IGF Transfer from Blood to Tissue.

Comparison of IGF-I with Analogs that Bind Poorly to Binding Proteins, Using a Vascular Perfusion Model.

A thesis submitted to

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FOREWORD

The theme of the thesis, together with a summary of the results and main conclusions drawn from the data, are outlined in the *Abstract*. The *Literature Review* contains information pertinent to the research presented in the experimental chapters, in addition to general background information to place the studies within a broader field of understanding. The *Literature Review* is followed by a *General Methods* chapter in which are described experimental techniques that are used repeatedly throughout the thesis; methods peculiar to just one or two chapters are presented in the Materials and Methods sections of those chapters, as appropriate. The research component of this thesis is embodied in five *Experimental Chapters,* each with an Introduction, Materials and Methods, Results and Discussion section. General comparisons and wider implications of the results contained in each experimental chapter are pursued in the *General Discussion* chapter.

To place the thesis in perspective with the state of knowledge as it was at the commencement of my research program, January 1989, citation of references in the *Literature Review* are mainly to publications that preceded this date. Where necessary, some recent citations are included to give adequate depth and clarity. More recent citations are generally made in the Introduction and Discussion sections of each experimental chapter and in the general discussion section, as appropriate.

In the interests of presentation and to facilitate the flow of text, addresses of contributors and of reagent and equipment manufacturers have not been included in the main body of the Thesis; the reader is referred to the *Appendices* for these details.

I wish the reader a good read!

Andrew P.D. Lord

QUOTATION

The poets eye, in fine frenzy rolling

Doth glance from heaven to earth, from earth to heaven;

And as imagination bodies forth,

The forms of things unknown, the poets pen

Turns them to shapes, and gives to airy nothing

A local habitation and name.

Shakespeare: A Midsummer Nights Dream

ABSTRACT

Insulin-like growth factor-I (IGF-I) circulates at high concentrations in blood, mainly complexed with IGF-binding proteins (IGFBPs). The main objective of this thesis was to determine the general role played by plasma IGFBPs in the regulation of IGF transfer from blood to tissues.

In a preliminary investigation, I characterised and compared the IGF-binding characteristics of plasma with those of lymph and CSF, fluids that are derived from the extravascular spaces and are representative of the extravascular milieu, to determine whether IGFs transferred to tissue would be subject to regulation by IGFBPs outside the vasculature. Lymph samples from the intestine, shoulder region and hind leg of lambs were compared with CSF and plasma in ligand blotting studies and also by size-exclusion chromatography following *in vitro* incubation with labelled IGFs. These comparisons employed radiolabelled IGF-II, IGF-I and the truncated analog, des(1-3)IGF-I. The ligand blotting and chromatographic characteristics of intestinal lymph were more similar to plasma than were those of either CSF or lymphs from the leg or shoulder regions. Thus CSF contained IGFBPs only of low molecular mass (≤35 kDa), while leg and shoulder lymphs contained proportionally more IGF-binding activity of low molecular mass than did plasma or intestinal lymph. These data reflected the results of the chromatographic analyses and suggest a greater similarity between the IGF-regulatory environment of the vasculature and the extravascular space of the intestine than between the vasculature and extravascular spaces in other tissues. This experiment also showed that the binding of des(1-3)IGF-I to IGFBPs was much weaker than that of either IGF-I or IGF-II.

In order to determine whether IGFBPs could regulate IGF transfer from plasma to tissue, a model was developed, in the sheep, to compare the transfer of IGF-I with that of analogs that had been shown to bind IGFBPs poorly. The analogs chosen for comparison with IGF-I were des(1-3)IGF-I and LR^3IGF-I, an IGF-fusion protein which has been shown to bind rat IGFBPs more poorly than des(1-3)IGF-I or IGF-I and, while binding receptors poorly, also demonstrates greater biological potency than either IGF-I or des(1-3)IGF-I. Radiolabelled

IGF-I, des(1-3)IGF-I or LR^{3}IGF-I was perfused into the arterial blood supply of a small intestinal segment at less than 1 ng/ml of plasma, for 45 min, with collection of all efferent blood and lymph and, afterwards, the intestinal contents. The perfused segment, mesentery, lymph node and adjacent intestine were excised and the radioactivity contained in all fluid and tissue samples was measured. IGF transfer to intestinal tissue was calculated as the proportion of total recovered radioactivity accounted for in the intestinal segment, lymph node, lymph and luminal contents, minus vascularly trapped radioactivity. TCA precipitability of radioligand was determined as an estimate of its intactness while the distribution of radioligand between the free state and the IGFBP regions in plasma was determined by size-exclusion chromatography. The results showed that 3-4% of the radioactivity was transferred and only a small reduction in intactness could be demonstrated. No statistically significant differences in transfer of the IGF peptides were found, although a trend towards greater transfer of des(1-3)IGF-I than of IGF-I or LR^{3}IGF-I was evident. The lack of differences in transfer of the IGF peptides was attributed to the unexpectedly small differences between the amounts of IGF-I, des(1-3)IGF-I and LR^{3}IGF-I that remained uncomplexed to IGFBPs in plasma.

A series of *in vitro* incubations was undertaken, using a constant amount of radiolabelled IGF peptide and varying amounts of unlabelled homolog, to select a ratio which resulted in increased proportions of the labelled analogs, but not labelled IGF-I, remaining unbound in plasma. Vascular perfusions were then performed using radioligand mixed with a 100-fold excess of homologous unlabelled IGF (Low Specific Activity perfusion). As had been found when radioligand was infused without unlabelled homolog (High Specific Activity perfusion), 3-4% of the radioactivity was transferred to intestine but no differences were apparent between peptides. There was, however, a trend toward greater transfer of des(1-3)IGF-I than of either IGF-I or LR^{3}IGF-I. Unexpectedly, the proportion of des(1-3)IGF-I that remained free of plasma IGFBPs was only slightly greater when perfused at Low than at High Specific Activity, although the proportion of free LR^{3}IGF-I was approximately double that attained in the High Specific Activity perfusions. These results do not support a hypothesis that IGFBPs inhibit transfer of IGF from the vasculature to tissue.

The remainder of the experiments presented in this Thesis explore the reasons for some of the unexpected findings obtained in the previous chapters. Thus the goals were to determine whether major species differences in the binding of analogs could account for the much greater binding of analogs in lamb plasma than has been reported previously for rat plasma, and to clarify the reasons for the greater binding of IGF analogs to IGFBPs in the vascular perfusion model than was found by *in vitro* incubation with lamb plasma. In comparisons between sheep and rats that were extended to the pig, chicken and man, binding of the three ligands and especially the analogs, differed for each species tested. Binding of analogs was poorest in rat plasma, intermediate in plasma from pigs, chickens and man, and greatest in sheep plasma. In rat plasma, labelled LR^3IGF-I was unable to displace endogenous IGFs from IGFBPs and binding of labelled des(1-3)IGF-I was negligible; in sheep plasma, both ligands, particularly LR^3IGF-I, were bound substantially. These differences occurred despite the demonstrated capacity of IGFBP-3, the most abundant IGFBP, from all mammalian species, to bind the analogs.

Another factor that contributed to the smaller than expected differences in binding of IGF-I and analogs in the vascular perfusions was an anaesthesia-induced rise in concentration of immunoreactive IGFBP-1, apparently as a result of depressed plasma glucose and insulin concentrations, and declines in circulating IGF-I and IGF-II. The net result of these changes was an expansion in the number of available IGF-binding sites, that could account for the increased binding capacity of plasma for des(1-3)IGF-I in the vascular perfusion study. These changes in IGF and IGFBP concentrations are previously undescribed effects of halothane anaesthesia.

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STATEMENT OF ORIGINALITY

The work presented in this thesis contains no material which has been accepted for the award of any other qualification in any university or other tertiary institution. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference is made in the text. I consent to a copy of this thesis, when deposited with The University of Adelaide Library, being available for photocopy and loan.

Dated at Adelaide ..November..4th..., 1993..

Signed

Andrew Peter Duncan Lord (B.Sc. Hons I)

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PUBLICATIONS ARISING FROM THESIS RESEARCH

Journal Entries

Lord APD, Martin AA, Walton PE, Ballard FJ & Read LC (1991) Insulin-like Growth Factor Binding Proteins in Tissue Fluids from the Lamb. *Journal of Endocrinology* **129:** 59-68.

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ABBREVIATIONS

BSA	bovine serum albumin
CSF	cerebrospinal fluid
freon	1,1,2,-trichloro-1,2,2,-trifluoroethane
GH	growth hormone
HFBA	heptafluoro butyric acid
HPLC	high pressure liquid chromatography
ID	inner diameter
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor-binding protein
mRNA	messenger ribonucleic acid
N-terminus	amino-terminus
OD	outer diameter
PVDF	polyvinylidine-difluoride
RIA	radioimmunoassay
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TFA	trifluoracetic acid

UNITS OF MEASUREMENT

cpm	counts per minute
g	gravity
IU	International Units
kDa	kiloDalton
M	moles per litre
min	minutes
MPa	mega pascal
P	partial pressure
sec	second
SEM	standard error of the mean
v	volume
w	weight

Chapter One

Literature Review

CHAPTER ONE: LITERATURE REVIEW

GENERAL INTRODUCTION

Growth is a dynamic process in which macromolecules are assimilated into a biological system, resulting in an increase to the size of that system. In the growing animal this may involve the accretion of tissue mass. Growth hormone (GH) has long been established as a potent stimulant of growth (Smith *et al;* 1955), but more recently it has become apparent that another group of macromolecules share a similar activity; the insulin-like growth factors (IGFs). Two species, IGF-I and IGF-II, have now been identified in plasma (Rinderknecht & Humbel; 1978*a* and 1978*b*). IGF-I appears to be under direct control of GH (Daughaday; 1981) and may therefore serve as a mediator of GH-stimulated growth. Certainly, receptors for the IGFs have been identified, in various degrees of abundance, in all organs and major tissues studied to date.

The IGFs circulate complexed with up to six IGF-binding proteins (IGFBPs), three of which had been identified at the time the present study was commenced. Implicit in their abilities to associate with the IGFs is the possibility that the IGFBPs may modify IGF bioactivity. Indeed, evidence from *in vitro* investigations has revealed the existence of both IGF-potentiating and inhibitory activities, suggesting that IGFBPs may have roles in the regulation of IGF bioactivity in the living organism. The main objective of the current study was to determine whether the transfer of IGF from the circulation to tissue is regulated by plasma binding proteins.

Included in this review of literature is some general background on the structure, characterisation, regulation and receptor binding properties of the IGFs (Sections **1.1-1.3**), in addition to description of their biological activities (Section **1.4**). IGFBPs are discussed as potential regulators of IGF bioactivity (Section **1.5**) after which the IGF analogs are introduced (Section **1.6**). Two experimental models that are potentially applicable to studying IGF transfer, are also compared (Section **1.7**).

1.1 STRUCTURE AND CHARACTERISATION OF IGFS

The insulin-like growth factors, IGF-I and IGF-II, are single chain polypeptides of 70 and 67 amino acids, respectively (Rinderknecht & Humbel; 1978*a* and 1978*b*). They belong to a family of structurally related peptides that includes insulin, proinsulin and the cervical dilatory hormone, relaxin (Blundell *et al;* 1978). Of these proteins, only insulin, proinsulin, IGF-I and IGF-II share any functional similarity and relaxin will therefore be excluded from further discussion. IGF-I, IGF-II and proinsulin are each composed of two regions, designated the 'A' and 'B' domains, that connect by a third ('C') domain; this C domain is cleaved from proinsulin during processing to insulin. In addition to the A, B and C domains, IGF-I and IGF-II each have a short carboxy-terminal extension termed the D-domain, that is not present in insulin. The A chain of human insulin bears 48% and 40% homologies with IGF-I and IGF-II, respectively, while its B chain is 52% and 57% homologous (Rinderknecht & Humbel; 1978*b*). Human IGF-I and IGF-II are themselves 62% homologous, with 75% and 77% of amino acids in the A and B chains, respectively, identical, but the homology between the C chain amino acids is only 25%. Based upon the structural similarities between the A and B chains of insulin and the IGFs, and the known tertiary structure of insulin, similar tertiary structures for IGF-I and IGF-II have been predicted (Blundell *et al;* 1972). Because the IGFs and insulin vary most at the C and D domains, these regions may confer receptor binding specificity. De Vroede *et al* (1985) have demonstrated the involvement of the B chain in binding of IGF-I to IGFBPs, since insulin analogs that contain this portion of IGF-I, but not native insulin, are able to bind IGFBPs.

A number of polypeptide growth factors have been described in the literature which, by amino acid sequence analysis, are now known to be the same as the IGFs. Thus somatomedin-C is identical to IGF-I (Rinderknecht & Humbel; 1978*b*, Klapper *et al;* 1983) while somatomedin-A differs from IGF-I by the deamination of a single amino acid (Enberg *et al;* 1984), perhaps a structural modification arising during purification. IGF-II also appears to have been isolated under alternative names. Indeed, human skeletal growth factor has identical biological activity to IGF-II and 100% amino acid sequence homology has been

demonstrated in the portion of human skeletal growth factor sequenced (Mohan *et al;* 1988). IGF-II has also been referred to as multiplication stimulating activity. The primary sequence of multiplication stimulating activity purified from the rat BRL-3A cell line has a 93% homology with human IGF-II, implying its identity as the rat homolog of human IGF-II (Marquardt *et al;* 1981).

1.2 REGULATION OF IGF-I SYNTHESIS

Biological activity that is now ascribed to the insulin-like growth factors, was first demonstrated by Salmon and Daughaday (1957) as a GH-dependent factor in rat serum that stimulated sulphate incorporation by cartilage *in vitro*. It appears that GH-control of plasma IGF-I is effected largely through hepatic IGF-I synthesis. Indeed, using a liver perfusion preparation, Phillips *et al* (1976) showed that hepatic IGF-I synthesis was reduced by hypophysectomy but could be restored by supplementation of the donor animal with GH. The responsiveness of hepatic IGF-I synthesis to GH has since been confirmed (Schwander *et al;* 1983) and reflects the findings obtained with cultured hepatocytes (Binoux *et al;* 1982). Nevertheless, GH-dependent IGF-I messenger ribonucleic acid (mRNA) synthesis has been demonstrated in other tissues, including kidney, lung, heart, testes, stomach and brain, although at much lower levels than in liver (Lowe *et al;* 1987). Accordingly, the circulating IGF-I pool probably represents accumulated synthesis by a range of tissues.

The control of IGF-I synthesis by GH may be contingent upon additional factors, for while the evidence for GH stimulation of plasma IGF is strong, conditions exist in which such responsiveness is attenuated. Plasma IGF-I concentrations are depressed during negative energy balance (Hintz *et al;* 1978, Phillips *et al;* 1988*a*, Breier *et al;* 1986) and appear to be resistant to GH under such conditions (Fekete *et al;* 1983). An additional factor that may influence the final production of IGF-I after GH stimulation relates to the IGF-I mRNA species induced. Thus Lowe *et al* (1987) have demonstrated the existence of multiple 5'-mRNA transcripts for IGF-I, which are each regulated by GH in a tissue-specific manner. The half lives of these transcripts and the efficiencies with which they are translated may also

vary between tissues. It is evident, therefore, that the GH-inducibility of IGF-I synthesis may be subject to a range of controls.

Multiple mRNA transcripts have also been described for IGF-II, with four expressed in rat brain, liver, lung and intestine (Lund *et al;* 1986). Thus while it is plausible that tissue-specific differences in IGF-II gene expression, mRNA processing and translation may play an integral role in the regulation of IGF-II expression, the factors that control plasma IGF-II concentration are not known. Nevertheless, plasma IGF-II and its mRNA are expressed at high levels in the fetus compared with the adult, suggesting a role in fetal growth (Lund *et al;* 1986).

1.3 RECEPTORS FOR IGFS

IGFs are capable of interactions with three distinct receptor species, namely the type-I, type-II and insulin receptors. The type-I and insulin receptors are structurally related, each composed of two α-subunits and two ß-subunits (Massague *et al;* 1981, Massague & Czech; 1982) structured as $\alpha_2\beta_2$ heterotetrameric complexes of molecular mass about 300 kDa (Czech; 1985, Rechler & Nissley; 1985). The α-subunits are disulphide-linked, reside outside the cell membrane and are responsible for ligand binding. Each subunit is linked via disulphide bonds to the extracellular domain of a ß-subunit which extends through the plasma membrane to an internal domain characterised by tyrosine kinase activity (Feltz *et al;* 1988). Ligand binding results in phosphorylation of tyrosine residues on the ß subunit (Jacobs *et al;* 1983), plausibly constituting the first step in a signal transduction mechanism. This could result either from the modified properties of the phosphorylated receptor or through phosphorylation of intracellular messengers, a possibility that is supported by the demonstration that a soluble cytosolic protein is phosphorylated as a result of binding at the insulin receptor (Madoff *et al;* 1988).

