milk LPL in a goat whereas, Kompiang *et al.* (1976) produced an inhibitory antiserum against chicken adipose LPL in the rabbit. Variations in the immunological cross-reactivities of mammalian LPLs have been previously studied using anti-LPL polyclonal antibodies. Goldberg *et al.* (1986) found that an inhibitory anti-human LPL polyclonal antibody failed to inhibit either bovine milk or bovine post-heparin plasma LPL. Shirai *et al.* (1982) found that an inhibitory anti-bovine milk LPL polyclonal antibody inhibited human and pig LPL but not that of the rat or dog. It, therefore, does not seem surprising that an anti-chicken LPL polyclonal antibody fails to cross-react with rat LPL as assessed by ELISA (Table 3.2) or by immunofluorescence on cryostat sections of rat cardiac tissue (Fig 3.5).

Monoclonal antibodies work well in cell staining techniques (requiring little fixation) where their purity and specificity yield low backgrounds. This was shown by the immunolocalization of LPL using Cal-11 and the anti-LPL antibodies secreted by hybridomas 1G9, 2A8 and 6A1, Fig 3.5. However, some monoclonal antibodies will not give satisfactory results in cell staining if the specific epitope is destroyed or masked during fixation. This may be the case with the anti-LPL antibodies secreted by hybridomas 1E7, 4F6, 5F2 and 5G3, all of which failed to localize LPL on acetone fixed cryostat sections of chicken cardiac tissue.

As was found with the anti-chicken LPL polyclonal, none of the anti-chicken LPL monoclonal antibodies showed any cross-reaction with rat LPL by immunofluorescence, Fig 3.5, or by ELISA, Table 3.2. Gershenwald *et al.* (1985) reported that the inhibitory anti-chicken monoclonal antibody Cal-11 failed to inhibit or immunoprecipitate human LPL. This suggests that there are considerable differences in the antigenic properties of chicken and rat LPL (and possibly LPL from other mammalian species). There is, however, considerable homology between the predicted primary sequences of chicken and mouse LPL

(Cooper *et al.*, 1989) the latter being shown to have similar antigenic properties to rat LPL (Soteriou, 1991). The lack of immunological cross-reactivity between chicken and rat LPL cannot be explained by species differences in glycosylation producing different antigenic properties. This is because, none of the monoclonal antibodies described in this Chapter were found to be specific for carbohydrate epitopes, Table 3.3, as defined by a loss of reactivity towards antigen treated with periodic acid (Lafferty *et al.*, 1991; Woodward *et al.*, 1985).

In conclusion, the work in this Chapter describes the production and partial characterization of both monoclonal and polyclonal antibodies against chicken LPL. However, the lack of cross-reactivity of the anti-chicken LPL antibodies with rat LPL does not allow the possibility of future comparisons between the rat and the chicken in the immunolocalization or immunoquantitation of LPL to be made.

CHAPTER 4

THE RELEASE OF LPL ACTIVITY FROM THE ISOLATED PERFUSED CHICKEN HEART BY HEPARIN

4.1 INTRODUCTION

The aim of the work described in this chapter was to use the isolated perfused chicken heart system to investigate the effect of nutritional status on the functional (ie: heparin-releasable) pool of LPL activity present in chicken cardiac tissue.

Langendorff introduced the isolated beating heart preparation in 1885 and the technique has since been used for a wide range of physiological and biochemical studies on the hearts of many species (Neely and Rovetto, 1975). However, there have been few studies on avians and only three studies have reported the use of such a preparation in the case of the chicken. Lindmar *et al.* (1982), for example, used the perfused chicken heart as a model system to investigate the interstitial washout and hydrolysis of acetylcholine. McCallum *et al.* (1989) investigated the effect of furazolidone on the level of myocardial vascular resistance, while Wack and Hamlin (1986) measured the left ventricular pressure generated during beating.

When isolated perfused rat hearts have been used as a model system for studying LPL at the capillary endothelium, it has been found that perfusion of the hearts with heparin caused an immediate release of LPL activity into the perfusate. This is because LPL has about a 40-fold greater affinity for heparin than for heparan sulphate (Bengtsson et al., 1980), the material through which it is bound to the endothelium. The short time required for release of the enzyme suggested that it was located at a site easily accessible to heparin, such as the surface of the capillary endothelium. In support of this idea, Borensztajn and Robinson (1970) found that perfusion with heparin reduced the ability of rat hearts to oxidise [^{14}C]-labelled chylomicron triacylglycerol by 80% and that fasting increased the size of the functional endothelial pool of LPL activity and the capacity of the heart to oxidise chylomicron triacylglycerol.

In isolated hearts, this rapid release of LPL activity by heparin is followed by a continuous slow phase of release, which can account for up to 50% of the total enzyme present in the tissue (Borensztajn and Robinson, 1970; Robinson and Jennings, 1965). It has been proposed that this fraction represents enzyme from the interstitial space in transit from the cardiac myocytes to the surface of the capillary endothelium (Atkin and Meng, 1972).

The proportion of total heart LPL activity that can be rapidly released by heparin tends to correlate positively with the level of total tissue LPL activity which is itself the subject of a variety of nutritional and physiological stimuli (Cryer, 1981). In mammals, changes in total tissue LPL activity have also been shown in response to such stimuli as fasting, exercise and lactation (Cryer, 1981). However, total LPL activity in avian tissues appears to be less responsive to nutritional changes than does that of mammalian tissues. Total LPL activity in chicken adipose tissue and skeletal muscle is only marginally affected by fasting, while the total activity of cardiac muscle increases only slightly under similar conditions (Evans, 1972; Husbands, 1972; Benson and Bensadoun, 1977; Hermier *et al.*, 1989; Griffin and Butterwith, 1988). Hermier *et al.* (1989) also found that, with 5 week old broiler chickens, fasting for 18 hrs failed to alter the level of heparin-releasable LPL activity from adipose tissue segments incubated *in vitro*. It is possible however, that the slight increase in total tissue activity (observed by Husbands, 1972 and Griffin and Butterwith, 1988) may mask much greater increases in the heparin-releasable pool of LPL activity thought to be located at the capillary endothelium.

In the present study, an attempt was made to relate the *in vitro* measurements of the functional LPL activity in the perfused chicken heart to the ability of the tissue to accumulate lipoprotein triacylglycerol NEFA *in vivo*. Wolfe *et al.* (1981) concluded from the results of several studies on the fate of radiolabelled VLDL triacylglycerol *in vivo*, that oxidation of VLDL derived NEFA did not involve their prior entry into the plasma pool of NEFA. Thus it has been thought that the hydrolysis of [^{14}C] labelled lipoprotein triacylglycerol and uptake of the released NEFA into tissues *in vivo* can be used as an indicator of the amount of functional LPL activity of tissues. For example, the technique has been previously used to assess differences in the uptake and oxidation of

VLDL triacylglycerol NEFA by the tissues of fat and lean broilers produced by divergent selection for plasma VLDL concentration (Griffin et al., 1989; Griffin *et al.*, 1991a) and differences in the uptake and turnover of VLDL triacylglycerol in the adipose tissue of broiler and layer chickens (Griffin *et al.*, 1991b). In the present study, the uptake and accumulation of NEFA derived from [^{14}C]-VLDL triacylglycerol was compared to the total LPL activity of individual tissues, to give a further indicator of the level of functional LPL in the muscle and adipose tissue of broiler chickens.

4.2 METHODS

4.2.1 *Animals*

Broiler chickens from a commercial strain (Ross Poultry Breeders Ltd, Newbridge) were fed *ad libitum* on standard broiler growing diets appropriate for age. A photoperiod of 14 hrs light and 10 hrs dark was maintained with lights off between 6 pm and 4 am. In all the experiments described in this Chapter, broilers of 4 to 6 weeks of age were used, with fed animals having free access to food and water. Animals designated as fat fed were given a diet of 10% (v/w) corn oil added to LS, mash *ad libitum* for 5 days. Fasted animals were allowed free access to water, but food was withdrawn 20 hrs before the birds were killed. This period of fasting was chosen because Benson *et al.* (1975) reported that the fasting of roosters for either 13 or 35 hrs produced a doubling in post-heparin plasma LPL activity.

Hisex Brown layer chickens (Ross Poultry Breeders Ltd.) were reared in the same manner as described for broilers using standard layer diets *ad libitum* appropriate for age.

Both strains of chickens were obtained as one day old chicks.

Mature male Sprague-Dawley rats were obtained from Harlan Olac Ltd, Shaws Farm, Blackthorn, Oxon and were maintained on a standard laboratory diet (Pilsbury 41B, Pilsbury, Birmingham, UK). Rats of between 250-300 g were used throughout the work described in this Chapter. Fed rats had free access to food and water while fasted rats had free access to water but the food was withdrawn 18 hrs before the animals were killed.

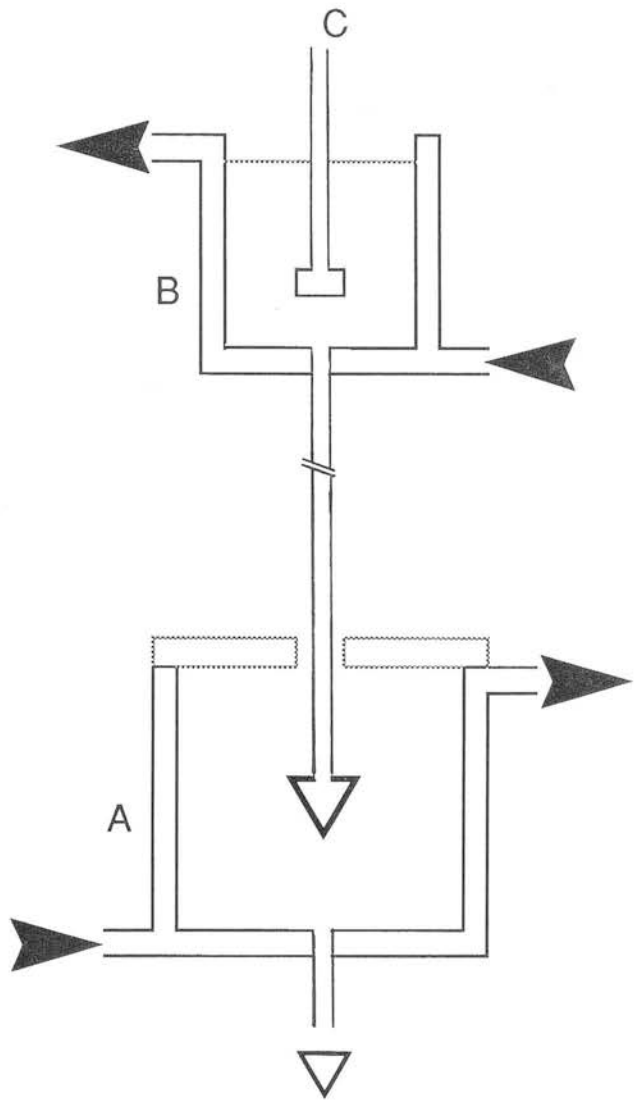
4.2.2 *Isolated perfused hearts*

Studies by Borensztajn (1987) have shown that through the day oscillatory changes occur in the amount of LPL activity present in the cardiac tissue of fully-fed and fasted rats. All perfusions were, therefore, performed between 10.00 and 12.00 to reduce possible diurnal changes in functional LPL levels. Any hearts which failed to reperfuse or maintain a steady beat were discarded. Hearts were perfused for 1 min to remove trapped blood from the coronary vasculature before the addition of heparin. Unless otherwise stated, perfusion was carried out by single pass, without the recirculation of perfusate, using the apparatus shown in Fig 4.1.

4.2.2.1 *Isolated perfused chicken hearts*

Hearts were perfused by the Langendorff technique. The method used was a modification of that described by Wack and Hamlin, (1986) for the perfusion of chicken hearts, including the addition of 1% (w/v) BSA to the perfusion medium (to provide oncotic pressure and to prevent the absorption of LPL to the apparatus). Birds were killed by cervical dislocation and the hearts were removed rapidly into physiological saline at 0°C to arrest the heart beat. The proximal portion of the aorta was cannulated using a short length of steel tubing (2.0 mm outside diameter) which was tied in place using surgical silk (Ethicon Ltd, Scotland). The right and left brachiocephalic arteries were also tied using the same material. The heart was then placed on the perfusion apparatus and retrograde perfusion commenced. Hearts were perfused with Krebs-Henseleit solution, pH 7.4, (Table 4.1) maintained at 41°C and equilibrated with 95% O_2/5% CO_2 in a reservoir set 100 cm above the level of the cannula. A flow rate of 12-15 ml/min of perfusion medium through the heart was typically achieved using this method. The release of LPL activity from the tissue preparation was initiated by the introduction of 30 units/ml of heparin (mucous injection BP, Leo Labs Ltd. Princes Risborough UK.) into the perfusion medium and fractions for assay were collected into tubes maintained at 0°C.

Figure 4.1 Diagramatic illustration of the Langendorff perfusion apparatus.



Essentially the apparatus consisted of a water-jacketed heart chamber (A), 7 cm internal diameter x 10 cm, and a water-jacketed reservoir (B), 3.5 cm internal diameter x 14 cm, the latter being set 100 cm above the level of the aortic cannula. To maintain the apparatus at a constant temperature a continual flow of water (indicated by solid arrow) was passaged through the equipment by means of a thermostatted circulator (Grant LTD 6, Grant Instruments Ltd. Cambridge, UK). The perfusate in the reservoir (B) was continually gased with a mixture of 95% O_2/5%CO_2 through a gas distribution tube (C). Silicone rubber tubing (BDH Ltd., Poole, Dorset) was used throughout, with the length connecting the heart chamber and the reservoir being of 8.0 mm outside diameter and the length connecting the aortic cannula and the reservoir being of 3.2 mm outside diameter. The perfusate (indicated by an open arrow) was collected into tubes maintained at 0°C or recirculated back to the reservoir by means of a rotary pump (Masterflex 7014, Cole-Parmer Instrument Co., Chicago, Ill.).

Table 4.1 Medium used for the perfusion of chicken hearts*

	m M
NaCl	110.0
KCl	10.0
$CaCl_2.2H_2O$	2.5
$NaHCO_3$	20.0
KH_2PO_4	1.2
$MgCl_2.6H_2O$	0.5
Glucose	12.7
BSA	1% w/v

*modified from Wack and Hamlin, (1986)

4.2.2.2 *Isolated perfused rat hearts*

Rats were stunned and killed by cervical dislocation. The hearts were removed rapidly and placed into physiological saline at 0°C to arrest the heart beat. The aorta was cannulated using a short length of steel tubing (1.2 mm outside diameter) secured using surgical silk and retrograde perfusion commenced. The perfusion medium, a Krebs-Henselite buffer (Table 4.2), was modified from that described by Neely and Rovetto (1975) by the addition of 1% (w/v) bovine serum albumin. The medium was held in a reservoir 100 cm above the cannula, maintained at 37°C and equilibrated with 95% O_2/5% CO_2. A flow rate of 4-6 ml/min of perfusion medium through the heart was typically achieved using this method. The release of LPL activity was initiated by the introduction of 5 units of heparin per ml into the perfusion medium with fractions for assay being collected into tubes maintained at 0°C.

Table 4.2 Medium used for the perfusion of rat hearts+

	m M
NaCl	118.0
KCl	4.7
$CaCl_2.2H_2O$	2.5
$NaHCO_3$	25.0
KH_2PO_4	1.2
$MgSO_4$	1.2
Glucose	11.0
BSA	1% w/v

+modified from Neely and Rovetto, (1975)

4.2.3 *LPL assay*

Chicken and rat LPL activity was measured as described in Chapter 2, with the exception that the Intralipid was activated for the measurement of rat LPL by incubation for 1 hr at 30°C with 3 volumes of sheep recalcified plasma. Residual LPL activity in the heart tissue was measured in fresh homogenates prepared using a Polytron homogenizer (Kinematica, Lucerne, Switzerland), in 0.233 M Tris/HCl buffer, pH 8.0 containing 0.25 M sucrose and 10 mg BSA, 1 mg sodium deoxycholate, 40 µg Nonidet P40, 20 µg heparin and 20 µg PMSF per ml (Iverius and Brunzell, 1985). Homogenates were centrifuged at 3000 *g* av for 5 min and LPL activity assayed in the supernatant. LPL activity has previously been measured in chicken tissues using this method (Griffin *et al.*, 1991b).

4.2.4 *Perfusion of chicken hearts with concanavalin A-FITC conjugate*

To assess the level of capillary reperfusion, isolated chicken hearts were perfused with concanavalin A-FITC conjugate which binds to the glycoproteins present at the luminal surface of the capillary endothelial cells. The chicken hearts were perfused as described in section 4.2.2 except that, after the blood had been washed free from the coronary vasculature, the hearts were perfused for 5 min in a recirculating system with medium containing 20 µg/ml of concanavalin A-FITC conjugate (Sigma Chemical Co.). They were then frozen in isopentane cooled by liquid nitrogen and 10 µm sections cut on a cryostat (Bright Ltd. UK) at -26°C. Sections were fixed for 5 min in acetone and mounted using 90% glycerol in PBS.

4.2.5 *Preparation of [^{14}C]-VLDL*

[^{14}C]-VLDL was prepared using the method of Griffin *et al.* (1989). Briefly, 9.25 MBq of [^{14}C]-palmitic acid (2 MBq/µmole; Amersham Int PLC, Amersham, Bucks, UK.) in 2 ml of 250 mM Tris/HCl buffer, pH 8.0, containing 10% (w/v) fatty acid depleted BSA, was injected intravenously into a fully-fed 6 week old broiler chicken. After 15 min, 1 ml of sheep anti-chicken LPL antiserum (section 3.3.1) was injected intravenously to block the LPL-mediated catabolism of VLDL triacylglycerol and the bird killed 45 min later by an intravenous injection of sodium pentobarbitone BP (Sanofi Animal Health Ltd., Herts., UK.). The blood was collected from the jugular vein and mixed with EDTA to give a 4 mM final concentration. Plasma was obtained by

centrifugation of the blood at 1000 *g* av for 10 min at 10°C and it was recovered by aspiration. VLDL was separated by overlaying the plasma with 0.9 % (w/v) sodium chloride and centrifuging at 100,000 *g* av for 20 hrs at 10°C. The floating layer of radiolabelled VLDL was then recovered by aspiration. Using this procedure approximately 97% of the radioactivity incorporated into the VLDL was present in the triacylglycerol component (Griffin *et al.*, 1989). Radiolabelled VLDL was used within 6 hrs of its preparation.

4.2.6 *Uptake of [^{14}C]-VLDL in vivo*

Labelled VLDL ($2x10^6$ dpm) was injected into the wing vein of 5 fully-fed broiler chickens and the birds were killed 30 min later by intravenous injection of sodium pentobarbitone. The tissues were removed rapidly, washed in ice cold saline, frozen in liquid nitrogen and stored at -70°C.

Total tissue [^{14}C] levels were measured by solubilizing tissue segments in 90% (v/v) hyamine hydroxide (Sigma Chem. Co.) at 60°C for 6 hrs. Colour was removed from the samples by the addition of 150 µl of 30% (v/v) hydrogen peroxide followed by a further 30 min incubation at 60°C. After cooling, 10 ml of Optiphase Hisafe II scintillation fluid (Pharmacia Wallac Ltd, Milton Keynes, Bucks, UK.) was added to each tube and samples dark adapted for 30 min to reduce chemiluminescence. The [^{14}C] levels in the tissue samples were then determined using liquid scintillation counting (Wallac 1410, Pharmacia Wallac Oy, Turku, Finland).

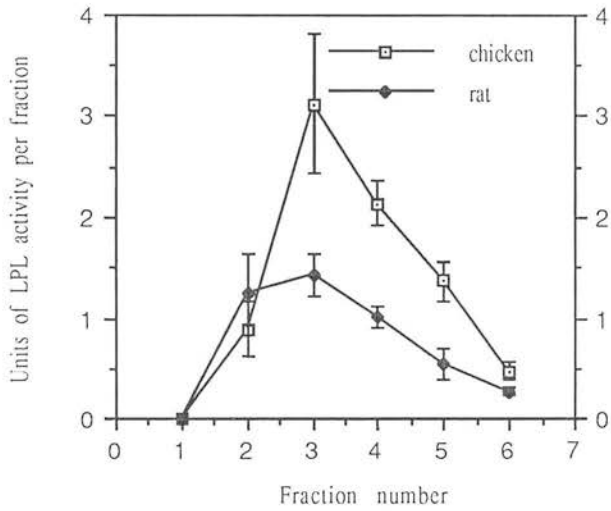
4.3 RESULTS

4.3.1 *Release of LPL activity by heparin*

A rapid release of LPL from perfused chicken hearts was produced by 30 units of heparin per ml of perfusion medium, Fig 4.1. Preliminary studies found that concentrations of heparin below this level did not release measurable levels of LPL activity, whereas concentrations above this failed to cause any increase in the amount of LPL activity released. Bensadoun and Koh, (1977) used 50 units of heparin per ml of medium to elute functional LPL activity in perfused chicken livers. These concentrations of heparin are considerably higher than those used in similar studies in rat tissues. For example, Chajek-Shaul et al. (1988)

found that 5 units of heparin per ml of medium caused the maximal release of LPL activity from the perfused rat heart and this concentration of heparin was also used by Gimenez-Llort *et al.* (1991) to release LPL activity from perfused newborn rat livers. In the present study 5 units of heparin per ml of medium was used to cause the rapid release of LPL activity in perfused rat hearts, Fig 4.1. This suggests a possible species difference between chickens and rats in the release of LPL by porcine mucous derived heparin.

Figure 4.1 Time course for the release of LPL activity by heparin in the isolated perfused chicken and rat heart model systems.



Chicken and rat hearts were perfused with heparin as described in sections 4.2.2. Perfusate fractions of 3.2 ml and 1.5 ml were collected from the chicken and rat hearts respectively. The values for the chicken are means ± SE of 9 perfusions of hearts from fully-fed birds. The values for the rat are means ± SE of the results of 5 perfusions of the hearts from fully-fed animals. Total LPL activity released per heart (Tables 4.3-4.5) was taken to be the cumulative amount of LPL activity present in fractions 2-5, which represented approximately 1 min of perfusion for both species.

4.3.2 *Effect of nutritional status on heparin-releasable LPL activity in perfused broiler hearts*

Hearts isolated from fully-fed, high-fat fed and fasted broilers were perfused with heparin as described in section 4.2.2.1. The total number of units of enzyme activity released by heparin from the isolated hearts of fully fed, high fat fed and fasted broiler chickens was 7.1, 9.5 and 11.5 (units per heart) respectively (Table 4.3). The 60% increase in the number of enzyme units released by heparin from the hearts of fasted birds was found to be significant ($p=0.0075$), while no significant

Table 4.3 The effect of nutritional status on the release of LPL activity by heparin from the isolated perfused broiler chicken heart.

Nutritional state	Heart weight (g)	Residual activity (units)	Activity released (units)	Total activity (units)	Percentage release
Fed	5.9±0.41	269.8±33.8	7.1±0.57	276.9±29.0	2.6±0.48
Fat fed	5.5±0.38	297.4±28.0	9.5±0.95	306.9±33.9	3.1±0.35
Fasted	5.1±0.21	294.3±41.3	11.5±0.36**	305.8±41.4	3.8±0.47

Broiler chicken hearts were isolated and perfused with heparin-containing medium, as described in section 4.2.2.1 The LPL activity released into the perfusate and that residual in the heart after heparin treatment was determined by the method described in section 4.2.3. Fat-fed birds were placed on a diet of 10% (v/w) corn oil in LS mash for 5 days prior to the experiment. Fasted birds had food removed 20 hours before perfusion. The values (expressed as total activity per heart) are means ±SE of the results from 4 birds at 4/5 weeks of age. Those values which are significantly different (Student's test) from those of the fully fed state are indicated by: **, $p<0.01$. The data was unchanged in pattern of expression when the values are expressed per g wet weight of tissue.

difference was found between the values for fed and high-fat fed birds. No significant difference was found between the different nutritional states in the measured level of residual or total tissue LPL activity when expressed per heart. The percentage of total tissue LPL activity released by heparin from hearts of fully-fed, high-fat fed and fasted birds was 2.6%, 3.1% and 3.8% respectively. No significant difference was discernible with respect to any of these values. The data was unchanged in pattern of expression when the values were expressed per g wet weight of tissue (data not shown).

4.3.3 *Comparison of the levels of LPL activity released by heparin perfusion of hearts isolated from broiler and layer chickens*

Due to selection for rapid growth over more than 40 generations, the plasma lipoprotein metabolism of broiler chickens may be atypical of chickens in general. Comparative experiments were, therefore, carried out on the percentage of total tissue LPL activity released by heparin perfusion of hearts isolated from broiler and layer chickens. For this study, 4 week old broilers and 6 week old layers were chosen because they have hearts of relatively comparable weights 4.7 g and 3.5 g respectively. The isolated hearts were perfused and treated with heparin as described in section 4.2.2.1. No significant difference was found between broilers and layers in either the levels of total tissue LPL activity or the activity residual in the heart after heparin perfusion (Table 4.4). Similarly, no significant difference was obtained between broilers and layers in the release of LPL activity by heparin or the percentage of total tissue LPL activity released (2.7% and 2.3% respectively).

4.3.4 *The effect of fasting on the release of LPL activity by heparin perfusion of isolated rat hearts*

As a means of validating the results obtained with the isolated perfused chicken heart system and as a comparison, a study was undertaken on the effect of fasting on the heparin-releasable levels of LPL activity in the isolated perfused rat heart.

The hearts of male Sprague-Dawley rats were isolated from fully-fed and fasted animals and perfused with heparin as described in section 4.2.2.2.

Table 4.4 The release of LPL activity by heparin from the perfused hearts of fully fed broiler and layer chickens.

Strain	Heart weight (g)	Residual activity (units/g)	Activity released (units/g)	Total activity (units/g)	Percentage release
Broilers	4.7±0.12	58.78±10.4	1.6±0.36	60.4±10.7	2.7±0.28
Layers	3.5±0.17**	44.56±6.97	1.1±0.18	45.6±7.12	2.3±0.15

Hearts were perfused with heparin containing-medium, as described in section 4.2.2.1 The LPL activity released into the perfusate and residual in the heart was determined by the method described in section 4.2.3. The values (expressed per g wet weight of tissue) are means ±SE of the results from 5 broilers at 3/4 weeks of age and 5 layers at 6 weeks of age. Those values for the layer chicken which are significantly different (student's test) from those of the broiler chicken are indicated by: **, $p<0.01$.

A significant difference (p=0.023) was found between the level of total tissue LPL activity (expressed per heart) in fed (70.5 units) and fasted (85.7 units) rats (Table 4.5). No significant difference was found between the levels of LPL activity residual in the tissue after heparin perfusion in either the fully-fed or fasted state. Fasting was found to cause a significant increase in the number of units of LPL activity and the percentage of total tissue LPL activity released by heparin-perfusion of the rat heart. Both these values were increased by a factor of 5 in the hearts of fasted animals. The data was unchanged in pattern of expression when the values were expressed per g wet weight of tissue (data not shown).

4.3.5 *Perfusion of chicken hearts with Concanavalin A-FITC conjugate*

In order to gain information on the state of reperfusion of the chicken heart concanavalin A-FITC conjugate was perfused through the isolated chicken heart as described in section 4.2.4. This was investigated because the apparently small percentage of the total tissue LPL activity released by heparin perfusion of the chicken heart by comparison with that seen from the fasted rat heart may have been due to a poor level of tissue reperfusion. The concanavalin A which binds to glycoproteins present at the luminal surface of the capillary endothelium effectively labels those blood vessels successfully reperfused. Frozen sections of cardiac tissue, prepared as described in section 4.2.4, from various portions of the heart were examined and found to have significant levels of label present in the capillary bed and over the luminal surface of the major blood vessels (Fig 4.1). Because of the high density of labelled capillaries observed in the sections studied, it was concluded that reperfusion of the chicken heart was effective and that heparin was being delivered to the capillary bed.

4.3.6 *Hydrolysis of [^{14}C] labelled lipoprotein triacylglycerol in vivo*

As a further indicator of the level of functional LPL activity in the muscle tissues and adipose tissue of broiler chickens, the uptake of [^{14}C] labelled VLDL triacylglycerol NEFA was studied *in vivo*. The [^{14}C]-VLDL was prepared as described in section 4.2.5. The radiolabelled VLDL was injected into the wing vein of 5 fully-fed broiler chickens and after 30 min the birds were killed and the level of [^{14}C] present in selected tissues determined, as described in section 4.2.6. The mean tissue [^{14}C] levels from 5 birds are given in Table 4.6. The method used in this

Table 4.5 The effect of fasting on the release of LPL activity by heparin from the isolated perfused rat heart

Nutritional state	Heart weight (g)	Residual activity (units)	Activity released (units)	Total activity (units)	Percentage release
Fed	1.21±0.09	66.1±2.80	4.4±0.37	70.5±3.48	6.2±0.64
Fasted	1.17±0.05	59.6±2.41	26.1±0.92**	85.7±2.9*	31.5±0.87***

Hearts were perfused with heparin containing-medium, as described in section 4.2.2.2 The LPL activity released into the perfusate and residual in the heart was determined as described in section 4.2.3. Fasted rats had food removed 18 hours before perfusion. The values (expressed per heart) are means ±SE of the results obtained from 5 rats. Those values for fasted rats which are significantly different (Student's test) from those of fed rats are indicated by *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. The data was unchanged in pattern of expression when the values are expressed per g wet weight of tissue.

Figure 4.1 Sections of chicken cardiac tissue obtained from hearts perfused with concanavalin A-FITC conjugate.

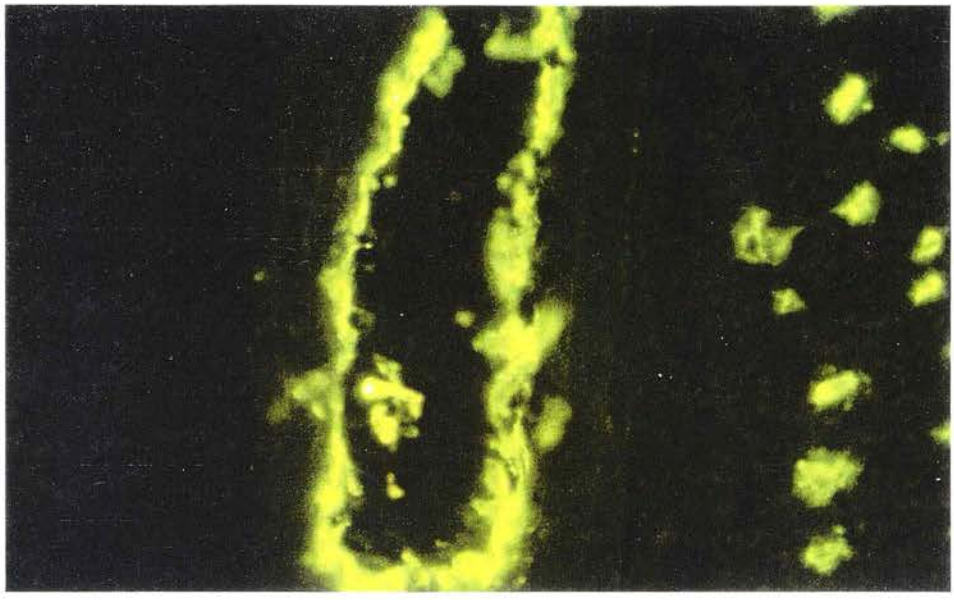
Cryostat sections of chicken cardiac tissue were obtained from hearts perfused with concanavalin A-FITC conjugate as described in section 4.2.5. The sections shown were taken from the left ventricular myocardium. Prints x 595.