The type-II receptor is structured quite differently to the type-I and insulin receptors. It is a single protein of molecular mass approximately 260 kDa (Massague & Czech; 1982)

and is able to bind both IGF-II and the structurally unrelated ligand mannose 6-phosphate at separate binding sites (Waheed *et al;* 1988, Braulke *et al;* 1988). Indeed, amino acid sequence analysis of the human type-II receptor reveals striking homology with the bovine mannose 6-phosphate receptor (Morgan *et al;* 1987). The type-II IGF receptor and mannose 6-phosphate receptor are now known to be the same protein. Polychronakos & Piscina (1988*a*) have shown that the simultaneous binding of both ligands results in enhanced endocytosis of IGF-II, but this apparent cooperativity probably results from the competitive removal of endogenous mannose-containing macromolecules that may otherwise cause a steric inhibition of IGF-II binding (Polychronakos *et al;* 1988*b*).

The type-I and insulin receptors are each able to bind IGF-I, IGF-II and insulin (Kasuga *et al;* 1981, Jacobs *et al;* 1983, Zick *et al;* 1984). The type-I receptor binds IGF-I and IGF-II with approximately similar affinities but its affinity for insulin is much lower. Since insulin circulates at about 0.1% of total circulating IGF-I and IGF-II concentrations in man (Baxter; 1986), it is unlikely to exert substantial direct effects at the type-I receptor. Nevertheless, one study has demonstrated that the type-I receptor may be phosphorylated as a result of insulin receptor occupancy (Beguinot *et al;* 1988), so that poor binding of insulin to the type-I receptor may not necessarily preclude significant activation by insulin. The insulin receptor binds both IGF-I and IGF-II, in addition to insulin (Czech; 1985), but whether the IGFs actually exert significant biological effects through this receptor, is uncertain. The type-II receptor predominantly binds IGF-II and bears very little affinity, if any, for insulin or IGF-I (Ballard *et al;* 1988). Earlier reports suggested that the type-II receptor was able to bind IGF-I (Ballard *et al;* 1986) but those data were obtained using IGF peptides purified from physiological sources, rather than the recombinant IGF peptides used by Ballard *et al* (1988), and it is likely that these observations arose from the co-purification of IGF-II with IGF-I.

IGF receptors have been detected in most tissues and cell types, including gut (Laburthe *et al;* 1988), smooth and rough muscle (Pfeifle *et al;* 1982, Beguinot *et al;* 1985, Zorzano *et al;* 1988), arteries and tissue capillaries (Bornfeldt *et al;* 1988, Bar *et al;* 1985, Bar *et al;* 1988, Duffy *et al;* 1988), and brain (Bohannon *et al;* 1988). Type-I receptors are

normally, however, undetectable in liver, although expression may occur during liver regeneration (Caro *et al;* 1988). The existence of receptors for IGFs in a wide range of tissues suggests that these tissues may be targets for circulating IGFs.

The type-II receptor is also found free in the circulation. This soluble form of the receptor was first described in plasma as a high molecular mass glycosylated IGF-binding species that was specific for IGF-II (Butler & Gluckman; 1986, Hey *et al;* 1987). High specificity for IGF-II is evident since binding at approximately 250 kDa can be demonstrated by incubation of plasma with radiolabelled IGF-II but not with labelled IGF-I, whereas unlabelled IGF-I competes with labelled IGF-II for binding with an affinity less than 0.5% that of unlabelled IGF-II (Gelato *et al;* 1989). Gelato *et al* (1989) also demonstrated that this protein was recognised by antibodies against the type-II/mannose 6-phosphate receptor, while other research has revealed it to be a carboxy-terminal truncated version of the membrane-bound receptor that lacks the transmembrane and intracellular domains (MacDonald *et al;* 1989). While abundant in the circulation, and more so in the fetus than in maternal serum or the circulation of the non-pregnant animal, this protein may also be found in urine (Causin *et al;* 1988, Gelato *et al;* 1989).

1.4 BIOLOGICAL ACTIVITIES OF THE IGFS

Two of the most widely recognised activities of the IGFs include enhancement of cellular protein and DNA synthesis. The data are derived largely from *in vitro* cell culture studies, in which cells such as fibroblasts (Corps & Brown; 1988, Rinderknecht & Humbel; 1976*a*), fetal calvariae (Hock *et al;* 1988), arterial capillary cells (Pfeifle *et al;* 1982) and granulosa cells (Baranao & Hammond; 1984), amongst others, have been shown to respond to IGFs through increased synthesis of DNA and/or total protein. IGFs are also effective inhibitors of protein breakdown (Ballard *et al;* 1986), so that the net protein accretion that results from treatment with IGFs is the combined result of increased protein synthesis and decreased protein breakdown.

At the commencement of research for this Thesis, demonstrations of *in vivo* IGF effects were few by comparison with the examples of *in vitro* effects, perhaps due to difficulties in obtaining the large amounts of IGF required for such studies. Nevertheless, in the limited number of studies published at this time, IGF-I had been shown to cause increased weight gain in normal and hypophysectomised rats (Schoenle *et al;* 1982, 1985, Phillips *et al;* 1988*b*). In addition, distinct effects of IGF-I on the weights of organs, including brain, liver, heart, testes, kidney and spleen had been demonstrated (Guler *et al;* 1988, Phillips *et al;* 1988*b*). These data suggest a general role for IGF-I in physiological growth.

The IGFs are also known to have a range of acute effects. Perhaps the first description of such effects came with the discovery of IGF as a factor in serum that caused an insulin-like stimulation of glucose uptake but which was not suppressible using antibodies against insulin (Froesch *et al;* 1963). Such effects have also been demonstrated *in vivo*, such that transient hypoglycaemia can be measured shortly after intravenous IGF infusion (Zapf *et al;* 1986). Direct demonstrations of such activity have been made in adipose tissue *in vitro* (Schwartz *et al;* 1985), while other insulinomimetic properties of the IGFs include the stimulation of glycogen synthesis and, at high concentrations, stimulation of lipogenesis (Bhaumick & Bala; 1988, Lewis *et al;* 1988).

1.5 INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPS)

The existence of circulating proteins able to bind specifically to IGFs (IGFBPs) is now well established. Incubation of radiolabelled IGF-I in plasma, followed by analytical gel-filtration chromatography and measurement of radioactivity in the eluate, reveals IGF-binding activity at two molecular mass regions (Kaufmann *et al;* 1977, Hintz & Liu; 1977). These binding regions have molecular masses of approximately 150 kDa and 50 kDa, compared with 8 kDa for free IGF.

In contrast to their larger apparent molecular masses by neutral gel chromatography, the molecular masses of the individual IGFBPs in electrophoretically separated plasma range between 24 and 42 kDa (Hardouin *et al;* 1987, Binoux & Hossenlopp; 1988). Electrophoresis of fractions collected during the size-exclusion chromatography of plasma has been used to determine the distribution of the IGFBP species between the binding regions. In human plasma for example, the predominant 38.5 and 41.5 kDa bands elute mainly in the 150 kDa high molecular mass region, while smaller bands of 34, 30 and 20 kDa elute within in the 50 kDa region (Hardouin *et al;* 1987).

1.5.1 IGFBP Structure and Nomenclature

As described above, the 50 kDa IGF-binding region contains mainly the 20-34 kDa IGFBPs, in addition to some 38.5-41.5 kDa IGFBPs. The discrepancy in size estimation by the two techniques is largely accounted for by an increase in molecular mass of IGFBPs, in the chromatographic studies, due to the binding of radioligand (electrophoretic separation is performed under dissociating conditions and therefore after the removal of any endogenous IGF ligand). The IGF-binding components of the 50 kDa binding region include IGFBP-2 and IGFBP-1, proteins with molecular masses of approximately 35 and 28 kDa, respectively, as estimated by electrophoresis (Baxter *et al;* 1987). In addition, IGFBP -4, -5, and -6 had yet to be identified when research was commenced towards this Thesis, but may also contribute binding activity to this peak since their molecular masses fall between 24 and 35 kDa (La Tour *et al;* 1990, Shimasaki *et al;* 1990, Shimasaki *et al;* 1991*a*, Shimasaki *et al;* 1991*b*).

The much greater size discrepancy between the 150 kDa complex and its 38.5-41.5 kDa IGF-binding subunits implies a more complex structure than a simple binary association of ligand and IGFBP. Wilkins & D'Ercole (1985) postulated that the 150 kDa complex was a hexamer of identical 24-28 kDa IGFBP subunits associated with a single IGF molecule, but the experimental data obtained to date do not support such a conformation. An alternative hypothesis was that the 150 kDa complex was comprised of an IGF molecule

bound to a single IGFBP subunit plus an additional, as yet uncharacterised subunit (Furlanetto & Van Wyk; 1978, Hintz & Liu; 1980). The putative third subunit was supposedly acid-labile in character, since radiolabelled IGF bound at 150 kDa in native plasma but associated only in the 50 kDa region following plasma-acidification and re-neutralisation. Furthermore, the elution position of the IGF-binding subunit shifted from 150 kDa to about 50 kDa (Moses *et al;* 1979, Furlanetto; 1980). The third subunit, a protein of 85 kDa (α-subunit), has subsequently been purified and is characteristically acid-labile (Baxter; 1988*a*). Interestingly, the α-subunit has no innate IGF-binding activity, nor does it bind the IGF-binding subunit in the absence of ligand. The 150 kDa complex may only form when the α-subunit binds to the binary complex between the high molecular mass binding protein (ß-subunit) and IGF (γ-subunit) (Baxter & Martin; 1989). The ternary model for the 150 kDa complex has since gained widespread acceptance.

The IGF-binding subunit of the 150 kDa complex has been designated IGFBP-3. However, a number of IGFBPs previously identified as 'BP 53', 'BP 29', 'growth hormone-dependent binding protein' and 'acid-stable subunit of the high molecular mass complex' are now also designated as IGFBP-3 under an accepted system of nomenclature (Ballard *et al;* 1989*b*). IGFBP-3 exists in multiple glycosylation states (Baxter *et al;* 1986) such that by electrophoresis it usually appears as a doublet comprising the IGFBPs in the 38-50 kDa range (Hardouin *et al;* 1987, Zapf *et al;* 1988*b*). *In vitro* deglycosylation of these IGFBPs followed by electrophoresis reveals a single band with only one N-terminal amino acid sequence (Zapf *et al;* 1988*b*).

Under the same system of nomenclature as that by which IGFBP-3 is classified, proteins such as 'placental protein 12' (Bohn & Kraus; 1980) and 'amniotic fluid binding protein' (Koistinen *et al;* 1986) are now identified as IGFBP-1 (Ballard *et al;* 1989*b*). Identity with a binding protein purified from Hep G2 culture medium has been demonstrated by amino acid sequence analysis (Povoa *et al;* 1985, Lee *et al;* 1988), and binding proteins referred to as 'BP-28' (Baxter & Cowell; 1987) and 'growth hormone-independent binding protein' (Yeoh & Baxter; 1988) are also IGFBP-1.

IGFBP-2 has also been known under other names. These include 'MDBK cell line-derived BP' (Szabo *et al;* 1988) and 'BRL 3A cell line-derived IGF BP' (Mottola *et al;* 1986).

1.5.2 IGFBP Regulation

Growth Hormone and IGF-I:

Growth hormone (GH) is an important factor in the regulation of circulating IGFBP levels. Perhaps the first indication of a relationship between IGF binding protein abundance and GH levels was the combined results of demonstrations that: 1) the circulating half life of IGF after acid dissociation from its binding proteins was 10-30 min compared with about 3 h for untreated plasma, and 2) the circulating half life of somatomedin activity in plasma from untreated hypophysectomised rats, was much lower than that of plasma from normal rats but could be restored by chronic treatment of hypophysectomised blood-donor rats with GH (Cohen & Nissley; 1976). Moses *et al* (1976) later directly confirmed that hypophysectomy caused a significant reduction in plasma IGF-binding activity in the rat and that this activity could be restored by administration of GH. In man, administration of GH has been shown to result in increased IGF-binding activity at about 150 kDa (White *et al;* 1981), while Baxter & Martin (1986) confirmed that GH-inducible IGF-binding activity is attributable to immunoreactive IGFBP-3. In acromegalics, whose plasma GH concentrations are typically well above those of normals, IGFBP-3 concentrations are also elevated. However, plasma IGFBP-3 is lower than normal in hypopituitary patients (Baxter & Martin; 1986, Hardouin *et al;* 1989). These data strongly support a role for GH in IGFBP-3 induction.

It is also possible that IGFBP-3 may be regulated indirectly by GH through IGF-I, since GH regulates IGF-I (Section **1.2**). Indeed, Zapf *et al* (1988*a*) have shown that IGFBP-3 can be induced by subcutaneous infusion of IGF-I in hypophysectomised rats. However, this does not appear to be the case for the α-subunit of the 150 kDa complex which, unlike IGFBP-3, appears to be regulated directly by GH and is not responsive to

IGF-I (Zapf *et al;* 1989). Thus while GH is clearly an important factor in the regulation of the 150 kDa complex, intermediate processes may also be involved.

Glucose / Insulin / Nutritional Factors:

While increased plasma IGFBP-3 is induced by GH, IGFBP-1 may be inversely regulated by GH. Thus the IGFBP-1 level tends to be depressed in acromegalics but is significantly higher than normal in the GH-deficient human (Drop *et al;* 1984, Busby *et al;* 1988*b*). Nevertheless, because GH has a significant hyperglycaemic potential (Gopinath & Etherton; 1989*a*, 1989*b*), it is plausible that GH stimulated glucose release and/or counter regulatory changes in insulin may in fact be responsible for modulating plasma IGFBP-1. In support of this, Suikkari *et al* (1988) showed that IGFBP-1 levels were less than 50% of normal in patients with insulinoma, while infusion of insulin with maintenance of euglycaemia caused a decline in plasma IGFBP-1 concentration by up to 70%. Furthermore, insulin-dependent diabetics have high plasma IGFBP-1 concentrations that fall when the hyperglycaemia is corrected by insulin administration (Brismar *et al;* 1988). However, increased IGFBP-1 levels are observed during induction of hypoglycaemia by insulin administration (Jacob *et al;* 1989). It is apparent, therefore, that a complex relationship exists between plasma insulin, glucose and IGFBP-1 concentrations.

Plasma IGFBP concentrations may also be responsive to changes in nutritional intake. Baxter & Cowell (1987) initially demonstrated a diurnal variation in plasma IGFBP-1 level, such that the concentration of IGFBP-1-immunoreactive protein in human subjects rose from midnight to peak at 7-8 am, after which it declined to basal levels over a 4 h period. Yeoh & Baxter (1988) subsequently showed that the morning fall in IGFBP-1 could be prevented if meals were omitted, so that IGFBP-1 remained at peak value for the entire day. This study showed also that plasma IGFBP-1 levels responded to oral glucose, such that immunoreactive-IGFBP-1 fell rapidly after glucose consumption. Hence, it is likely that the reduced level of IGFBP-1 observed in response to altered nutritional intake is a consequence of lower plasma glucose level.

Nutritional state appears to be an important factor in the regulation of other IGFBP species. While plasma IGFBP-3 does not vary diurnally (Baxter & Cowell; 1987, Yeoh & Baxter; 1988), IGFBP-3 concentration is sensitive to altered nutrition. Thus, a marked decrease in plasma IGFBP-3 occurs in newborn pigs as a result of 24 h food deprivation (McCusker *et al;* 1989). McCusker *et al* (1989) also showed that smaller molecular mass IGFBPs, similar in size to those of IGFBP-2 (35 kDa; McCusker *et al;* 1991) and IGFBP-4 (24 kDa; Shimasaki *et al;* 1990), declined markedly with starvation, while a 29 kDa IGFBP, identified as IGFBP-1 (McCusker *et al;* 1991), was elevated.

1.5.3 IGFBP Ligand Binding

Although IGFs circulate at concentrations in the order of several hundred ng/ml, much higher than the circulating concentrations of most hormones, very little IGF is found free in the circulation (Daughaday *et al;* 1982, Clemmons *et al;* 1983). Most appears to be associated with IGFBP-3. In support of this, a 1:1 molar ratio can be demonstrated between IGFBP-3 and total IGF-I and IGF-II (Baxter & Martin; 1986, Baxter; 1988*b*). Moreover, plasma IGFBP-3 is detected predominantly at 150 kDa (Baxter & Martin; 1986), a size at which it does not elute unless complexed with IGF and the α-subunit (Baxter; 1988*a*). In addition, ligand-binding to IGFBP-3 appears to be favoured more than to some other IGFBPs. The association constants reported for IGFBP-1, for example, are just 10-20% of those reported for IGFBP-3 (Martin & Baxter; 1986, Baxter *et al;* 1987).