A) Considerable amounts of label were associated with the endothelial cell surface and lumen of the major blood vessels.

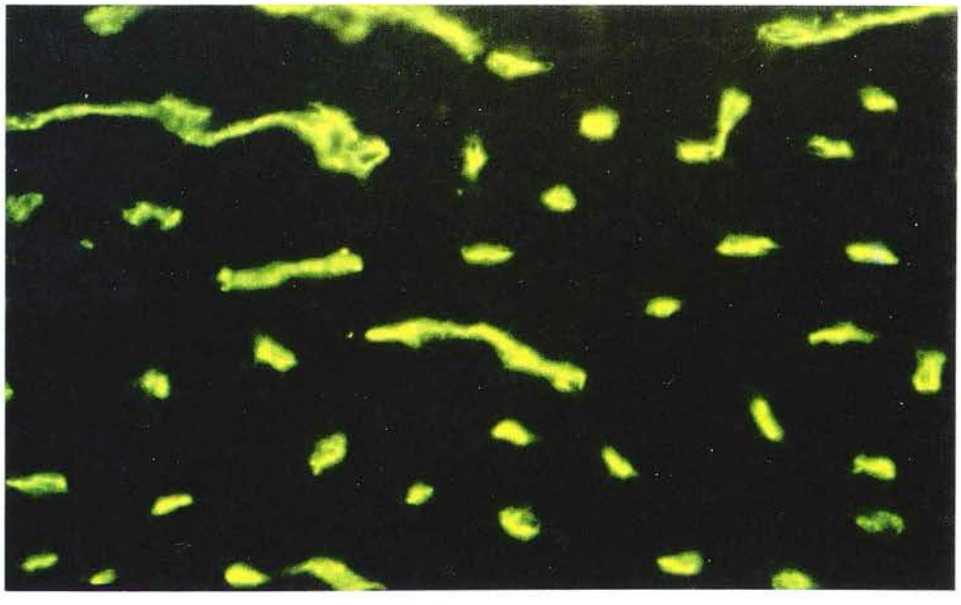
B) A high density of labelled capillaries was observed throughout the heart.

The Langendorff preparation adopted in the present study was therefore considered to effectively reperfuse the capillary bed of the isolated chicken heart.

A



B



study to assess tissue radioactivity, ie: hyamine hydroxide solubilization, would include measurement of aqueous components and bicarbonate levels within tissues/plasma whereas previous studies have only examined lipid extractions of tissues (Griffin *et al.*, 1989; Griffin *et al.*, 1991a; 1991b). After 30 min, assuming the plasma volume to be 4.7% of body weight for 6 week old broiler chickens (Griffin *et al.*, 1989), 3.4% of the injected dose of radiolabel remained in the plasma. This correlates closely with previous studies which reported the half life of VLDL in broiler chicken plasma to be 5 min (Griffin *et al.*, 1991b). The total tissue LPL activities shown in Table 4.6 were obtained from Guo (1988) and had been measured in 9 fully fed broiler chickens at 6 weeks of age.

Table 4.6 Tissue accumulation of radiolabelled NEFA from [^{14}C]-VLDL triacylglycerol in relation to total tissue LPL activities.

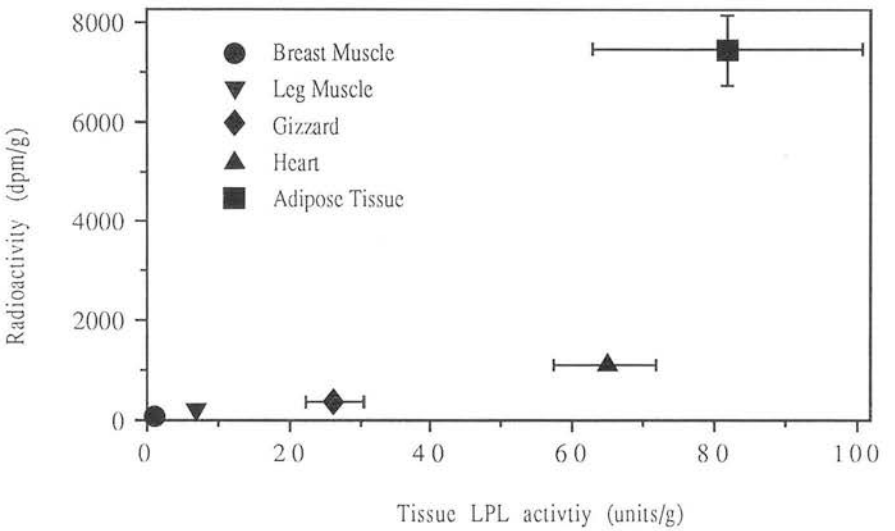
Tissue	Tissue LPL activity (units/g)	Accumulation of [^{14}C] NEFA (dpm/g)
Breast muscle	0.6±0.2	56±5
Leg muscle	7.2±1.5	143±16
Gizzard	26.5±4.2	361±68
Heart	64.5±7.2	1088±24
Abdominal fat	82.0±19.0	7440±701
Liver	1.2±0.6	8268±416
Plasma	0.0	900±50

Radiolabelled VLDL was injected into the wing vein of 5 fully-fed 6 week old broiler chickens and after a period of 30 min the birds were killed and the level of [^{14}C] determined in selected tissues (as described in section 4.2.6). The total tissue LPL activities shown were obtained from Guo (1988) where they were measured in 5 fully-fed broiler chickens at 6 weeks of age. In both cases the values are means ±SE and are expressed per g wet weight of tissue or per ml of plasma. 1 unit of LPL activity was taken to be the release of 1 µmole of NEFA/hr in the assay system described in section 2.2.2.

Comparison of radiolabel accumulation and total tissue LPL activity shows a linear relationship for all the muscle tissues studied (Fig. 4.2). However, for adipose tissue considerably more radiolabel (7441 dpm/g wet weight of tissue) was accumulated than would have been expected from the level of total tissue LPL activity (82 units/g wet weight of tissue). The present study found the muscle tissues (breast muscle, leg

muscle, gizzard and heart) to accumulate an average of 36 dpm of [^{14}C] lipoprotein triacylglycerol NEFA per unit of total tissue LPL, while the abdominal fat pad was found to accumulate 90 dpm of [^{14}C] triacylglycerol NEFA per unit of total tissue LPL activity.

Figure 4.2 Tissue accumulation of NEFA from [^{14}C]-VLDL in relation to total tissue LPL activities of fully-fed broiler chickens.



Radiolabelled VLDL was injected into the wing vein of 5, fully-fed, 6 week old brolier chickens and after 30 min the birds were killed and the level of [^{14}C] determined in selected tissues (as described in section 4.2.6). the total tissue LPL activities were obtained from Guo (1988) and were measured in fully-fed broiler chickens at 6 weeks of age (see Table 4.6). The values are means ±SE and are expressed per g wet weight of tissue.

4.4 DISCUSSION

Studies using the isolated perfused rat heart as a model system have shown significant changes in the level of heparin-releasable (ie: functional) LPL activity and smaller changes in total cardiac tissue LPL activity during fasting and carbohydrate or fat feeding (eg: Borensztajn and Robinson, 1970; Pedersen and Schotz, 1980; Kronquist *et al.*, 1980). Results described in this Chapter also demonstrate a significant increase in the percentage of total tissue LPL activity released by heparin from the isolated perfused rat heart during fasting, ie: from 6.2% to 31.5%. Similar results were obtained by Bagby and Corll (1989) who found that the heparin-releasable fraction of total tissue LPL activity in the isolated perfused rat heart rose from 3.7% to 30.2% during fasting.

Significant differences were not observed in the percentage of total tissue LPL activity released by heparin perfusion of hearts isolated from fully-fed, high fat-fed and fasted broiler chickens. This would seem to correlate with the response of total tissue LPL activities in avian species which also seem not to be significantly altered during fasting. The latter was found to be the case in ducks (Evans, 1972) and pigeons (Cryer *et al.*, 1979). Chicken adipose tissue and skeletal muscle LPL activity is only marginally affected by fasting, while total activity in cardiac muscle increases only slightly (Husbands, 1972; Benson and Bensadoun, 1977; Hermier *et al.*, 1989; Griffin and Butterwith, 1988). Hermier *et al.* (1989) also found that fasting 5 week old broiler chickens for 18 hrs failed to alter the level of heparin releasable LPL activity from adipose tissue segments *in vitro*.

Griffin *et al.* (1987) have reported that the LPL activity in chicken adipose tissue is rate limited by substrate concentration, within the normal range of plasma VLDL levels found in the fully-fed state. The concentration of plasma triacylglycerol from fasted chickens has been reported to be between 30% and 50% of that of fully-fed chickens (Kim et al., 1991; Leclercq *et al.*, 1984). These observations have led to the suggestion that in birds in the fasted state the supply of substrate is the critical regulator of triacylglycerol uptake rather than changes in functional LPL activity (Butterwith, 1988).

Plasma NEFA concentrations in broiler chickens have been observed to double during periods of fasting (Annison, 1971). It is possible that during fasting the muscle tissues derive a significant proportion of their energy requirements from the elevated plasma pool of NEFA, thereby reducing the requirement for increases in the amount of functional LPL activity in these tissues. Adipose tissue is the major source of the plasma NEFA during fasting, derived from increased rates of lipolysis (Annison, 1971). The level of product inhibition of LPL activity (Baginsky and Brown, 1977; Bengtsson and Olivecrona, 1980) caused by the localized build-up of NEFA may overcome the requirement for a reduction in the level of functional LPL at the capillary endothelium of adipose tissue in the fasted state (ie: plasma lipoprotein triacylglycerol NEFA are directed to the muscle tissues due to the inhibition of adipose tissue functional LPL rather than by a reduction in the amount of LPL

functional at the capillary bed of adipose tissue or by an increase in the amount of LPL functional at the capillary bed of muscle tissues). This would seem to correlate well with the lack of responsiveness of functional LPL activity in broiler cardiac and adipose tissue to alteration in nutritional status, seen both here and elsewhere. However, similar changes in plasma triacylglycerol and NEFA levels are also seen in rats (Bertin et al., 1985, Larue-Achagiotis and LeMagnen, 1983, Menahan and Sobocinski, 1983) where significant changes in the functional LPL activity of cardiac tissue has been documented, in the present and earlier studies (Borensztajn and Robinson, 1970, Bagby and Corll, 1989).

The selection of broilers for rapid growth over 40 generations has caused a 2-fold increase in the proportional level of carcass fat in broiler as opposed to layer chickens. Because plasma lipoprotein metabolism in broiler chickens may therefore be atypical of chickens in general, the level of functional LPL activity in the cardiac tissue of fully-fed broiler and layer chickens was compared. However, in the present study, no significant difference was found between the percentage of total tissue LPL activity released by heparin perfusion from the hearts of broiler and layer chickens (2.7% and 2.3% respectively).

Wolfe *et al.* (1981) concluded from several *in vivo* studies on the fate of radiolabelled VLDL triacylglycerol that oxidation of VLDL derived NEFA did not involve their prior entry into the plasma pool of NEFA. The hydrolysis of [^{14}C] labelled lipoprotein triacylglycerol *in vivo* and the uptake of the released NEFA can, therefore, give a comparative indication of the amount of LPL activity functional in a given tissue. The low amounts of radiolabel found in muscle tissues, by comparison to adipose tissue (Table 3.6), were not due to the rapid oxidation of the [^{14}C]-NEFA to CO_2 in these tissues since less than 4% of the administered dose, in fully-fed broilers, was expired as CO_2 over the experimental period used in the present study (Griffin *et al.*, 1991a). From Table 4.6, it can be seen that the total tissue LPL activity of the muscle tissues of broiler chickens is very variable between individual muscles. Similar variations in the LPL activity of mammalian muscle tissue have been attributed to differences in the muscle type ie: high in red muscle fibres which have a high capacity for fatty acid oxidation and low in white muscle fibres (Brown and Layman, 1988). Following the injection intravenously of [^{14}C]-VLDL *in vivo,* a linear correlation between total

tissue LPL activity and [^{14}C] lipoprotein triacylglycerol NEFA accumulation in the broiler muscle tissues was observed (Fig 4.2). Similarly, several early studies found that the uptake of radioactivity following an intravenous injection of radiolabelled triacylglycerol was greater in muscle containing mainly red fibres, which have higher LPL levels than in those containing mainly white fibres (Linder *et al.*, 1976; Tan *et al.*, 1977). However, recent studies by Brown and Layman (1988) found no such correlation between total tissue LPL activities and the hydrolysis *in vivo* of [^{14}C]-labelled chylomicron triacylglycerol in female rats. They concluded that tissue and plasma post-heparin LPL activities were not a measure of the ability of individual tissues to accumulate triacylglycerol NEFA or the rate of plasma chylomicron triacylglycerol clearance. Similarly, Marin *et al.* (1990) also found little correlation between total adipose tissue LPL activity and the *in vivo* uptake of radiolabelled lipoprotein triacylglycerol in man and concluded that factors other than LPL are also important for adipose tissue uptake of lipoprotein triacylglycerol. These may include the regulation of blood flow to a given tissue or fat depot (West *et al.*, 1989).

The correlation between total tissue LPL activity and [^{14}C] lipoprotein triacylglycerol hydrolysis in the broiler muscle tissues studied suggests that the percentage of total tissue LPL activity functional in the heart is typical of the percentage of functional LPL in the other muscle tissues. The relatively low level of heparin-releasable LPL activity in the perfused chicken heart (2.7% in the fully-fed state) also correlates with the relatively low level of uptake and accumulation of NEFA derived from [^{14}C] lipoprotein triacylglycerol by the muscle tissues (Fig 4.2). In rats, up to 40% of plasma lipoprotein triacylglycerol was taken up and oxidised by muscle tissues (Bragdon and Gordon, 1958). Whereas in broiler chickens this figure was between 20-25% over a similar time period (Griffin *et al.*, 1991b). This species difference may be due to lower levels of functional LPL activity in the muscle tissues of broilers in comparison to rats, as was found to be the case in the present study. Although, levels of total cardiac tissue LPL activity (per g wet weight of tissue) were found to be similar in fully-fed broiler chickens and rats, the percentage of total tissue LPL activity released by heparin was 2-fold higher in the rat heart than in the chicken heart.

The adipose tissue of broiler chickens appears, in relation to the muscle tissues, to be able to accumulate considerably more triacylglycerol NEFA than would be expected from measurement of the total tissue LPL activity (Fig 4.2). Several studies have confirmed that the mammalian adipose tissue is also particularly adept at the incorporation of lipoprotein triacylglycerol NEFA. Within 10 min of an intravenous administration of radiolabelled chylomicrons, Bragdon and Gordon (1958) found that carbohydrate-fed rats incorporated 32% of the label into adipose tissue. Similar studies in dogs (Nestel *et al.*, 1962) found 27.5% of the label in adipose tissue 45 min after administration of radiolabelled chylomicrons. This high level of uptake is not due to greater amounts of lipoproteins being delivered to the fat depots by the bloodstream, as adipose tissue has a relatively low rate of blood flow when compared to other tissues (Rosell and Belfrage, 1979). This also suggests that broiler chicken adipose tissue not only has more LPL per g wet weight of tissue, but also has a higher percentage of total tissue LPL activity functional at the capillary bed. The density of LPL at the luminal surface of the endothelium may play a crucial role in the kinetics of lipoprotein triacylglycerol hydrolysis at the capillary bed.

The 60% increase in the number of units released from the hearts of fasted broilers correlates with the observation that LPL activity in post-heparin plasma of fasted chickens is twice that of fully fed birds (Benson *et al.*, 1975; Hermier *et al.*, 1989). The heart may therefore be similar to other chicken muscle tissues in its response to fasting. This assumes that functional LPL activity in adipose tissue *in vivo* is unchanged, as was found to be the case by Hermier *et al.* (1989) *in vitro*.

In conclusion, the work described in this Chapter characterizes the perfused chicken heart as a model system for the study of LPL, although the proportion of the functional (ie: heparin-releasable) enzyme activity was found to be low. From this, the preparation may potentially be used as the model system for attempts to immunoquantify functional LPL by perfusion with an iodinated anti-chicken LPL monoclonal antibody.

CHAPTER 5

STUDIES ON THE IMMUNOQUANTITATION OF FUNCTIONAL LPL USING AN IODINATED ANTI-CHICKEN LPL MONOCLONAL ANTIBODY

5.1 INTRODUCTION

Chapter 4 described the isolated perfused chicken heart as a model system for studying the heparin-induced release of LPL activity. The work in this Chapter extends this by attempting to measure the amount of LPL protein available at the capillary endothelium using an iodinated anti-LPL monoclonal antibody, either perfused through the isolated heart or injected intravenously *in vivo*.

Several studies have investigated the relationship between total tissue LPL activities and LPL protein levels. Jansen *et al.* (1978), for example, correlated nutritionally-induced changes in rat adipose tissue LPL activity with changes in the LPL immunoreactive mass present and concluded that alterations in enzyme activity were due to changes in the amount of enzyme protein present. Similar work by Kronquist *et al.* (1980), using the isolated perfused rat heart, correlated changes in the release of functional endothelial LPL activity by heparin with the concomitant release of LPL immunoreactive mass. These authors concluded that, in the rat, changes in functional LPL activity at the capillary lumenal surface were due to alterations in the amount of enzyme present at this site rather than to the operation of any local activation/inactivation processes.

A considerable number of studies have been published on the use of iodinated antibodies for the radioimmunodetection *in vivo* of tumours expressing a given antigen (eg: Van Herle *et al.*, 1991; Peters *et al.*, 1990; Harwood *et al.*, 1990) and the diagnostic and therapeutic uses of passive immunization with radiolabelled antibodies *in vivo* has recently been reviewed by Thomas *et al.* (1990) and Bourdon *et al.* (1984). Similarly, Huettinger *et al.* (1984) injected an iodinated anti-LDL receptor monoclonal antibody intravenously in order to measure the levels of LDL receptor protein present *in vivo* at the vascular endothelium of the tissues of normal and Wantanabe Heritable Hyperlipadaemic rabbits. On this basis, the similar use of an iodinated anti-LPL monoclonal antibody,

injected intravenously *in vivo*, may allow the measurement of LPL protein in the capillary bed of individual tissues. Because measurements of enzyme protein can be related directly to enzyme activity (Jansen *et al.*, 1978; Kronquist *et al.*, 1980), the technique would therefore assess the functional LPL activity of individual tissues *in vivo*.

Preliminary studies utilised the isolated perfused chicken heart as a model system. It was proposed that successful measurement of LPL protein in this system and the determination of its relationship with heparin-induced release of LPL activity could act as a feasibility study for subsequent experiments *in vivo*. Subject to feasibility being shown, it would then become possible to use the technique for the assessment of tissue specific changes in functional LPL *in vivo*, using animals in different nutritional states, varying developmental stages and in animals from selected fat and lean lines.

The first application of anti-LPL antibodies in the study of functional LPL in the perfused heart was reported by Schotz *et al.* (1977), who perfused rat hearts with an inhibitory polyclonal antibody. Following this treatment the hearts were found to be incapable of oxidising chylomicron triacylglycerol. Similarly, following perfusion with an anti-LPL antiserum, LPL was visualised at the capillary endothelium of isolated rat hearts using immunocytochemistry (Pedersen *et al.*, 1983). The estimated coverage of the capillary lumen surfaces by immunoreaction product was found to correlate positively with previously reported changes in heparin-releasable LPL activity. Intravenous injection of polyclonal anti-LPL antibodies into chickens (Bensadoun and Kompiang, 1979; Griffin *et al.*, 1989) and monoclonal anti-LPL antibodies into monkeys (Goldberg *et al.*, 1988a) has also been shown to rapidly block the catabolism of lipoprotein triacylglycerol *in vivo*. These studies indicate that the fraction of tissue LPL that is functional in lipoprotein triacylglycerol hydrolysis is readily available to circulating inhibitory antibodies in the perfused heart and *in vivo*.

In the present study, an iodinated anti-chicken LPL monoclonal antibody (Cal-11) was perfused through isolated chicken hearts or injected intravenously *in vivo* and the amount of radiolabel released by heparin used to estimate the amount of specifically bound antibody. Cal-11 (Gershenwald *et al.*, 1985) was chosen because it had a relatively

high association constant ($2.4.x10^{10}$ M^{-1}). It has been reported that the association constants for monoclonal antibodies range between 10^6-10^{12} M^{-1} (Liddell and Cryer, 1991). Larson (1985) suggested that only monoclonal antibodies with affinity constants of 10^{10} M^{-1} or above be used for the analogous technique of *in vivo* radioimmunodetection of tumour associated antigens. Cal-11 also inhibits LPL activity in the presence of heparin (Gershenwald *et al.*, 1985), indicating recognition of an epitope available when LPL is bound by proteoglycans at the vascular endothelium.

5.2 METHODS

5.2.1 *Monoclonal antibody purification*

Ascitic fluid containing an anti-chicken LPL monoclonal antibody (Cal-11) was a gift from Professor A. Bensadoun, Cornell University, Ithaca. The production of the antibody was detailed previously by Gershenwald *et al.* (1985). The antibody was isolated initially by ammonium sulphate precipitation. For this, sufficient saturated ammonium sulphate solution, pH 7.2, (prepared by dissolving 100 g of ammonium sulphate in 100 ml of water at 50°C) was added to the ascitic fluid, at 0°C, to achieve a 40% (v/v) final concentration. The samples were then incubated for 1 hour at 0°C and centrifuged at 1600 *g* av for 15 min. The protein precipitate was then washed with a 40% (v/v) saturated solution of ammonium sulphate by resuspension and re-centrifugation. The protein precipitate was then dissolved in 1/3 of the original volume of 0.01 M phosphate buffer, pH 7.2 and dialysed against 3 changes of the same buffer, overnight at 4°C.

The protein containing solution was then applied to a 10 ml DEAE-Sephadex (A50) anion-exchange column (Sigma Chemical Co.), previously equilibrated with 0.01 M phosphate buffer, pH 7.2. The column was eluted with this buffer at a rate of 20 ml/hour and 2.5 ml fractions of eluate collected. The void volume fractions containing the IgG peak, determined by measuring the absorbance at 280 nm, were pooled and aliquots stored at -70°C. At each stage of the purification procedure, samples were taken for analysis by SDS-PAGE, as described in section 2.2.5.

5.2.2 *Inhibition of LPL activity*

Chicken adipose LPL (0.5 units) partially purified by heparin-Sepharose affinity chromatography, as described in section 2.2.4, was pre-incubated with increasing amounts (1-20 μg) of purified Cal-11 IgG in 100 μl of 10 mM phosphate buffer, pH 7.4, containing 20% (v/v) glycerol to stabilize the enzyme activity. After the pre-incubation periods, the mixtures were assayed for LPL activity as described in section 2.2.2. LPL activity in control tubes in which Cal-11 IgG was substituted with non-immune mouse IgG (Sigma Chemical Co.) was also determined under identical pre-incubation conditions.

5.2.3 *Iodination of Cal-11 IgG using the chloramine T method*

Iodination using chloramine T was carried out using a modification of the method of McConahey and Dixon (1980). Briefly, 20 μg of Cal-11 IgG, 1 mCi of carrier free Na[^{125}I] (93.7 mCi/ml Amersham International plc. Amersham, Bucks, UK), 20 μl (0.5 mg/ml) chloramine T and 10 μl of 0.3 M phosphate buffer, pH 7.6, were mixed continuously, using a magnetic stirrer, for 30-60 seconds. The reaction was then stopped by the addition of 20 μl (1mg/ml) sodium metabisulphite. The mixture was then applied to a 5 ml Sephadex G25 gel filtration column (PD-10, Pharmacia Ltd., Milton Keynes, Bucks, UK) which had previously been equilibrated with 1% (w/v) BSA in PBS to minimize the absorption of tracer. The column was eluted using PBS containing 0.1% BSA and eluate fractions of 0.5 ml were collected. The relative amounts of radioactivity contained in each fraction were assessed using a gamma counter (Mini Assay 6-20, Mini Instruments Ltd., Burnham on Crouch, Essex). The void volume fractions containing the [^{125}I]-IgG were pooled and the specific activity of the fractions (μCi/μg) determined using a Wallac 1261 gamma counter (Pharmacia Wallac Oy, Turku, Finland). Radiolabelled IgG was stored in aliquots at -20°C and used within 4 weeks of preparation.

5.2.4 *Iodination of IgG using the lactoperoxidase-glucose oxidase method*

Enzymobead reagent (Biorad Labs, Richmond, California, USA) was reconstituted in 100 μl of distilled water, mixed with 25 μg of Cal-11 IgG and 500 μCi of carrier free Na[^{125}I]. The reaction was then initiated by the addition of 25 μl of 1% (w/v) β-D-glucose. After a 20 min incubation period, the reaction was stopped by the separation of the mixture on a gel filtration column, as described in section 5.2.3. The [^{125}I]-IgG

containing fractions were pooled and the specific activity of the preparation determined as described in section 5.2.3. Radiolabelled IgG was stored in aliquots at -20°C and used within 4 weeks of preparation.

5.2.5 *Assessment of antibody binding to LPL by dot-blotting*

A dilution series of chicken LPL (1.0-0.06 μg), purified by heparin-Sepharose and concanavalin A-Sepharose affinity-chromatography as described in section 3.2.3, was bound to a nitrocellulose filter (0.45 μm, Schleicher and Schuell, Dassel, Germany) using a dot blot apparatus (Hybridot Manifold, BRL, Gaithersburg, USA.). The filter was then removed from the apparatus and the remaining reactive sites on the membrane blocked by incubation with 3% (w/v) BSA, 1% (v/v) horse serum and 0.5% (v/v) Tween-20 in PBS for 2 hours at 20°C. After washing with PBS containing 0.05% (v/v) Tween-20 (PBS-T), the filter was cut into strips and incubated for 2 hours at 20°C with 2 ml of either 0.05 μg/ml Cal-11 IgG or 0.05 μg/ml non-immune mouse IgG in PBS-T. The strips were then washed with PBS-T and incubated for 1 hour with 5 ml of a 1/2000 (v/v) dilution of HRP-conjugated anti-mouse IgG (Sigma Chemical Co.) in PBS-T. Following exhaustive washing with PBS-T, the presence of bound HRP-conjugated antibody was visualized by incubation with 50 ml of PBS containing 25 mg of 3,3' diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and 50 μl of 30% (v/v) hydrogen peroxide. To ensure that the binding of primary antibody observed by this procedure was due to radiolabelled Cal-11, autoradiography was also carried out. The autoradiographs were developed overnight at -70°C on Hyperfim-MP (Amersham International plc, Amersham, Bucks) in an X-Omatic cassette (Eastman Kodak, Rochester NY) using intensifying screens.

5.2.6 *Perfusion of Cal-11 through the isolated heart*

Broiler chickens (reared as described in section 4.2.1) were fasted for 16 hours prior to the experiment in order to enhance the LPL activity present at the capillary endothelium of cardiac tissue (section 4.3.2). Hearts were isolated and perfused for 1 min with a modified Krebs-Henselite buffer to remove trapped blood from the vasculature, as previously described in section 4.2.2. The hearts were then transferred immediately to a second perfusion apparatus containing perfusion medium to which Cal-11 (100,000 dpm [^{125}I]-IgG/ml) had been added. Perfusion with the iodinated antibody was continued using either a

single pass or a recirculating system (Fig 4.1). The hearts were then transferred back to the initial perfusion apparatus and perfused with a minimum of 100 ml of perfusion medium to remove unbound antibody before the perfusion medium was supplemented with 30 units/ml of heparin. Perfusate fractions of 2.5 ml were collected throughout the course of the experiment. The amount of antibody associated with the perfusate fractions (before and after heparin perfusion) and residual in the heart tissue was determined using a Wallac 1261 gamma counter.

5.2.7 *Clearance of iodinated Cal-11 in vivo*

Fully-fed broiler chickens (reared as described in section 3.2.1) of 4 weeks of age were injected via the wing vein with 36 ng of Cal-11 (1.10^6 dpm [^{125}I]-IgG) in PBS containing 1% (w/v) BSA. Serial blood samples were then taken to monitor the disappearance of the label from the blood stream. After injection of the radiolabelled IgG, 1 min was allowed for the mixing of the tracer in the bloodstream, before removal of the first blood sample. The level of radioactivity in this sample was defined as the 100% value from which subsequent disappearance was calculated. After a time interval of either 5 or 30 min, an intravenous injection of 1000 units of heparin/Kg (mucous injection BP, Leo Labs Ltd, Princes Risborough, UK) body weight was given and blood samples again taken 2 and 4 min later to monitor the presence of the label in the blood stream. This amount of heparin has previously been shown to cause maximal release of LPL activity into the plasma of broiler chickens within 1 min and that post-heparin plasma LPL activity remained at this level 5 min after heparin injection (Guo, 1988). For each sample, the radioactivity was present in 150 µl aliquots of blood was determined in triplicate using a Wallac 1261 gamma counter.

5.3 RESULTS

5.3.1 *The purification of Cal-11 from ascitic fluid*

A pure preparation of monoclonal IgG was required for iodination to reduce any potential non-specific interactions which may arise from the labelling of contaminant proteins.