IGFBP -1, -2 and -3 all vary in their relative affinities for IGF-I and IGF-II. Thus IGFBP-3 and especially IGFBP-2 have greater affinities for IGF-II than for IGF-I while IGFBP-1 binds each ligand with a similar affinity (Scott *et al;* 1985, Martin *et al;* 1986, Forbes *et al;* 1988). The results obtained in such competition experiments are, however, dependent upon the species of radiolabelled IGF used. For example, IGF-I competes 30-40% as well as IGF-II for binding to IGFBP-2 against ^{125}I-IGF-I but only 5-10% as well as IGF-II using ^{125}I-IGF-II (Forbes *et al;* 1988). Because there are differences in the affinities

of the endogenous IGFs for some IGFBP species, however, it is plausible that IGF-I and IGF-II may be differentially regulated by IGFBPs.

1.5.4 IGFBP Effects on IGF Bioactivity

Roles in both the inhibition and potentiation of IGF activity have been attributed to the IGFBPs, but such effects may vary according to experimental conditions. For example, De Mellow & Baxter (1988) have shown that IGFBP-3 inhibits IGF-I-stimulated DNA synthesis when added to cultured human skin fibroblasts simultaneously with IGF-I, but also showed that it may potentiate IGF-I-stimulated DNA synthesis if preincubated with the cells for 48 h prior to the addition of IGF-I. While the IGF-inhibitory effects could be explained as competitive inhibition of receptor binding, the possibility that the potentiating effect was the result of receptor up-regulation, arising from the sequestering of endogenously secreted IGFs by added IGFBP-3 and, therefore, increased sensitivity to IGF-I, cannot be excluded. In support of this possibility, Conover & Powell (1991) have since demonstrated that IGFBP-3 prevents IGF-I induced receptor down-regulation of the type-I receptor in cultured bovine fibroblasts. However, while De Mellow and Baxter (1988) demonstrated maximal inhibition of DNA synthesis with equimolar amounts of human IGFBP-3 and IGF-I, the opposite effects have been demonstrated in hamster kidney fibroblasts; DNA synthesis is maximal in the presence of equimolar amounts of IGF-I and human IGFBP-3 (Blum *et al;* 1989). These combined data suggest, therefore, that the effects of human IGFBP-3 on IGF-I activity may vary according to tissue or organ type, or in a species-specific manner.

As was found from *in vitro* studies using IGFBP-3, the IGF-regulatory role of IGFBP-1 is not clearcut. Thus Elgin *et al* (1987) have shown that IGFBP-1 may potentiate IGF-I-stimulated DNA synthesis and cell replication in a number of cell types, while others have demonstrated inhibitory effects of purified IGFBP-1 upon binding of IGF-I to cell surface receptors and upon IGF-I-stimulated DNA synthesis (Knauer & Smith; 1980, Rutanen *et al;* 1988). The discrepancy between these studies could perhaps be explained if there were differences in the IGFBP preparation used. This may indeed be the case, since

two forms of IGFBP-1 have now been isolated, one of which is capable of potentiating IGF-I-stimulated DNA synthesis by smooth muscle cells while the other inhibits its activity (Busby *et al;* 1988*a*). Interestingly, this study also showed that the IGF-potentiating binding protein species was able to associate with the cell surface, while the inhibitory IGFBP remained in solution and competed with the cell-associated species for ligand binding. Perhaps demonstrations of inhibitory actions by IGFBP-1 have resulted from the use of IGFBP preparations containing a greater abundance of the soluble than of the cell surface-binding IGFBP species.

While IGFBPs may be capable of modulating the effects of IGF-I on DNA synthesis in the cell, other IGF activities are also modified. Thus IGF-stimulated glucose incorporation is inhibited by IGF-binding proteins (Drop *et al;* 1979) and IGF-I-induced amino acid uptake is inhibited by an IGFBP-1 that is unable to associate with cell surfaces (Ritvos *et al;* 1988). Perhaps the opposite effects might have been obtained using the cell surface-associating IGFBP-1 species isolated by Busby *et al* (1988*a*). Other studies show that IGFBPs specifically block the insulin-like effects of IGFs, since IGF-stimulated but not insulin-stimulated glucose uptake and lipogenesis can be blocked by addition of IGF-binding proteins (Meuli *et al;* 1978, Zapf *et al;* 1979, Knauer & Smith; 1980, Walton *et al;* 1989*b*).

1.5.5 Hypothesised Roles for IGFBPs *In Vivo*

Maintenance of Circulating IGF Reservoir:

A potential role for the IGFBPs, particularly for the high molecular mass complex, may be to prolong the half life of IGFs in the blood stream. Indeed, the circulating half lives of IGF-I and IGF-II in the high molecular mass complex of sheep have been reported as 545 and 351 min, respectively, 16 and 36 times their respective half lives in the small molecular mass IGF-binding region (Davis *et al;* 1989, Hodgkinson *et al;* 1989*a*). Furthermore, free IGF peptide falls to negligible levels within about 15 min of vascular infusion (Davis *et al;* 1989, Hodgkinson *et al;* 1989*a*). Oxidisation of IGF-I to prevent its association with plasma

IGFBPs results in a half life of about 0.85 h in sheep compared with a 5-7 h half life for native IGF-I (Francis *et al;* 1988*a*). The circulating half lives for radiolabelled IGF-I injected into normal and hypophysectomised rats, a condition in which plasma IGFBP-3 is at reduced concentrations (see Section **1.5.2**), have been reported as 4 h and 20-30 min, respectively (Zapf *et al;* 1986). Cohen and Nissley (1975) have shown that IGF activity in lymph is approximately half that in plasma, suggesting that the prolongation of circulating half life as a result of IGFBP-interaction may be partly accounted for by restricted access to extravascular target tissues.

Prevention of Insulin-like Effects by IGFs:

The IGFs circulate at high concentrations compared with insulin and are capable of causing insulin-like effects (see Section **1.4**). Indeed, with an *in vivo* insulin-like potency at about 6% that of insulin (Guler *et al;* 1987), the circulating IGF pool represents a large hypoglycemic potential. However, although vascular infusion of IGF-I has been shown to effect a hypoglycemic state, the full insulin-like potential of the endogenous IGF pool is not expressed, presumably because of association with circulating IGFBPs. Recent data support this concept. Thus, infusion of IGFBP-1 results in a state of hyperglycaemia, presumably through additional buffering of the tonic insulin-like activity of endogenous IGFs (Lewitt *et al;* 1991). Furthermore, infusion of IGF-I causes an insulin-like fall in blood glucose concentration that can be prevented by co-infusion of equimolar amounts of IGFBP-1 (Lewitt *et al;* 1991). Since IGFBP-1 concentration increases in response to reduced blood glucose (Section **1.5.2**), one role for this binding protein may be to buffer undesirable insulin-like effects of endogenous IGFs.

IGF Shuttle; Transfer of IGFs from Blood to Tissues:

A number of studies have established that the degree to which a circulating macromolecule is able to access the extravascular space is inversely proportional to its molecular mass (Mayerson *et al;* 1960, Garlick & Renkin; 1970, Simionescu *et al;* 1972, Firrell *et al* ; 1982). Hence, because binding proteins such as IGFBP-1 and IGFBP-2 have smaller molecular masses than the 150 kDa complex (Section **1.5.1**), they are likely to have

greater access to the extravascular space than the ternary complex. This is supported by the findings of Binoux & Hossenlopp (1988) whose data show that, compared with plasma, the smaller IGFBP species are more abundant than IGFBP-3 as a proportion of the total IGFBPs present in human leg lymph. In addition, binding of radiolabelled IGF at 150 kDa in leg lymph was negligible. These data suggest, therefore, that the α-subunit and the high molecular mass complex are unable to cross the capillary wall in the human leg and that IGFs associated with smaller molecular mass IGFBPs are more likely to gain access to extravascular tissue. It is not clear, however, whether this applies to all tissues or species, since some binding of labelled IGFs has been detected at 150 kDa in ovine mammary lymph (Hodgkinson *et al;* 1989*b*). Nevertheless, the greater relative abundance of smaller molecular mass IGFBPs in lymph than in plasma suggests that transport of IGFBPs bound in this form would be greater than that of IGFs in the high molecular mass complex, although the extent of such differences may vary in a tissue- or species-specific manner. An alternative explanation for the greater relative abundance of small molecular mass IGFBPs in lymph is that this may reflect synthesis of IGFBPs by the tissues drained by lymph.

Tissue Targeting:

IGFBP-1 and IGFBP-2, but not IGFBP-3, feature an "RGD" sequence that consists of the tripeptide -Arg-Gly-Asp- (Brewer *et al;* 1988, Brown *et al;* 1989). This sequence, which in proteins such as fibronectin, fibrinogen and collagens is known to be involved in attachment to RGD receptor sites on the extracellular matrix (Pierschbacher *et al;* 1982, Rouslahti & Pierschbacher; 1987), may be involved in the targetting of IGFBPs to tissues expressing RGD receptor sites. Brewer *et al* (1988) have demonstrated the potential involvement of this sequence in binding of IGFBPs to the cell surface, since synthetic RGD peptide competitively inhibits cellular attachment by IGFBP-1. That cellular attachment by IGFBPs may serve a role in IGF activity is suggested by the demonstration that IGFBP-1 which are able to bind to cell surfaces promote IGF activity, but that IGFBP-1 isolates that fail to associate with the cell surface inhibit its activity (Busby *et al;* 1988*a*). Taken together, these data suggest a role for the IGFBP RGD site in the targetting and regulation of IGF activity.

1.6 ANALOGS OF IGF-I THAT BIND POORLY TO IGFBPS

A variant form of IGF-I, des(1-3)IGF-I, has now been isolated from a variety of physiological sources that include bovine colostrum (Francis *et al;* 1986, 1988*b*),.the human brain (Carlsson-Skwirut *et al;* 1986) and the pig uterus (Ogasawara *et al;* 1989). Des(1-3)IGF-I is structurally identical to IGF-I except for the absence of the first three amino acids (Gly-Pro-Glu) present in IGF-I. The variant has a 5-10 fold greater potency than IGF-I in most cultured-cell bioassays, an effect which cannot be accounted for by greater receptor affinity. Instead, it appears to be entirely the result of reduced affinity for IGFBPs (Francis *et al;* 1986, Ballard *et al;* 1987). This suggests an overall inhibitory role for IGFBPs in the regulation of IGF activity.

The importance of the amino terminus of IGF-I in its interaction with IGFBPs was first demonstrated by Szabo *et al* (1988) who found that des(1-3)IGF-I was unable to compete with radiolabelled IGF-I or IGF-II for binding to IGFBP-2. In fact, the third amino acid residue, Glu, is critical for the binding of IGF-I to this binding protein, since removal of either Gly or Gly-Pro causes no change in affinity for IGFBP-2, whereas the absence of the Gly-Pro-Glu tripeptide virtually eliminates its capacity to bind IGFBP-2 (Bagley *et al;* 1989). The relatively poor affinity of des(1-3)IGF-I, compared with IGF-I, for IGFBP-2 has also been demonstrated for a number of other IGFBPs. Thus Forbes *et al* (1988) showed that des(1-3)IGF-I binds poorly to both IGFBP-1 and IGFBP-3, and confirmed its negligible affinity for IGFBP-2. They also showed that the extent to which binding of IGF-I and des(1-3)IGF-I differ depends upon the IGFBP. Thus the efficiency with which des(1-3)IGF-I competes with radiolabelled IGF-I for binding is about 1% for IGFBP-1 but 30-40% for binding to IGFBP-3.

There now exists a range of IGF-I variants designed to bind IGFBPs poorly and which may be employed in comparisons with IGF-I and des(1-3)IGF-I to determine the role of reduced IGFBP interaction in the regulation of IGF activity. Bayne *et al* (1988) have produced four IGF-I variants that bind IGFBPs poorly, including a mutant in which the first

16 residues of IGF-I are replaced by the first 17 of insulin. However, this protein has a 4 fold higher affinity for the insulin receptor, a 100 fold lower affinity for the type-II receptor and a 2 fold lower affinity for the type-I receptor. The IGF-I analog [Gln^3, Ala^4, Tyr^{15}, Leu^{16}]IGF-I has a 600 fold lower affinity for serum IGFBPs than native IGF-I but is equipotent to IGF-I at the type-I receptor. However, it has a 10 fold greater affinity than IGF-I for the insulin receptor which may limit its usefulness for comparative studies (Bayne *et al;* 1988). A third analog ([Tyr^{15}, Leu^{16}]IGF-I) has a 4 fold lower affinity for IGFBPs and is bound normally to IGF receptors, but its binding to the insulin receptor is also 10 fold greater than that of IGF-I. The fourth analog ([Gln^3, Ala^4]IGF-I) has an affinity for serum IGFBPs that is 4 fold lower than that of IGF-I, and because it binds normally to all three receptor types, it may be the most suitable of the above analogs for comparison with IGF-I and des(1-3)IGF-I to determine the effect of reduced IGFBP-interaction on IGF transfer.

Another analog is Long(Arg^3)IGF-I (LR^3IGF-I). This protein differs from IGF-I by the substitution of Arg for Glu at the third residue, and by an N-terminal extension that consists of an eleven amino acid fragment of methionyl porcine GH (met-pGH[1-11]) (Francis *et al;* 1992). In designing this peptide, the third residue was replaced because of studies demonstrating its importance in binding IGFBPs (see above). Unlike des(1-3)IGF-I, the affinity of LR^3IGF-I for the type-I receptor is some 3-4 fold lower than that of IGF-I (Francis *et al;* 1991). While its affinity for the insulin receptor had not been tested at the time I began my research, binding of LR^3IGF-I to this receptor is unlikely to differ from that of IGF-I since the residues equivalent to amino acids 15 and 16 of IGF-I, which appear to be involved in binding the insulin receptor (Bayne *et al;* 1988), remain unaltered. Francis *et al* (1992) showed that the potency of LR^3IGF-I in an L6 myoblast protein synthesis assay was almost double that of des(1-3)IGF-I and even more so than IGF-I, despite the reduced affinity of LR^3IGF-I for the type-I receptor. In addition, it exhibited a much lower affinity for IGFBPs than did des(1-3)IGF-I. Combined with its poorer receptor binding, these data suggest that the advantages of lower IGFBP interaction outweigh any disadvantage of lower receptor affinity (Francis *et al;* 1992). Nonetheless, it is apparent that there exists a point beyond which reduced receptor affinity may compromise biological activity, since

Hodgkinson *et al* (1989*c*) have shown that the binding affinities of incorrectly folded N-met IGF-I for IGFBPs and the receptor are 40 fold and 200 fold lower, respectively, than those of the correctly folded peptide. Interestingly, despite the large reduction in receptor binding, the bioactivity of this misfolded analog was reduced by only 15 fold.

These data show that the absence of the N-terminal tripeptide from IGF-I does not result in poorer binding to the type-I IGF-receptor (Bagley *et al;* 1991), such that des(1-3)IGF-I could be a useful peptide for comparison against IGF-I in determining the effect of reduced IGFBP interaction on tissue transfer of plasma IGFs. LR^{3}IGF-I also appears to be a useful peptide for such comparisons since it exhibits even lower IGFBP affinity and is even more potent than des(1-3)IGF-I despite its lower affinity for the type-I receptor.

1.7 MEASUREMENT OF MACROMOLECULAR TRANSFER FROM BLOOD TO TISSUE

Two methods hold particular promise with regards to their usefulness as models with which to study transfer from the vasculature to tissue. One technique involves the infusion of a test substance into the vascular supply of a surgically isolated tissue bed or organ, with the blood supply substituted for by an oxygenated nutrient solution, infused under conditions of controlled temperature, pressure and/or flow rate. This method has been employed in organs such as the liver (Linzell *et al;* 1971), testis (Linzell & Setchell; 1969) and mammary gland (Linzell *et al;* 1972), usually to study substrate utilisation. However, because the organ is removed from the animal, it is not subject to the same neuronal and hormonal controls as the *in situ* organ. A more physiological method for studying transfer to tissue is the *in situ* perfusion, in which the vascular supply and nervous connections are maintained. This method involves infusion of a test substance directly into the arterial blood supply of a tissue, through a very fine cannula, without interruption to the normal blood supply. During perfusion, the efferent blood flow from the tissue may be collected to prevent recirculation of any test substance not utilised in a single pass through the tissue. Simultaneous collection of lymph may reveal transfer of an infused agent from the

vasculature, since lymph is derived from the extravascular space. Following perfusion, the tissue may be excised to determine transfer of the infused test substance. Windmueller & Spaeth (1977) have previously developed an *in situ* vascular perfusion model for the intestine, but employed it to study substrate utilisation, rather than transfer to tissue.

Bar *et al* (1985, 1988) have employed a non-recirculating perfusion method in the isolated beating heart to study transfer of radiolabelled IGFs from the arterial supply to heart tissue. Their method involved the infusion of radiolabelled IGF-I, dissolved in an oxygenated nutrient buffer, into the coronary artery, with collection of the efferent flow via a slit in the right ventricle. Subsequently, tissue sections were prepared and autoradiographed to determine the tissue distribution of the infused radioligand. This study demonstrated uptake of labelled IGF-I by the heart tissue but at the time I commenced research towards this Thesis, the model had not been employed to estimate percentage IGF-transfer from the vasculature (Bar; 1988). Subsequently, however, Bar *et al* (1990*b*) showed that 3-5% of IGF infused into the heart was transferred to heart tissue.