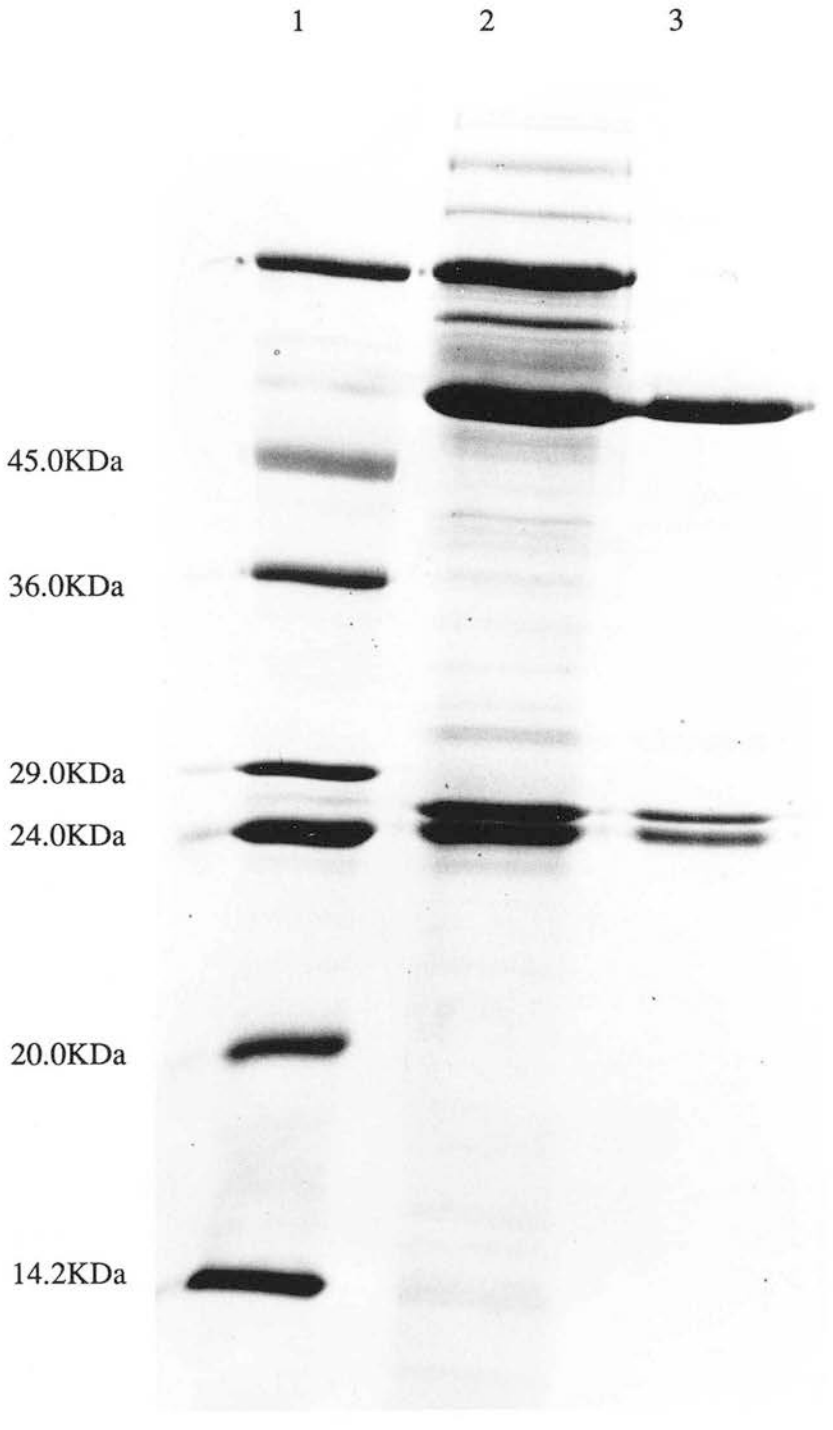
The initial step in the purification of IgG from ascitic fluid was ammonium sulphate precipitation. The immunoglobulin fraction following this procedure was analysed by SDS-PAGE and found to contain a large number of proteins in the 20-60 KDa molecular weight range (Fig. 5.1). The partially purified IgG obtained by ammonium sulphate precipitation was further purified by chromatography on an anion-exchange column, as described in section 5.2.1. Following this step, 3 IgG component proteins, of molecular weight 52.5 KDa which corresponded with the expected size of the 2 murine IgG heavy chains and 2 proteins with molecular weights of 24.5 KDa and 25 KDa, which corresponded with the expected size of the 2 component IgG light chains, were observed as the sole major constituents of material analysed by SDS-PAGE under reducing conditions (Fig 5.1).

5.3.2 *Inhibition of LPL activity by Cal-11*

To determine how rapidly maximal binding of the antibody to the enzyme occurred, the ability of the purified anti-chicken LPL monoclonal antibody, Cal-11, to inhibit partially purified LPL under various pre-incubation conditions was investigated. To represent equilibrium conditions, a pre-incubation period of 16 hours at 4°C was chosen, whereas pre-incubation periods of 5 and 15 min at 41°C were chosen to represent the conditions and possible times of circulation of the antibody in the perfused heart system or *in vivo*.

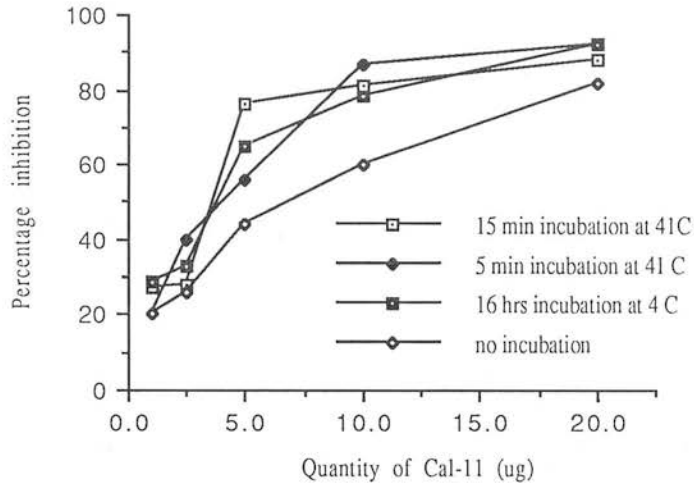
No difference in the ability of Cal-11 to inhibit LPL activity was found between the different pre-incubation periods studied (Fig 5.2). When the pre-incubation period was omitted, the curve of enzyme inhibition versus antibody concentration (Fig 5.2) was shifted slightly to the right. This may represent a time lag for binding to occur or competition for the epitope on the enzyme between the antibody and either the activator protein (apo CII) or the substrate.

Figure 5.1 Polyacrylamide gel electrophoretic analysis of samples obtained during the purification of the anti-chicken LPL monoclonal antibody, Cal-11, from mouse ascitic fluid.



Samples were obtained at each stage in the procedure described in section 5.2.1 for the purification of Cal-11. The samples were analysed by SDS-PAGE under reducing conditions on a 12% polyacrylamide gel, as described in section 2.2.5.
Lane 1. Electran molecular weight markers (Sigma Chemical Co.). Lane 2. material following ammonium sulphate precipitation (40 µg). Lane 3. material following anion exchange chromatography (10 µg).

Figure 5.2 The effect of pre-incubation conditions on the inhibition of LPL activity by the anti-chicken LPL monoclonal antibody, Cal-11.



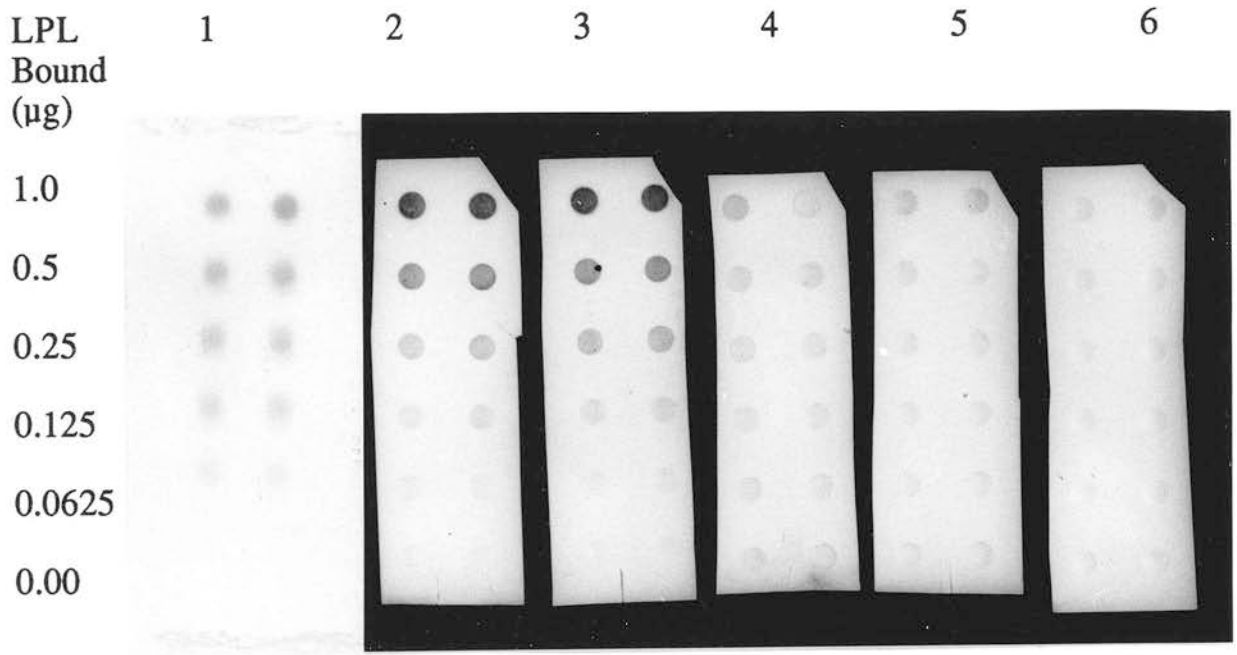
Chicken adipose LPL (0.5 units), partially purified by heparin-Sepharose affinity chromatography (section 2.2.4), was pre-incubated with increasing amounts (1-20 μg) of purified Cal-11 IgG in 100 μl of phosphate buffer as described in section 5.2.2. The mixtures were then assayed for LPL activity (section 2.2.3) and the percentage inhibition calculated against control tubes pre-incubated with non-immune mouse IgG.

5.3.3 *Assessment of antibody binding to LPL after radiolabelling*

Using the dot-blot assay described in section 5.2.5, the binding of radiolabelled preparations of Cal-11 to LPL were compared with the binding of non-iodinated Cal-11 in order to determine whether any loss of reactivity towards the enzyme had occurred during the labelling procedure. The results obtained are shown in Fig 5.3.

Cal-11 iodinated by the lactoperoxidase method (lane 3) appeared to exhibit an identical pattern of binding to that seen with non-iodinated Cal-11 (lane 2). However, both preparations of Cal-11 iodinated by the chloramine T method (lanes 4 and 5) showed significant reduction in the level of binding when compared to non-iodinated Cal-11 (lane 2). Reducing the specific activity of the chloramine T preparations from 39.2 μCi/μg (lane 5) to 20.0 μCi/μg (lane 4), thereby reducing the level of substitution from 2.6 to 1.2 [^{125}I] molecules per IgG, was found to increase the level of Cal-11 binding. Non-immune mouse IgG was found to give no detectable binding in the assay system. Autoradiography of lactoperoxidase iodinated Cal-11 (lane 1) indicated that the binding of

Figure 5.3 Comparison of the effect of lactoperoxidase and chloramine T methods of iodination on the reactivity of Cal-11 towards LPL bound to a nitrocellulose filter.



Samples of purified chicken LPL were bound, in duplicate, to a nitrocellulose filter and incubated with radiolabelled Cal-11. The binding of Cal-11 to LPL was visualized either by the use of a secondary peroxidase-conjugated antibody procedure (2-6) or by autoradiography (1), as described in section 5.2.4. The strips of nitrocellulose shown above, were incubated with the following preparations of Cal-11:

1. Cal-11 iodinated using the lactoperoxidase method (12.4 µCi/µg IgG).
2. Unlabelled Cal-11.
3. Cal-11 iodinated using the lactoperoxidase method (12.4 µCi/µg IgG).
4. Cal-11 iodinated using the chloramine T method (20.0 µCi/µg IgG).
5. Cal-11 iodinated using the chloramine T method (39.9 µCi/µg IgG).
6. Unlabelled non-immune mouse IgG.

Cal-11 to LPL, detected by the secondary HRP conjugated antibody system, was due to the binding of iodinated IgG.

Chloramine T iodination has on many occasions been shown to reduce the reactivity of labelled antibodies either by oxidative damage or by oversubstitution. For example, Trucco and Petris (1981) found that after the iodination of anti-HLA monoclonal antibodies by chloramine T, only 15-20% of the labelled antibodies were able to bind to the target cells at saturation. Matzku and Zoller (1977) compared lactoperoxidase iodination with chloramine T iodination and found improved reactivity with the more gentle enzymatic procedure. The lactoperoxidase method was used for the iodination of Cal-11 in all subsequent studies described in this Chapter.

5.3.4 *Heparin releasable binding of iodinated Cal-11 in the perfused heart system*

Iodinated Cal-11 (100,000 dpm [^{125}I]-IgG/ml) was perfused through the isolated chicken heart, as described in section 5.2.6 and the amount of radioactivity in the perfusate fractions (before and after heparin treatment) and residual in the heart was determined. The results of the study are summarised in Table 5.1. The Table shows that Cal-11, iodinated by the chloramine T method, failed to produce any specific binding (heparin-releasable) when perfused through the isolated chicken heart. However, when Cal-11 iodinated by the lactoperoxidase method was perfused through the isolated chicken heart, between 12,426 and 7,296 counts were subsequently released by heparin, (Table 5.1). The number of counts released by heparin was related to the time of exposure to the antibody, ie: lower with shorter circulation times. The number of residual counts in the heart after heparin perfusion (88,557 to 33,349) was also found to correlate positively with the period of perfusion. The ratio of heparin-releasable to heparin-residual counts ranged from 14.0 to 21.9, This ratio was higher with shorter periods of antibody circulation and little changed by the inclusion of non-immune serum from several species as a source of IgG to block non-specific binding. In all cases, the residual counts measured in the heart after heparin perfusion, represented between 1% and 2% of the total number of counts either added to the system (in the case of perfusate recirculation) or perfused through the heart (in the case of single-pass perfusions).

Table 5.1 Heparin releasable binding of iodinated Cal-11 in the perfused chicken heart model system.

Period of circulation (minutes)	Type of perfusion	Addition of serum (v/v)	Counts released by heparin (dpm)	Heparin residual counts (dpm)	Ratio of heparin releasable to heparin residual counts
10	R	----	0.0	50,492	0.0*
10	R	----	0.0	60,120	0.0**
10	R	----	12,426	88,557	14.0
10	R	1% HS	11,533	74,291	15.5
5	R	1% HS	12,949	81,169	15.9
1.5	R	1% HS	7,090	45,672	15.5
1.5	SP	1% HS	7,958	50,886	15.6
1.5	SP	1% CS	8,074	54,014	14.9
1.5	SP	5% CS	9,617	56,002	17.2
1.5	SP	1% MS	7,296	33,349	21.9

Iodinated Cal-11 (100,000 dpm[^{125}I]-IgG/ml medium) was perfused through the isolated chicken heart and the levels of iodinated antibody released into the perfusate and residual in the heart following a subsequent period of heparin perfusion were measured by the method described in section 5.2.4. The effect of altering the period of circulation of the antibody and of the addition of non-immune serum on the levels of heparin-releasable and heparin-residual binding were investigated.
Perfusions denoted by R, were performed in a recirculating system while SP denotes single-pass pefusion of the antibody in a non-recirculating system. All perfusions were carried out using a Cal-11 preparation (12.4 uCi/ug IgG) iodinated using the lactoperoxidase method except those denoted by * and **, where Cal-11 preparations iodinated using the chloramine T method and with specific activities of 20.0 and 39.2 uCi/ug IgG respectively were used. HS, non-immune horse serum; CS, non-immune chicken serum; MS, non-immune mouse serum.

5.3.5 *Clearance of iodinated Cal-11 monoclonal antibody in vivo*

The levels of radioactivity present in the bloodstream of fully-fed broiler chickens, injected intravenously with lactoperoxidase iodinated Cal-11, after various periods of circulation, are shown in Table 5.2.

Table 5.2 The clearance of radiolabelled Cal-11 from the bloodstream of broiler chickens.

	Time after injection (minutes)	Radioactivity in 150 µl of blood (dpm)	Total counts in circulation
Bird 1	1	2595	925,714
	2	2307	822,976
	5	2244	800,502
	6	IV heparin injection	
	8	2453	875,058
	10	2528	901,813
Bird 2			
	1	2142	863,783
	10	1773	714,980
	20	1686	679,896
	30	1582	637,957
	31	IV heparin injection	
	33	1727	696,430
	35	1712	690,381

Fully-fed 4 week old broiler chickens were injected via the wing vein with lactoperoxidase iodinated Cal-11 ($1x10^6$ dpm [^{125}I]-IgG). The level of radioactivity in the circulation was determined in serial blood samples before and after an intravenous injection of heparin (1000 units/Kg). Plasma volume was assumed to be 4.7% of body weight (Griffin *et al.*, 1989).

In the case of Bird 1, following a 5 min period of circulation in the bloodstream, 13% of the iodinated Cal-11 (ie: 125,212 dpm) had been removed from the circulation. Of this, 101,421 dpm (81%) was released back into the bloodstream within 4 min of an intravenous heparin injection. In the case of Bird 2, following a 30 min period of antibody circulation in the bloodstream, 26% of the radioiodinated Cal-11 (ie: 225,826 dpm) was removed from the circulation. Of this, only 56,456 dpm (25%) was released back into the circulation within 4 min of an intravenous injection of heparin (Table 5.2). It was found that in all samples >97% of the measured radioactivity in the bloodstream was precipitable with trichloroacetic acid. This indicates that little degradation of the antibody occurs over the time period of the experiment.

5.4 DISCUSSION

The characteristics of Cal-11 made it useful for the work described in this Chapter. This is shown by the rapid binding and inhibition of LPL activity *in vitro* (section 5.3.2) and the high reactivity of Cal-11 towards LPL, after iodination using the lactoperoxidase method (section 5.3.3). The release by heparin of iodinated Cal-11 both *in vitro* (section 5.3.4) and *in vivo* (section 5.3.5) shows that rapid specific binding of the antibody to capillary associated LPL does occur.

The low level of functional heparin-releasable LPL in the perfused heart system (Chapter 4) made it less than ideal as a model system for the *in vitro* testing of iodinated Cal-11 and perhaps this alone may explain the low levels of specific (heparin releasable) binding in relation to non-specific (heparin residual) binding. The chloramine T iodinated preparations of Cal-11 serve as a suitable control for these experiments because the same IgG molecule was labelled with the same substance except using a procedure which reduces the specific binding of the antibody to the antigen (Fig 5.3). Such preparations when perfused through isolated hearts showed no heparin-releasable binding while producing considerable amounts of non-specific (heparin-residual) binding (Table 5.1). This indicated that the heparin-releasable binding of lactoperoxidase iodinated Cal-11 is indeed the result of specific binding to LPL.

One of the few precedents for the present study was the work of Huettinger *et al.* (1984) who reported the use of a radiolabelled monoclonal antibody to estimate the number of LDL receptors *in vivo*. The rate of clearance of lactoperoxidase iodinated Cal-11 from the bloodstream of broilers *in vivo* is similar to that obtained by Huettinger *et al.* (1984) with iodinated anti-LDL receptor monoclonal antibody in rabbits ($t_{1/2}$ 1.5 hours). Huettinger and his co-workers concluded that the specific accumulation of the antibody by the tissues depends on the recycling property of the LDL receptor. The LDL receptor cycles in and out of the cells about once every 10 min (Goldstein *et al.* (1979). If a monoclonal antibody is bound to the receptor, it moves into the cell with the receptor and is deposited in an endosome where the LDL and the antibody dissociate from the receptor. The receptor then returns to the

surface to bind more LDL and antibody. The radiolabelled antibody in the endosomes is eventually transferred to lysosomes and degraded, the rate of uptake and degradation being proportional to the number of recycling receptors. Although small amounts of [^{125}I] derived from the degraded antibody may slowly be transported from the cell, over the experimental period the tissues will gradually accumulate label from the plasma.

However, in the case of LPL, antibody binding to the enzyme at the capillary endothelium *in vivo* will eventually be released into the circulation and degraded by the liver. In the isolated perfused rat heart LPL at the capillary endothelium is turned over with a $t_{1/2}$ of 10 min (Bagby, 1983). Therefore, despite similar clearance rates, the extrahepatic tissues will not accumulate anti-LPL antibodies to the same extent as anti-LDL receptor antibodies.

In the case of anti-LPL antibodies, longer periods of antibody circulation in the bloodstream will not result in an increased accumulation of specifically bound antibody present in the extrahepatic tissues. This is shown by the percentage of iodinated Cal-11 cleared from the bloodstream which can be released by heparin after circulation periods of 5 and 30 min. Following a 5 min circulation period, 81% of the antibody taken up from the bloodstream was released by heparin, while after a 30 min circulation period, this percentage is 25%. Although, in both cases, this represents a similar percentage of the original antibody level in the bloodstream, 11% and 7% respectively.

The initial aim of the study was to achieve saturation binding of the functional pool of LPL activity by iodinated antibody in the whole animal. However, *in vitro* studies described in section 5.3.2 indicated that, for saturation binding of the antibody to the enzyme, a large molar excess of antibody to LPL is required. The 0.5 units of enzyme activity used in the study, which represented approximately 25 ng of LPL protein (Gershenwald *et al.*, 1985), required 20 µg of Cal-11 IgG to produce 90% inhibition of enzyme activity. Similar results were reported by Gershenwald *et al.* (1985) who found that with a pre-incubation of 18 hours at 4°C, 9 µg of Cal-11 (purified on an LPL-affinity column) was required for the complete inhibition of 51 ng of purified LPL. This correlates with previous experiments *in vivo* which have used

polyclonal (Bensadoun and Kompiang, 1979; Griffin *et al.*, 1989) and monoclonal (Goldberg *et al.*, 1988a) anti-LPL antibodies to rapidly block the catabolism of plasma lipoproteins. In all these cases molar excesses of antibody to functional LPL levels were injected to produce saturation binding of the antibody to the enzyme. Goldberg *et al.* (1988a) infused 200 mg of polyclonal or 4-8 mg of monoclonal anti-LPL IgG into Cynomolgus monkeys at hourly intervals to cause acute inhibition of LPL activity. Therefore, in the present study, trace (36 ng) quantities of anti-LPL monoclonal IgG were used in an attempt to achieve rapid and complete uptake of the antibody.

From the data obtained by the preliminary *in vivo* studies (Table 5.2), if a circulation time of 5 minutes is followed by vascular perfusion to remove the large amount of non-bound antibody (87% of the injected dose), the residual tissue label (13% of the injected dose) would, in theory, comprise mainly of specifically bound antibody. Vascular perfusion of all organs except the lungs can be performed using the method of Hayat (1981) whereby heparinized-saline is introduced into the left ventricle under pressure of 120 mmHg and vented via the vena cava. However, vascular perfusion of broilers, using heparin as an anti-coagulant results in many tissues reperfusing poorly and with high individual variation (M. Gentle, personal communication). This problem would be exacerbated by the omission of heparin from the perfusion medium, which would be a requirement for any studies of functional LPL.

The problem of large amounts of non-bound antibody present in the bloodstream may alternatively be overcome by the use of a double label injected simultaneously with the iodinated Cal-11. Either, non-immune mouse [^{131}I]-IgG (Huettinger *et al.*, 1984) or [^{51}Cr] labelled red bloodcells (Wallinder *et al.*, 1984) may potentially be used. This would enable specific antibody binding within tissues to be calculated in relation to non-bound antibody present in the tissue blood volume. However, it is unlikely whether the maximal 11% specific binding (of the $1x10^6$ dpm injected) achieved *in vivo* using Cal-11 would be sufficient to allow such studies to proceed.

CHAPTER 6

IMMUNOLOCALIZATION AND IMMUNOQUANTITATION OF LPL IN CHICKEN CARDIAC TISSUE

6.1 INTRODUCTION

The measurement of LPL activity released from the isolated chicken heart by perfusion with heparin, reported in Chapter 4, showed that a relatively small percentage (2.7%) of the total tissue LPL activity, based on the criterion of heparin releasability at least, was located at the luminal surface of the capillary endothelium. It, therefore, became important to investigate the overall location of the LPL within chicken cardiac tissue. For this purpose, the technique of immunocytochemistry using anti-chicken LPL antibodies was applied and the results of such studies in the chicken heart are described in this Chapter.

Immunocytochemistry was first used to localize LPL by Pedersen *et al.*, (1983) in a study of the rat heart. In that study the functional pool of LPL was visualized in hearts perfused with primary anti-LPL antibody, secondary peroxidase conjugated antibody and diamidinobenzidine substrate followed by electron micrographic observation. A positive correlation was found between the amount of immunoreaction product present in the hearts of rats under various nutritional states and previously observed changes in heparin-releasable LPL activity. That is to say, the estimated percentage coverage of immunoreaction product, increased in fat fed rats and decreased in glucose fed rats. However, despite these findings the method has been criticised (Vilaro *et al.*, 1988) because of the possible high levels of non-specific antibody binding. In partial support of these criticisms, relatively high levels of non-specific antibody binding were found following the perfusion of iodinated non-immune IgG through isolated chicken and rat hearts in the present study (Chapter 5).

Other immunocytochemical studies have been carried out on a more quantitative basis by Blanchette-Mackie *et al.* (1989), who used the technique of immunogold labelling followed by electron micrographic observation to localize LPL in the hearts of mice at a cellular and subcellular level. In particular, they found that for mice in the fed state,

78% of the specific antibody binding was found over the myocytes, 3-6% in the extracellular space and 18% over the capillary endothelium (ie: from basement membrane to luminal cell surface). The LPL in the myocytes was found to be located in the sarcoplasmic reticulum, Golgi vesicles and secretory vesicles at the cell periphery. The LPL found in the extracellular space was present near the opening of myocyte secretory vesicles and in the space between the myocytes and the capillary endothelium. At the endothelium, the highest density of gold particles was at the surface of luminal projections with the lowest density at the basal plasma membrane. Fasting, with its attendant increase in total cardiac LPL activity, resulted in a 2.7-fold increase in overall labelling, with the greatest increase (5-fold) occurring at the surface of intraluminal endothelial projections.

More recently, Camps *et al.* (1990) immunolocalized LPL in the cardiac tissue of fully-fed guinea pigs and found immunofluorescence over the capillary walls, at the periphery of muscle cells and at the endothelium of larger blood vessels.

The work in this Chapter covers the immunolocalization and immunoquantitation of LPL in the chicken myocardium using a purified sheep anti-chicken LPL polyclonal antibody and murine anti-chicken LPL monoclonal antibodies. Production of these antibodies was described in Chapter 3. Comparison of the amounts of LPL associated with the capillary network and myocyte basement membrane/extracellular matrix was achieved using image analysis to measure the area covered by immunoreaction product and the relative intensity of immunostaining over each of these compartments.

Many of the previous studies on the immunolocalization of LPL resulted in poor histological preservation of the sections because of the use of low titre antibodies which required the inclusion of detergents in incubation buffers and long incubation periods. Significant improvements in the sensitivity of immunostaining were achieved in the present study by the development of high titre polyclonal and monoclonal anti-chicken LPL antibodies. Improvement in the quality of the sections was also obtained by the inclusion of an acetone fixation step rather than the use of either paraformaldehyde as a fixative (Camps *et al.*, 1990; Jensen *et al.*, 1991) or the omission of a fixation step

(Jonasson *et al.*, 1984; Fager *et al.*, 1990).

6.2 MATERIALS AND METHODS

6.2.1 *Purification of sheep IgG by protein G affinity chromatography*

10 ml of the sheep anti-chicken LPL recalcified plasma, produced as detailed in section 3.2.1, was centrifuged and filtered through a 0.22 μm membrane (Gelman Sciences Ltd., Northampton, UK). It was then loaded, at a flow rate of 0.5 ml per minute, onto a 5 ml column of Protein G-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) previously equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was then washed with 10 column volumes of equilibration buffer before the bound IgG was eluted using 0.1 M glycine-HCl buffer, pH 2.7. The eluted fractions (1.0 ml) were collected into tubes containing 1 M Tris-HCl buffer (pH 9.0), such that the final pH of the fractions was approximately neutral. Fractions eluted by the low pH buffer which contained IgG (as determined by absorbance at 280 nm) were pooled and stored in aliquots at -70°C. At each stage of the purification procedure, samples were analysed by SDS-PAGE (as described in section 2.2.5) to monitor the protein constituents. The same procedure was used for the purification of the IgG fraction from non-immune sheep recalcified plasma for use as a control.

6.2.2 *Absorption of sheep anti-chicken LPL IgG on a column containing immobilized chicken plasma protein.*

To reduce potential non-specific interactions, the purified sheep IgG (section 6.2.1) was exposed to chicken plasma proteins immobilized on a solid phase support. For this, 2.4 g of Cyanogen-Bromide activated Sepharose 4B (Sigma Chemical Co.) was washed and hydrated overnight in 15 ml of 1 mM HCl, at 4°C. The gel was then washed and resuspended in 15 ml of 0.1 M $NaCO_3/NaHCO_3$ buffer, pH 8.0, and mixed with 2 ml of whole plasma from a six week old broiler chicken. The mixture was then incubated at 4°C overnight with continuous stirring. After washing the gel with 0.1 M $NaCO_3/NaHCO_3$ buffer, any remaining reactive groups on the matrix were deaminated by the addition of 0.75 ml of ethanolamine. The mixture being incubated with stirring for 4 hours at 4°C. The gel was then washed with 200 ml of distilled water, 200 ml of 1.2 M NaCl and again with 200 ml of distilled water before being packed into a 10 ml column. After equilibration with PBS, the column was loaded with 7.5

mg of purified sheep IgG dissolved in 2.0 ml of PBS. The column was eluted with PBS and the fractions containing sheep IgG, determined by the absorption measured at 280 nm, were pooled and stored at -70°C at a protein concentration of 2 mg/ml.

6.2.3 *Western blotting and immunodetection of LPL*

The solubilization of the LPL present in acetone diethyl-ether dried powders of chicken cardiac, adipose and liver tissue (prepared as described in section 2.2.1) was achieved by homogenizing the powders in 10 mM phosphate buffer (pH 6.5) containing 30% (v/v) glycerol and 1.2 M NaCl, as described in section 2.2.3. Before preparation for SDS-PAGE, the extracts were dialysed against 10 mM phosphate buffer, pH 6.5, containing 30% (v/v) glycerol to reduce the NaCl concentration. SDS-polyacrylamide gel electrophoresis was carried out as described in section 2.2.5. The transfer of proteins from SDS polyacrylamide gels onto nitrocellulose (Schleicher and Schuell, Dassel, Germany) was achieved by semi-dry western blotting using a electrophoretic transfer unit (Multiphor II Novablot 2117-005, LKB, Stockholm, Sweden). The buffer system was as follows: 30 mM Tris/HCl buffer, pH 10.4, containing 20% (v/v) methanol (Anode buffer 1), 25 mM Tris/HCl buffer, pH 10.4, containing 20% (v/v) methanol (Anode buffer 2) and 25 mM Tris/HCl buffer, pH 9.4, containing 20% (v/v) methanol and 40 mM 6-amino-n-hexanoic acid (Cathode buffer). The transfer was achieved at 0.8 mA/cm^2 for 50 min and the nitrocellulose sheet was then washed and placed into PBS. The nitrocellulose was then incubated in PBS containing 0.5% Tween-20 for 2 hours at 20°C to block any remaining reactive sites and then incubated overnight at 4°C with purified absorbed sheep anti-chicken LPL IgG (4µg/ml) diluted in PBS containing 0.05% Tween-20 (PBS-T). After subsequent washing with PBS-T, the nitrocellulose sheet was incubated for 2 hours at 20°C with biotinylated anti-sheep IgG antibody (Sigma Chemical Co.) diluted (1/500 v/v) in PBS-T. Following washes in PBS-T, the nitrocellulose was incubated subsequently (for 1 hour at 20°C) with streptavadin-peroxidase complex (Amersham Int plc., Amersham, Bucks, UK) diluted (1/200 v/v) in PBS-T. Antibody binding was visualized by incubation in 50 mM Tris/HCl buffer, pH 7.6, containing 4-chloro-1-napthol (0.5 µg/ml) and 30% (v/v) hydrogen peroxide (1 µl/ml), the reaction being stopped by washing in distilled water. The pattern of total protein bound to the nitrocellulose was determined by staining using 0.1% (w/v) amido black in 50% (v/v)

methanol, 10% (v/v) acetic acid and destained with 90% (v/v) methanol, 2% (v/v) acetic acid.

6.2.4 *Staining of cryostat sections using immunofluorescent labelled secondary antibodies.*

The chicken left ventricular myocardium was removed and tissue segments (0.5 cm cubes) were frozen in isopentane cooled using liquid nitrogen, the blocks of tissue being subsequently stored at -70°C in sealed containers. Tissue sections of 10 µm thickness were cut from the blocks using a cryostat (Bright Ltd, UK.) at -26°C. The sections were mounted on to slides previously coated with 0.1% poly-L-lysine (Sigma Chemical Co.) and fixed by immersion in acetone for 5 min at -20°C. The mounted sections were then incubated for 15 min with 20% (v/v) normal serum (Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire) in PBS, to saturate any non-specific IgG binding sites present in the tissue. In all cases, reference to normal serum refers to serum from the species which was the donor of the second antibody preparation. Sections were then incubated for 2 hours at 20°C with primary antibody preparations, ie: sheep anti-chicken LPL IgG (4 µg/ml) or the anti-chicken LPL monoclonal antibody, Cal-11 (1 µg/ml) diluted in PBS containing 10% (v/v) normal serum.

For detection of antibody binding by immunofluorescence, the sections were washed with PBS and incubated for 1 hour at 20°C with either anti-sheep or anti-mouse IgG-FITC antibody conjugate (Sigma Chemical Co.) diluted 1/100 (v/v) in PBS containing 10% (v/v) normal serum. Sections were then washed with PBS (3 changes each of 15 min) and mounted in 90% (v/v) glycerol and 2.5% (w/v) 1,4 diazabicyclo-[2.2.2]octane in PBS.

In certain experiments the structural detail of sections, previously immunostained for LPL, was provided by the use of one of the following methods. In some cases, the nuclei of tissue sections were counterstained with diamidino phenyl indole, DAPI, (1 µg/ml in MacIlvane's buffer, 0.62 M citric acid and 0.77 M Na_2HPO_4, pH 4) for 1 hour at 20°C in the dark. The luminal surface of the vascular endothelium was labelled in some sections by perfusing the isolated chicken heart with concanavalin A-FITC conjugate (as described in section 4.2.5) prior to cryosectioning and immunostaining for LPL.

For the detection of endothelial cells in some experiments, specific labelling of non-LPL endothelial antigens was achieved. For this a technique of double immunostaining was employed in which sheep anti-human von Willebrand Factor (vWF) polyclonal antibodies (Scottish Antibody Production Unit, Law Hospital, Lanarkshire, Scotland) were used at a dilution of 1/100 (v/v), to identify the presence of Factor VIII related antigen, an endothelial cell marker, on the sections. The anti-vWF antibody was detected using an anti-sheep IgG-FITC conjugated secondary antibody (Sigma Chemical Co.) at a dilution of 1/100 (v/v). LPL was immunostained on the same section using Cal-11 as the primary antibody (as described above) and an anti-mouse IgG-Texas Red secondary antibody (Vector Laboratories Inc., Burlingame, CA., USA) at a dilution of 1/150 (v/v).

6.2.5 *Staining on cryostat sections using the technique of silver-enhanced immunogold*

For the silver-enhanced immunogold staining, cryosections were mounted onto slides previously coated with 1% (w/v) gelatine which were then processed, as described above for immunofluorescence labelling, up to the stage of secondary antibody addition. At this stage, the sections were incubated for 1 hour at 20°C with either biotinylated anti-sheep- or biotinylated anti-mouse-IgG antibody (Sigma Chemical Co.) diluted 1/200 (v/v) in PBS containing 10% (v/v) normal serum. After washing in PBS, the sections were incubated for 1 hour at 20°C with streptavadin-gold (5 nm diameter) complex (Janssen Life Science Products, Beerse, Belgium) diluted 1/150 (v/v) in PBS containing 10% (v/v) normal serum. After washing, the sections were post-fixed with 2.5% (v/v) glutaraldehyde (E.M grade, TAAB Laboratories Equipment Ltd. Reading U.K.) in PBS for 15 min at 20°C and washed in distilled water before incubation for 7 min in silver enhancer reagent (Sigma Chemical Co.). The sections were then washed in distilled water and post-fixed by immersion for 3 min in 3% (w/v) sodium thiosulphate. The sections were then counterstained by immersion in Mayer's haematoxylin for 5 min, followed by a brief exposure in 70% (v/v) alcohol containing 1% (v/v) HCl, to reduce non-specific background staining. The sections were then rinsed under running tap water for 5 min and stained by immersion for 3 min in 1% (w/v) Eosin. The sections were finally dehydrated in graded alcohols, cleared in CMP 30 (Pentone Ltd., Cramlington, Northumberland, UK) and mounted using DPX (BDH

Laboratory Supplies, Poole, UK).

6.2.6 *Photography and image analysis*

The immunostaining was visualized and photographed using a fluorescence photomicroscope (Olympus BH2, Olympus Optical Co, Tokyo, Japan). Fluorescent markers were photographed using a fast film, Kodak Ektachrome P800/1600, while the silver staining was photographed using standard Kodak Ektachrome 64-T film (Eastman Kodak, Rochester, NY.).

Image analysis was carried out on the negatives (x166.7 magnification) of immunofluorescent stained sections using a charged-coupled camera (Cambridge Instruments Ltd., Cambridge, UK) linked to a Quantimet 570 image analysis system (Cambridge Instruments Ltd.). Quantitative analysis was made on 30 representative fields of view. The total area covered by and the intensity of immunostaining was determined in each field. The interstitial capillary elements (ie: the endothelial cells, pericyte-like cells, basement membrane and sub-endothelial space) were then defined manually. This allowed measurement of the area covered by and the intensity of the immunostaining associated with the capillary network and the myocyte basement membrane/extracellular matrix.

6.2.7 *Tissue processing for electron microscopy*

To gain more information on the subcellular localization of LPL within chicken cardiac tissue and to quantify the level of functional LPL at the lumen of the capillary endothelium, immunogold labelling followed by electron micrographic observation was attempted on tissue sections processed for electron microscopy by Araldite/LR White embedding or ultrathin cryosectioning.

6.2.7.1 *Tissue processing for Araldite embedding*

1 mm cubes of the left ventricular myocardium were fixed by immersion in 4% (w/v) paraformaldehyde or 4% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4,, for 2 hours at 4°C. The tissue was then washed in PBS and left overnight in PBS, before being incubated with 2% (v/v) osmium tetroxide for 3 hours. The tissue blocks were then washed with distilled water and dehydrated in progressive acetone/water mixtures and

incubated overnight in a 1:1 (v/v) mixture of acetone and Araldite resin (TAAB Laboratories Equipment Ltd. Reading U.K.). Following incubation in a mixture of 75% (v/v) Araldite resin, 25% (v/v) acetone for 6 hours, the tissue blocks were left overnight in 100% Araldite resin. After incubation in fresh Araldite for 2 hours, the resin was polymerized by heating to 60°C for 48 hours.

6.2.7.2 *Tissue processing for embedding in LR White resin*

Hearts were fixed by perfusion for 20 min with Zamboni's fixative (4% (w/v) paraformaldehyde, 15% (v/v) saturated picric acid in PBS), as described in section 4.2.2. This fixative was previously found not to effect the LPL immunoreactivity of chicken cardiac cryostat sections processed and labelled as described in section 6.2.4. The left ventricular myocardium was then cut into 1 mm^3 blocks and further fixed by immersion in Zamboni's fixative for one hour at 4°C. The blocks were then placed directly into 70% (v/v) ethanol (3 changes for 20 min each) before being placed into a mixture of equal quantities of LR White hard grade resin (London Resin Co, UK.) and 70% (v/v) ethanol for 1 hour at 4°C. The blocks were then incubated with a 2:1 (v/v) mixture of LR White resin and 70% (v/v) ethanol for one hour at 4°C and then 100% LR White resin for one hour at 4°C, before an overnight incubation in fresh 100% LR White resin. Following a final change of the LR White resin, the resin was either polymerized by incubation at 50°C for 24 hours or accelerator cured at 0°C for 4 hours (Newman, 1989).

For both Araldite and LR White embedded tissues samples, 1 μm sections were cut using an microtome (Ultrotome III, LKB, Stocholm, Sweden) and mounted on to gelatine coated slides. The mounted sections were then stained using the immunogold or immunofluorescence staining techniques as described in sections 6.2.5 and 6.2.4 respectively. Araldite was removed from the tissue sections by immersion in 50% (w/v) saturated NaOH in ethanol for 10 min before being subjected to immunostaining.

6.2.7.3 *Ultrathin cryostat sectioning*

The procedure followed was essentially that described by Tokuyasu (1980). 1 mm^3 blocks of chicken myocardium were fixed for 1 hour at 4°C in 0.1% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The blocks were then washed overnight in PBS and incubated in 2.3 M

sucrose in 0.1 M phosphate buffer, pH 7.4, for 1 hour at 4°C before being frozen in liquid nitrogen. Ultrathin cryostat sections were cut at -110°C on a Reichert Ultracut E microtome (Reichert-Jung, Germany) and transferred on drops of 2.3 M sucrose to nickle grids. Any free aldehyde groups present in the sections were quenched using a 10 min incubation in 0.02 M glycine. These sections were then washed in PBS and immunostained as described in section 6.2.5 for immunogold labelling by transferring the grids between drops of the antibody reagents diluted in PBS. Sections were then treated with 2% (w/v) neutral uranyl acetate and embedded in 2% (w/v) methyl cellulose (Methocel, Fluka A.G., Buchs, Switzerland) for viewing using an electron microscope (JEM 1005, Jeol Inc., Tokyo, Japan).

6.3 RESULTS

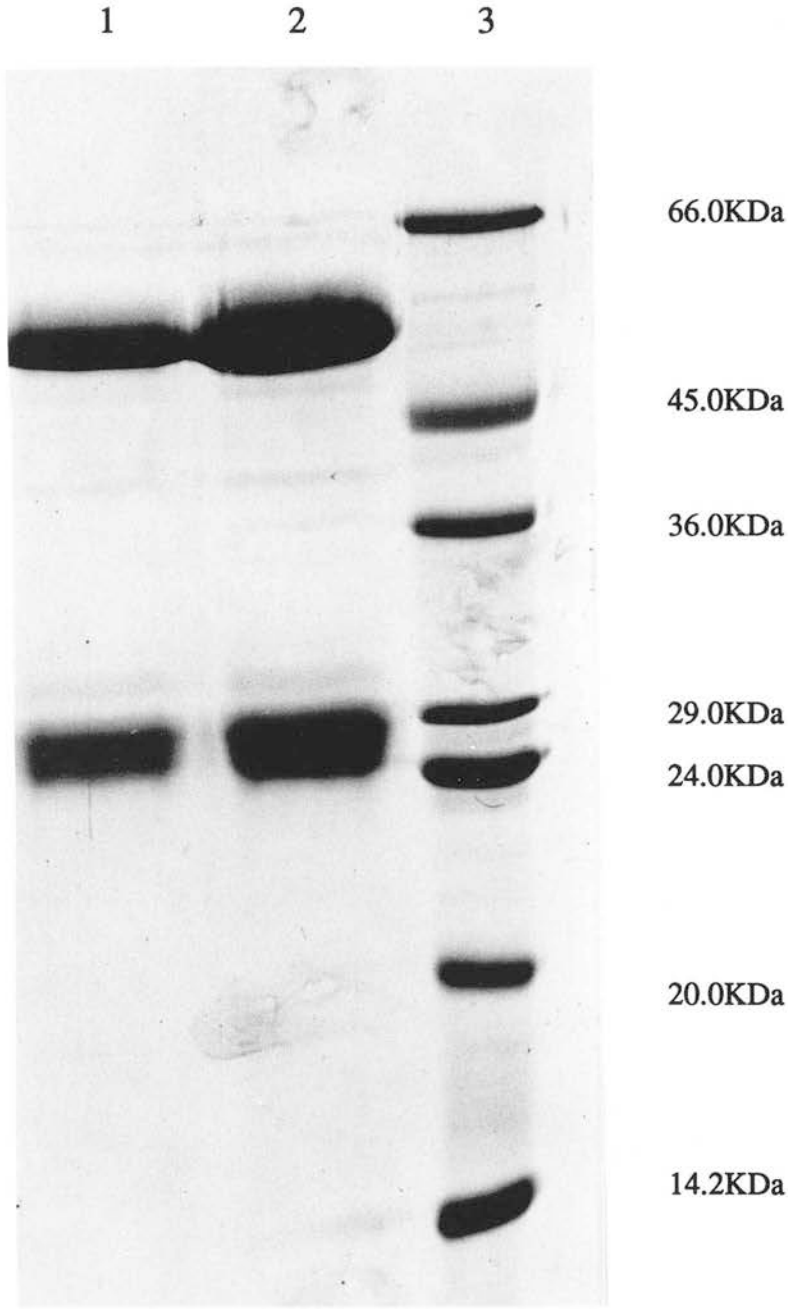
6.3.1 *The purification of sheep IgG*

The IgG fraction was purified from sheep recalcified plasma by a one step procedure using Protein G affinity chromatography as described in section 6.2.1. Analysis of the IgG-containing Protein G eluate by SDS-PAGE (Fig 6.1, lane 2) revealed the presence of 2 major protein bands, with approximate molecular weights of 53 KDa and 25 KDa. After absorption of the purified sheep IgG on a chicken plasma protein-column, as described in section 6.2.2, the same 2 bands were evident as the major constituents by SDS-PAGE (lane 1). Because the gel was run under reducing conditions, the protein band of higher molecular weight corresponds to the component sheep IgG heavy chains, while the protein band of lower molecular weight corresponds to the component light chains.

6.3.2 *Immunoblotting of chicken tissue extracts with sheep anti-chicken LPL IgG*

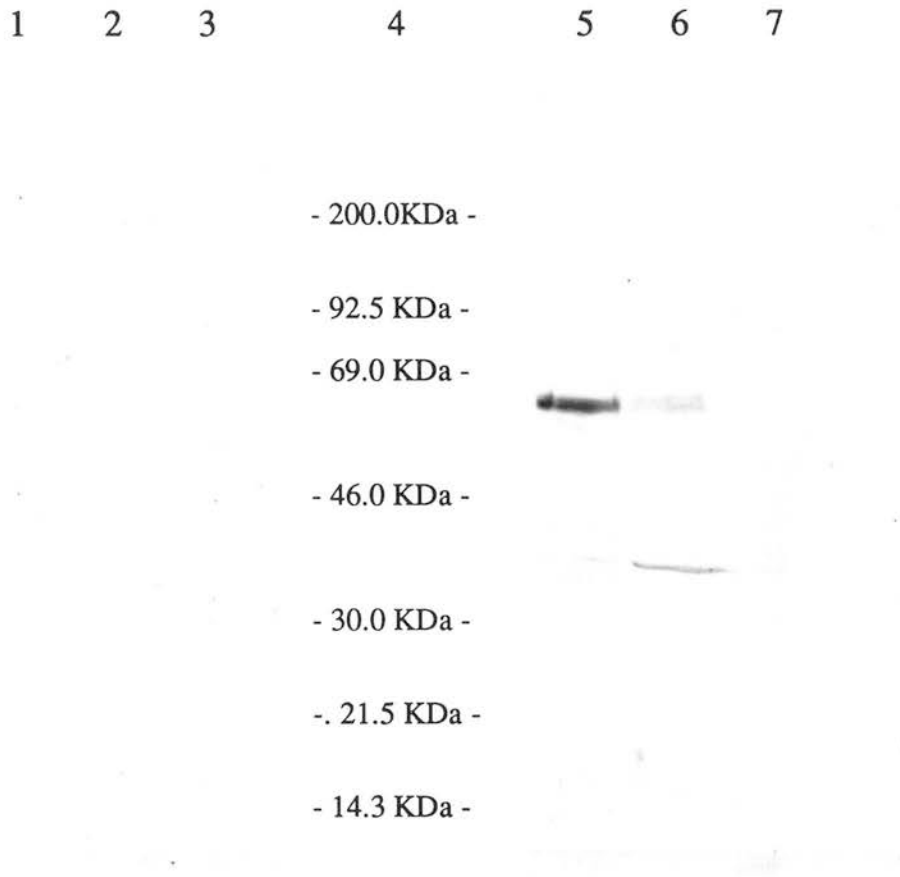
Immunoprobing soluble extracts of acetone diethyl-ether powders of adipose tissue, heart and liver with absorbed sheep anti-chicken LPL polyclonal IgG revealed the presence of immunoreactivity in the former two tissues only (Fig 6.2). The major protein band identified in adipose tissue and heart corresponds in molecular weight (ie: 61 KDa) to that of purified chicken LPL (sections 2.3.2 and 2.3.3). This confirms that there was no difference in the molecular weight or apparent immunoreactivity between LPL derived from chicken heart and adipose

Figure 6.1 Analysis by polyacrylamide gel electrophoresis of samples obtained during the purification of the IgG fraction from sheep recalcifed plasma.



Samples were obtained at each stage in the purification and absorption procedures of the IgG from sheep recalcified plasma, described in sections 6.2.1 and 6.2.2 respectively. The samples were analysed by SDS-PAGE under reducing conditions on a 12% polyacrylamide gel, as described in section 2.2.5, with amounts of protein indicated per lane.
Lane 1. purified sheep IgG following adsorption on a chicken plasma protein-column (30 µg). Lane 2. Sheep IgG eluted from a protein-G affinity column (45 µg).
Lane 3. Electran molecular weight markers (Sigma Chemical Co.).

Figure 6.2 Immunoblotting of solubilized extracts of chicken liver, cardiac and adipose tissue acetone diethyl-ether dried powders using absorbed sheep anti-chicken LPL IgG



Lane 1. adipose tissue
Lane 2. cardiac tissue
Lane 3. liver
Lane 5. adipose tissue
Lane 6. cardiac tissue
Lane 7. liver
Lane 4. Rainbow molecular weight markers (Amersham Plc.).

LPL was extracted from chicken tissue acetone diethyl-ether dried powders using 10 mM phosphate buffer (pH 6.5) containing 1.2 M NaCl and 30% (v/v) glycerol. The extracts were then dialysed overnight against this buffer without NaCl before being prepared for SDS-PAGE, as described in section 2.2.5, with 20 µg of protein being loaded into each well. LPL was detected after western blotting onto nitrocellulose by incubation with purified and absorbed sheep anti-chicken LPL IgG (Lanes 4-6) or by incubation with non-immune sheep IgG purified and absorbed in an identical fashion (Lanes 1-3). The position of the proteins of known molecular weight was determined by staining the nitrocellulose strip with amido black, as described in section 6.2.3.

tissue. The minor band identified by the antibody in the samples of both tissue extracts corresponded in molecular weight (35 KDa) to a known breakdown product of LPL (Kern *et al.*, 1988; Socorro and Jackson, 1985) and was also observed in highly purified preparations of chicken heart LPL (Fig 2.4). The lack of detectable LPL immunoreactive mass in the liver of 5 week old broilers correlates with the lack of measurable LPL activity in the liver at this age reported by Guo *et al.* (1988).

From these results it was concluded that the processes of immunization, purification and absorption in the production of the sheep anti-chicken LPL IgG resulted in an antibody which was highly specific towards LPL and potentially suitable for the immunodetection of the enzyme in tissue sections. This procedure could not be repeated to test the specificity of Cal-11 since previous investigations by Gershenwald *et al.* (1985) found that the antibody did not detect LPL by immunoblotting, probably due to the loss or masking of the specific epitope involved.

6.3.3 *Immunocytochemical localization of LPL*

When sections of chicken left ventricular myocardium were labelled using the immunofluorescence procedure described in section 6.2.4., the pattern of immunoreactivity obtained (Fig 6.3, A) was identical when either sheep anti-chicken LPL IgG or Cal-11 IgG was used as the source of the primary antibody. Two distinct pools of immunoreactivity for LPL were observed, one composed of intensely staining elliptical shaped structures (which were thought to represent pools of enzyme either associated with the nuclei of the myocytes or concentrated around the interstitial capillary elements) and the other thought to be due to the accumulation of the enzyme at the myocyte basement membrane and the surrounding extracellular matrix. When non-immune sheep or mouse serum or the respective purified non-immune IgGs were substituted for the anti-LPL antibodies, no specific staining of the chicken myocardium was observed (Fig 6.3, B).

The effect of heparin on the pattern of immunoreactivity was then investigated. Heparin (30 units/ml of medium) was perfused through the isolated chicken heart as described in section 4.2.5 or injected intravenously (1000 units/Kg) into six week old broiler chickens as described in section 5.2.6. The introduction of heparin both *in vitro* or *in vivo* was found to have no substantial observable effect on the pattern

Figure 6.3

A) The immunolocalization of LPL in cryosections of chicken cardiac tissue.

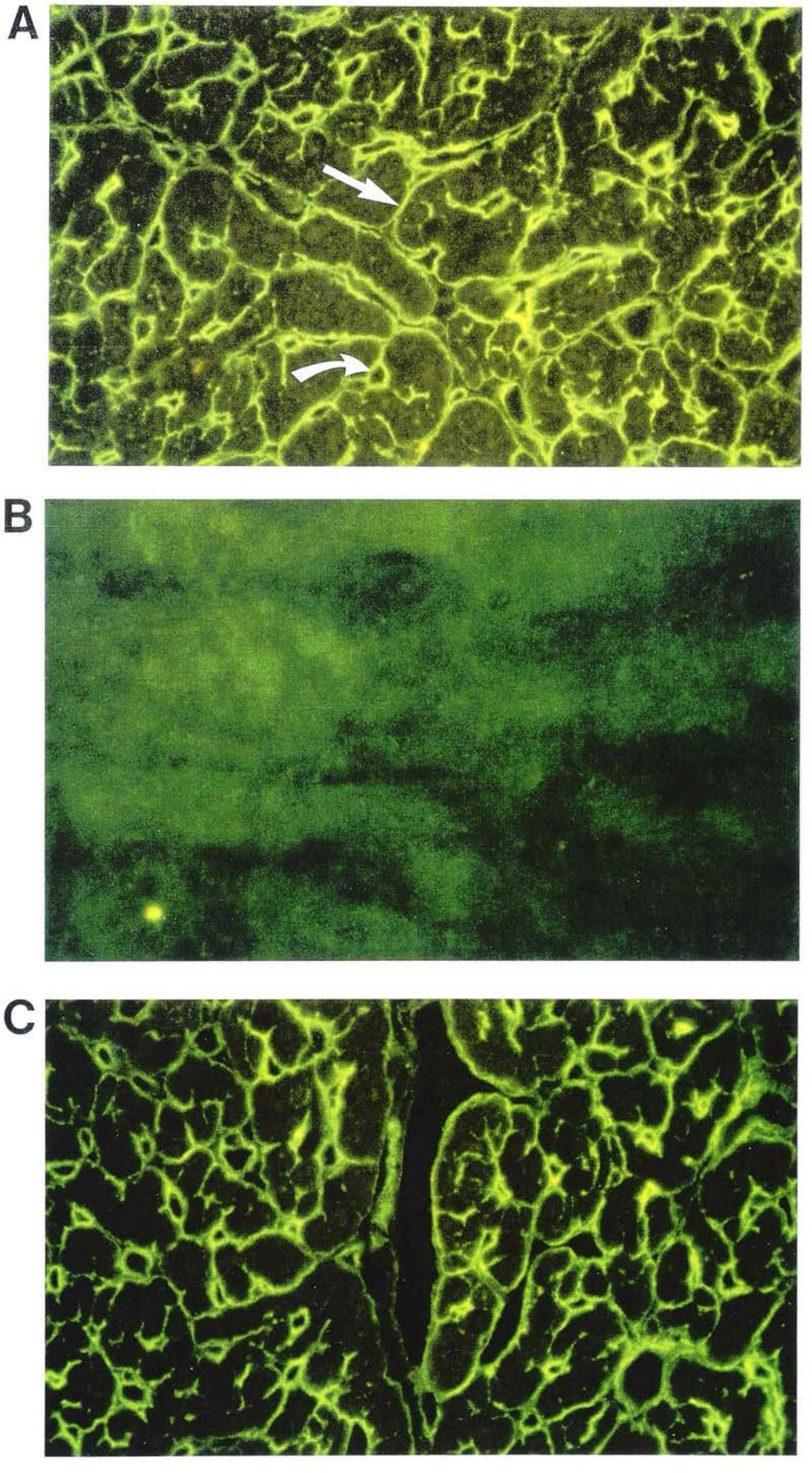
LPL was localized in cryostat sections of the chicken left ventricular myocardium using sheep anti-chicken LPL IgG in the immunofluorescence staining procedure described in section 6.2.4. The print shows immunofluorescence in association with interstitial capillary elements (curved arrow) and the ECM/myocyte basement membrane (straight arrow). Print x 595

B) Non-immune control for the immunolocalization of LPL in chicken cardiac tissue.

Cryostat sections of the chicken left ventricular myocardium were incubated with non-immune sheep IgG and processed using the immunofluorescent labelling procedure described in section 6.2.4. No specific immunofluorescence was detected over the section. Print x 595.

C) The immunolocalization of LPL in sections of chicken cardiac tissue obtained from hearts perfused with heparin.

Chicken hearts were perfused with heparin as described in section 4.2.2. LPL was localized in cardiac tissue cryostat sections obtained from heparin-perfused hearts using sheep anti-chicken LPL IgG in the immunofluorescence staining procedure described in section 6.2.4. Heparin perfusion was found to have no effect on the pattern of LPL immunoreactivity (as compared to A). Similarly an IV injection of heparin *in vivo* was also found to have no effect on the pattern LPL immunoreactivity of chicken cardiac tissue. Print x 595.


A
B
C

of LPL immunofluorescence (Fig 6.3, C).

The elliptical shaped structures which showed considerable LPL immunoreactivity were conclusively shown to be capillaries by perfusion of the heart with concanavalin A-FITC conjugate followed by cryosectioning and immunofluorescent labelling using anti-LPL IgG (Fig 6.4, A). The concanavalin-A binds to the glycoproteins present at the luminal surface of the vascular endothelium and shows the extent of reperfusion of the tissue by effectively labelling the capillary bed (Chapter 4). Because few capillaries, revealed by immunostaining for LPL, did not contain concanavalin-A, the technique described in section 4.2.2, therefore produces a high level of reperfusion in the isolated chicken heart. A similar association was found between LPL immunoreactivity and structures immunostained with the anti-human von Willebrand factor (vFW) antibody (Fig 6.4, B). The identification of chicken vascular endothelial cells by the expression of a von Willebrand-like factor has previously been demonstrated (Yablonka-Reuveni, 1989) with the use of a cross-reacting anti-human vFW antibody.

From these co-localization studies it was concluded that a considerable portion of LPL in the chicken heart myocardium is associated with the capillary network.

Although it has been proposed that LPL is a cryptic enzyme, stored in the cisternae of both adipocytes and myocytes (Vannier et al., 1989; Pradines-Figueres *et al.*, 1990), no peri-nuclear localized pool of LPL immunoreactivity within the cardiac myocytes could be detected in association with DAPI, a fluorescent nuclear probe (Fig 6.5, A). The examples of an association between DAPI and LPL immunofluorescence are due to the presence of nucleated chicken red blood cells within capillaries. The lack of a peri-nuclear localized pool of LPL in the cardiac myocytes was confirmed by the sensitive silver-enhanced immunogold technique. At high magnifications, x1509, it can be shown clearly that the haematoxylin stained nuclei of the cardiac myocytes are not associated with concentrations of LPL immunoreactivity (Fig 6.5, B). This was by contrast to the substantial amounts of LPL immunoreactivity observed in association with the interstitial capillary elements and the myocyte basement membranes.

Figure 6.4

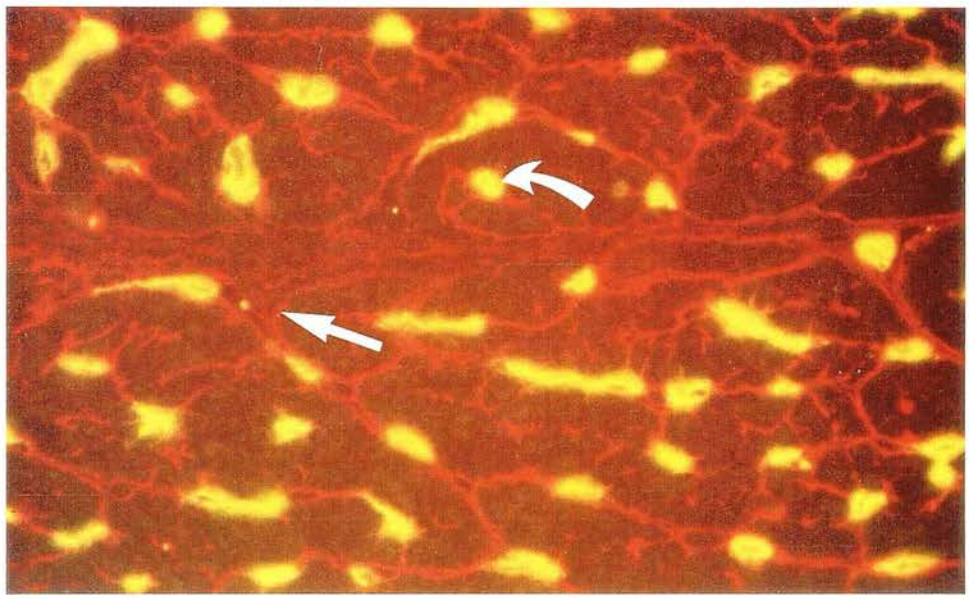
A) The localization of LPL on sections of chicken cardiac tissue from hearts perfused with concanavalin A-FITC conjugate.

Cryostat sections of the chicken cardiac left ventricular myocardium were obtained from hearts perfused with concanavalin A-FITC conjugate (yellow) as described in section 4.2.5. LPL (red) was localized in such sections using Cal-11 and an anti-mouse IgG Texas Red antibody conjugate in the immunofluorescence staining procedure described in section 6.2.4. The print shows that the elliptical shaped structures which stain intensely for LPL are capillaries (curved arrow) and that considerable amounts of label are also found in association with the ECM/myocyte basement membrane (straight arrow). Print x 595.

B) The co-localization of LPL and von Willebrand like-factor antigens in sections of chicken cardiac tissue.

LPL (red) and von Willebrand like-factor (vWf) antigens (green) were localized on cryostat sections of the chicken left ventricular myocardium using the double-immunofluorescent staining procedure described in section 6.2.4. The co-localization of LPL and vWf antigens in association with the capillary endothelium (arrow), was obtained. Print x 595.