Prosser *et al* (1991) and Prosser & Fleet (1992) have recently employed a derivation of the above-described *in situ* perfusion model to study IGF transfer from the circulation to milk. Thus radiolabelled IGFs were continuously infused into the vascular supply of the goat mammary, with determination of transfer to milk performed over a 720 min period. However, unlike the *in situ* perfusion model described above, these studies did not involve collection of all efferent blood from the perfused gland, such that infused IGF that was not transferred to milk in a first pass through the tissue was recirculated. Thus radioligand reached both glands, with about 5% of infused radioactivity recovered in the total milk. Nevertheless, this may not reflect net transfer from blood since the mammary tissue may plausibly have retained some transferred IGF.

The *in situ* vascular perfusion method has not previously been used to study IGF transfer to tissue. As a model with which to determine the role of plasma IGFBPs in regulating IGF transfer, I chose to employ a non-recirculating *in situ* vascular perfusion system. The specific characteristics of this system would be as follows:

1) an IGF-responsive tissue would be perfused with IGFs.

2) the tissue would be perfused under conditions that were close to the physiological; perfusion would be performed with maintenance of all nervous connections and with the blood supply intact to allow normal auto regulation of blood flow; IGF peptides would be infused into the arterial blood that supplied the tissue; blood pressure and heart rate would be maintained in the physiological range by infusion of donor blood to replace that lost during blood sampling.

3) the system would allow the quantitative recovery of infused growth factor: it would be a one-pass perfusion with occluding cannulae in the efferent blood and lymph vessels draining the tissue bed, for quantitative collection of all vascular and lymphatic outflow so that no infused peptide reached the general circulation or was recirculated through the tissue.

On the basis of these criteria, the intestine was selected. This organ was an appropriate tissue in which to measure transfer because it demonstrates growth in response to infusion of IGF and because the segmented nature of the blood supply to this organ allows for the isolation of discrete regions of tissue for perfusion, obviating the need to perfuse the whole organ and the attendant requirement for large amounts of replacement donor blood. In addition, the tissue could be easily recovered after perfusion to measure the amount of infused peptide retained. Moreover, the method of Read *et al* (1990), designed to measure absorption of luminally infused peptides by the ovine intestine, was best suited to modification to study intestinal transfer of vascularly infused IGFs.

1.8 STATEMENT OF PROBLEM

IGFs are found at high concentrations in the circulation, mostly in association with IGFBPs. Circulating IGFs are bioactive and are presumed capable of stimulating growth of tissues outside the vasculature, to do which they must be transferred from the blood stream to the tissue. Receptors for IGFs are distributed in tissues and organs throughout the body, so that a general role for IGF in growth is inferred. The present investigation centres on the delivery of circulating IGFs to tissues.

The Literature Review reveals that IGFBPs may enhance or inhibit the effects of IGFs *in vitro*. However, a general role for the plasma IGFBPs in regulating IGF transfer from the circulation is not known. Nevertheless, IGF-I variants that bind poorly to IGFBPs exhibit greater biological potency than IGF-I *in vivo*. Since IGFs exert their effects as a direct result of binding to receptors, I hypothesised that transfer of the variants would be greater than that of IGF-I.

The specific aims of this project were:

1) to characterise, in a preliminary study, the IGFBPs contained in lymph collected from small intestine and other anatomical sites, and also in CSF and plasma, to determine whether extravascular IGF activity was likely to be regulated by IGFBPs in a tissue-specific manner;

2) to develop a vascular perfusion model that would enable the transfer of circulating IGFs from blood to tissue to be studied;

3) to use the model to compare transfer of IGF-I with that of IGF(s) that bind poorly to IGFBPs;

4) if unexpected results were obtained, perform limited investigations to determine the reasons for those results.

Chapter Two

General Methods

CHAPTER TWO: GENERAL METHODS

Contained within this section are methods common to three or more experimental chapters, in addition to a small experiment, the purpose of which was to select an appropriate anticoagulant treatment for samples collected in latter studies.

2.1 RADIOLABELLED PEPTIDES

Radiolabelled peptides were required for a variety of techniques that included *in vitro* ligand binding studies, ligand blotting experiments, *in vivo* vascular perfusion studies and for the assay of plasma IGF-I, IGF-II and insulin concentrations. Sources of peptides included Genentech Inc. (hIGF-I and des[1-3]IGF-I), GroPep Pty. Ltd. (hIGF-I, hIGF-II, des[1-3]IGF-I and LR^3IGF-I) and Lilly Research Laboratories (bIGF-II and ovine insulin).

Iodination of peptides was performed using the chloramine T method described by Van Obberghen-Schilling & Pouyssegur (1983). 10 µg of peptide was dissolved in 50 µl of 10 mM HCl over 1 h at room temperature and was then mixed with $Na^{125}I$ (Amersham International plc, or Nordion International Inc.). The volume of $Na^{125}I$ stock added was altered according to the specific activity of the iodide solution (approximately 100 mCi/ml) and ranged from 8-12 µl/mCi for 1 mCi of ^{125}I. The tube contents were buffered using 500 mM Na_2HPO_4 (50 µl, pH 7.5) and the reaction catalysed by addition of a chloramine T solution (20 µl; Sigma Chemical Co.). Chloramine T was added to a final concentration of 0.10 mM when used with iodide purchased from Amersham International plc, and was increased to 0.32 mM for iodide acquired from Nordion International Inc because the efficiency of iodide incorporation into the peptide differed according to the source. The mixture was gently swirled and allowed to react for 45 sec before addition of sodium metabisulphite (20 µl, 0.19 mM final concentration) to stop the reaction. The sample was mixed with 70 µl of Elution Buffer (50 mM NaH_2PO_4, pH 6.5, 0.25% [w/v] bovine serum albumin [BSA; RIA-grade, Sigma Chemical Co.]) in preparation for its application to a size-

exclusion column. Sephadex G50 size-exclusion gel chromatography was used to separate growth factors from high and low molecular mass reaction products and unreacted reagents.

A Sephadex G50 size-exclusion column was freshly prepared for the purification of each iodination. 2.5 g of dry Sephadex G50 gel beads (Pharmacia Fine Chemicals) were swelled in water overnight at room temperature, according to directions of the manufacturer. The swollen gel was suspended by trituration using a glass pasteur pipette and approximately 23 ml of the slurry was poured into a plastic column (1 x 30 cm); this column contained an end-filter frit and incorporated a 3-way stop cock for the control of column outflow. The supernatant was withdrawn at intervals of 2-5 min as the gel partially settled, and the column was topped up with slurry. This procedure was repeated until the gel bed reached a height of 28 cm. Finally, the supernatant was withdrawn from the top of the column and the gel bed was carefully overlayed with 2 ml of Elution Buffer. The column was then equilibrated at a flow rate of 0.25 ml/min for 1 h, using a peristaltic pump (2232 Microperpex S; LKB Bromma).

For application of the sample mixture to the gel, buffer was allowed to drain from the column under gravity until the meniscus declined to the level of the gel bed. Flow was arrested using the 3-way stop cock and the iodination mixture (approximately 170 µl) was applied to the column. Residual contents of the iodination reaction vial were washed onto the column using 100 µl of Elution Buffer. The total sample size of 270 µl was equivalent to about 1% of column volume and was therefore well within the maximum 5% (v/v) ratio recommended for optimal separation by this gel (Pharmacia Fine Chemicals). The sample was then allowed to run into the gel bed under gravity and was washed in using a further 0.5 ml of Elution Buffer. The column was then topped up with 2 ml of Elution Buffer and the sample eluted at a flow rate of 0.25 ml/min, with fractions collected at 2 min intervals for a period of 2 h. To determine the elution profile of radioactivity, the counts contained in 5 µl aliquots were measured in a gamma counter (1261 Multigamma; LKB Wallac).

Radioactivity eluted in three peaks, timed at about 16 min (4 ml), 25 min (6.5 ml) and 40 min (10 ml). Since iodinations are performed routinely at the Child Health Research

Institute, it had previously been found that the radiolabelled IGFs eluted in the second fraction (6.5 ml). Nevertheless, in all iodinations performed for this thesis, TCA precipitability of fractions from the second peak was measured (see below) to establish that this radioactivity was bound to protein. TCA precipitability of the 4 - 6 fractions of highest activity was generally about 97% while, in limited comparisons, maximum precipitability of fractions in the first and third regions was only 80% and 20%, respectively. Fractions in which radioactivity was at least 95% precipitable were combined, aliquoted and stored at -20°C.

2.2 TRICHLOROACETIC ACID (TCA) PRECIPITABILITY

TCA precipitability of iodinated peptides and of radioactivity contained in physiological tissue and fluid samples was measured as an estimate of radioligand intactness. Thus radiolabelled IGFs should be highly precipitable, but their breakdown products and free iodide should be poorly precipitable. Radioiodinated IGF peptides (5 µl) were mixed with 900 µl of ice cold Elution Buffer (see above) after which 100 µl of ice cold 100% (w/v) TCA was added. Following a 20-30 min incubation on ice, precipitated proteins were pelleted by centrifugation for 20 min at 2000 x *g* and 4°C. The supernatant was withdrawn and placed in a separate tube and the pellet solubilised in 2 M NaOH to a similar volume as that of the supernatant. Radioactivity was measured in a gamma counter and TCA precipitability was calculated as follows:

$$\text{Precipitability} = 100 \times (\text{cpm in pellet}) / (\text{cpm in pellet} + \text{cpm in supernatant})$$

2.3 ANALYTICAL GEL FILTRATION

Gel filtration chromatography allows the separation of macromolecules on the basis of molecular size. I used this technique to analyse the binding of radiolabelled IGFs to IGFBPs in plasma, lymph and CSF *in vitro* and in samples collected from lambs that had been infused with radiolabelled IGFs. Chromatography was performed at room temperature on either a Superose-6 or -12 column (1 x 30 cm; Pharmacia Fine Chemicals) using an FPLC system (Pharmacia Fine Chemicals). This consisted of a P-500 high precision pump, automatic injector (MV-7), UV-monitor (UV-M), fraction collector (Frac 100) and chart recorder (REC-482), with all components coordinated using an LCC-500 programmable chromatography controller.

Prior to chromatography and so as to prevent rapid column blockage, lipid was extracted from samples using 1,1,2-trichloro-1,2,2-trifluoroethane (freon) (Francis *et al;* 1990). After mixing with an equivalent volume of freon, samples were vigorously vortexed (30 sec - 1 min) then centrifuged (5 min at 10000 x *g* and 4°C) to separate the aqueous supernatant from the freon-lipid mixture. The supernatant was withdrawn for Superose chromatography.

Columns were equilibrated with heparinised PBS (50 mM NaH_2PO_4, 150 mM NaCl, 0.2 g/l NaN_3, 10 IU/ml heparin, pH 7.2) and samples (100-200 µl) were loaded into the automatic injector (500 µl sample loop) from where they were applied to the column. Sample elution rate was 1.0 ml/min for Superose-12 and 0.5 ml/min for Superose-6; a reduced flow rate used for the latter column because of the lower resilience of its gel matrix. Protein absorbance (280 nm) was recorded and fraction collection was commenced after a programmed delay; the delay corresponded to the time required for sample to flow from the absorbance detection unit to the collection tube and was incorporated so as to simplify alignment of radioactivity in fractions with the protein absorbance trace. To calculate this delay, the internal tubing volume between the UV detector and fraction collector was measured and divided by the sample elution rate. Radioactivity collected in the fractions was measured in a gamma counter.

Superose columns were standardised using protein and radioactive markers, with respective elution volumes measured by UV absorbance and elution of radioactivity in fractions. The elution volume for thyroglobulin in each column was used to determine column void volume, since the molecular mass of this protein (600 kDa) is beyond the limit of macromolecular separation for either column (Pharmacia Fine Chemicals). Values were 7.7 ml and 7.5 ml for Superose-6 and Superose-12, respectively. Calibration of the columns with smaller protein markers and radiolabelled IGFs was performed so that the molecular masses of radioactivity peaks could be calculated; markers included human γ-globulin (150 kDa), BSA (69 kDa), chicken egg ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and radiolabelled IGFs (8 kDa). With the exception of IGFs, all markers were obtained from Sigma Chemical Co. Radioactivity in fractions was counted using a gamma counter. Elution positions for radioactive and protein markers are shown against the elution profiles of radiolabelled IGFs, as appropriate.

2.4 PERIPHERAL BLOOD COLLECTION AND PLASMA PREPARATION

2.4.1 Cannulation Technique

Peripheral veins in lambs, either the saphenous or jugular vein, were cannulated for the convenient collection of multiple blood samples, and also for the purpose of donor blood infusion. The cranial tibial artery was catheterised for experiments in which monitoring of heart rate, arterial blood pressure, arterial blood gases and pH were required.

Sterile Technique:

All cannulations performed in which animals were to be revived at the end of the experiment, were carried out using sterile technique. The skin was shaved and was then sterilised using Betadine antiseptic (Faulding Pharmaceuticals). Surgical instruments and catheters were sterilised by autoclave or in 70% ethanol, while solutions used to flush and fill the catheters were sterile-filtered (0.22μm Millex; Millipore Corp.). Following cannulation,

the wound was closed by application of sutures and Savlon antiseptic cream (ICI Australia Operations Pty. Ltd.) was applied to prevent bacterial infection.

Saphenous Vein:

This vessel is located on the outer aspect of each leg and was cannulated in the metatarsal area. In animals which were not under general anaesthesia at the time of this procedure, local anaesthetic (0.5-1.0 ml Lignocaine; Apex Laboratories) was injected subcutaneously at multiple sites adjacent to the vein and was gently massaged for 3-5 min to aid dispersal into the tissues. A 3 cm incision was made in the skin adjacent to the vessel and blunt-tipped forceps were used to isolate it from surrounding connective tissue. Two silk sutures (4-0) were passed under the vessel and the proximal ligature was tightened to arrest blood flow. The vessel was lifted by the distal ligature and butterfly scissors were used to make an incision in its wall at a position between both sutures. A catheter (inner diameter [ID] 1.0 mm and outer diameter [OD] 1.5 mm), filled with heparinised saline (NaCl at 9 g/l, heparin at 10 IU/ml), was inserted into the vein in the dorsal direction for a distance of 10-20 cm. The ligatures were tightened to secure the catheter in position and the wound was sutured closed. The end of the catheter was attached to a blunted needle (18G) and was plugged to prevent loss of blood or entry of air bubbles and foreign matter into the bloodstream.

Jugular Vein:

In some animals, the jugular vein was cannulated instead of the saphenous vein. Digital pressure was applied to the lower portion of the jugular to cause the vessel to swell and a 14 or 16G plastic-sheathed needle (Surflo, Terumo Corp.) was inserted. The needle was then withdrawn and a polyethylene indwelling cannula (1.0 mm ID, 1.5 mm OD), filled with heparinised saline (see above), was inserted into the jugular to a depth of approximately 20 cm. The sheath was then withdrawn from the vessel and passed back over the catheter for removal. The catheter was attached to a blunted 18G needle and was plugged using a removable plastic stopper.

Cranial Tibial Artery:

This vessel is located between the flexor digitorum profundus and the extensor digitorum lateralis muscles in the hind leg, and its cannulation required similar procedures to those used for catheterisation of the saphenous vein. In lambs not already under halothane anaesthesia, local anaesthetic (0.5 - 1.0 ml Lignocaine) was injected subcutaneously around the target area, prior to cannulation. An incision was made in the skin and blunt dissection was employed to separate the two muscle groups and clear connective tissues from the artery. Forceps were used to separate any co-lateral veins from the artery, which was then cannulated in the dorsal direction as described for the saphenous vein.

2.4.2 Selection of Anticoagulant for *In Vitro* Analyses

Because the aim of the subsequent study (Chapter **3**) is to compare binding of IGFs to IGFBPs in plasma with those in lymph and CSF, fluids in which IGFBPs of high molecular mass constitute a smaller proportion of the total IGF-binding activity than in plasma (Binoux & Hossenlopp; 1988, Hodgkinson *et al;* 1989*b*), it was particularly important to select conditions in which interference with radioligand binding in these regions was minimised. It had been reported that association of radiolabelled IGFs with IGFBPs may be inhibited in the presence of heparin (Clemmons *et al;* 1983), therefore a preliminary comparison of anticoagulants was made to select an appropriate procedure.

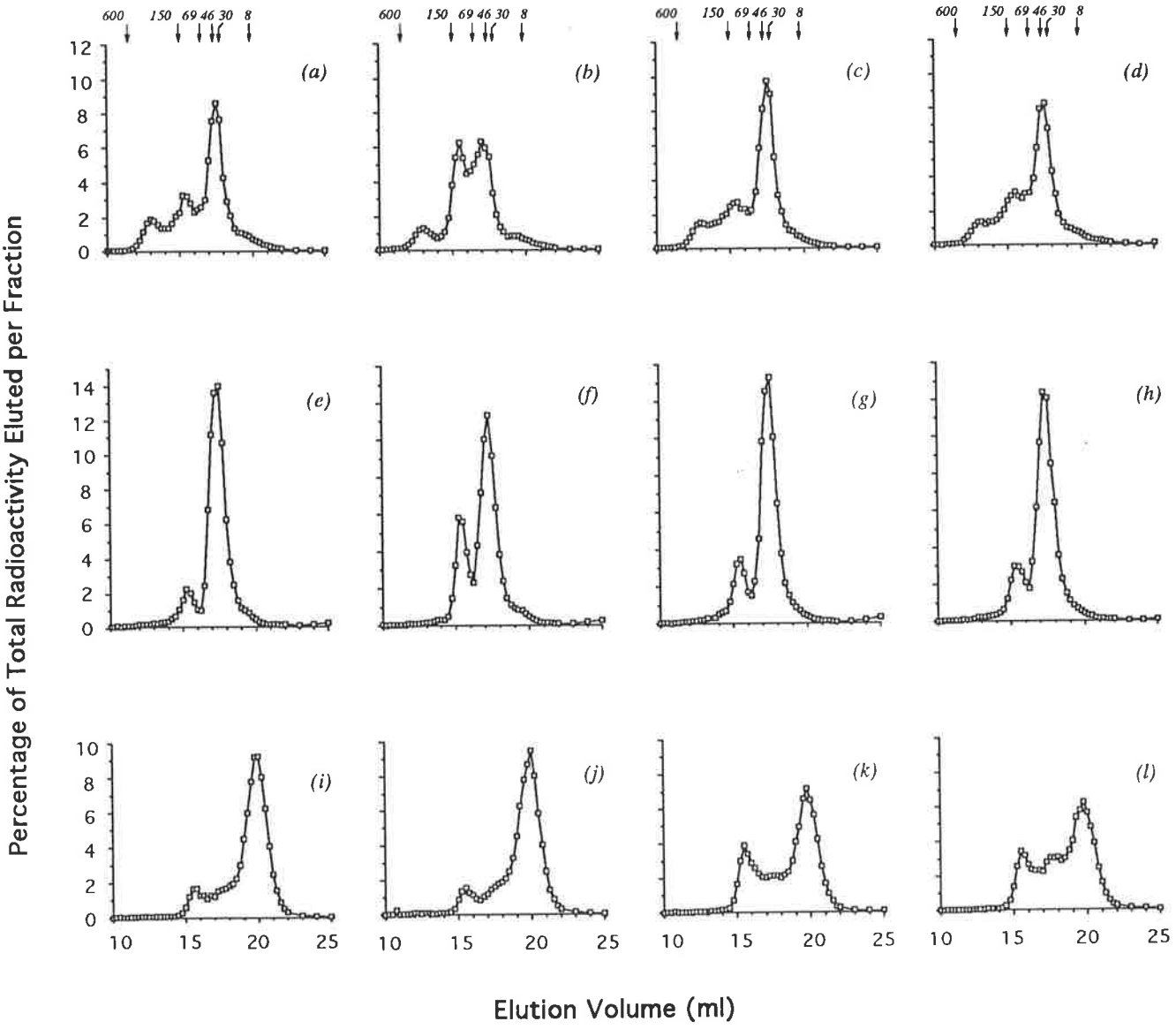
Venous blood was collected from 3- to 6-week-old Merino-Dorset crossbred lambs, of either sex, which were not weaned at the time of sampling but which had access to lucerne chaff as part of the mother's diet. Lambs were instrumented with saphenous or jugular vein cannulae (Section **2.4.1**), with the assistance of Dr A.A. Martin. Blood was collected and was either allowed to clot to obtain serum, or was treated with anticoagulant (EDTA, heparin or potassium citrate solution [pH 7.4] at final concentrations of 1.5 mg/ml, 10 IU/ml or 3.1 mg/ml, respectively) to obtain plasma. The latter blood samples were centrifuged for 20 min at 1800 x *g* and 4°C after which plasma was aspirated and frozen in liquid nitrogen,

prior to storage at -80°C. Serum was similarly prepared and stored after blood had been allowed to clot overnight at 4°C. These samples were used for *in vitro* radioligand binding studies to determine the effects of the various treatments on association of radiolabelled IGFs with binding proteins.

Samples (1.5 ml) of serum or plasma, from either one of two animals, were thawed and defatted (Section **2.3**) prior to incubation for 4, 15 or 40 h at 4°C with approximately 1.4 x 10^6 cpm of labelled IGF-I, IGF-II or des(1-3)IGF-I. Plasma was then rapidly frozen in liquid nitrogen and stored at -80°C until size-exclusion chromatographic analysis was performed. Samples were thawed individually and, after centrifugation for 1 min at 15000 x *g* to pellet any insoluble precipitates, a 100 µl portion of the supernatant was applied to a Superose-6 size-exclusion column for chromatography as described in Section **2.3**. The eluate was collected in 0.25 ml fractions. All heparinised samples were applied to the column after pre-equilibration with phosphate buffer containing heparin 10 IU/ml, and were eluted in the same buffer. Heparin in the phosphate buffer was replaced by citrate (3.1 g/l) or EDTA (1.5 g/l) for chromatography of citrate-treated or EDTA-treated samples, respectively. Serum was chromatographed as for EDTA-treated samples.

The profiles of radiolabelled IGFs in plasma and serum, which underwent a 15 h incubation, are shown in Fig. 2.1. Size-exclusion chromatography of serum and plasma samples after incubation with labelled IGF-II revealed three ligand-binding regions with apparent molecular masses of approximately 250, 130-150 and 30-50 kDa (Fig. 2.1*a-d*). The profile of radioligand association was similar in all fluids except in heparinised plasma, in which the percentage of tracer that bound in the 130-150 kDa region was greater than in the other fluids and binding at 30-50 kDa was reduced. When incubated with radiolabelled IGF-I (Fig.2.1*e-h*), as for IGF-II, a greater percentage of IGF-I tracer associated at the 130-150 kDa region in heparinised plasma than in serum or the other plasma groups, although IGF-I did not bind at 250 kDa in any fluid. Association of IGF-I and IGF-II with binding proteins in all samples was near complete, as very little radioactivity eluted at the position of free tracer. On the other hand, less than 50% of labelled des(1-3)IGF-I was bound in any sample, although this ligand, like IGF-I, bound at only the 130-150 and 30-50 kDa regions.

Fig. 2.1 *Superose-6 chromatography of 100 µl portions of lamb serum (a, e, i), heparin-plasma (b, f, j), EDTA-plasma (c, g, k) or citrate-plasma (d, h, l) after incubation with radiolabelled insulin-like growth factors (IGFs) at 4°C for 15 h.* The radioligands used were IGF-II (*a-d*), IGF-I (*e-h*) and des(1-3)IGF-I (*i-l*). The plots show recovery of radioactivity in 0.25 ml fractions as a percentage of total eluted radioactivity. The column was pre-equilibrated in phosphate elution buffer (50 mM NaH_2PO_4, 150 mM NaCl, 0.02% (w/v) sodium azide; pH 7.2) which contained heparin (10 IU/ml) for heparinised plasma samples, citrate (0.31% [w/v]) for citrate-plasma or EDTA (1.5 g/l) for serum and EDTA-plasma. The elution positions of thyroglobulin (600 kDa), human γ-globulin (150 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and radiolabelled IGFs (8 kDa) are shown.


600 150 69 46 30 8
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Percentage of Total Radioactivity Eluted per Fraction
Elution Volume (ml)

Unlike the binding of both IGF-I and IGF-II, however, association of des(1-3)IGF-I at 130-150 kDa was greatest in citrate-treated and EDTA-treated plasma samples (Fig. 2.1*i-l*). The absence of the increase in binding of des(1-3)IGF-I at 130-150 kDa in the presence of heparin is clearly not due to saturation of binding sites. Since both citrate and EDTA are chelators of divalent metal ions, the greater binding of des(1-3)IGF-I in plasma that contains these anticoagulants suggests that such ions may inhibit ligand association with IGFBPs.

The binding of radiolabelled IGF-I, IGF-II and des(1-3)IGF-I in the serum and plasma samples was qualitatively similar for incubation times of 5 and 40 h to that found at 15 h, although the distribution of radioligand between the peaks did vary slightly at different time points. The distribution of radioactivity between the 250, 130-150, 30-50 and 8 kDa regions is shown for IGF-I (Fig 2.2), IGF-II (Fig 2.3) and des(1-3)IGF-I at the three time points (Fig. 2.4). Calculation of this distribution was estimated as the proportion of ligand eluting at 11-14 ml (250 kDa), 14.25-16 ml (130-150 kDa), 16.25-18.5 ml (30-50 kDa) and 18.75-22 ml (8 kDa). 15 h was selected as an appropriate incubation period because association of radioligands with IGFBPs had largely stabilised at this time.

Since the purpose of the present study was to select a sample treatment that interfered least with binding of IGF-I and IGF-II at 130-150 kDa, heparin was the treatment of choice. Thus comparisons of the binding profiles for IGF-I and IGF-II in the four sample types revealed that association in the 130-150 kDa region was substantially greater in heparinised plasma than in the other plasmas or in serum, although the lack of a similar finding when des(1-3)IGF-I was used is not easily explained. Nonetheless, use of heparin for sample collection combined with use of radiolabelled IGF-I and especially IGF-II for binding studies, would afford the greatest opportunity to detect the 130-150 kDa IGFBP complex in extravascular fluids of the lamb, should it be present. Furthermore, since the aims of Chapters **4** & **5** are to compare tissue transfer of IGF-I with that of analogs which bind poorly to IGFBPs, the lack of an increase in binding of des(1-3)IGF-I to IGFBPs in the presence of heparin also makes heparin an appropriate choice of anticoagulant. Accordingly, heparin was used in all subsequent sample collections.

Fig. 2.2 *Distribution of radiolabelled IGF-I between 250 kDa (solid), 130-150 kDa (diagonal stripe), 30-50 kDa (grey) and 8 kDa (white) regions in serum and plasma containing heparin, EDTA or citrate.* Incubations were performed at 4°C for periods of 4, 15 and 40 h, after which size-exclusion chromatography was performed as described in Fig. 2.1. Distribution of radioactivity between the four molecular mass regions was calculated as described in Section **2.4.2.**

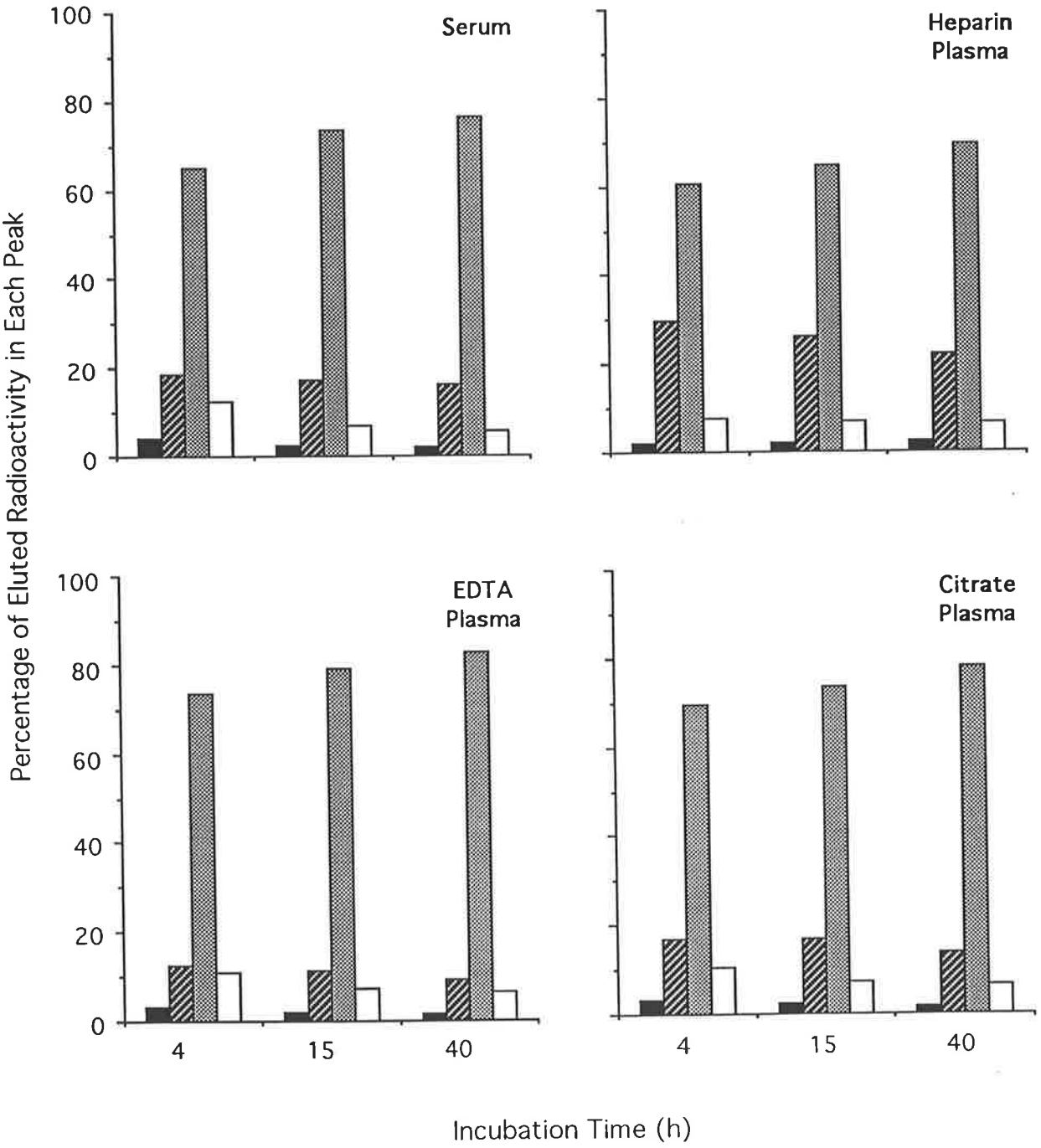

Serum
Heparin Plasma
EDTA Plasma
Citrate Plasma
Percentage of Eluted Radioactivity in Each Peak
100
80
60
40
20
0
4
15
40
Incubation Time (h)

Fig. 2.3 *Distribution of radiolabelled IGF-II between 250 kDa (solid), 130-150 kDa (diagonal stripe), 30-50 kDa (grey) and 8 kDa (white) regions in serum and plasma containing heparin, EDTA or citrate.* Incubations were performed at 4°C for periods of 4, 15 and 40 h, after which size-exclusion chromatography was performed as described in Fig. 2.1. Distribution of radioactivity between the four molecular mass regions was calculated as described in Section **2.4.2.**