A



B

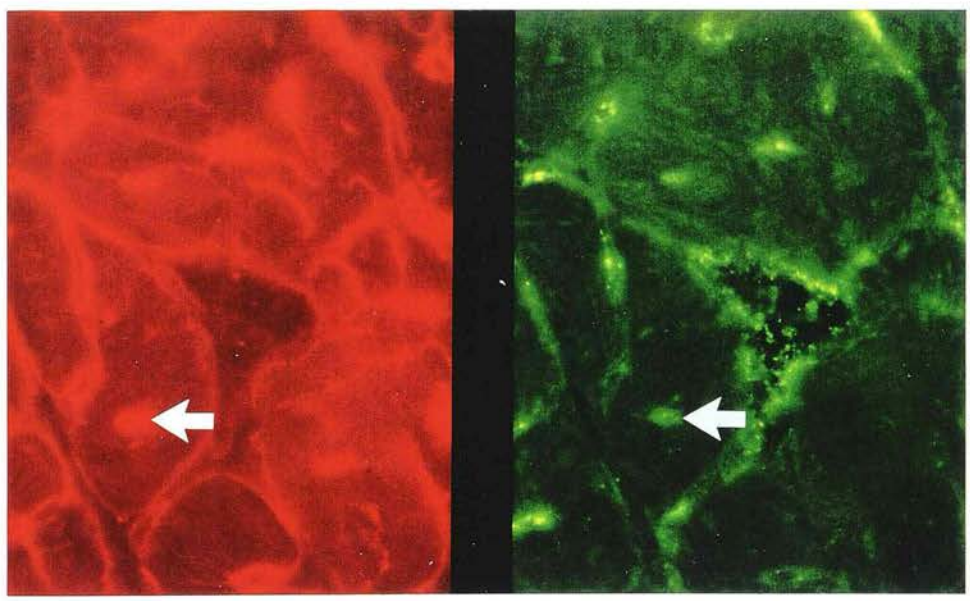


Figure 6.5

A) The lack of peri-nuclear localized LPL within chicken cardiac myocytes.

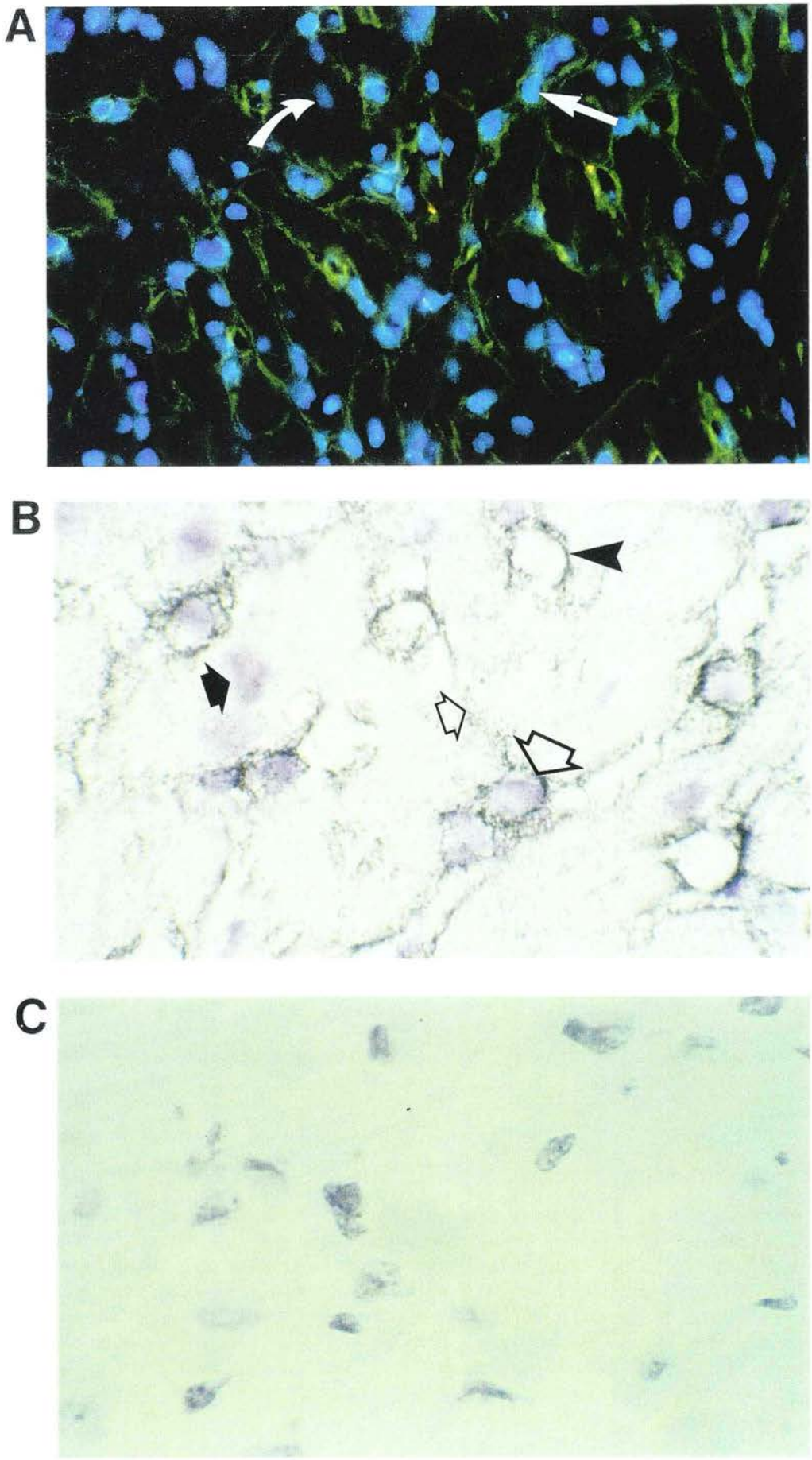
LPL (green) was localized in cryostat sections of the chicken left ventricular myocardium using sheep anti-chicken LPL IgG in the immunofluorescence staining procedure described in section 6.2.4. The sections were then counterstained using DAPI (blue), a fluorescent nuclear marker, as described in section 6.2.4. No immunofluorescence for LPL was found in association with DAPI in the myocytes (straight arrow), suggesting no peri-nuclear accumulation of LPL occurs in these cells. The examples of an association between DAPI and LPL immunofluorescence are due to the presence of nucleated chicken red blood cells within capillaries (curved arrow). Print x 595.

B) The localization of LPL in chicken cardiac tissue using the technique of silver enhanced immunogold.

LPL was localized in cryostat sections of the chicken left ventricular myocardium using sheep anti-chicken LPL IgG and the technique of silver enhanced immunogold labelling followed by counterstaining with haematoxylin, as described in section 6.2.5. This technique confirms the accumulation of LPL in association with interstitial capillary elements (➤) and the ECM/myocyte basement membrane (small open arrow). No concentration of LPL immunoreactivity in association with the nuclei of the cardiac myocytes (➤) was observed and those examples of an association between the two are due to the presence of nucleated chicken red blood cells within capillaries (large open arrow). Print x 1509.

C) Non-immune control for the immunolocalization of LPL in chicken cardiac tissue.

Cryostat sections of the chicken left ventricular myocardium were incubated with non-immune sheep IgG and processed using the technique of silver enhanced immunogold labelling followed by counterstaining with eosin, as described in section 6.2.5. No specific immunoreactivity was detected over the section. Print x 1509.


A
B
C

Despite the apparent concentration of LPL in association with the interstitial capillary elements, no LPL immunoreactivity could be detected over the endothelium of larger blood vessels in the chicken myocardium using either the immunofluorescence or silver-enhanced immunogold staining procedures (Fig 6.6, A and B respectively). By contrast, labelling with anti-vFW antibodies resulted in intense immunofluorescence over the endothelium of the larger blood vessels in the chicken myocardium (Fig 6.6, C). This shows that the sectioning, fixation and staining procedures adopted in the present study maintain the endothelial epitopes of major blood vessels successfully for antibody binding.

6.3.4 *Image analysis*

Image analysis was used to determine the percentage of the area of immunostaining associated with the interstitial capillary elements and with the myocyte basement membrane/extracellular matrix. The technique was also used to assess the intensity of immunostaining for LPL over these tissue compartments. The results of the study are summarised in Table 6.1.

Table 6.1 The quantitative immunolocalization of LPL within the chicken myocardium

	Percentage area immunostained:		Mean intensity of immunostaining (units of greyness)
	of field of view	of area stained	
Immunostaining associated with capillary network	6.23±0.28	46.4	152.62±5.85
Immunostaining associated with myocytes/ECM	7.20±0.61	53.6	116.34±5.78
Total staining of field of view	13.44±0.68	100	136.60±5.37

Image analysis using a Quantimet 570 System was carried out on the negatives of cryosections of the chicken left ventricular myocardium, immunostained for LPL using sheep anti-chicken LPL IgG and the immunofluorescence procedure described in section 6.2.4. The values are means±SE derived from the measurement of 10 fields of view of cardiac tissue sections from each of 3 fully fed 5 week old broiler chickens (n=30). The units of greyness (0-256) give a comparative indication of the intensity of staining over the tissue compartments, although the number of units positively correlates with the intensity of immunostaining, linearity cannot be assumed.

Figure 6.6

A) The lack of LPL immunofluorescence over the endothelium of major blood vessels of chicken cardiac tissue.

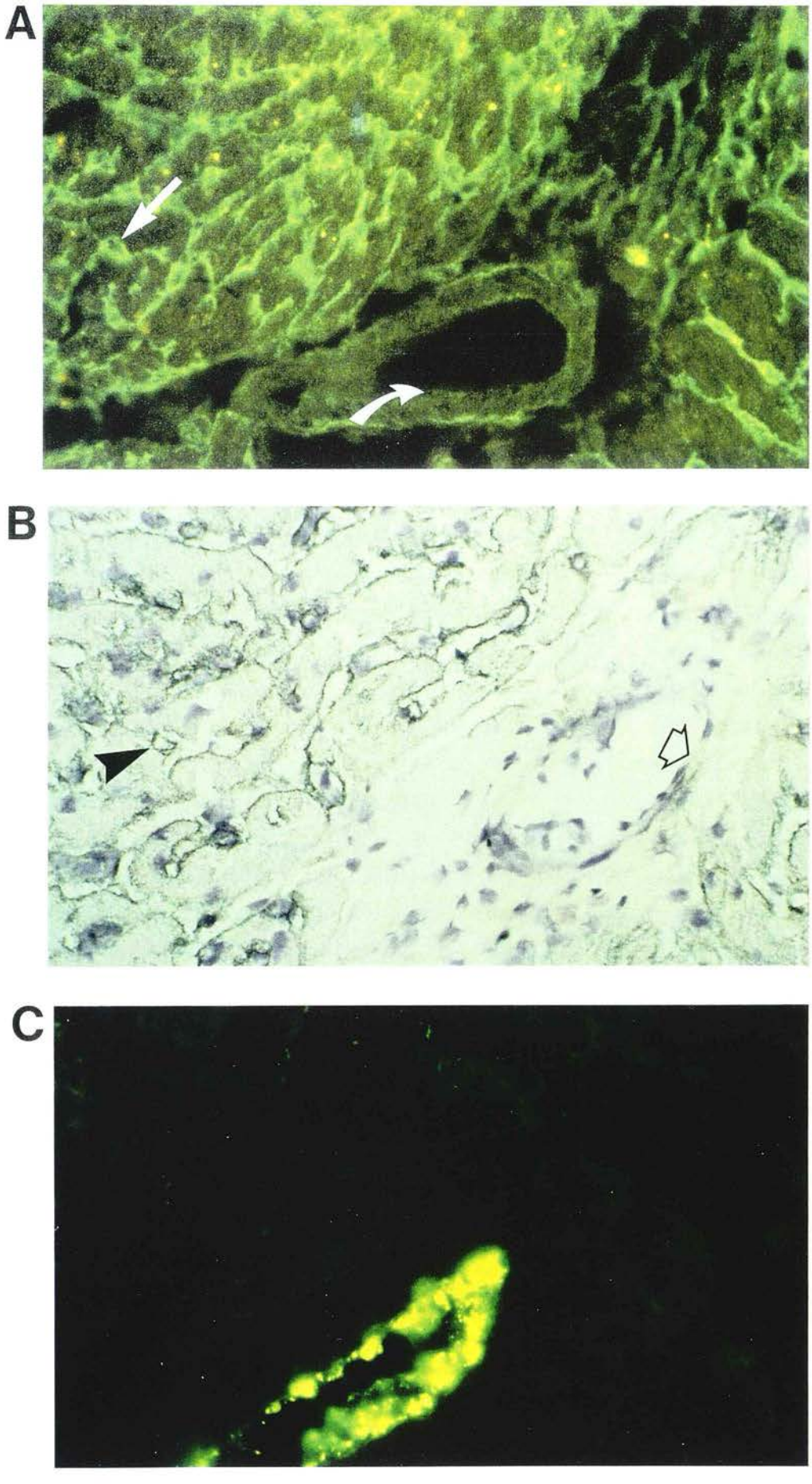
LPL was localized in cryostat sections of the chicken left ventricular myocardium using sheep anti-chicken LPL IgG in the immunofluorescent staining procedure described in section 6.2.4. No LPL immunoreactivity was detected over the endothelium of major blood vessels in chicken cardiac tissue (straight arrow) despite the presence of LPL immunoreactivity at the interstitial capillary elements (curved arrow). Print x 425.

B) The lack of LPL immunoreactivity over the endothelium of major blood vessels of chicken cardiac tissue.

LPL was localized in cryostat sections of the chicken left ventricular myocardium using sheep anti-chicken LPL IgG and the technique of silver enhanced immunogold labelling followed by counterstaining with haematoxylin, as described in section 6.2.5. This technique confirms the lack of LPL immunoreactivity over the endothelium of major blood vessels in chicken cardiac tissue (open arrow) despite the presence of LPL immunoreactivity at the interstitial capillary elements (closed arrow). Print x 595.

C) The presence of von Willebrand factor antigens over the endothelium of major blood-vessels in sections of chicken cardiac tissue.

von Willebrand factor (vWf) antigens were localized in cryostat sections of the chicken left ventricular myocardium using anti-human vWf IgG in the immunofluorescence staining procedure described in section 6.2.4. Intense immunofluorescence for vWf was obtained over the endothelium of major blood vessels, showing that endothelial antigens are available for antibody binding after tissue processing. Print x 595.


A
B
C

The area covered by immunofluorescence in association with the interstitial capillary elements (ie: over the endothelial cells, pericyte-like cells, vascular basement membrane and sub-endothelial space) was 6.2% of the total area (46.4% of the area immunostained). The area covered by immunofluorescence in association with the myocyte basement membrane and the extracellular matrix was 7.2% of the total area (53.6% of the area immunostained). The intensity of immunofluorescence for LPL which was associated with the interstitial capillary elements (mean grey level, 152.62) was found to be greater than the intensity of immunofluorescence for LPL which was associated with the myocyte basement membranes and extracellular matrix (mean grey level, 116.34).

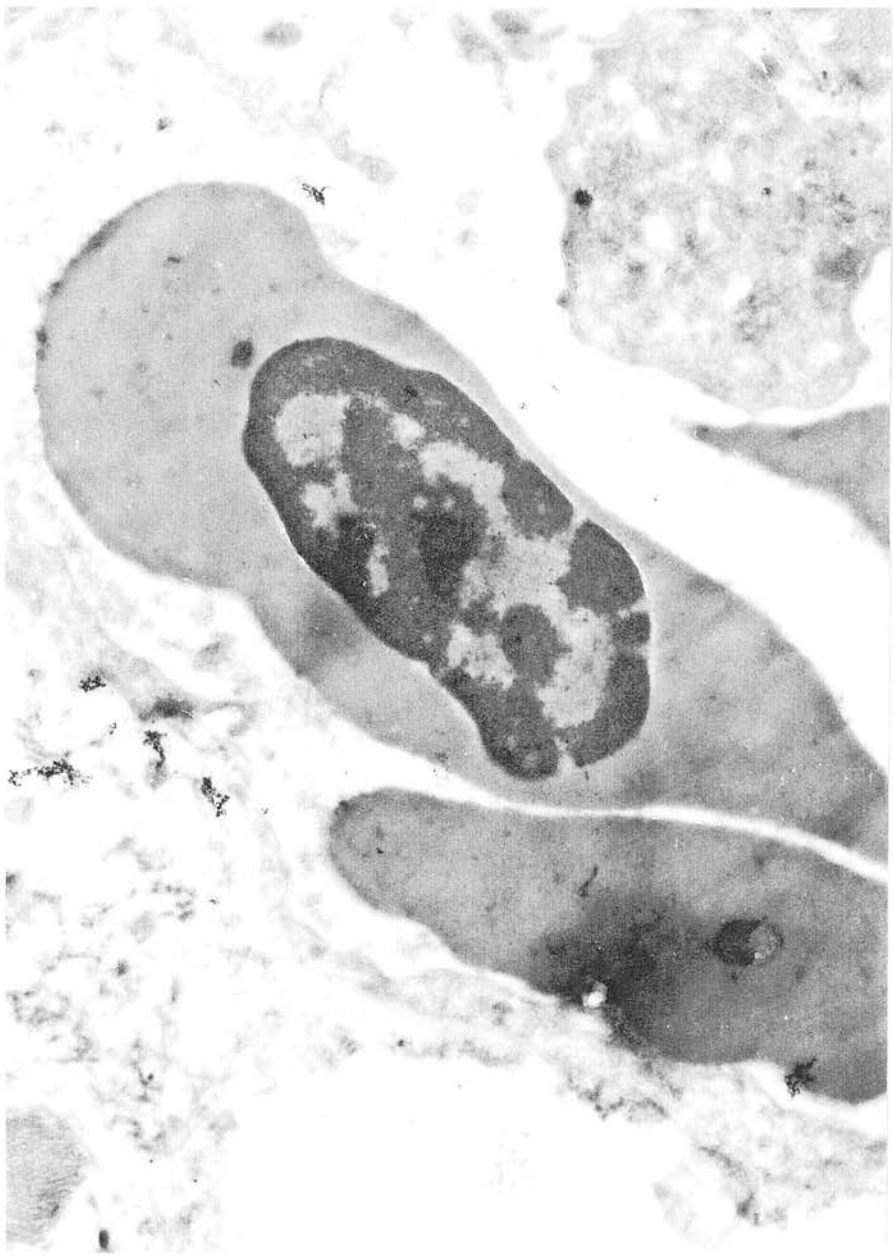
6.3.5 *Electron microscopy*

Attempts were made to define the subcellular localization of LPL at the capillary endothelium using immunogold electron microscopy. However, no immunoreactivity could be found after processing the tissue for electron microscopy using either post-embedding techniques involving Araldite or LR White resin embedded tissues sections, or when using ultra-thin cryosections of unembedded tissues. This was found to be the case following incubation with either the sheep anti-chicken LPL polyclonal antibody or the murine anti-chicken LPL monoclonal antibody, Cal-11. The photomicrograph (Fig 6.7) of an ultra-thin cryostat section shows a nucleated red blood cell in a minor blood vessel, no immunogold labelling for LPL could be detected at higher magnifications (x50,000) in association with either the capillary endothelial cells or within the cardiac myocytes.

6.4 DISCUSSION

The present study is the first use of an image analysis system for the study of LPL distribution within a tissue. Blanchette-Mackie *et al.* (1989) previously quantified the distribution of LPL within mouse cardiac tissue by immunogold labelling followed by electron micrographic observation. This technique, which requires the counting of individual gold particles in the field of view, is laborious, requires the use of strong fixatives (ie: glutaraldehyde) and considerable tissue processing, resulting in the possible loss of immunoreactivity over various tissue compartments.

Figure 6.7 Electron photomicrograph of an ultrathin cryostat section of chicken cardiac tissue.



Ultrathin cryostat sections of the chicken left ventricular myocardium were prepared and processed for immunogold labelling, as described in section 6.2.7.3. The photomicrograph is printed at a magnification of x17,500 and shows nucleated chicken red blood cells in a minor blood vessel. No immunogold labelling was observed on inspection of such sections at this or higher magnifications.

In the chicken myocardium the area covered by immunofluorescence in association with the capillary network was found to be 6.2% of the total area (46.4% of the area immunostained). The interstitial capillary-associated cellular elements in the myocardium comprises fibroblasts, pericytes and endothelial cells. Frank and Langer (1974) found that these cells accounted for 6% and the myocytes 69% of the total volume of the rabbit myocardium, as determined by stereological quantitation. From these figures it can be concluded that, despite possible species differences, the entire area comprised of the interstitial capillary elements is positively immunostained for LPL in the chicken myocardium. This observation is in agreement with the results of Camps *et al.*, (1990) who reported significant levels of immunoreactive LPL associated with the capillaries of the guinea pig myocardium. However, the amount of capillary-associated labelling found in the chicken myocardium is much greater than the 18% of total tissue label found over the capillary endothelium in mice hearts using immunogold electron microscopy (Blanchette-Mackie *et al.*, 1989).

In the present study no LPL immunoreactivity was associated with the endothelium of any of the larger blood vessels of the chicken myocardium (Fig 6.5). This contrasts with previous studies in the guinea pig which found considerable levels of LPL immunoreactivity over the endothelium of the major blood vessels of the myocardium (Camps *et al.*, 1990). This potential species difference is discussed in greater detail in section 7.3.

The lack of response of the LPL immunolocalized in association with the interstitial capillary elements to heparin both *in vitro* and *in vivo* corresponds closely to the low percentage of heparin-releasable activity in the isolated perfused chicken heart (Chapter 4). However, this differs from the observation that in humans an intravenous injection of heparin caused a drastic reduction of immunoreactivity over blood vessels in adipose tissue (Jonasson *et al.*, 1984). This difference could be explained by a much greater level of functional LPL activity being found in human adipose tissue than in chicken cardiac tissue. Capillary associated LPL in chickens is either present predominantly at a position other than at the luminal surface of capillary endothelial cells (non-functional), perhaps being concentrated at the basement membrane, or is predominantly

bound to capillary endothelial cells in a non-heparin dependent fashion (functional). These possibilities are fully discussed in section 7.3.

In the present study, the area covered by immunofluorescence for LPL in association with the basement membrane of the cardiac myocytes and the extracellular matrix was found to be 7.2% of the total area of the chicken myocardium (53.6% of the area immunostained). The relative lack of reactivity within chicken cardiac myocytes and the relatively high levels of immunoreactivity detected at the myocyte basement membrane or in an extracellular position contrasts with data reported for the mouse heart (Blanchette-Mackie *et al.*, 1989) where 78 % of the total tissue label was found over the myocytes (although a significant proportion of it was located at the cell periphery) and only 3-6 % in the extracellular space. These latter figures do not take into account low levels of non-specific background labelling which would increase the relative amounts of the label over the larger tissue compartments. However, the distribution of LPL obtained in the chicken myocardium agrees with the work of Camps *et al.*, (1990), who reported that LPL associated with the cardiac myocytes of the guinea pig myocardium was located at the periphery of such cells, however, the exact site of interaction could not be ascertained using standard fluorescent microscopy.

The present study using highly sensitive silver enhanced immunogold staining and immunofluorescence techniques shows that LPL accumulates at interstitial locations in association with the myocyte basement membrane/extracellular matrix and the capillary elements in chicken cardiac tissue *in vivo*. This confirms the results of numerous previous studies on the distribution of LPL in the cardiac tissue of several species.

LPL activity in cardiac tissue has been observed in association with rat cardiac myocytes (Chohan and Cryer, 1978; Bagby and Corll, 1988; Rajaram *et al.*, 1980). However, Chajek *et al.* (1977) found that in cultures of neonatal rat heart cells, LPL was predominantly synthesized by mesenchymal cell types derived from the vascular and interstitial elements rather than by the cardiac myocytes. Therefore, it is possible that in cardiac tissue the myocytes and/or cells of mesenchymal origin may serve as a source of endothelial LPL.

Recently, Stein *et al.* (1991) concluded from *in situ* hybridization studies that the main site of LPL synthesis in the adult rat heart may not be the cardiac myocytes but the interstitial cells of the capillary bed, such as the pericytes. The expression of LPL mRNA was found to be strong only in mesenchymal cell types (capillary and perivascular) with sparse reaction over the cardiac myocytes. This is at variance with the findings of Camps *et al.* (1990) who found significant hybridization for LPL mRNA over the cardiac myocytes in the fully-fed guinea pig heart.

The suggestion of an interstitial rather than intracellular location for much of the residual LPL activity (non heparin-releasable) was proposed by Hulsmann *et al.* (1982) who found that a substantial portion of the residual LPL activity was not localized in the myocytes but in an extracellular compartment which was opened during Ca^{2+}-free perfusion. Jansen *et al.* (1980) also demonstrated the presence of interstitial LPL by collecting separately the coronary and interstitial post-heparin effluents from the perfused heart model system. The LPL activity present in the coronary effluents from the heart of fasted rats was higher than in those of fed rats, while the opposite was true for the enzyme activity in the interstitial effluents. However, these experiments failed to demonstrate that LPL activity was found in the interstitial compartment of cardiac tissue under normal conditions *in vivo*.

Liu and Olivecrona (1991) using pulse-chase studies in perfused guinea pig hearts, concluded that LPL moves rapidly from the myocytes by the so-called default pathway (Pelham, 1989) with no obligatory delay. In the default pathway, non-selective bulk flow to the cell surface is thought to be driven by a process in which the contents of each secretory compartment are transferred to the next, with diversion from the pathway or retention in a given compartment being signal-mediated. The relatively low level of LPL reactivity found within chicken heart myocytes in this study is consistent with the rapid movement of LPL out of the cells by this mechanism. However, the data produced by Liu and Olivecrona (1991) does not support a model whereby LPL is transported directly to the luminal surface of capillary endothelial cells and is released into the medium in the presence of heparin. When heparin was present throughout a 105 minute perfusion period, 32% of the pulse-labelled LPL appeared in the medium while

only 9% of the labelled LPL was released by heparin at the end of a similar period of perfusion without heparin. The total recovery of labelled LPL was similar with or without heparin in both cases. This data indicated that LPL does indeed accumulate at some point between the myocytes and the luminal surface of capillary endothelial cells, as shown by the present study of chicken tissues. The transport of LPL from the basal membrane and other interstitial structures where it accumulates may be aided by heparin or responsive to the depletion of LPL at the capillary lumen, caused by the addition of heparin to the perfusion medium.

Blanchette-Mackie *et al.* (1989) concluded from immunocytochemical studies that LPL did not appear to move as a soluble protein in mouse heart tissue and there was only limited accumulation of LPL around the capillary network or other interstitial structures. The work in this Chapter indicates that LPL does indeed accumulate around the interstitial capillary elements possibly in association with the vascular basement membrane and at other interstitial structures extracellular to the cardiac myocytes. The accumulation of LPL at these sites within cardiac tissue has important implications for the means by which the enzyme is transported from the myocytes to the capillary lumen. The differences between the results of the present study and those of Blanchette-Mackie *et al.* (1989) may be due to the different protocols used for tissue processing and fixation. Blanchette-Mackie *et al.* (1989) used glutaraldehyde as a fixative while in the present study mild fixation in acetone at -20°C was used to preserve LPL antigenicity across all the tissue compartments.

The technique of immunogold labelling combined with electron microscopy would have allowed the subcellular localization of LPL within the capillary endothelium and quantitation of functional LPL. The lack of reactivity found using post-embedding techniques can be explained by the processing required, since dehydration and resin embedding often result in both a reduction of antigenicity and linked accessibility to the antigen. Embedding in Araldite (an impermeable hydrophobic epoxy resin) and post-fixation with osmium has been shown to strongly reduce immunoreactivity of tissue sections (Newman and Hobot, 1989). Embedding in LR White, a hydrophilic acrylic resin, which does not require complete dehydration of the tissue and is

permeable to antibodies so does not require etching, frequently improves immunoreactivity (Newman, 1989). However, due to the lack of LPL immunoreactivity of LR White embedded tissue, the technique of ultra thin cryosectioning of unembedded tissue was attempted. The technique has previously been used to immunolocalize LPL in the mouse heart (Blanchette-Mackie *et al.*, 1989) and requires little tissue processing with the sections being fully hydrated. Despite this no LPL immunoreactivity could be detected on ultra-thin cryosections (Fig 6.10). This may be due to the use of glutaraldehyde as a fixative (even though it was used by Blanchette-Mackie *et al.*, 1989) which can cause destruction of epitopes by steric hindrance or by changes in conformation when the fixative binds to the epitope and also reduces antibody penetration (Kellenberger *et al.*, 1987). However, this lack of detectable LPL immunoreactivity may also be due to differences in the reactivities of the antibodies that are available for use against chicken LPL.

CHAPTER 7

THE IMMUNOLOCALIZATION OF LPL IN CHICKEN TISSUE, DEVELOPMENTAL CHANGES IN THE HEART AND LIVER DURING HATCHING

7.1 INTRODUCTION

The validation of methods for the immunolocalization of LPL in chicken cardiac tissue was described in Chapter 6. The pattern of immunoreactivity that was observed indicated that LPL accumulated around the interstitial capillary elements, mainly in a fashion not releasable by heparin perfusion, and was also present, to a quantitatively important extent, at the basement membrane surrounding the cardiac myocytes. In this Chapter, the distribution of LPL within other tissues of the chicken was examined and compared with that observed in the heart. The use of the technique to investigate tissue-specific post-hatching developmental changes in the chicken is also described.

In the first comparative study of several tissues in one species, Camps *et al.* (1990) reported the immunolocalization of LPL protein and the detection of LPL mRNA using *in situ* hybridization in the adipose tissue, heart, aorta, diaphragm, mammary gland and kidney of the guinea pig. In the tissues studied LPL immunoreactivity was found to be present at the endothelium of all blood vessels. In the study of Camps *et al.* (1990), the total LPL immunoreactivity in adipose tissue was found to be relatively intense when compared with other tissues, with the prominent staining of the capillaries and larger blood vessels being associated with subendothelial cells, pericyte-like cells and endothelial cells. LPL was also found at the periphery of adipocytes. In the heart, there was LPL immunoreactivity in both the capillary walls and at the periphery of myocytes, a pattern repeated in diaphragm muscle with strong labelling at the muscle cell periphery. The parenchymal cells of muscle and adipose tissues were also found to express LPL mRNA using *in situ* hybridization. The aorta showed immunoreactivity over the endothelium with some reactivity being present in the cells of the smooth muscle layer for both LPL mRNA and protein. In the kidney there was strong immunofluorescence at the vascular endothelium,

especially in the glomeruli, but little LPL mRNA could be detected in the tissue by *in situ* hybridization.

The synthesis and distribution of LPL in the guinea pig lung, liver and spleen, where the overall LPL activity is relatively low, was reported in a second paper by Camps *et al.* (1991). In these tissues the enzyme was expressed in scattered cells such as macrophages in the lung and spleen. In the liver significant levels of LPL immunoreactivity was found in Kupffer cells, in association with sinusoids and inside some of the cells in the periportal area. However, because of the the low level of LPL mRNA detected in the liver, it was concluded that the LPL protein which was present was taken up from the bloodstream rather than synthesized by the hepatocytes.

Previous studies have ignored the potential contribution to whole body LPL activity by enzyme associated with the bone marrow. Wood (1967) reported that adipocytes are the most conspicuous element of the marrow mass in chickens and Nir and Lin (1982) estimated, from measurements of the incorporation of [1-^{14}C] acetate into lipids *in vivo*, that the total lipogenic activity of the chicken skeleton was approximately half that of the liver and that the lipogenic activity of isolated bone marrow *in vitro* was approximately two thirds that of the hepatic activity. Gimble *et al.* (1990) found that the cloned bone marrow stromal cell line, BMS2, synthesized LPL *in vitro* after undergoing adipocyte differentiation. However, despite these findings, no study of the distribution or level of LPL activity has been made on this major lipid storing tissue, in either chickens or mammalian species. To this end, work in this Chapter also describes the use of immunocytochemistry to localize LPL activity within chicken bone marrow, amongst the range of tissues studied.

Many studies have shown that developmental changes occur in the level of LPL activity present in mammalian tissues (Cryer, 1987). For example LPL activity in the liver of neonatal (1 day old) rats has been found to be 2-3 times higher than that in the liver of adult rats (Burgaya *et al.*, 1989; Peinado *et al.*, 1990). This activity has also been seen to decrease progressively during the postnatal period of development to reach the lower values typical of adults at weaning (Lopez-Tejero *et al.*, 1988). However, such a developmental pattern is the reverse of that in rat

heart, where LPL activity was very low in the foetus and rises to adult levels during suckling (Planche *et al.*, 1980; Cryer and Jones, 1978). Little work has been recorded on the tissue specific developmental changes in LPL activity of non-mammalian species and only one such study has been reported in chickens (Speake *et al.*, 1992).

Work in this chapter describes the use of immunocytochemistry to follow the developmental changes in chicken liver and heart LPL activity and distribution, from the embryo to 6 weeks of age.

7.2 METHODS

7.2.1 *Immunohistochemistry*

For the work described in this Chapter, fully-fed broiler chickens of 5/6 weeks of age and reared as described in section 4.2.1 were used throughout. Tissue preparation and sectioning was carried out as described in section 6.2.4. The immunofluorescence and silver enhanced immunogold staining procedures were performed as described in sections 6.2.4 and 6.2.5 respectively. For the release of LPL from the vascular endothelium *in vivo,* 1000 units/Kg of heparin were injected intravenously as described in section 5.2.6. The birds were killed 2 or 8 min after the administration of heparin and the tissues were collected and placed into PBS at 0°C before being frozen by the method described in section 6.2.4.

7.2.2 *Lipid staining*

A saturated stock solution of Oil Red O (Sigma Chemical Co.) was prepared by mixing 0.5% (w/v) Oil Red O in isopropyl alcohol at 56°C for 1 hour. This stock solution was subsequently stored at room temperature. Immediately before use, 6 ml of this stock solution was diluted with 4 ml of distilled water, allowed to stand for 10 min and then filtered using Whatman filter paper No.1 (Whatman Int plc, Maidstone, Kent, UK). Unfixed frozen sections of bone marrow were prepared as described in section 6.2.4. The bone marrow sections were washed well with water before being placed in the stain for 10 minutes. Non-specific background staining was reduced by immersing the sections for 3 min in 70% (v/v) alcohol, washed in tap water and counterstained lightly with Mayer's haematoxylin as described in

section 6.2.5. The tissue sections were mounted in 90% (v/v) glycerol in PBS for viewing as described in section 6.2.6.

7.3 RESULTS

7.3.1 *Immunostaining of chicken muscle tissues, adipose tissue and bone marrow*

Cryostat sections from chicken leg muscle, breast muscle, gizzard, adipose tissue and bone marrow were prepared and the potential presence of LPL in them investigated using the immunofluorescence procedure described in section 5.2.4. The pattern of immunostaining obtained using either sheep anti-chicken LPL IgG or Cal-11 IgG as the primary antibody was identical in all of the tissues studied. When non-immune sheep or mouse serum or purified non-immune IgG was substituted for anti-LPL antibodies, no staining was observed in any of the tissues studied (see control sections in each case).

In the skeletal muscles, breast (superficial pectoral) and leg (gastrocnemius), a staining pattern similar to that obtained in cardiac tissue was observed, with staining in the extracellular matrix, at the basement membrane surrounding the myofibrils and in association with the interstitial capillary elements (breast muscle Fig 7.1, A and gastrocnemius Fig 7.2, A). Antibodies against LPL were found to stain the endomysium of chicken skeletal muscle (gastrocnemius) strongly while showing only a weak reaction over the perimysium (Fig 7.3, A).

The pattern of LPL immunoreactivity was also similar in sections of adipose tissue taken from the abdominal fat pad, with intense staining in association with the interstitial capillary elements and staining at the adipocyte basement membrane (Fig 7.4, A).

In the gizzard (cranioventral) muscle, strong immunofluorescence was obtained in association with the interstitial capillary elements and myocytes of the semi-autonamous muscle layer (Fig 7.5, A). No immunoreactivity was observed over the glands of the mucous membrane or over the cuticle lining of the gizzard (data not shown).

Figure 7.1

A) The immunolocalization of LPL in sections of chicken breast muscle.

LPL was localized in cryostat sections of chicken breast (superficial pectoral) muscle using sheep anti-chicken LPL IgG in the immunofluorescence labelling procedure described in section 6.2.4. LPL immunoreactivity was confined to the basement membrane surrounding the myofibrils (straight arrow) and in association with the interstitial capillary elements (curved arrow). Print x 595.

B) The immunolocalization of LPL in sections of chicken breast muscle following an IV injection of heparin.

LPL was localized in cryostat sections of chicken breast (superficial pectoral) muscle, from a bird killed 2 min after an IV injection of heparin, using sheep anti-chicken LPL IgG in the immunofluorescence labelling procedure described in section 6.2.4. The pattern of LPL immunoreactivity was unchanged by heparin administration (ie: from that shown above). Print x 595.

C) Non-immune control for the immunolocalization of LPL in chicken breast muscle.

Cryostat sections of chicken breast (superficial pectoral) muscle were incubated with non-immune sheep IgG and processed using the immunofluorescent labelling procedure described in section 6.2.4. No immunofluorescence was detected over the sections. Print x 595.

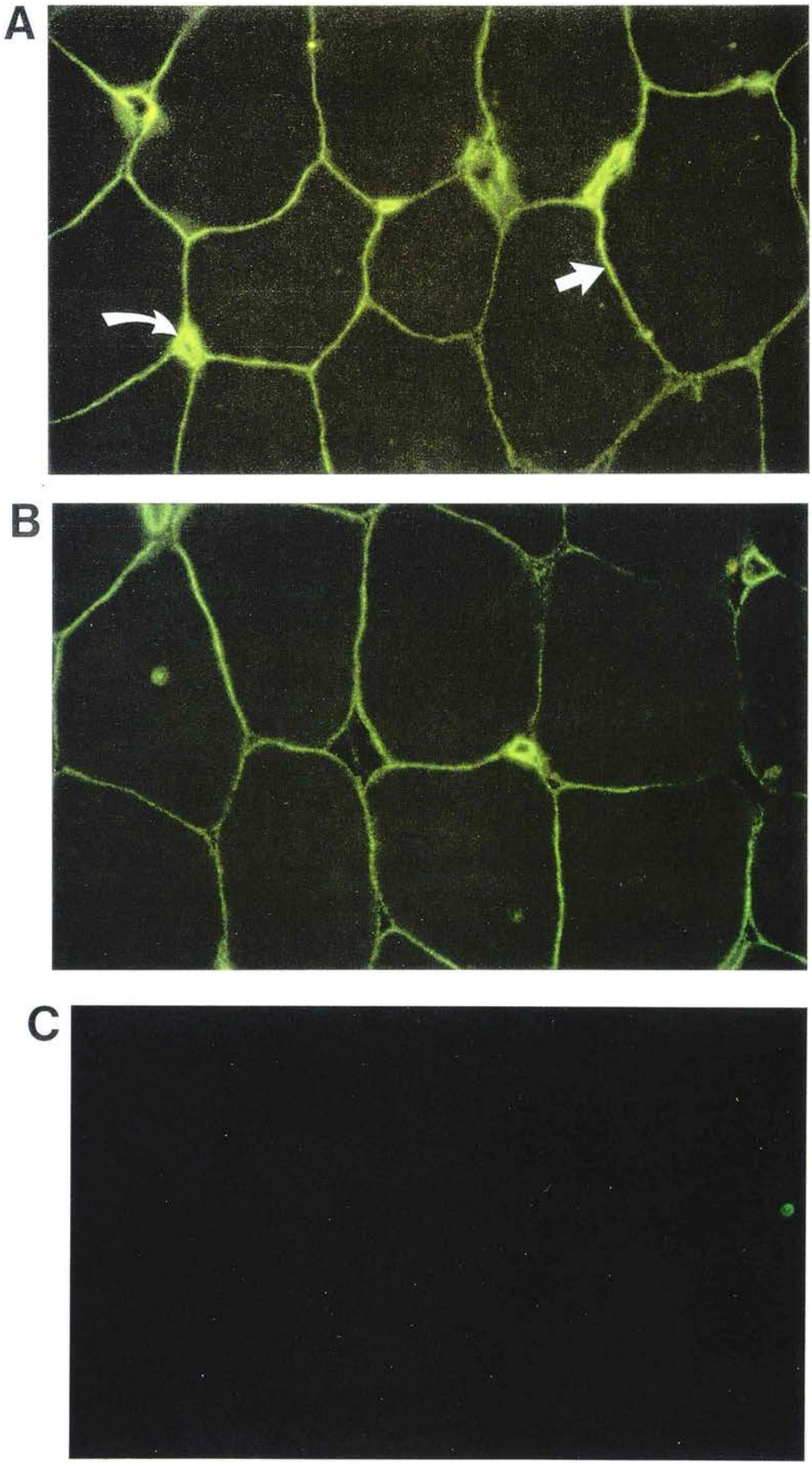

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Figure 7.2

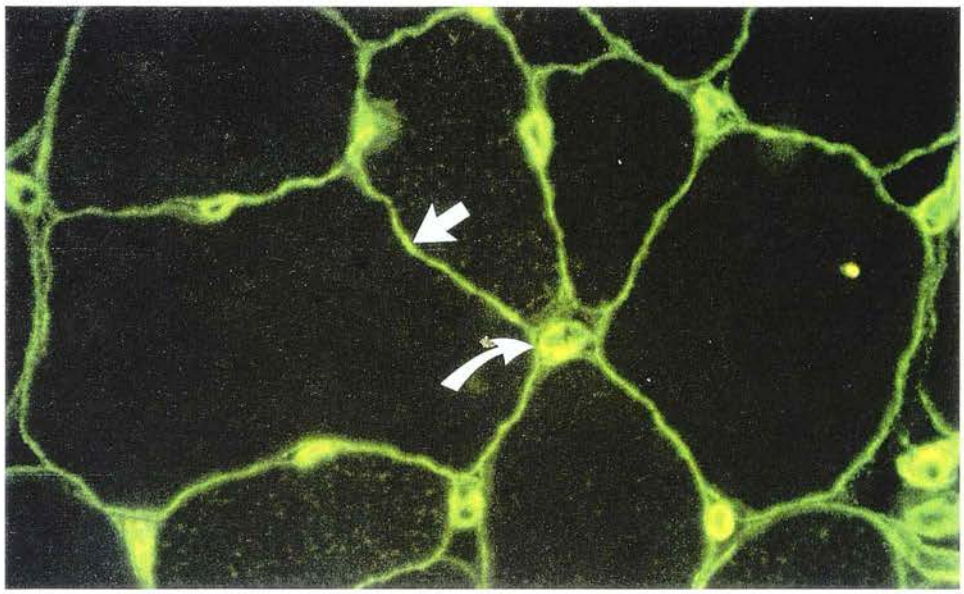
A) The immunolocalization of LPL in sections of chicken leg muscle.

LPL was localized in cryostat sections of chicken leg (gastocnemius) muscle using sheep anti-chicken LPL IgG in the immunofluorescence labelling procedure described in section 6.2.4. The pattern of LPL immunoreactivity was confined to the basement membrane surrounding the myofibrils (straight arrow) and in association with the interstitial capillary elements (curved arrow). Print x 595.

B) The immunolocalization of LPL in sections of chicken leg muscle following an IV injection of heparin.

LPL was localized in cryostat sections of chicken (gastocnemius) muscle, from a bird killed 2 min after an IV injection of heparin, using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. The pattern of LPL immunoreactivity was unchanged by heparin administration (ie: to that shown above). Print x 595.

A



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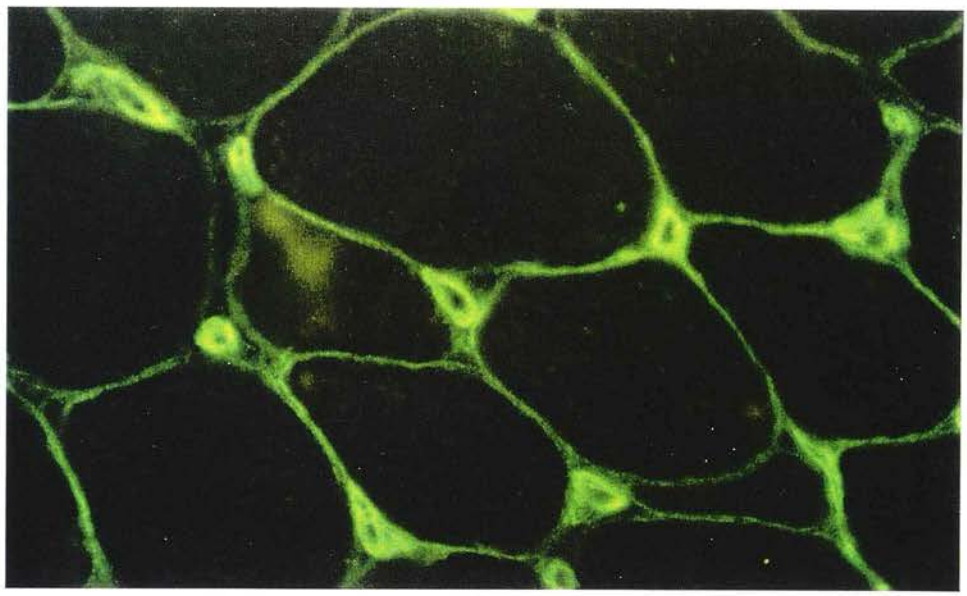


Figure 7.3

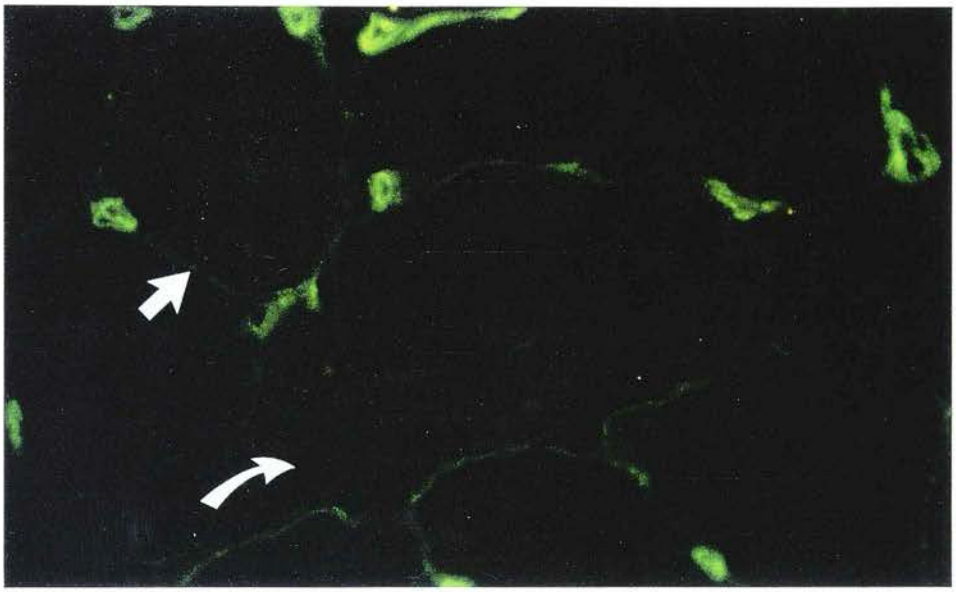
A) The immunolocalization of LPL in sections of chicken leg muscle.

LPL was localized in cryostat sections of chicken leg (gastocnemius) muscle using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. Antibodies against LPL were found to give strong immunofluorescence over the endomysium (straight arrow) but little reaction was observed over the perimysium (curved arrow). Print x 595.

B) Non-immune control for the immunolocalization of LPL in chicken leg muscle.

Cryostat sections of chicken leg (gastrocnemius) muscle were incubated with non-immune sheep IgG and processed using the immunofluorescent labelling procedure described in section 6.2.4. No immunofluorescence was detected over such sections. Print x 595.

A



B

Figure 7.4

A) The immunolocalization of LPL in sections of chicken adipose tissue.

LPL was localized in cryostat sections of chicken adipose tissue using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. Immunofluorescence for LPL was intense in association with the interstitial capillary elements (curved arrow). Immunoreactivity was also found at the periphery of the adipocytes (straight arrow). Print x 595.

B) The immunolocalization of LPL in sections of chicken adipose tissue following an IV injection of heparin.

LPL was localized in cryostat sections of chicken adipose tissue, from a bird killed 2 min after an IV injection of heparin, using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. The pattern of LPL immunoreactivity was unchanged by heparin administration (ie: to that shown above). Print x 595.

C) Non-immune control for the immunolocalization of LPL in chicken adipose tissue.

Cryostat sections of chicken adipose tissue (from the abdominal fat pad) were incubated with non-immune sheep IgG and processed using the immunofluorescent labelling procedure described in section 6.2.4. No immunofluorescence was detected over the sections. Print x 595.

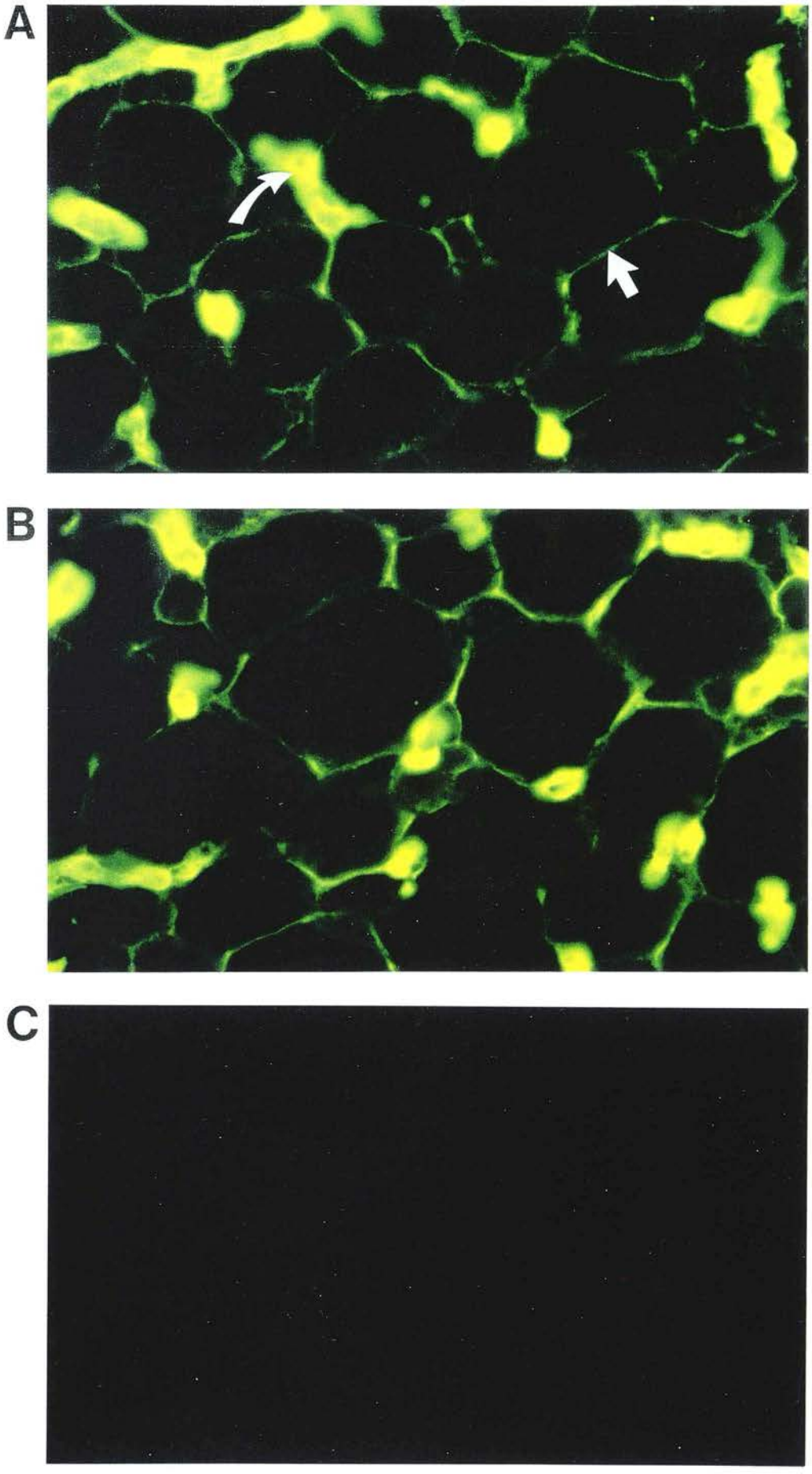

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Figure 7.5

A) The immunolocalization of LPL in sections from the gizzard muscle of the chicken.

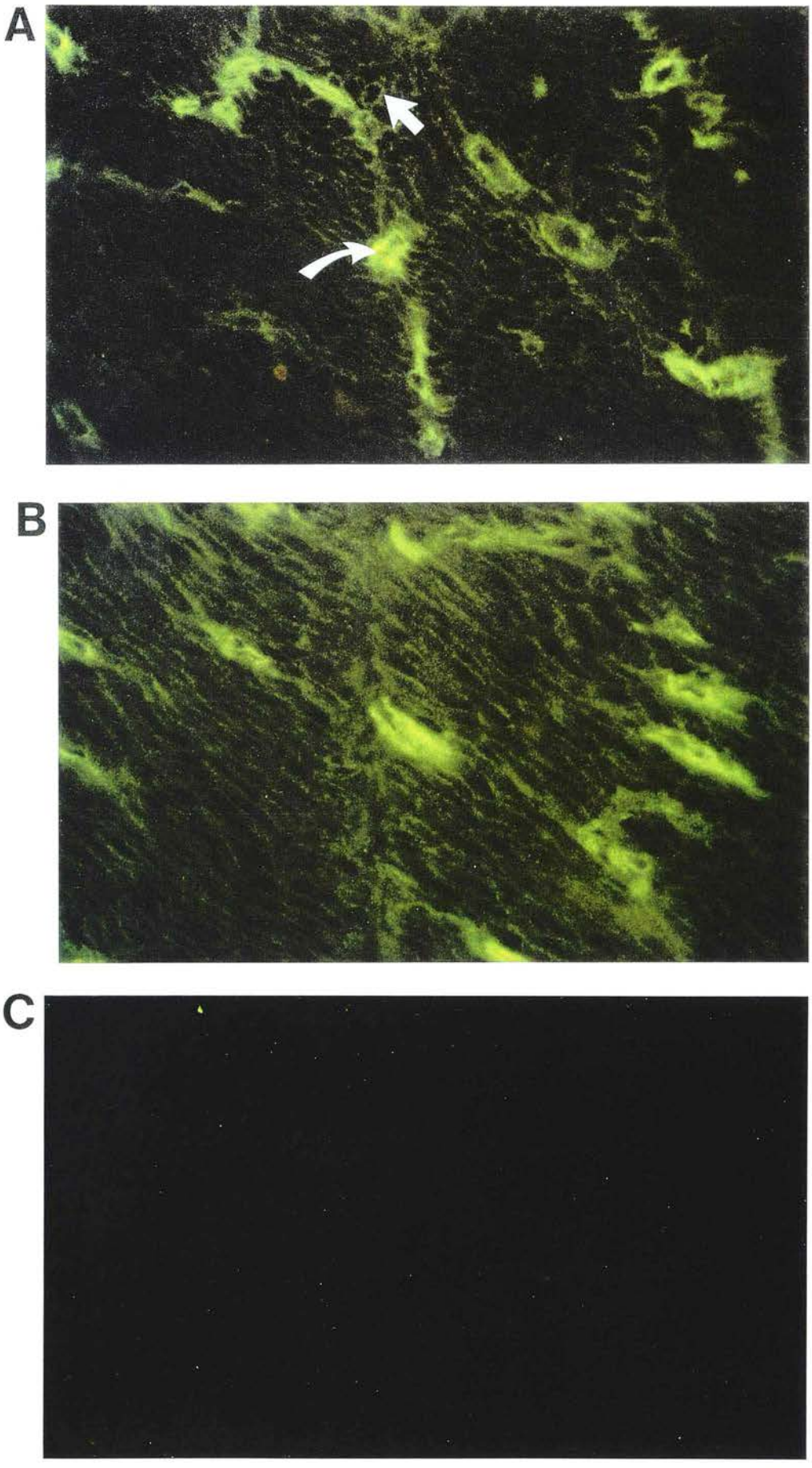
LPL was localized in cryostat sections of chicken gizzard (cranioventral) muscle using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. LPL immunoreactivity was confined to the basement membrane surrounding the muscle cells (straight arrow) and in association with the interstitial capillary elements (curved arrow). Print x 595.

B) The immunolocalization of LPL in sections from the gizzard muscle of the chicken following an IV injection of heparin.

LPL was localized in sections of chicken gizzard (cranioventral) muscle, from a bird killed 2 min after an IV injection of heparin, using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 5.2.4. The pattern of LPL immunoreactivity was unchanged by heparin administration (ie: to that shown above). Print x 595.

C) Non-immune control for the immunolocalization of LPL in chicken gizzard muscle.

Cryostat sections of chicken gizzard (cranioventral) muscle were incubated with non-immune sheep IgG and processed using the immunofluorescent labelling procedure described in section 6.2.4. No immunofluorescence was detected over the sections. Print x 595.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Intense immunofluorescence was detected in the bone marrow of the tibiotarsus (Fig 7.6, A and B) but because of the complex and irregular nature of the tissue interpretation of the pattern of immunoreactivity was difficult without counterstaining. Therefore, the technique of silver enhanced immunogold was used in conjunction with haematoxylin and haematoxylin counterstaining, in the further study of bone marrow. This technique revealed little immunoreactivity over the dense haematopoietic tissue (Fig 7.7, A). However, intense staining was observed at the periphery of lipid containing cells which were selectively stained with the lipid dye Oil Red O (Fig 7.7, C) and in association with the vascular elements (Fig 7.7, A).

In all of the tissues studied, no immunoreactivity was detected in association with the endothelium of larger blood vessels.
The relative intensity of tissue immunoreactivity determined by eye, correlated positively with the level of total tissue LPL activity (Table 4.6) as measured in six week old broiler chickens. The order of reaction was as follows : adipose tissue > heart > leg muscle/gizzard muscle > breast muscle. The immunoreactivity of bone marrow was similar in intensity to that of adipose tissue, indicating the possibility that a high level of total tissue LPL activity was present in chicken bone marrow.
The capillary associated LPL in all extrahepatic tissues was found to be predominantly heparin-insensitive, since the pattern of LPL immunofluorescence of the tissues (Figs 7.1, A - 7.5, A) was unchanged following an intravenous injection of heparin *in vivo* (Figs 7.1, B - 7.5, B).

No immunoreactivity could be detected in the liver of six week old broiler chickens using either immunofluorescence (Fig 7.8, A) or silver enhanced immunogold (data not shown). However, within 2 min of an intravenous injection of heparin the liver showed immunoreactivity for LPL which closely followed the contours of the sinusoidal spaces, with no label being detected in the hepatocytes themselves (Fig 7.8, B). If the bird was killed 8 min after an intravenous heparin injection and the liver removed and sectioned, substantial levels of LPL immunoreactivity were observed over the sinusoids and other blood vessels (Fig 7.9, A and B). After this longer period of heparin circulation, additional immunofluorescence for LPL was also observed within the lumen of the larger blood vessels but at no stage was significant reaction observed

Figure 7.6

A and B) The immunolocalization of LPL in sections of chicken bone marrow.

LPL was localized in cryostat sections of chicken bone marrow taken from the tibiotarsus bone using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. No immunoreactivity for LPL was found over the haematopoietic tissue (arrow). print x 595.

C) Non-immune control for the immunofluorescent localization of LPL in chicken bone marrow.

Cryostat sections of chicken bone marrow taken from the tibiotarsus bone were incubated with non-immune sheep IgG and processed using the immunofluorescent labelling procedure described in section 6.2.4. No immunofluorescence was detected over the section. Print x 595.