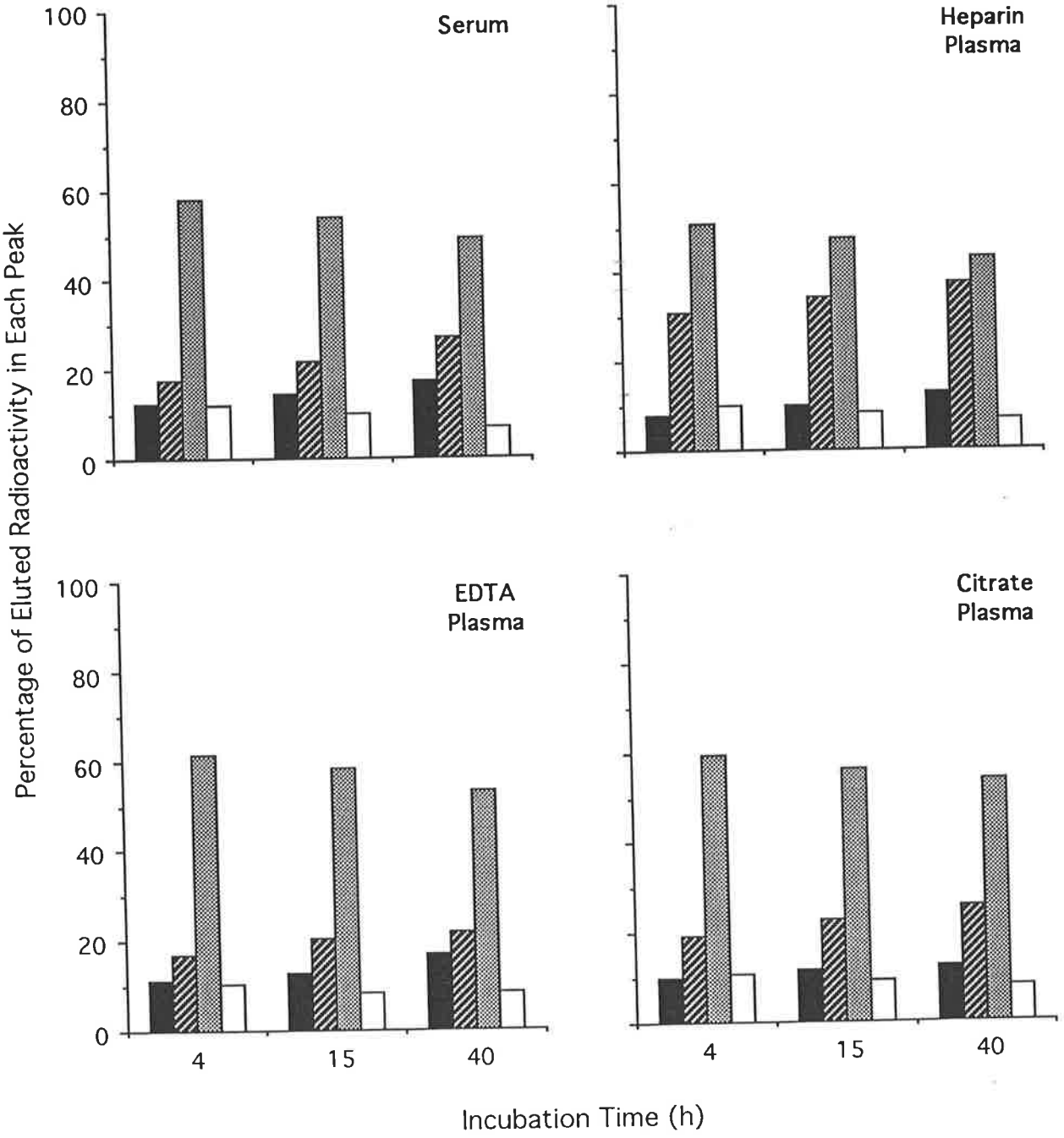
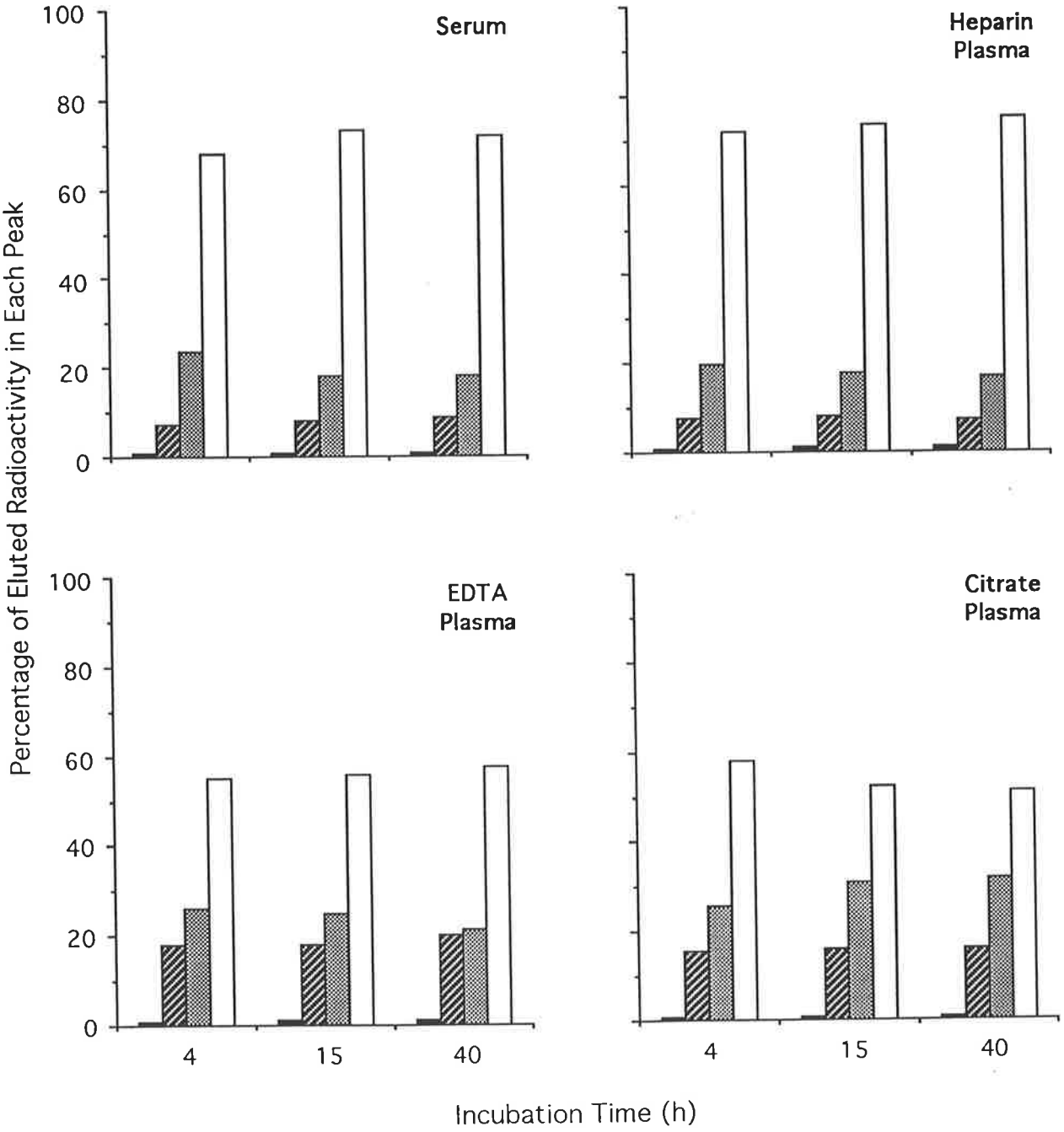

Serum
Heparin Plasma
EDTA Plasma
Citrate Plasma
Percentage of Eluted Radioactivity in Each Peak
100
80
60
40
20
0
4
15
40
Incubation Time (h)

Fig. 2.4 *Distribution of radiolabelled des(1-3)IGF-I between 250 kDa (solid), 130-150 kDa (diagonal stripe), 30-50 kDa (grey) and 8 kDa (white) regions in serum and plasma containing heparin, EDTA or citrate.* Incubations were performed at 4°C for periods of 4, 15 and 40 h, after which size-exclusion chromatography was performed as described in Fig. 2.1. Distribution of radioactivity between the four molecular mass regions was calculated as described in Section **2.4.2.**


Serum
Heparin Plasma
EDTA Plasma
Citrate Plasma
Percentage of Eluted Radioactivity in Each Peak
100
80
60
40
20
0
4
15
40
Incubation Time (h)

The demonstration of almost complete binding for IGF-I and IGF-II in the presence of heparin differs from results obtained when this anticoagulant is used in human serum. Thus Clemmons *et al* (1983) found that addition of heparin to serum, at only one tenth the concentration used in the present experiment, caused a 70% reduction in ^{125}I-labelled IGF-I binding to IGFBPs. The different results of the two studies may perhaps be attributed to differences in the ligand-binding properties of IGFBPs between species or to the use, in the present experiment, of heparinised plasma rather than heparinised serum.

2.5 LIGAND BLOTTING

Ligand blots were used to determine the molecular masses of IGFBP species in plasma, lymph and CSF and to characterise these IGFBPs with respect to binding of radiolabelled IGF-I, IGF-II and the IGF-I analogs des(1-3)IGF-I and LR^{3}IGF-I. Samples and molecular mass standards (Rainbow ^{14}C-protein molecular weight markers; Amersham International plc) which had been diluted in heparinised PBS (Section **2.3**), were incubated at 65°C for 15 min with an equal volume of Dissociating Buffer (w/v; 3.02% Tris base, 8% sodium dodecyl sulphate, 0.004% bromophenol blue and [v/v] 20% glycerol, pH 6.8) and applied to a 1.5 mm thick discontinuous sodium dodecyl sulphate-polyacrylamide gel (Laemmli, 1970). Dissociated ^{14}C-markers (15 µl), and unknowns (15 or 20 µl) containing the equivalent of 1 - 10 µl of sample, were stacked through 4% (w/v) polyacrylamide and separated in 10% (w/v) polyacrylamide at 15 mA (Hoefer SE 250 apparatus; Hoefer Scientific Instruments). The electrophoresed proteins were then transferred to nitrocellulose sheets (Schleicher and Schuell Inc.) for 45 min at 300 mA (Hoefer TE series Transphor) by the method of Towbin *et al* (1979). Subsequent steps were performed at 4°C. The nitrocellulose sheets were wetted for 15 min (150 mM NaCl, 1% [w/v] Triton X-100 [Ajax Chemicals]) and non specific binding sites blocked during a 2 h incubation with 1% (w/v) BSA in Saline/Tween (150 mM NaCl, 0.1% [w/v] Tween 20 [BDH Limited]). The sheets were then washed for 10 min in Saline/Tween and probed for 40 h with 1.5 - 10 ng of radiolabelled IGF in Saline/Tween with 1% (w/v) BSA. The nitrocellulose was washed in

two changes of Saline/Tween (30 min and 2 h), dried, and visualised by autoradiography (X-ray film; Konica).

2.6 GENERAL ANAESTHESIA

In experiments for which invasive surgical techniques were employed (cannulations of peripheral blood vessels are excluded from this category), animals were maintained under halothane anaesthesia (ICI Pharmaceuticals). Lambs were rendered unconscious either by intravenous administration of sodium pentathione (Pentothal; Bomac Laboratories Pty. Ltd.; 15 mg/kg body weight) or by administration of halothane through a face mask (5% in O_2, 2.0 l/min), as specified in the text. With the aid of a laryngoscope (Penlon), lambs were then intubated using an endotracheal tube (5-0 - 6-0; Portex Ltd.) after which they were maintained under halothane anaesthesia (1-3% in O_2 or air at 1.0 - 3.0 l/min) for the duration of the required surgical procedures. During anaesthesia, lambs were regularly tested for an eye reflex by gentle application of digital pressure to the eyeball; animals were assessed as fully anaesthetised when the eye reflex was very weak or absent.

Chapter Three

IGFBPs in Tissue Fluids of the Lamb

CHAPTER THREE: IGFBPS IN TISSUE FLUIDS OF THE LAMB

3.1 INTRODUCTION

IGFBPs are found not only in the circulation but also in lymph (Binoux & Hossenlopp; 1988) and cerebrospinal fluid (CSF; Roghani *et al;* 1989; Rosenfeld *et al;* 1989), fluids that are derived from the interstitial milieu of the potential extravascular target tissues for circulating IGFs. Because of the proximity of these IGFBPs to target tissue, and because IGFBPs have been shown to affect IGF activity (De Mellow & Baxter; 1988, Ballard *et al;* 1989*a*; Busby *et al;* 1989, Gopinath *et al;* 1989), it is likely that the bioactivity of any IGF that is transferred from the circulation will be modified by IGFBP interactions.

IGFBPs have been characterised in lymph derived from peripheral regions such as the leg in man and the ovine mammary gland, and also in CSF. These studies have shown that IGFBPs are much less abundant in lymph and CSF than in blood, and that lymph and particularly CSF are especially deficient in the higher molecular mass IGFBPs (Binoux & Hossenlopp; 1988, Hodgkinson *et al;* 1989*b*, Roghani *et al;* 1989; Rosenfeld *et al;* 1989). Thus the regulation of IGF activity by IGFBPs outside the vasculature is likely to differ to that within the vasculature.

The IGFBPs in intestinal lymph have not previously been characterised. Having selected intestine as a tissue in which to study the transfer of circulating IGFs, I chose to compare the IGF-binding characteristics and relative abundances of IGFBPs in intestinal lymph with those in plasma, using radiolabelled IGF-I, IGF-II and des(1-3)IGF-I; this would provide information as to which of the IGFs, if transferred to the extravascular space, would be subject to regulation by IGFBPs and also which IGFBPs could be involved in such regulation. Furthermore, comparisons between plasma and lymph from multiple sites have not previously been made; differences in the relative abundances of IGFBPs and the IGF-binding characteristics of these fluids would suggest tissue specificity in the regulation of IGF activity. Therefore intestinal lymph, lymphs from the hind-leg and the shoulder regions, and CSF, were compared against plasma.

The specific objectives of this research were to compare:

1) the IGFBP profile of intestinal lymph with that of plasma, lymph from peripheral sites, viz. prescapular and popliteal, and that of extravascular fluid from brain/spinal cord (CSF), by ligand blotting;

2) also by ligand blotting, the relative ligand-binding specificities of IGFBPs in these fluids, using radiolabelled IGF-I, IGF-II and des(1-3)IGF-I;

3) the IGF-binding characteristics of intestinal lymph with plasma, peripheral lymphs and CSF, using the above radioligands in competitive binding experiments;

4) the immunoreactivities of IGFBPs in different fluids using antiserum against IGFBP-2 in immunoblotting studies.

3.2 MATERIALS AND METHODS

3.2.1 Radiolabelled IGFs

Radiolabelling of peptides for the work presented in the current study was performed using $Na^{125}I$ from Amersham International plc and was catalysed by chloramine T at a final concentration of 0.10 mM, as described in Section **2.1**. Peptides used were recombinant human IGF-I, des(1-3)IGF-I (Genentech Inc.) and recombinant bovine IGF-II (Lilly Research Laboratories), iodinated to specific activities in the range 25-80 μCi/μg.

3.2.2 Collection of Blood, Lymph and CSF for *In Vitro* Analyses

Plasma, lymph and CSF samples were collected from 3- to 6-week-old Merino-Dorset crossbred lambs of either sex which were not weaned at the time of sampling but had access to lucerne chaff as part of the mother's diet. As preliminary procedures in the preparation of lambs for sample collections, wool was sheared from the hind-leg, shoulder,

neck, abdomen and lumbar regions and anaesthesia was induced by halothane (5% in O_2) delivered via a face mask. Animals were intubated with an endotracheal tube (5.0) and maintained under halothane anaesthesia (1-2% in O_2) for the duration of the surgery and sample collections, as described in Section **2.6**.

Collection of Blood and CSF:

Blood was obtained through a jugular catheter that was installed as previously described (Section **2.4.1**). Upon collection, blood was treated with heparin (10 IU/ml) for plasma preparation. To catheterise the sub-arachnoid space for CSF collection, the lamb was placed on its side and the hind legs were extended cranially to expand the inter-vertebral spaces in the lumbar region. A Tuohy spinal needle (18G; Portex Ltd.) was inserted between the vertebrae at a position that was central to the sacral tuberoscities of the pelvis, and an epidural catheter was passed into the sub-arachnoid space. CSF was drained from the mid-spinal region and heparinised as for blood samples, then placed on ice.

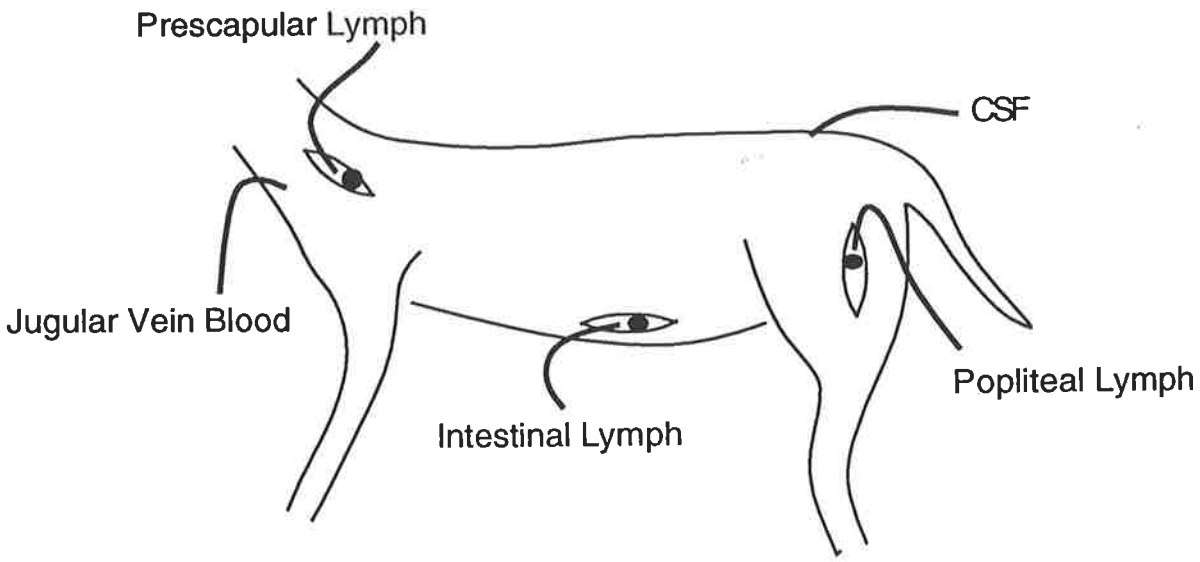
Lymph Collections:

For comparison of the IGFBP characteristics of lymph with those of plasma and CSF, lymph was collected from the efferent lymphatic ducts of the popliteal and prescapular nodes in the hind leg and shoulder regions, respectively, and from a jejuno-ileal lymph node in the small intestine. These collection points are illustrated schematically in Fig. 3.1. Access to these nodes was gained as follows:

Popliteal lymph node; With the lamb anaesthetised and laying on its side, the interface between the semitendinosis and femoral bicep muscles was located by digital palpation and an incision made in the skin directly over it. The skin was reflected away from the wound and hemostats were employed to clear connective tissue from the region. Surgical retractors were positioned to separate the two muscle groups and the popliteal lymph node was found in the opening, within a pocket of fatty tissue that was immediately caudal to the gastrocnemius muscle.