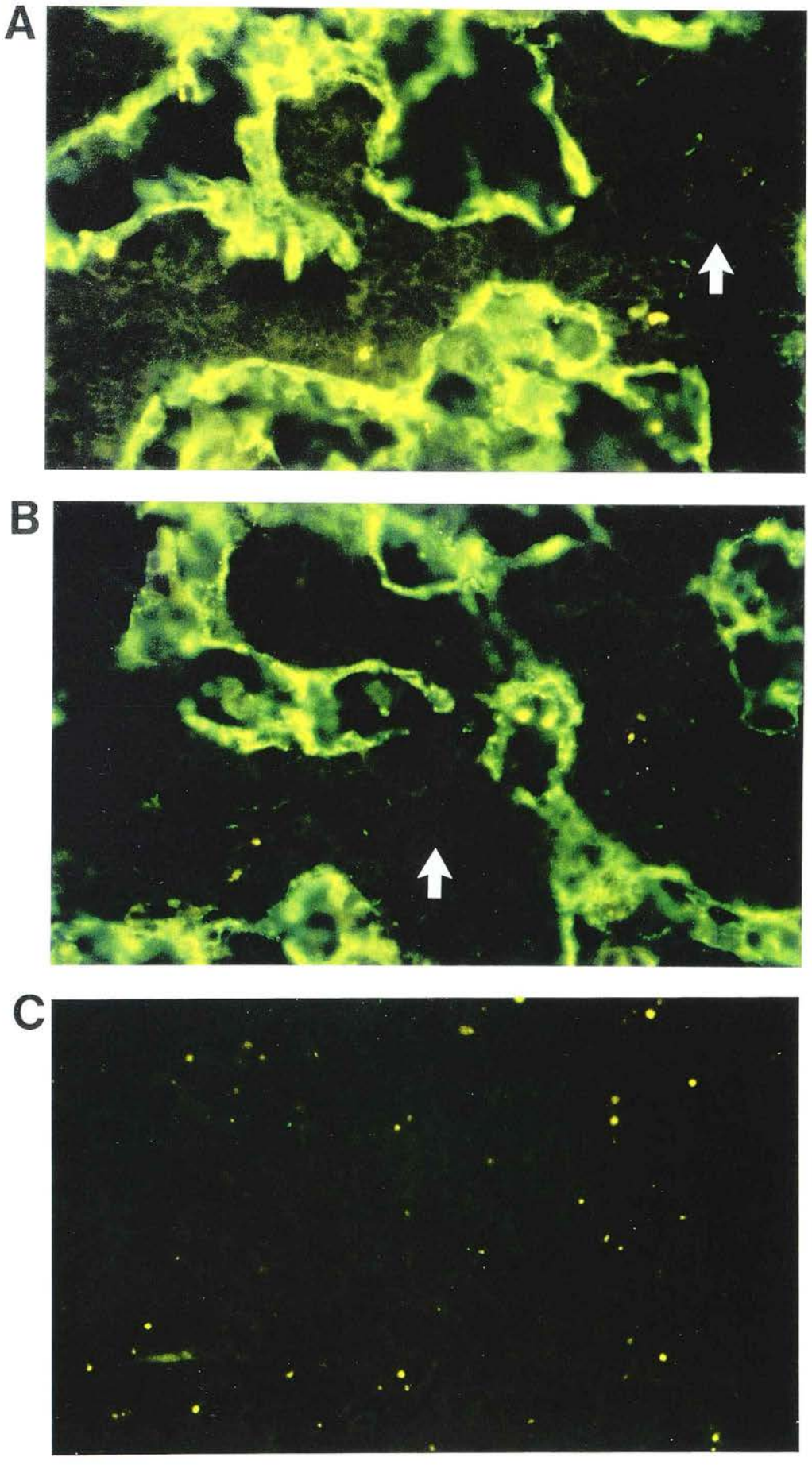
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Figure 7.7

A) The immunolocalization of LPL in sections of chicken bone marrow.

LPL was localized in cryostat sections of chicken bone marrow taken from the tibiotarsus bone using sheep anti-chicken LPL IgG in the silver enhanced immunogold labelling procedure followed by counterstaining with haematoxylin, as described in section 6.2.5. Immunoreactivity for LPL was confined to the adipocyte periphery (open arrow) and the associated vascular elements (black arrow). Little immunoreactivity was detected over the dense haematopoietic tissue (white arrow). Print x 595.

B) Non-immune control section for the immunolocalization of LPL in chicken bone marrow.

Cryostat sections of chicken bone marrow from the tibiotarsus were incubated with non-immune sheep IgG and processed using the silver enhanced immunogold labelling procedure described in section 6.2.5. No immunoreactivity was detected over the section. Print x 595.

C) The selective staining of chicken bone marrow sections, for lipid, using Oil Red O.

Accumulations of lipid in cryostat sections of chicken bone marrow from the tibiotarsus bone were stained using Oil Red O, as described in section 7.2.2. Considerable numbers of lipid containing adipocytes (arrow) were associated with the dense haematopoietic tissue (asterisk). Print x 595.

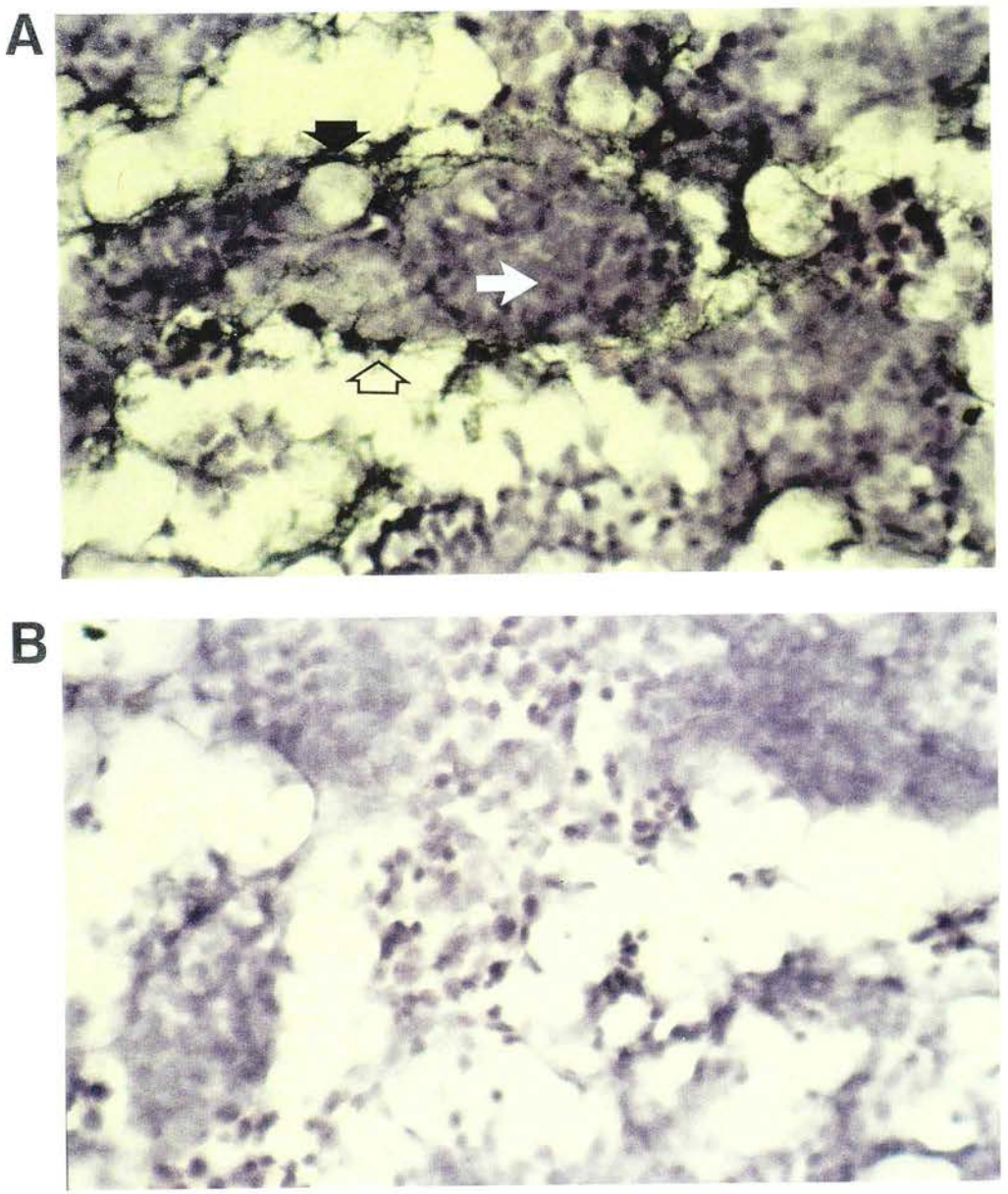
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Figure 7.8

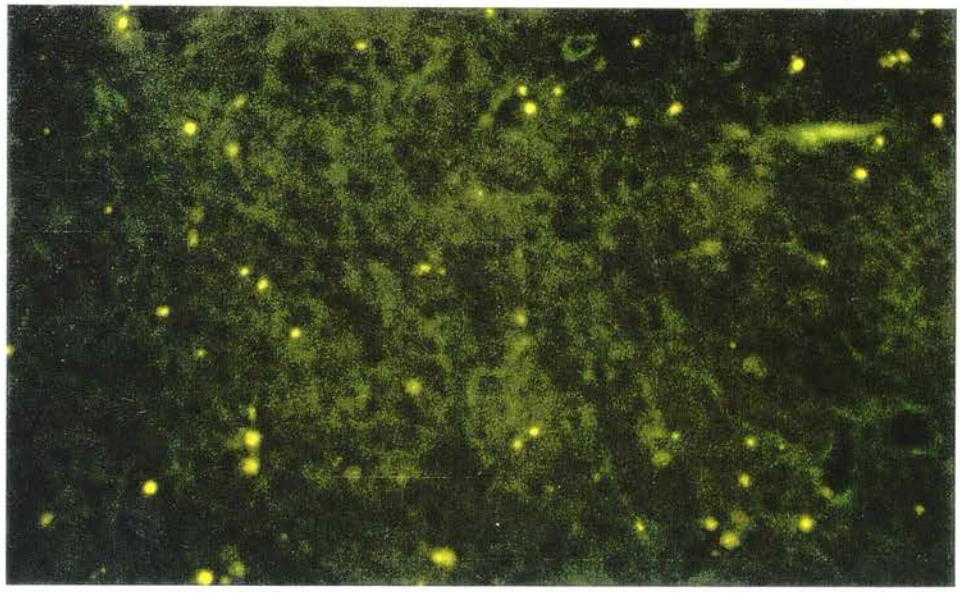
A) The lack of LPL immunoreactivity in the liver of 6 week old broiler chickens.

Cryostat sections of 6 week old broiler chicken liver were incubated with sheep anti-chicken LPL IgG and processed using the immunofluorescent labelling procedure described in section 6.2.4. No immunofluorescence was detected over the section. Print x 595.

B) The immunolocalization of LPL in sections of the liver from a 6 week old broiler chicken killed 2 min after an intravenous injection of heparin.

Chickens were injected with heparin and killed after 2 min as described in section 7.2.1. LPL was localized in cryostat sections of the liver from such birds using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. Immunofluorescence for LPL closely followed the contours of the sinusoidal spaces (arrow). Print x 595.

A



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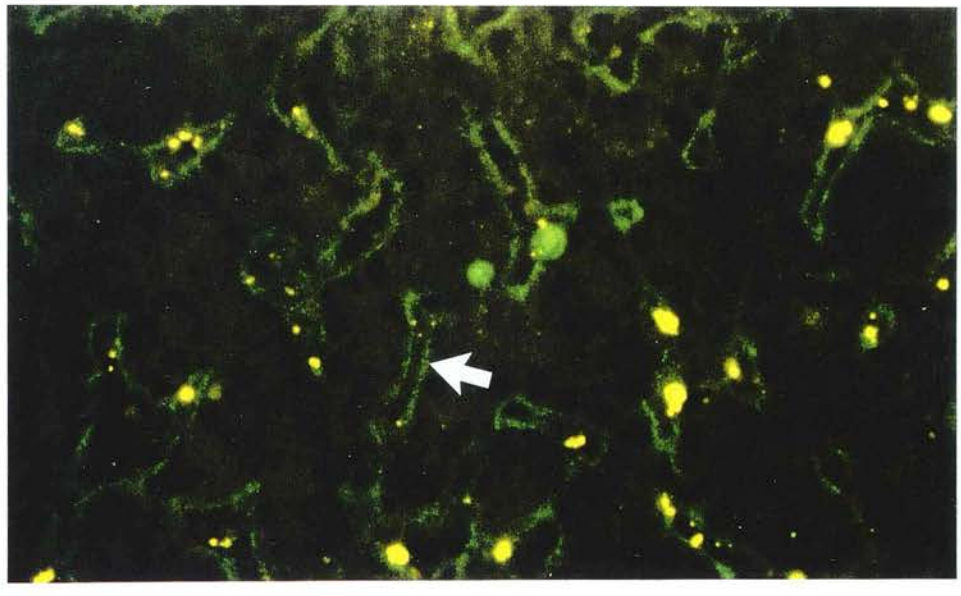


Figure 7.9

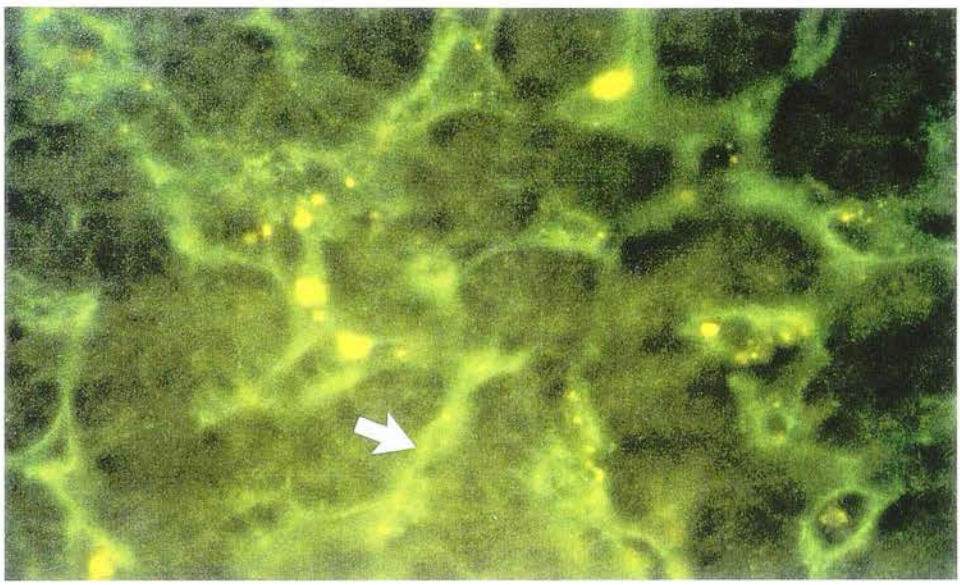
A) The immunolocalization of LPL in sections of the liver from a 6 week old broiler chicken killed 8 min after an intravenous injection of heparin.

Chickens were injected with heparin and killed after 8 min as described in section 7.2.1. LPL was localized in cryostat sections of the liver from such birds using sheep anti-chicken LPL in the immunofluorescent labelling procedure described in section 6.2.4. LPL immunofluorescence was associated with sinusoidal spaces (arrow) and was found to be more intense than that observed in the liver 2 min after heparin administration (Fig 7.8, B). Print x 595.

B) The immunolocalization of LPL in sections of the liver from a 6 week old broiler chicken killed 8 min after an intravenous injection of heparin.

Chickens were injected with heparin and killed after 8 min as described in section 7.2.1. LPL was localized in cryostat sections of the liver from such birds using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. LPL immunofluorescence was associated with the sinusoidal spaces (curved arrow) as well as the endothelium (open arrow) and lumen of larger hepatic blood vessels (straight closed arrow). Print x 595.

A



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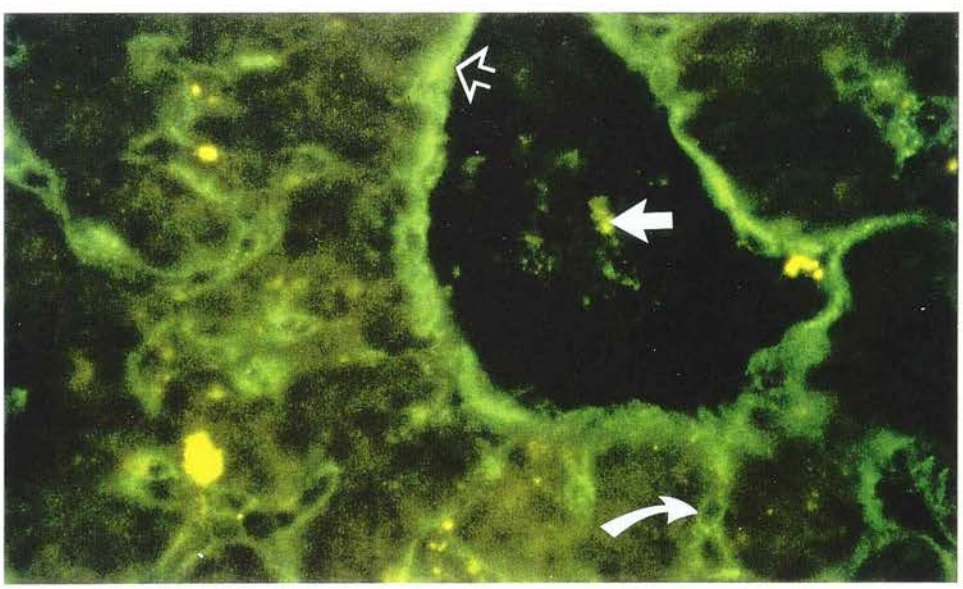


within the hepatocytes themselves (Fig 7.9, B).

7.3.2 *Developmental changes*

Cryostat sections from the heart and liver of chicken embryos and 1 to 6 day old chicks were prepared and the LPL localized using the immunofluorescence procedure described in section 6.2.4.

The livers of day old chicks, 20 day and 15 day old embryos showed strong immunoreactivity over the sinusoids, with little staining at the hepatocyte periphery, and some reaction over the endothelium of major blood vessels (Fig 7.10, A). There was no alteration in the distribution or the intensity of immunofluorescence for LPL in the embryonic liver or the liver from one day old chickens. However, hepatic LPL immunoreactivity rapidly diminished after hatching and the liver of six day old chicks showed only a little reactivity over the hepatic sinusoids (Fig 7.10, B). This corresponds to the complete lack of LPL immunoreactivity in the liver of 6 week old broiler chickens seen previously using immunohistochemistry (Fig 7.8, A) or immunoblotting (Fig 6.2) techniques.

The hearts from 15 day old embryos showed staining within the myocytes themselves with no relative concentration of LPL in association with interstitial elements (Fig 7.11, A), which was by contrast with the distribution of LPL in one day post-hatch tissue (Fig 7.11, C). This early embryonic pattern of LPL distribution changed gradually, with the 20 day old embryo heart and day old chick heart (Fig 7.11, B and C) being an intermediate stage between the early embryonic pattern (Fig 7.11, A) and that observed in the 6 week old broiler (Fig 6.3, A). That is to say the myocyte associated staining was reduced and confined solely to the basement membrane while LPL was also concentrated in association with the interstitial capillary elements. The general level of total tissue LPL reactivity, estimated by eye, appeared to increase from the 15 day old embryo heart to the 20 day old embryo heart and remain high thereafter. This was because of an apparent increase in the intensity of and area covered by the immunolabelling associated with the interstitial capillary elements.

Figure 7.10

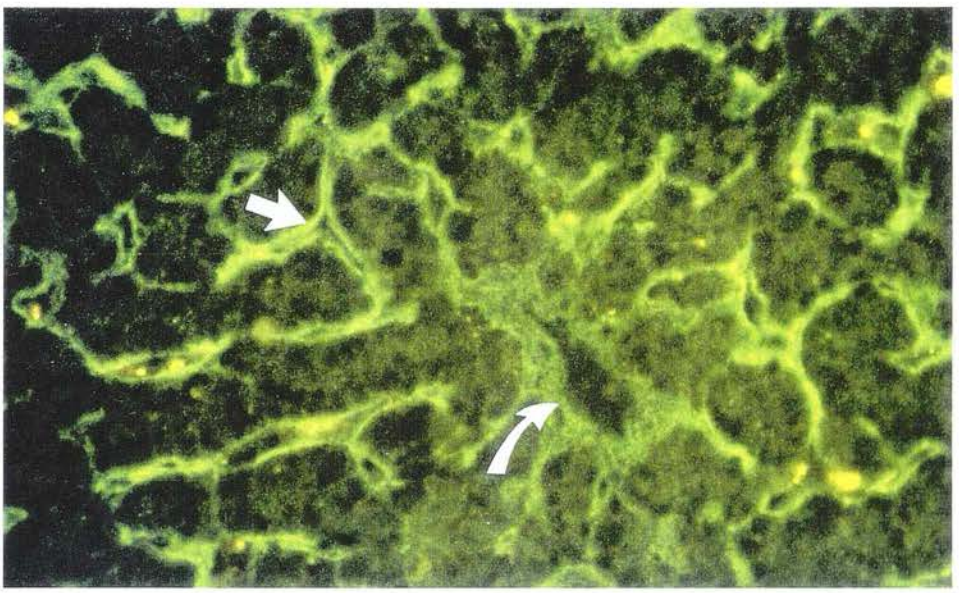
A) The localization of LPL in tissue sections of the liver from day old broiler chickens.

LPL was localized in cryostat sections from the liver of day old broiler chickens using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. Immunofluorescence for LPL closely follows the contours of the sinusoidal spaces (straight arrow) and is also present, to a lesser extent, over the endothelium of hepatic blood vessels (curved arrow). The immunofluorescent staining pattern obtained in the liver of day old chickens was typical of that found in the liver of 15 and 20 day old chicken embryos. Print x 595.

B) The localization of LPL in tissue sections of the liver from 6 day old broiler chickens.

LPL was localized in cryostat tissue sections from the liver of 6 day old broiler chickens using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. Low levels of immunoreaction were detected in association with the sinusoidal spaces (arrow). Print x 595.

A



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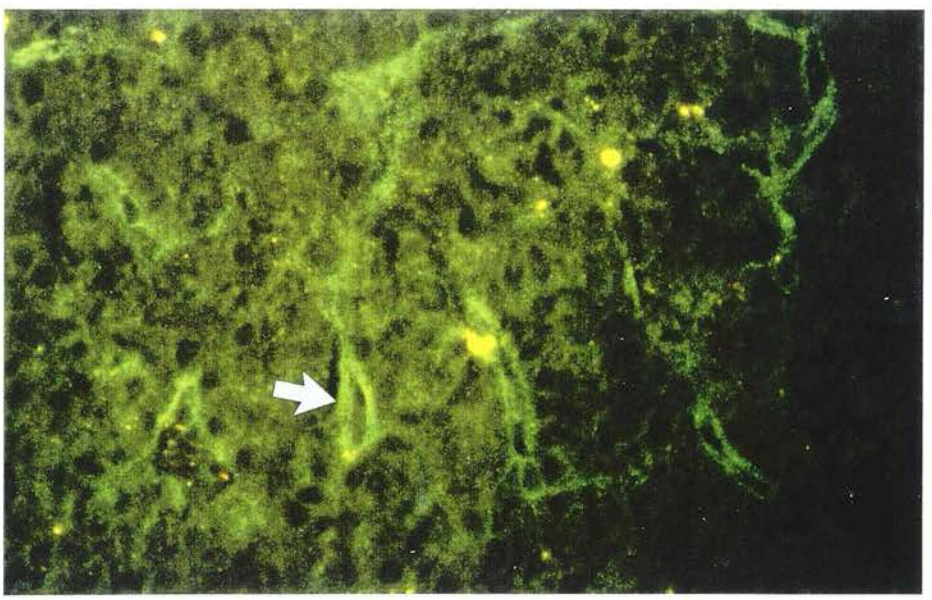


Figure 7.11

A) The localization of LPL in tissue sections of the heart from a 15 day old chicken embryo.

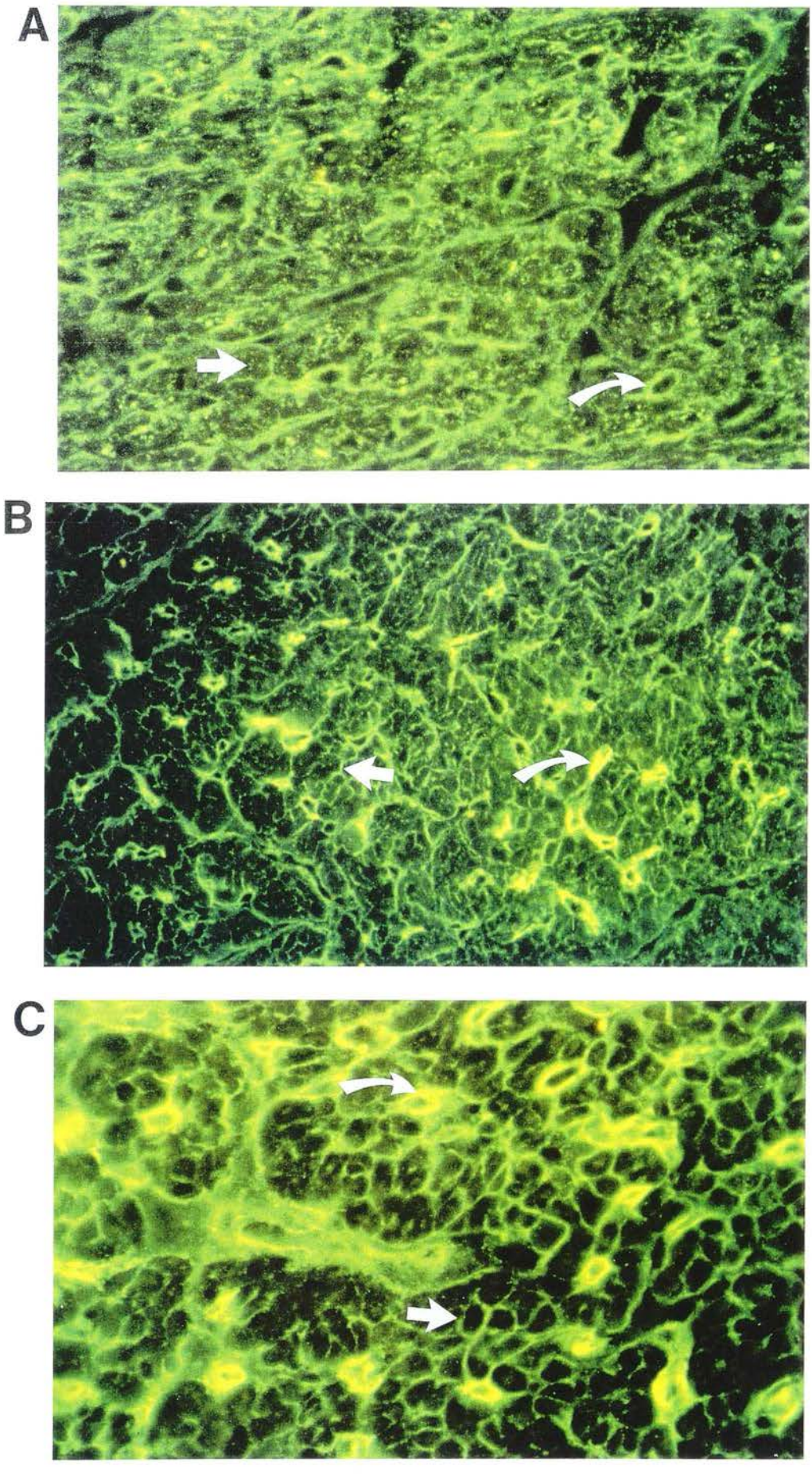
LPL was localized in cryostat sections of embryonic heart tissue using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. Immunofluorescence for LPL was associated with equal intensity with the cardiac myocytes (straight arrow) and interstitial capillary elements (curved arrow). Print x 425.

B) The localization of LPL in tissue sections of the heart from a 20 day old chicken embryo.

LPL was localized in cryostat tissue sections from the heart of day old broiler chickens using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. Immunofluorescence for LPL was concentrated at the myocyte basement membrane/ECM (straight arrow) and at the interstitial capillary elements (curved arrow). Print x 425.

C) The localization of LPL in tissue sections of the heart from day old broiler chickens.

LPL was localized in cryostat tissue sections from the heart of day old broiler chickens using sheep anti-chicken LPL IgG in the immunofluorescent labelling procedure described in section 6.2.4. Immunofluorescence for LPL was concentrated at the myocyte basement membrane/ECM (straight arrow) and at the interstitial capillary elements (curved arrow). Print x 595.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7.4 DISCUSSION

The general pattern of LPL distribution observed by immunofluorescence in the chicken heart (Fig 6.3, A) was found, by the results of work described in the present Chapter, to be typical of chicken muscle and adipose tissues. In both breast muscle and leg muscle, LPL was found mainly at the basement membrane of the myofibrils and in association with interstitial capillary elements (Fig 7.1, A - 7.2, A), a pattern similar to that found in the guinea pig diaphragm muscle by Camps *et al.* (1990).

In the chicken relatively high levels of LPL immunoreactivity were found in association with adipocytes in both adipose tissue (Fig 7.4, A) and in the bone marrow (Fig 7.6, A and B; Fig 7.7,.A) In these cases, immunoreactivity was observed at the periphery of adipocytes and associated with the interstitial capillary elements in the case of adipose tissue or vascular sinusoids in the case of bone marrow. Wood (1967) observed that each adipocyte in chicken bone marrow made at least one connection with the sinusoidal endothelium. This relates adipose tissue in the bone marrow to other fat depots where each adipocyte makes some contact with a capillary, thus providing a rich blood supply in relation to the volume of metabolically active cytoplasm (Wood, 1967). It is, however, unclear whether bone marrow adipocytes play an active role in energy metabolism, if they regulate lympho-heamatopoiesis, or if they simply occupy space (Tavassoli, 1984; Sorrell and Weiss, 1980). The localization of substantial levels of LPL in association with the adipocytes and blood sinusoids of the bone marrow suggests however, a potential role in the energy metabolism of the tissue. The bone marrow may, therefore, represent a sizable and as yet relatively uncharacterized pool of functional LPL in the chicken and possibly in mammalian species. The low level of LPL immunoreactivity over the haemopoietic cells of the chicken bone marrow, seen in Figs 7.6 and 7.7, correlates with the report by Burgaya *et al.* (1989) that the haemopoietic cells of the neonatal-rat liver did not contain LPL activity. However, Camps *et al.* (1991) localized LPL protein and mRNA to a minority of scattered macrophage and related cell types in the adult guinea pig spleen. The spleen, as an important haemopoietic organ, is functionally closely related to bone marrow. LPL has previously been localized on the

surface of human monocyte-derived macrophages using an immunogold technique (Goldberg *et al.*, 1988b). The presence of scattered macrophages synthesizing LPL in bone marrow would be difficult to detect because of the high levels of LPL apparently produced by the bone marrow adipocytes.

No immunoreactivity could be observed in the liver of 6 week old broiler chickens (Fig 7.8, A). This contrasts with the observation of Camps *et al.* (1991) who found strong staining at the sinusoids and in the periportal area in the livers of 2-3 month old guinea pigs. In both cases this can be related to the level of total tissue LPL activity in the liver of each species. In the liver of 6 week old broiler chickens, little or no LPL activity can be detected (Guo *et al.*, 1988), while in the liver of 2-3 month old guinea pigs the LPL activity per gram of tissue is approximately 10% of that found in the heart or in adipose tissue.

In the guinea pig, Camps *et al.* (1990, 1991) found strong immunoreactivity for LPL over the endothelium of the larger blood vessels of the extrahepatic tissues and over the liver sinusoids. This led to the suggestion that LPL moves away from LPL synthesizing tissues along the vascular endothelium from one binding site to the next carried by the blood., the LPL in the general circulation being kept low by degradation in the liver. Therefore, it was proposed that the distribution of functional LPL was governed not only by synthesis, but by the distribution of LPL binding sites within tissues. Clearly in 6 week old broiler chickens, there was no evidence from the present study for such a circulatory pool of LPL activity. No LPL immunoreactivity was found in the liver (Fig 7.8) or over the larger blood vessels of the extrahepatic tissues studied (Fig 6.6, A and B). It is possible that in the chicken the turnover of LPL at the capillary endothelium of the extrahepatic tissues is less rapid than in the guinea pig and/or that the liver is more efficient in the removal and degradation of LPL from the blood stream and this may represent a more general difference between avians and mammals. However, Moskowitz and Moskowitz (1965) and Hausman (1982) both failed to observe LPL activity over the endothelium of larger blood vessels in the mouse or pig respectively, using an enzyme histochemical technique, which may suggest that it is the guinea pig which is atypical in this respect.