Prescapular lymph node; This node was located by digital palpation of the tissues between the shoulder tip and the base of the ear, and was identified as a semi-mobile subcutaneous tissue mass that lay approximately midway between these points. An incision was made

Fig 3.1 *Schematic diagram indicating positioning of cannulae for collection of blood, CSF, and popliteal, prescapular and intestinal lymphs.*


Prescapular Lymph
CSF
Jugular Vein Blood
Popliteal Lymph
Intestinal Lymph

in the skin over the tissue mass, in line with the ear and shoulder tip, and the two flaps of skin were reflected to expose a fatty tissue mass. The node was located on the underside of this body.

Jejuno-ileal lymph node; The animal was layed on its back and was propped with sandbags to provide lateral support. After making a midline incision in the lower abdominal wall, the small intestine was extruded through the wound. Lymph nodes draining the intestinal tissues were located adjacent to the superior mesenteric blood vessels and emptied into a single efferent lymphatic vessel. Following catheterization of this lymphatic (see following paragraph), the intestines were either returned to the peritoneum or were moistened with physiological saline solution (0.9% [w/v] NaCl) to prevent desiccation of the tissues.

Cannulation of the lymphatics was performed according to previously published methods (Lascelles and Morris, 1961). Blunt dissection techniques were used to clear connective tissue from a 5 mm portion of the lymphatic and a silk ligature (7-0) was secured around its distal aspect to cause the vessel to swell slightly. The lymphatic was nicked using butterfly scissors, and a bevelled vinyl cannula (0.5 mm ID, 0.8 mm OD) was inserted for lymph collection. The catheter was secured inside the vessel using additional silk ligatures and samples were collected continuously for a period of approximately 1 h. Lymph samples were heparinised after collection (10 IU/ml). At the end of collections and while under halothane anaesthesia, lambs were euthanased by intravenous administration of saturated KCl (20 ml); animals were not recovered because of the invasive and disruptive nature of the lymphatic cannulations.

Preparation of Plasma, Lymph and CSF:

All samples were maintained on ice until centrifugation for 20 min at 4°C and 1800 x *g* to sediment blood cells and lymphocytes. Supernatants were rapidly frozen in liquid nitrogen and were stored at -80°C until analysis. Lymph and CSF samples in which precipitated red blood cells were visible after centrifugation were discarded.

3.2.3 *In vitro* Ligand Binding Comparisons

The *in vitro* ligand binding characteristics of heparinised plasma were compared with those of the lymph and CSF samples. Fluids (125 µl) from three animals were incubated with 1.5-3.0 x 10^4 cpm IGF-II (10 µl; equivalent to approximately 0.7 ng ligand) and 15 µl heparinised PBS (Section **2.3**) containing 1% (w/v) BSA for 15 h at 4°C, frozen in liquid nitrogen and stored at -80°C. For analysis of radioligand distribution between IGFBPs, samples were thawed, de-fatted and applied to a Superose-6 size-exclusion column at room temperature, as described in Section **2.3**. Samples from another animal were incubated with ^{125}I-labelled IGF-II, IGF-I or des(1-3)IGF-I and, in some fluids, unlabelled IGF-I or IGF-II (15 µl, containing approximately 0.7 µg ligand) was added in place of buffer to demonstrate competitive binding.

3.2.4 Western Ligand Blot Comparisons

The IGFBPs contained in plasma, lymph and CSF from three animals were characterised by the ligand blot method (Section **2.5**). Molecular mass standards (15 µl) and samples (20 µl) containing the equivalent of 2.0 µl plasma, 5 µl lymph or 10 µl CSF, were run in parallel lanes through a 10% (w/v) polyacrylamide gel and were transferred to nitrocellulose for blotting with either radiolabelled IGF-I, IGF-II or des(1-3)IGF-I. The blood, lymph and CSF volumes used in this ligand blotting experiment had been selected on the basis of a preliminary experiment to determine the relative volumes at which a 35 kDa IGF-binding protein band, common to all sample types, would be similar in each fluid type. After probing, washing and drying the nitrocellulose, the positions of radioactive bands were determined by exposure to X-ray film. The time required to obtain an X-ray image of radioligand-binding bands varied according to specific activity of the radioligand at time of use, and the radioligand species used for probing. Exposure times for autoradiography are indicated in the figure legends.

Alternate fractions collected during the Superose-6 chromatography of plasma, lymph and tenfold concentrated CSF (Speed Vac evaporator; Savant Inc.), from one animal, were also ligand blotted. Equal volumes of fraction and of Dissociating Buffer (Section **2.5**) were mixed and incubated, and 15 µl of this dissociated sample was blotted using labelled IGF-II, as above.

3.2.5 Western Immunoblotting

Immunoblotting was performed at 4°C using samples from two animals. Samples were electrophoresed and transferred to nitrocellulose but before immunoblotting, the nitrocellulose was ligand blotted with IGF-II (as described above) so that alignment of the radioligand-binding material with immunoreactive bands could be used to identify those binding proteins which also bound antibody. After re-wetting of the ligand-blotted nitrocellulose filters for 15 min in 150 mM NaCl containing 1% (w/v) Triton X-100, non-specific binding sites were blocked during an overnight incubation in 150 mM NaCl containing 1% (w/v) BSA. Filters were then washed in three changes (3 x 15 min) of 150 mM NaCl containing 0.1% (w/v) Tween 20 and the nitrocellulose was incubated overnight in rabbit anti-bovine IGFBP-2 polyclonal antiserum (1/1000 in 150 mM NaCl, containing 10 mM Tris and 0.1% [w/v] Tween 20 at pH 7.4) kindly provided by Dr. B. Forbes. The filters were again washed as above, before incubation with a goat anti-rabbit alkaline phosphatase conjugate (Sigma) for 3.5 h. Further washing steps were performed, followed by development of the colour reaction for which the nitrocellulose sheets were bathed in a buffered solution containing enzyme substrate (100 mM NaCl, 100 mM Tris, 5 mM $MgCl_2$ at pH 9.5, plus 0.03% [w/v] tetrazolium blue and 0.02% [w/v] 5-bromo-4-chloro-3-indoyl phosphate). The reaction was stopped after 3-5 min by washing in EDTA (100 mM).

3.3 RESULTS

3.3.1 Western Ligand Blots

Ligand blotting was used to characterise the molecular masses and binding-specificities of IGFBPs in plasma, CSF and lymph. Six prominent IGF-binding species, with estimated molecular masses of 52, 46, 35, 28, 23.5 and 22 kDa, were revealed using radiolabelled IGF-II (Fig. 3.2*a*), and additional tracer binding was detected at 18 kDa after a long exposure. The distribution of these IGFBPs varied according to fluid type. Thus, all IGF-binding species were evident in prescapular and popliteal lymphs and all but the 22 kDa IGFBP were detected in plasma and intestinal lymph, while neither the 52 nor the 46 kDa IGFBPs were detectable in CSF . It was also apparent that the abundances of IGFBPs in lymph and in CSF were generally lower than in plasma, because the intensities of bands in the ligand blots were generally comparable when plasma, lymph and CSF were electrophoresed at 2, 5 and 10 µl, respectively. The 52 and 46 kDa bands were present at similar levels in intestinal and prescapular lymph but were present at reduced levels in popliteal lymph. The relative abundance of the 28 kDa IGFBP between the lymphs declined from intestinal lymph through prescapular and popliteal lymphs, while the 35 kDa band appeared more intensely in the latter lymphs than in intestinal lymph. Furthermore, the 35 kDa IGFBP accounted for a greater proportion of total radioligand-binding activity in prescapular lymph, popliteal lymph and particularly in CSF, than it did with plasma and intestinal lymph samples.

Ligand blots performed using labelled IGF-I detected all but the 22 and 18 kDa bands demonstrated using IGF-II ligand (Fig. 3.2*b*). The relative intensities of the 35 and 23.5 kDa bands were much lower compared with those of the 52, 46 and 28 kDa bands when IGF-I ligand was used than they were using IGF-II, indicating the greater relative affinity of the 35 and 23.5 kDa IGFBPs for IGF-II. The relative abundances of the respective IGF-I-binding bands in plasma, lymph and CSF were similar to those demonstrated when IGF-II was used as ligand.

Fig. 3.2. *Ligand blotting of IGFBPs in physiological fluids of the lamb.* Samples were electrophoresed on 10% polyacrylamide gels, transferred to nitrocellulose and autoradiographed after probing with ^{125}I-labelled IGFs as described in Section **3.2.4**. Blots with labelled IGF-II (*a*), IGF-I (*b*) and des(1-3)IGF-I (*c*) were exposed for 4, 6 and 16 h, respectively. The lanes contain 2 µl plasma (*lane 1*), 5 µl intestinal lymph (*lane 2*), 5 µl prescapular lymph (*lane 3*), 5 µl popliteal lymph (*lane 4*) and 10 µl CSF (*lane 5*). Estimated molecular masses of the IGFBPs are indicated, at *left*.

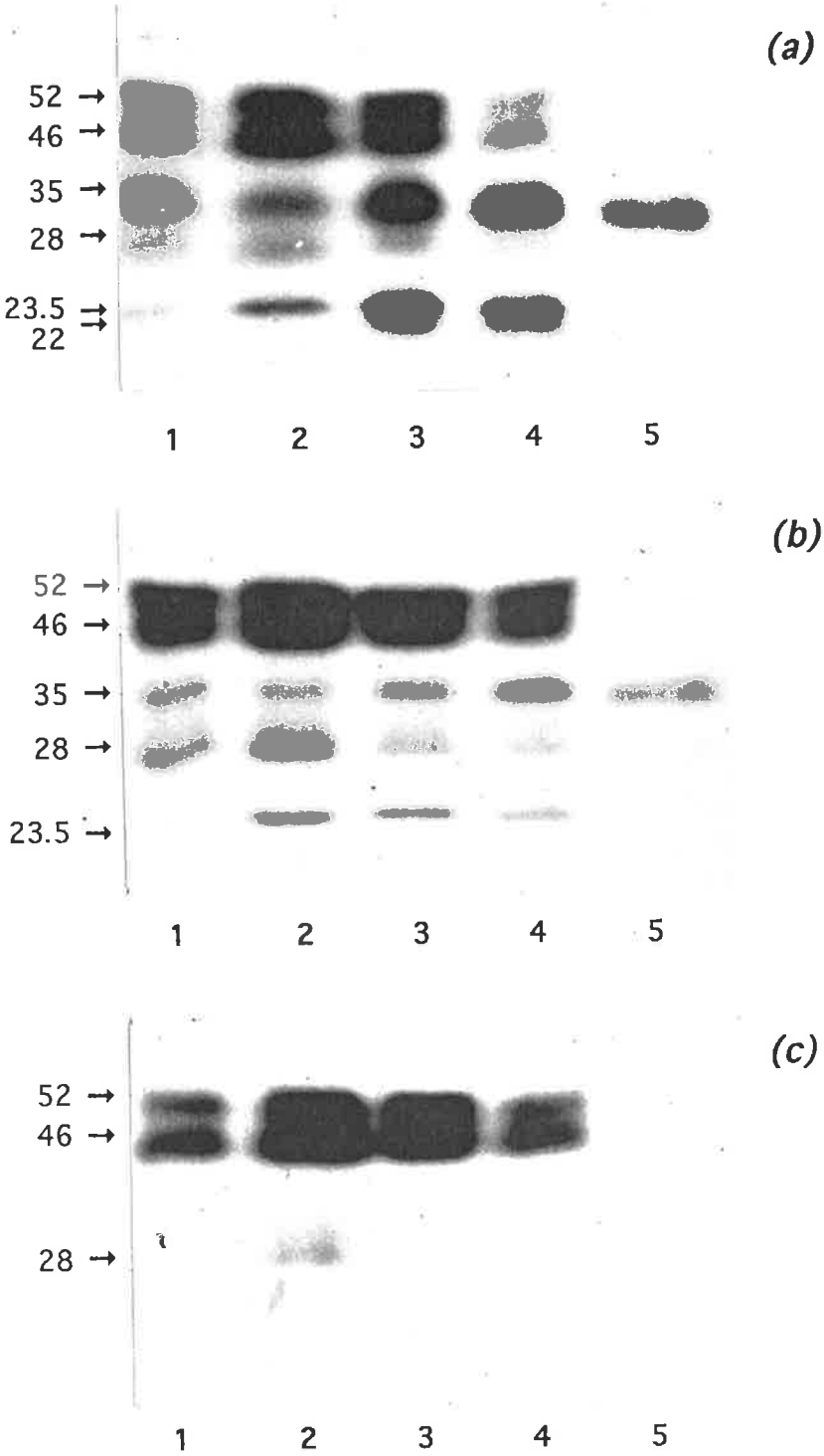
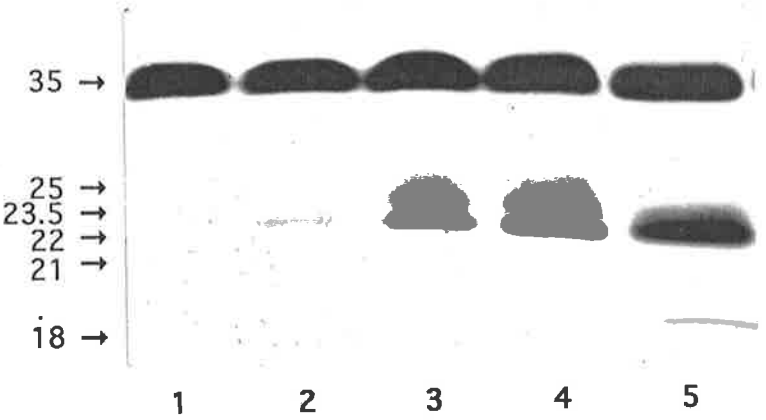

(a)
52
46
35
28
23.5
22
1
2
3
4
5
(b)
52
46
35
28
23.5
1
2
3
4
5
(c)
52
46
28
1
2
3
4
5

Fig. 3.3. *Western immunoblot of physiological fluid samples of the lamb using antiserum prepared against bovine IGFBP-2.* The lanes contain 2 µl plasma (*lane 1*), 5 µl intestinal lymph (*lane 2*), 5 µl prescapular lymph (*lane 3*), 5 µl popliteal lymph (*lane 4*) and 10 µl CSF (*lane 5*). The positions of immunoreactive proteins were detected by a colour reaction, as described in Section **3.2.5**. The positions of molecular masses of the antibody-binding bands are indicated, at *left*.


35
25
23.5
22
21
18
1
2
3
4
5

Radiolabelled des(1-3)IGF-I was used as a probe in the ligand blotting of physiological fluids to determine which of the IGFBPs did not require the three-amino acid amino-terminus of IGF-I in order to bind the ligand. It was shown (Fig. 3.2*c*) that while the 52, 46 and 28 kDa IGFBPs were able to bind labelled des(1-3)IGF-I, the 35 and 23.5 kDa IGFBPs failed to bind this ligand. Nevertheless, the relative intensity of ligand binding at the respective 52, 46 and 28 kDa IGFBP bands in different fluids was similar using des(1-3)IGF-I as a ligand, as found using IGF-I or IGF-II.