In all of the extrahepatic tissues of the chicken studied, pools of heparin-insensitive LPL existed in association with the capillaries and the interstitial extracellular matrix. This pattern differs from that observed by Jonasson *et al.* (1983) who found that, in humans, an intravenous injection of heparin caused a drastic reduction in the LPL immunoreactivity over the blood vessels in adipose tissue. There is, however, considerable evidence that LPL does indeed interact with the components of the extracellular matrix and that a pool of LPL activity exists at the subendothelial basement membrane. Enerback *et al.* (1988) concluded that heparin and other glycosaminoglycans probably interact with a cluster of positively charged amino-acid residues on the enzyme and that the extracellular matrix contains a large number of binding sites for LPL. Chajek-Shaul *et al.* (1990) studied the interaction of LPL with the subendothelial extracellular matrix, using iodinated LPL and cultured endothelial cells. From their results, it was concluded that, *in vivo*, the subendothelial basement membrane, in common with the extracellular matrix, sequesters and stabilises LPL secreted into the subendothelial space by non-endothelial cells. The sub-endothelial basement membrane serves as a solid phase reservoir of LPL on its way from its site of synthesis towards its site of action. Saxena *et al.* (1991a), using a similar model system *in vitro*, proposed that a receptor-mediated transport system, which requires heparan sulphate proteoglycans and is inhibited by high concentrations of NEFA on the basal side of the endothelial cells, appears to exist for the transport of enzymically active LPL across the vascular endothelium. Therefore, regulation of LPL transport to the luminal surface of the endothelium from the basement membrane may help to modulate the size of the physiologically active pool of LPL *in vivo*. Similarly, Scow and Chernick (1987) reported that LPL can be stored in the basement membranes of lactating mammary glands followed by translocation to the surface of the ductal epithelium for subsequent release into the milk. The observation of a heparin insensitive pool of LPL in association with the capillary network in all the extrahepatic tissues of the chicken *in vivo* supports these studies.

After heparin injection, immunoreactivity *in vivo* was found over the sinusoids of the liver (Fig 7.8, A; Fig 7.9, A and B) in a pattern similar to that described by Camps *et al.* (1991) in the liver of untreated guinea pigs. This result confirms the specificity of immunostaining, as this

observation can be correlated with the appearance of LPL activity in the chicken liver after an intravenous injection of heparin. Guo *et al.* (1988) reported that before an intravenous injection of heparin, no LPL activity could be detected in the liver of 6 week old broiler chickens, however, within 2 min of such an injection, 25.6 units of LPL activity were measured per gram wet weight of liver tissue. The present study also confirms the presence of heparin-insensitive LPL-binding sites in the sinusoids and blood vessels of the chicken liver *in vivo* (Fig 7.9, B). Such binding sites were previously observed by Vilaro *et al.* (1988) who perfused bovine LPL through isolated rat livers and identified heparin-sensitive and heparin-insensitive LPL-binding sites in the rat liver, the latter being present only within the liver sinusoids. That heparin-insensitive binding sites for LPL were observed at the endothelium of all blood vessel types in the chicken liver may relate to experimental differences between the present study and that of Vilaro *et al.* (1988), rather than to a possible species difference. Vilaro perfused non-saturating levels of LPL from a different species through the isolated rat liver *in vitro*, whereas in the present study potentially saturating levels of chicken LPL were released into the circulation to be absorbed by the chicken liver *in vivo*.

The reported tissue specific developmental changes in LPL activity (Cryer, 1987) may well be a response to the metabolic transitions which occur during development. In rats, metabolic transitions occur at birth from a mainly carbohydrate and NEFA-derived calorie supply (available from the maternal circulation) to a primarily triacylglycerol-derived supply during suckling (Cryer, 1987) and at weaning, when milk is substituted by rat chow with a 70% carbohydrate and <5% lipid content. In birds, on hatching, the metabolic transition is from a predominantly triacylglycerol energy supply derived from the yolk sack to a basically carbohydrate diet, as broiler starter diets contain over 70% carbohydrate and <5% lipid.

Many studies have shown the presence of elevated levels of LPL activity in neonatal rat liver (Burgaya *et al.*, 1989, Peinado *et al.*, 1990). Gimenez-Llort *et al.*, (1991), using the isolated perfused rat liver as a model system, concluded that the LPL activity present in the neonatal rat liver was functional in that it had the capacity to hydrolyze circulating triacylglycerol. Recently, Speake *et al.* (1992) proposed a

major role for LPL in the tissue-specific utilization of yolk-derived lipid in the chicken embryo. In the present study, significant levels of LPL immunoreactivity were found in the embryonic and day old broiler chicken liver (Fig 7.10, A). The low level of immunoreactivity in the chicken liver 6 days after hatching (Fig 7.10, B) was similar to the observed fall in LPL activity of rat liver during postnatal development (Lopez-Tejero, 1988) although the reduction is considerably more rapid in the liver of chickens. This observation may be related to the initiation of hepatic lipogenesis in mammalian and avian species ie: the point at which the liver becomes a net exporter of triacylglycerol. In chickens the onset of hepatic lipogenesis is associated with hatching (Goodridge, 1968) while in rats it is associated with weaning (Henning, 1981). The distribution of LPL within embryonic and day old chicken liver agrees with that observed by Vilaro *et al.* (1988) in new born rats, with LPL being present within the sinusoids and to a lesser extent over the endothelium of larger blood vessels. Camps *et al.* (1990) postulated that immunoreactivity associated with the extracellular matrix may be a characteristic of tissues where LPL is being synthesized and delivered to the vascular endothelium. The presence of LPL only at the vascular endothelium (as seen in the embryonic and day old chicken liver, Fig 7.10, A) is therefore an indication of little LPL synthesis occurring within that tissue. The amount of LPL mRNA in the liver of day old broiler chickens has been compared to that present in the liver of 6 week old broiler chickens (where no LPL activity or immunoreactivity can be detected). Northern blot and dot blot analysis of total tissue mRNA using a riboprobe specific for chicken LPL mRNA has revealed the presence of only small amounts of LPL mRNA at both stages of development, with the levels of LPL mRNA being slightly higher in the liver of 6 week old broilers (personal communication S.C. Butterwith). From this, it can be concluded that only low levels of LPL protein are synthesized in the chicken liver at any stage of development. However, the relatively high levels LPL present in the liver of day old broiler chickens cannot be accounted for by synthesis in constituent liver cells. This is in agreement with work in rats which concluded that the low levels of LPL mRNA detected in the liver prenatally or on day one could not account for the substantial LPL activity detected in the rat liver at these times and the enzyme activity must originate from other tissues and be anchored on the hepatic vascular endothelium (Semenkovich *et al.*, 1989). A similar conclusion was reached by Camps *et al.*, (1991) who

found significant levels of LPL protein in the liver of the guinea pig but no detectable amounts of LPL mRNA. There is also considerable similarity in the distribution of LPL in the embryonic or day old chicken liver (Fig 7.10, A) and in the liver of 6 week old broilers injected intravenously with heparin (Fig 7.9, A and B). In the latter case, the enzyme is known to be derived by absorption from the blood stream of LPL released from the extrahepatic tissues by heparin. The absorption of LPL from the blood stream is therefore a possible source of the sinusoidal associated LPL in the embryonic and day old chicken liver. The elevated levels of LPL in the embryonic or day old chicken liver in relation to the liver of 6 week old broilers may be due to either a faster turnover of LPL at the vascular endothelium of the extrahepatic tissues causing increased levels of circulating LPL or by reduced degradation of LPL in the liver itself.

In the present study, an increase in the intensity of total tissue LPL immunoreactivity from the 15 day old embryo heart to the 20 day old embryo heart (Fig 7.11, A-C) was observed and this is similar to changes which occur in the measured levels of LPL activity. Speake *et al.* (1992) found the total LPL activity of chicken cardiac tissue to double between days 12 and 16 of embryonic development. A similar developmental pattern has also been recorded in the LPL activity and LPL mRNA content of rat cardiac tissue. Semenkovich *et al.* (1989) found that in the rat heart LPL mRNA was detected at low levels 6 days before birth and increased 278 fold as the animal grew to adulthood. Tavangar *et al.* (1992) also found LPL mRNA in foetal and neonatal rat hearts to be low and to increase 11 fold by 60 days remaining high thereafter. Corresponding developmental patterns are seen in the levels of cardiac tissue enzyme activity, with LPL very low in the foetus and rising to adult levels during suckling (Planche *et al.*, 1980; Cryer and Jones, 1978). In the chicken heart the increase in LPL appears to be associated with embryonic development and the onset of hatching, while in the rat these changes are associated with postnatal development and suckling.

The developmental changes in the pattern of LPL distribution that occur in the embryonic chicken heart are similar to those observed by Hausman (1982) in the pig foetus using an enzyme histochemical technique. Capillaries were stained for LPL activity only in 85 day old and older foetuses and in young pigs. Hausman concluded that in pig

foetal adipose tissue the cellular synthesis of LPL and capillary staining were not concurrent events as the adipocytes were capable of LPL synthesis before transport of the LPL to or across the capillary wall was possible. Similarly, in the 15 day old chicken embryo a low level of LPL was associated with the capillaries of the heart although considerable activity was associated with the cardiac myocytes (Fig 7.11, A). At this stage of development the capillaries of the embryonic chicken heart are associated with a basement membrane (Kitten *et al.*, 1987). It is therefore possible that, as described by Hausman in the pig, the cellular synthesis of LPL and capillary accumulation of the enzyme are not concurrent events in the development of the embryonic chicken heart. The nature of the developmental trigger for LPL transport to and accumulation at the interstitial capillary elements is presently uncharacterized, although it may perhaps require the expression of the LPL receptor, recently characterized by Saxena et al. (1991b), by the capillary endothelial cells.

Stein *et al.* (1991) and Chajek *et al.* (1977) have correlated the postnatal morphological changes of the rat heart with the rise in the level of heart LPL activity over a similar period. These workers found that, from 3 days before to 20 days after birth, there was a progressive increase in the number of capillaries and in the area covered by the intercellular spaces in sections of rat cardiac tissue. Similarly, the increase in LPL immunoreactivity of the chicken heart, from that observed in the 15 day old embryo (Fig 7.11, A-C), may also relate to such morphological changes which occur in the capillary bed and in the size of the extracellular spaces of chicken cardiac muscle as well as the initiation of transport of LPL from the cardiac myocytes to these compartments. In support of this suggestion, the myocardial capillary bed and extracellular spaces, which are responsible for almost all the LPL immunoreactivity of mature chicken cardiac tissue, appear to progressively increase in relative size and intensity of immunostaining from that observed in the 15 day old embryo to the day old chick (Fig 7.11, A-C).

CHAPTER 8

GENERAL DISCUSSION AND FUTURE WORK

As discussed in Chapter 2, the precise quantitative role played by LPL in the partitioning of lipoprotein triacylglycerol between tissues which may use the NEFA for oxidation and/or storage is unclear. Similarly, the exact quantitative relationship between the measured LPL activity of adipose tissue and the rate of fat deposition in birds has yet to be defined. Recent reviews by Bensadoun (1991) and Griffin *et al.* (1992) have both highlighted the requirement for the development of new methods and approaches to assess the functional LPL capacity of a given tissue and its contribution to the lipoprotein triacylglycerol NEFA uptake *in vivo*. Such measurements may lead to the full characterization of the role played by the enzyme in plasma triacylglycerol partitioning and fat deposition.

The initial objective of the project, described in the previous Chapters, was the development of a technique for the measurement of the amount of LPL protein at the luminal endothelial surface of the capillary bed of individual tissues *in vivo*. The immunological technique attempted required the production and use, *in vivo,* of an iodinated anti-chicken LPL monoclonal antibody and would in theory have allowed the reproducible and rapid measurement of the amount of endothelial surface LPL in many different tissues from a single individual. Chapters 2 and 3 described work necessary for the production of anti-chicken LPL monoclonal antibodies. Although useful monoclonal antibodies were produced, the monoclonal antibody (Cal-11), which had been prepared and extensively characterized by Gershenwald *et al.* (1985), was used to investigate the possibilities of the technique.

In both the perfused heart model system and *in vivo,* the specific binding (heparin-releasable) of the iodinated antibody to capillary LPL was demonstrated. However, the levels of specific binding were low and unable to give an accurate indication of the amount of LPL present at the luminal surface of the capillary endothelium of individual tissues.

Although attempts to measure functional LPL levels *in vivo* using the binding of labelled antibodies were judged unsuccessful, the release of

LPL activity by heparin perfusion of the isolated heart was successful and was used to assess the relative sizes of the functional pool of LPL bound by heparan-sulphate proteoglycans at the luminal surface of the vascular endothelium of cardiac tissue taken from chickens in a variety of nutritional states. Using this technique it was found that a low percentage (2-3%) of the LPL activity present in chicken cardiac tissue was releasable by heparin-perfusion in either the fully-fed or fasted states. Although the technique has been used by others to investigate the functional pool of LPL activity in, for example, rat cardiac tissue (Bagby, 1983; Borensztajn and Robinson, 1970), the work reported here represented the first such investigation of the putative endothelial pool of LPL activity in any avian tissue. Direct comparative studies using the technique showed that significant species differences occur between rats and chickens in the nutritional regulation of functional LPL activity. The present study also found the hydrolysis of [^{14}C] labelled lipoprotein triacylglycerol and the uptake of the released NEFA, to be low in the muscular tissues, by comparison with the adipose tissues, of fully-fed broiler chickens. The low percentage of total tissue LPL activity released by heparin in chicken cardiac tissue correlates with the low level of lipoprotein triacylglycerol NEFA uptake by the muscle tissues in comparison to their total tissue LPL activities. This indicates that in broiler chickens the relative levels of functional LPL activity may play a role in plasma lipoprotein triacylglycerol NEFA partitioning between muscle and adipose tissue.

Due to the low percentage of total tissue LPL activity released by heparin-perfusion of the isolated chicken heart, it became important to investigate the precise location of the LPL within chicken tissues. Using the anti-chicken LPL antibodies (the production of which is described in Chapter 3) coupled with immunofluorescent or silver enhanced immunogold detection systems, no observable difference in the pattern of LPL immunoreactivity was observed in chicken extrahepatic tissues before or after an intravenous injection of heparin *in vivo* or heparin perfusion *in vitro*. This confirms the results of the perfused heart studies which indicated that a low percentage of total tissue LPL activity is heparin-releasable in chicken cardiac tissue.

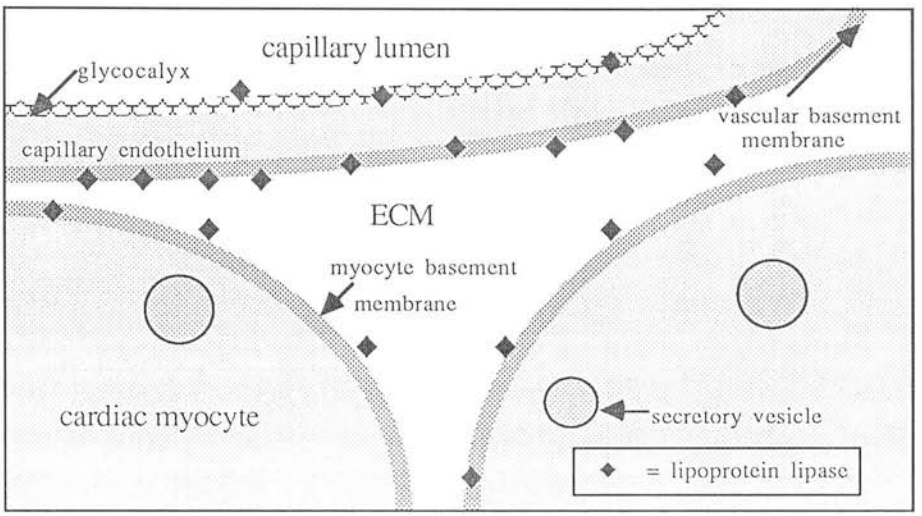
The immunocytochemical studies showed the enzyme to be predominantly located in an extracellular location, at the basement membrane of the tissue parenchymal cells or in association with interstitial capillary elements. Using image analysis, the interstitial capillary elements, which represented approximately 6% of the field of view of sections of the chicken myocardium, accounted for 46% of the area immunostained for LPL. This compartment was also found to give the strongest reaction to anti-LPL antibodies, indicating the specific accumulation of LPL at this site.

The distribution of LPL found in the present study of chicken tissues was similar to the distribution of basement membrane heparan sulphate proteoglycans, surrounding the tissue parenchymal cells and the interstitial capillary elements, observed in previous studies by Couchman and Ljubimov (1989) in the mouse; Fernandez *et al.* (1991) in the chicken; Schleicher *et al.* (1989) in man and Soroka and Farquhar (1991) in the rat. Antibodies against LPL were found to stain the endomysium of the chicken gastocnemius muscle strongly while showing only a weak reaction over the perimysium. Similarly, Fernandez *et al.* (1991) found that heparan sulphate proteoglycans are located predominantly in the endomysium of the chicken gastocnemius muscle.

From these studies and the accumulated evidence of others, several conclusions can be made on the secretion, transport and regulation of LPL within chicken tissues and Fig 8.1 represents a diagramatic summary of distribution of LPL within chicken cardiac tissue.

That LPL is probably transported rapidly out of the tissue parenchymal cells in which it is synthesized was shown by the low observable levels of LPL immunoreactivity within the cytoplasm of the parenchymal cells of chicken tissues in the present study and in guinea pig tissues (Camps *et al.*, 1990, 1991). Similarly, Liu and Olivecrona (1991) concluded from the results of pulse chase experiments using the isolated perfused guinea pig heart that LPL was transported rapidly out of the cardiac myocytes. The lack of a peri-nuclear pool of LPL does not support the proposal that LPL is a cryptic enzyme and stored in the cisternae of both adipocytes and muscle cells as proposed by Vannier *et al.*, (1989) and

Figure 8.1 Proposed distribution of LPL within chicken cardiac tissue.



From the results of immunocytochemical studies of LPL distribution in chicken cardiac tissue (section 6.3.3) and heparin perfusion studies of the isolated chicken heart (section 4.3.2), a model for the distribution of LPL within chicken cardiac tissue can be proposed.

Quantitatively important pools of LPL were found in association with the basement membrane and ECM surrounding the cardiac myocytes, which themselves contained low levels of the enzyme.

Another substantial pool of the enzyme was found in association with the interstitial capillary elements, probably concentrated at the sub-endothelial ECM or at the vascular basement membrane (Chajek-Shaul *et al.*, 1990).

Enzyme at these sites was not released by perfusion with heparin and is presumably non-functional.

Only a small percentage of the total cardiac tissue LPL activity was releasable by heparin perfusion of the isolated chicken heart. It has been proposed that this heparin-releasable fraction of enzyme activity represents the functional pool of enzyme, present at the luminal surface of the capillary endothelium (Fielding and Higgins, 1974; Rogers and Robinson, 1974).

Pradines-Figueres *et al.* (1990). Indeed, only when cultured adipocytes were treated with monensin, which blocks the transport of secretory proteins at the level of the cisternae of the Golgi, has the peri-nuclear immunolocalization of LPL been observed (Vannier *et al.*, 1985).

The proposed co-localization of LPL with heparan sulphate proteoglycans suggests that LPL secreted from the tissue parenchymal cells rapidly associates with these and other LPL binding components of the basement membranes and extracellular matrix. The accumulation of the enzyme at these structures suggests the extracellular storage of LPL in association with heparan sulphate proteoglycans may occur in chicken tissues. It has previously been proposed, from studies on bovine endothelial cells *in vitro*, that the subendothelial basement membrane serves as a solid-phase reservoir of LPL on its way from its site of synthesis towards its site of action (Chajek-Shaul *et al.*, 1990).

In chicken cardiac tissue the large accumulation of LPL present at the interstitial capillary elements contrasts with the low levels of LPL activity released by heparin perfusion of the isolated heart, the latter presumably representing enzyme located at the luminal surface of the capillary endothelium. The large pool of enzyme associated with the interstitial capillary elements may therefore be concentrated at the vascular basement membrane or at the sub-endothelial extracellular matrix (where it is inaccessible to heparin in the plasma or perfusate) in the presence of large amounts of heparan sulphate proteoglycans (Fig 8.1). It is possible that in chickens one of the controls of the level of functional LPL may be exercised by the rate of transport of the enzyme from the subendothelial pool to the luminal surface of the capillary endothelial cells. This transportation process may be affected by the localized NEFA concentration at the basal endothelial cell surface, potentially allowing the functional pool of LPL to respond rapidly to the physiological requirements of the tissue. Such a mechanism for the regulation of functional LPL levels has previously been proposed by Saxena *et al.*, (1991a) from the results of studies using cultured endothelial cells. In this *in vitro* system, these authors found that high concentrations of NEFA on the basal side of the cells inhibited the heparan sulphate proteoglycan dependant translocation of the enzyme to the apical cell surface. Any regulatory changes occurring in the rate of synthesis or secretion of LPL by chicken parenchymal cells would

simply add to the substantial pools of enzyme activity present in the extracellular matrix or in association with the interstitial capillary elements and not to the functional pool of enzyme present at the luminal surface of the capillary endothelial cells. In support of this, Doolittle *et al.* (1990) concluded that, in rat adipose tissue, the nutritional regulation of LPL activity *in vivo* occurred post-translationally with the redistribution of LPL within adipose tissue.

Although it appears that a transport based regulation mechanism may well be common to both chickens and mammalian species, the response to a period of fasting on the functional pool of LPL activity is different in rat and chicken cardiac tissue. Upon fasting, no significant difference in the percentage of total tissue LPL activity released by heparin perfusion of chicken hearts was obtained. In rats, fasting was found to cause a 5-fold increase in the percentage of total tissue LPL activity released by heparin. This increase in the heparin-releasable LPL activity was due almost completely to the redistribution of LPL within the rat heart, because fasting resulted in no detectable change in the heparin-residual LPL activity of the tissue and only a 22% increase in the level of the total cardiac tissue LPL activity. Jansen *et al.* (1980) effectively demonstrated the redistribution of LPL in the hearts of fasting rats by separately collecting the coronary and interstitial post-heparin perfusates. The LPL activity present in the coronary effluents from the hearts of fasted rats were higher than those of fed rats, while the opposite was true for the enzyme activity in the interstitial effluents. The redistribution of LPL activity within rat cardiac tissue during fasting is consistent with the operation of a transport based regulatory mechanism for functional LPL rather than the occurrence of regulatory changes in LPL synthesis and/or secretion by the cardiac myocytes.

The results of the work described in Chapter 7 suggest that LPL may play an important role (common to both avians and mammals) in the maturation and development of individual tissues. There have been few previous studies published on the role played by LPL in the growth and maturation of tissues during early development in birds. In the present study, considerable similarities were found in the tissue specific changes in LPL distribution in chickens during early development and those previously reported in rats and pigs (Vilaro *et al.*, 1988; Hausman, 1982). This is despite the differing nutrient transitions which occur

between the chicken on hatching and in mammals during birth, suckling and weaning. It has been suggested that LPL may play a role in the accumulation of lipid in the rat liver during gestation (Jamdar *et al.*, 1978) and the enzyme may therefore play a similar role in the reported accumulation of lipid in the embryonic chicken liver (Noble, 1986). The tissue specific changes in the level and distribution of LPL observed in the present study of chicken heart and liver may be due to changes in the expression or distribution of LPL binding heparan sulphate proteoglycans or other LPL binding molecules. Developmentally regulated tissue specific changes in heparan sulphate proteoglycan expression have been recently reported by David *et al.* (1992) and Fernandez *et al.* (1991). For example, in the present study, it was found that between day 15 and day 20 of chick heart embryonic development, LPL accumulates at the myofibril endomysium. This observation may be compared with the reports by Fenandez *et al.* (1991) that between day 13 and day 17 of embryonic development, the endomysium of chicken skeletal muscle becomes strongly positive for the presence of heparan sulphate proteoglycans. Accumulation of the enzyme at the interstitial capillary elements of the myocardium prior to hatching may also be due to the expression of LPL binding heparan sulphate proteoglycans at this site. Similarly, the loss of LPL from the liver sinusoids may also be due to changes in the pattern of LPL binding proteoglycans synthesized by the hepatocytes.

There have been no reported studies on the distribution or level of LPL activity in bone marrow, despite the presence of numerous adipocytes in the marrow mass and the result of immunocytochemical studies of the LPL distribution in chicken bone marrow was described in section 7.3.1. Considerable levels of LPL were found in association with the adipocytes of the chicken bone marrow and this strongly suggests that bone marrow adipocytes do play an active role in energy metabolism. Previous studies have found that mammalian bone marrow adipocytes failed to respond to starvation or insulin (Tavassoli *et al.*, 1974; Ailhaud, 1982). The present study indicates that the bone marrow may make a substantial contribution to whole body levels of functional LPL activity in the chicken.

Overall, the present study has characterized the pools of LPL activity located in various chicken tissues, suggesting a potential regulatory point for the control of LPL activity at the luminal surface of the capillary endothelium and found evidence of tissue specific developmental changes in LPL activity. The importance of LPL binding proteoglycans in the distribution, storage, transport and developmental regulation of the enzyme *in vivo* has also been highlighted. However, despite this work, the requirement for a technique which can measure the capacity of a tissue or whole organism to hydrolyze lipoprotein triacylglycerol *in vivo* still exists and this represents the most significant future challenge in the study of the role played by LPL in plasma lipoprotein kinetics.

Future work

Further attempts to measure LPL protein at the luminal endothelial surface *in vivo* using a labelled monoclonal antibody would require the production and characterization of a monoclonal antibody against chicken LPL with a high level of affinity which may, potentially, give rise to higher levels of specific binding. The affinity of such an antibody would have to be greater than the affinity of Cal-11, which has been reported to be 2.4×10^{10} M^{-1} (Gershenwald *et al.*, 1985). The required antibody would therefore possess an affinity close to the maximal value for monoclonal antibody affinity constants, which range between 10^{6}-10^{12} M^{-1} (Liddell and Cryer, 1991). Substantial amounts of such an iodinated high-affinity anti-LPL monoclonal antibody intravenously injected, *in vivo*, may give high levels of specific antibody binding in the short period of circulation required for the study of LPL. The technique may also need to incorporate an appropriate double label and be followed by vascular perfusion to allow the assessment of specifically bound antibody. An initial starting point for this work would be to purify the anti-chicken LPL antibodies secreted by the murine hybridomas (the production of which is detailed in Chapter 3) and determine their affinity constants by Scatchard analysis. However, it is likely that the production, purification and characterization of large numbers of anti-LPL monoclonal antibodies would be necessary before a suitable candidate antibody was found. The entire procedure would therefore be very labour intensive and time consuming and could not be contemplated within the context of the present study.

Future work using the immunocytochemical techniques developed in the present study may include comparative studies on the distribution of LPL in rat tissues using the immunolocalization procedure adopted in the present study of chicken tissues. Such experiments may potentially characterize the redistribution of LPL within rat cardiac tissue during periods of fasting. Direct comparison could also be made of the tissue specific maturational changes in the distribution of LPL which occur in rat tissues during the early stages of development with those observed in the present study in embryonic and post-hatch chicken tissues. However, the production of a high-titre specific anti-rat LPL antibody would be a requirement for this work. The role played heparan sulphate proteoglycans in the observed tissue-specific developmental changes in the distribution of LPL may also be studied using the relevant specific primary antibodies for co-localization immunofluorescent studies of embryonic tissues at various stages of maturation. The results of the present study and those of Fenandez *et al.* (1991) indicate that developmental changes of heparan sulphate proteoglycans distribution appear to immediately precede similar changes in the distribution of LPL.

To further investigate the level of functional LPL present at the blood sinusoids of the bone marrow, *in vivo* studies using [^{14}C]-VLDL triacylglycerol, as described in Chapter 4, could be carried out. Such experiments would allow the levels of functional LPL present in the bone marrow from different sites to be compared with those of adipose tissue depots and of muscle tissues. The turnover of triacylglycerol in the adipocytes of chicken bone marrow could also be assessed using this method as has previously been reported by Griffin *et al.* (1991b) for the turnover of triacylglycerol in chicken abdominal fat pads. The results of such experiments would characterize the contribution of the bone marrow adipocytes to energy metabolism and fat storage. This could lead then to studies on the relationship between the LPL activity of the marrow mass and the immune response of challenged birds.

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Appendix 1 Production of NEFA-depleted bovine serum albumin.

7 g of BSA (Boehringer Mannheim Uk Ltd, Lewes, East Sussex) was dissolved in 70 ml of distilled water to which 3.5 g of activated charcoal (Sigma Chemical Co.) was added. The pH of the mixture was then lowered to 3.0 by the addition of 2 M HCl and the mixture was stirred on a magnetic stirrer for 1 hour at 4°C. Following centrifugation at 20,000 *g* av for 20 min, to remove the charcoal, the clarified solution was adjusted to pH 7.0 by the addition of 1 M NaOH. The BSA was then freeze dried and stored at 4°C until required.

Appendix 2 Autoanalyser reagents.

The colour reagent was prepared by dissolving 1.5 g of 1,5-diphenylcarbazide (Sigma Chemical Co.) in a mixture consisting of 2.5 ml glacial acetic acid and 500 ml of isopropanol and was stored at 4°C until required. Copper reagent A, consisted of 0.9% (w/v) cupric sulphate pentahydrate. Copper reagent B, was prepared by dissolving 100 g of NaCl in a mixture of 40 ml glycerol; 40 ml piperdine; 5 ml glacial acetic acid and 600 ml of water. The working copper reagent was prepared, freshly before use, by mixing the copper reagents A and B (1:1 v/v).

Appendix 3 Preparation of heparin-Sepharose affinity matrix.

Heparin (porcine mucosal, Sigma Chem Co.) was covalently linked to Sepharose 4B-CL by the method of Iverius (1971). Cyanogen bromide activated Sepharose 4B-Cl (Sigma Chem Co.) was suspended in distilled water (30 g/150 ml) and swollen over a period of 24 hours at 4°C. The hydrated gel was then washed in a sintered glass funnel with 1 l of 0.1 M sodium hydrogen carbonate and 3 l of distilled water.
The gel was then suspended in a solution of heparin in 0.1 M sodium hydrogen carbonate (205 mg/100 ml) and mixed by end over end rotation for 16 hours at 4°C. Following this incubation period unbound heparin was then removed by filtration on a sintered funnel. Any residual unreacted sites on the Sepharose matrix were blocked by incubating the gel with 10 ml of ethanolamine and mixing for 4 hours at 4°C by end over end rotation. The gel was then washed in the sintered funnel with 1.5 l of distilled water, 1.5 l of 0.5 M sodium chloride and a further 3 l of water.

Appendix 4 Phosphate buffered saline.

PBS was prepared by dissolving 8.0 g NaCl; 0.2 g KH_2PO_4; 2.9 g $Na_2HPO_4.12H_2O$; 0.2 g KCl; in 1000 ml of distilled water. The solution was adjusted to pH 7.4 and stored at 4°C until required.