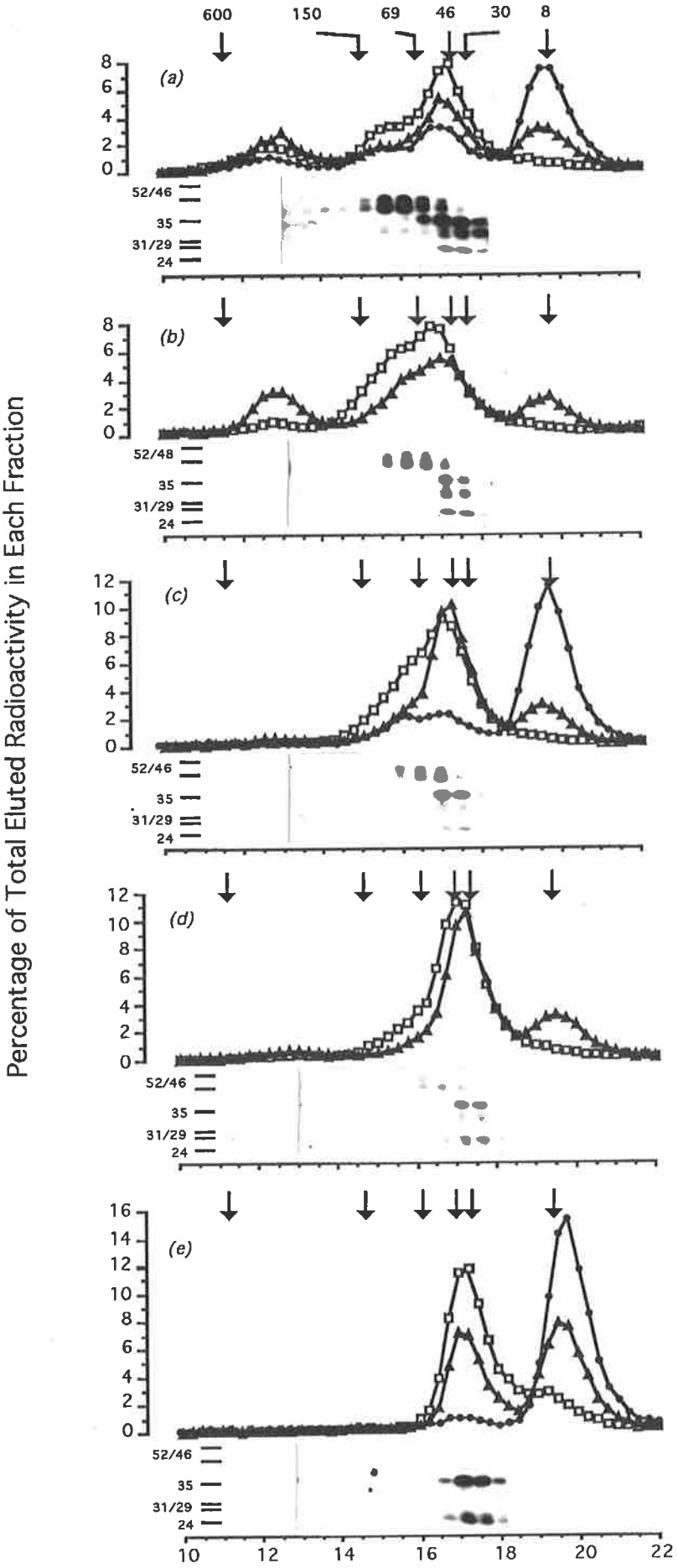
3.3.2 Western Immunoblots

Immunoblotting was performed to determine whether any of the IGFBPs identified by ligand blotting were immunologically related to IGFBP-2. The major region of antibody-binding coincided with the 35 kDa IGFBP detected in plasma, CSF and each lymph type by ligand blotting (Fig. 3.3). The antibody also cross-reacted with the 23.5, 22 and 18 kDa bands that were previously shown to bind ligand, plus bands of 25 and 21 kDa which were detected only in prescapular and popliteal lymphs.

3.3.3 *In vitro* Radioligand Binding

The *in vitro* IGF-binding characteristics of plasma, lymphs and CSF were also compared using gel filtration chromatography. Plasma and lymph bound labelled IGF-II at three regions, with estimated molecular masses of 250, 130-150 and 30-50 kDa (Fig. 3.4*a-d*), while CSF bound ligand only at the 30-50 kDa region (Fig. 3.4*e*). Radioligand binding was essentially complete in plasma and the lymphs, but some ligand eluted free of IGFBPs when incubated with CSF. The proportion of labelled IGF-II that associated at each region differed according to the fluid tested, with plasma and intestinal lymph demonstrating greater binding at the two higher molecular mass peaks than either the prescapular or popliteal lymphs (Fig. 3.4). A trend was apparent between the three lymph types, in which the

Fig. 3.4. *Elution of radioactivity from Superose-6 chromatography of lamb fluid samples incubated with radiolabelled IGF-II (squares) and also excess unlabelled IGF-I (triangles) or IGF-II (circles).* The plots show the recovery of radioactivity in 0.25 ml fractions, as a percentage of total eluted radioactivity, for plasma (*a*), intestinal lymph (*b*), prescapular lymph (*c*), popliteal lymph (*d*) and CSF (*e*). Samples (100 µl) were chromatographed as described in Section **3.2.3**. The molecular masses of protein markers are indicated. Following chromatography, alternate fractions of plasma and lymph and of tenfold concentrated CSF were ligand blotted using IGF-II tracer, as described in Section **3.2.4**. The binding proteins contained in these fractions are shown aligned according to elution volume, with estimated molecular masses indicated at *left*. X-ray exposures were 6 days for plasma and lymphs, and 2 days for CSF


600
150
69
46
30
8
(a)
(b)
(c)
(d)
(e)
52/46
35
31/29
24
52/48
Percentage of Total Eluted Radioactivity in Each Fraction
Elution Volume (ml)
10
12
14
16
18
20
22

proportion of ligand that associated at the 130-150 kDa binding region was greatest in intestinal, intermediate in prescapular and least in popliteal lymph. Binding activity at the 250 kDa region was apparent in intestinal lymph but was barely detectable in prescapular or popliteal lymph.

Inclusion in the incubations of unlabelled IGF-I at 1000 fold the amount of IGF-II tracer enabled comparisons of the relative affinities of each ligand-binding region to be made between the fluids (Fig. 3.4). In plasma and in intestinal lymph, the proportion of IGF-II tracer eluting at 130-150 and 30-50 kDa was decreased, while that at 250 kDa and as free ligand were increased. Similar competition experiments in prescapular and popliteal lymph resulted in decreased association of tracer at 130-150 kDa, increased free ligand and slightly increased binding at 250 kDa, but failed to reduce the proportion of tracer eluting at 30-50 kDa. In some fluids, additional competition experiments demonstrated specificity of binding by labelled IGF-II, since addition of excess unlabelled IGF-II resulted in reduced radioligand binding at all size regions.

Ligand blotting of every second fraction eluted from the Superose-6 column was carried out using labelled IGF-II in order to compare the elution positions of IGFBP species in each fluid with the *in vitro* IGF-binding profiles. It was shown (Fig. 3.4) that the 52 and 46 kDa IGFBPs eluted predominantly within the 130-150 kDa region but that a smaller proportion eluted at 30-50 kDa with the lower molecular mass IGF-binding species. The volume at which the 52 and 46 kDa species eluted varied between fluids, with peaks between 15.5 and 16.5 ml in plasma and intestinal lymph but somewhat later, 16.0 to 17.0 ml, in prescapular and popliteal lymph. Elution of the smaller IGFBP species peaked between 17.0 and 17.5 ml in all fluids.

In plasma and lymph, radiolabelled IGF-I associated at the 130-150 and 30-50 kDa regions (Fig. 3.5*a-d*), with binding near complete in each fluid. In CSF, however, IGF-I bound only at 30-50 kDa (Fig. 3.5*e*) and a significant proportion of the radioactivity eluted at the position of free ligand. In plasma and lymph, most labelled IGF-I was bound at 30-50 kDa with the remainder eluting at 130-150 kDa. Binding at 130-150 kDa was

greatest in prescapular lymph, intermediate in intestinal lymph and least in popliteal lymph, a pattern that differed to the trend observed when IGF-II was employed (Fig. 3.4). Elution of radiolabelled IGF-I at the high molecular mass region was approximately 0.5 ml later in these lymphs than in plasma.

As was shown using IGF-I, radiolabelled des(1-3)IGF-I associated only at 30-50 and 130-150 kDa (Fig. 3.6). The proportion of this ligand that eluted at 130-150 kDa was similar in all lymphs and was higher than in plasma, although a greater proportion was bound at 30-50 kDa in plasma compared with the lymphs. Association of labelled des(1-3)IGF-I was incomplete in all fluids tested, particularly in CSF in which it failed to bind altogether. With the inclusion of excess unlabelled IGF-I in the incubation, the proportion of radioactivity that eluted free in the plasma and lymph incubations was increased to include most labelled des(1-3)IGF-I.

The protein absorbance profile in eluates from Superose-6 chromatography was used to compare the albumin concentrations between fluids as an index of total protein content. Thus the relative abundance of the eluted albumin peak at 280 nm in fluids from two sheep averaged 1.0 for plasma, 0.49 for intestinal lymph, 0.63 for prescapular lymph, 0.44 for popliteal lymph and 0.05 for CSF.

Fig. 3.5. *Elution of radioactivity from Superose-6 chromatography of lamb plasma (a), intestinal lymph (b), prescapular lymph (c), popliteal lymph (d) and CSF (e) incubated with labelled IGF-I (squares) and with the addition of excess unlabelled IGF-I (triangles) to some fluids.* The plots show the recovery of radioactivity in 0.25 ml fractions, as a percentage of total eluted radioactivity. Samples were chromatographed as described in Section **3.2.3**. The molecular masses (kDa) of protein markers are indicated.

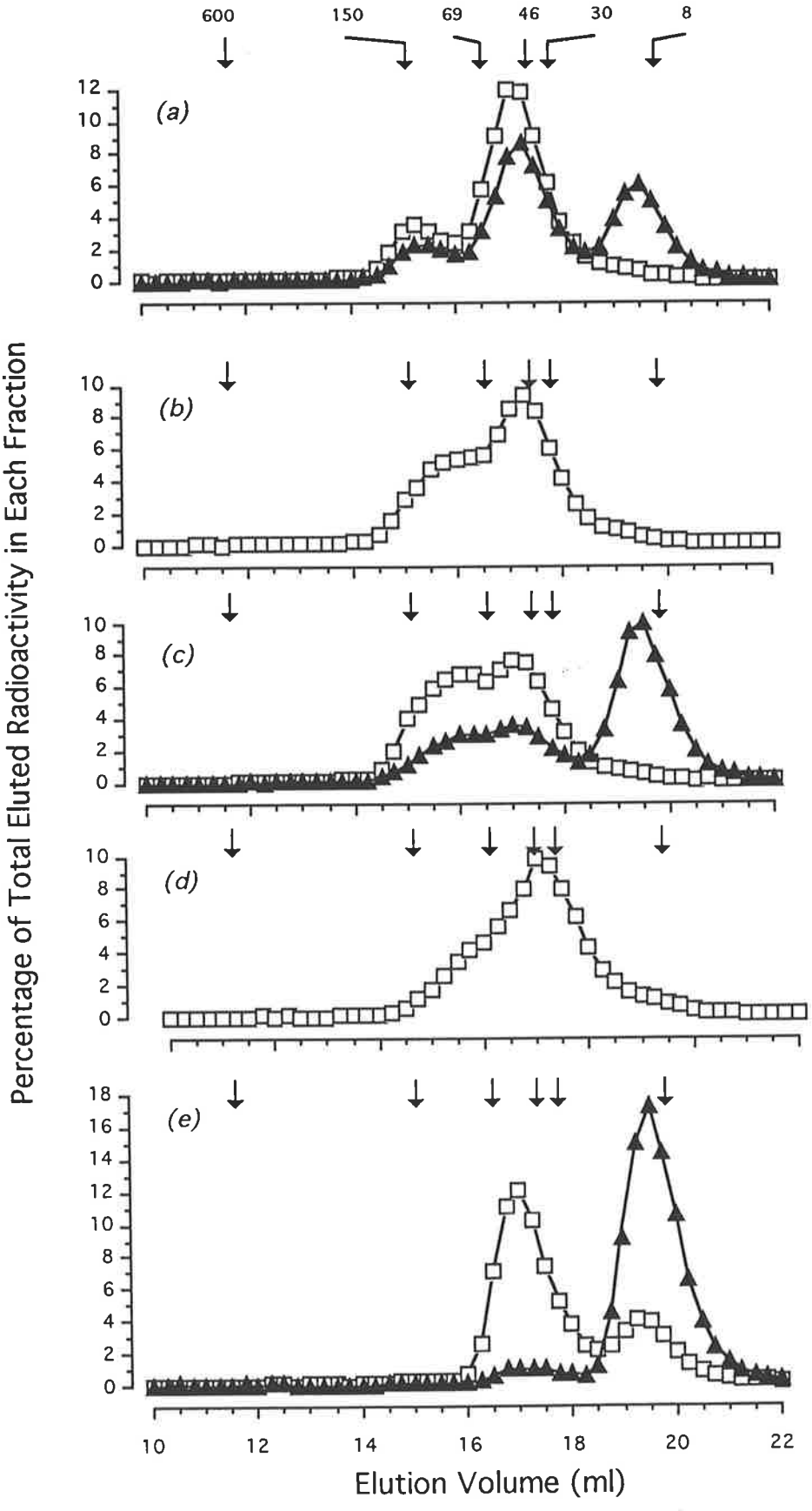
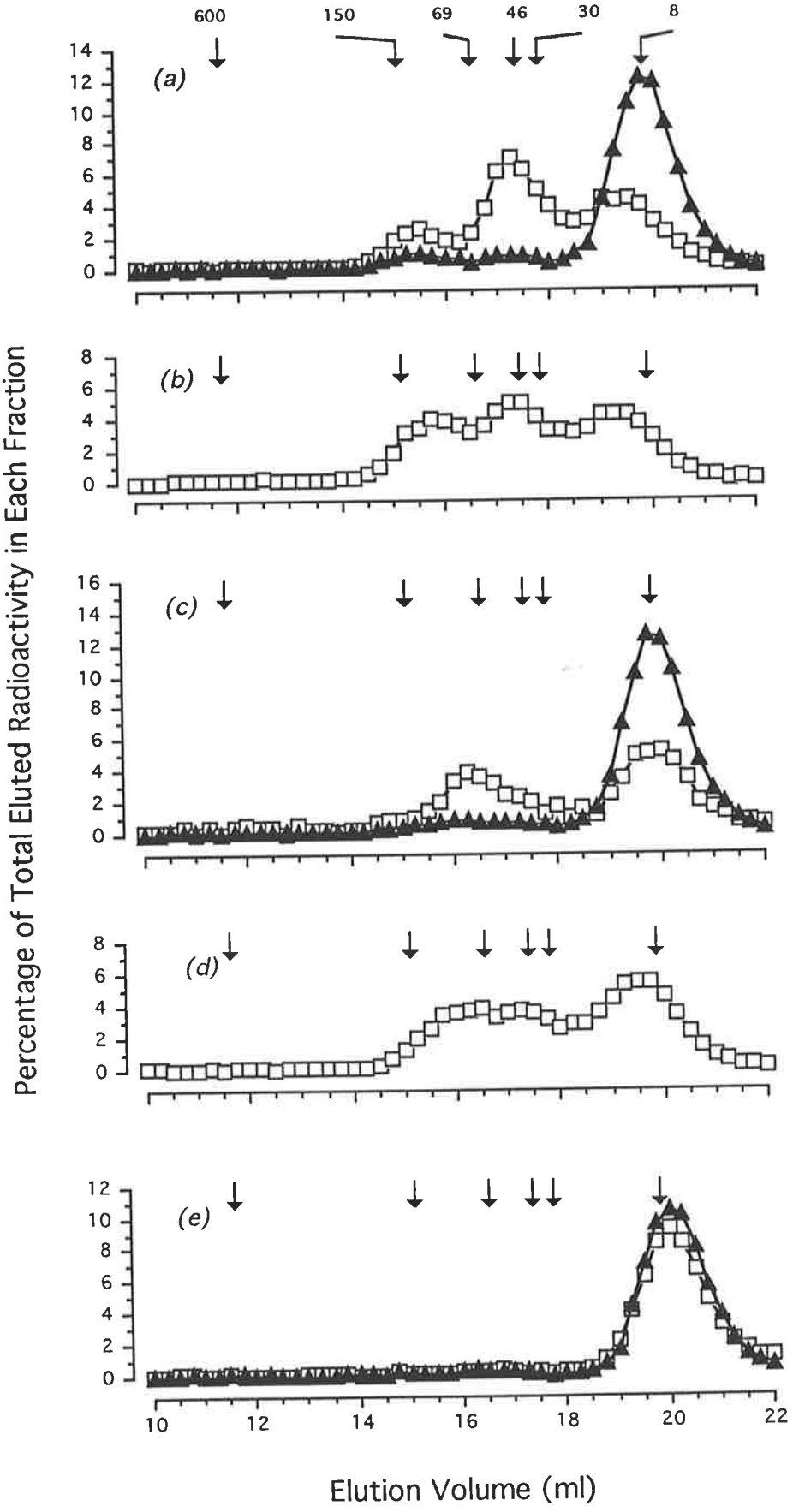

600
150
69
46
30
8
(a)
(b)
(c)
(d)
(e)
Percentage of Total Eluted Radioactivity in Each Fraction
Elution Volume (ml)
10
12
14
16
18
20
22

Fig. 3.6 *Elution of radioactivity from the Superose-6 chromatography of lamb fluid samples incubated with labelled des(1-3)IGF-I (squares) and with the addition of excess unlabelled IGF-I (triangles) to some fluids.* The plots show the recovery of radioactivity in 0.25 ml fractions for plasma (*a*), intestinal lymph (*b*), prescapular lymph (*c*), popliteal lymph (*d*) and CSF (*e*), as a percentage of total eluted radioactivity. Samples were chromatographed as described in Section **3.2.3**. The molecular masses (kDa) of protein markers are indicated.


600
150
69
46
30
8
(a)
(b)
(c)
(d)
(e)
Percentage of Total Eluted Radioactivity in Each Fraction
Elution Volume (ml)

3.4 DISCUSSION

This study compares the IGF-binding characteristics of plasma with those of CSF and lymph from the intestinal, prescapular and popliteal nodes, using radiolabelled IGF-I, IGF-II and des(1-3)IGF-I. The data show the existence in plasma and lymph of IGF-binding activity at three molecular mass regions (250, 130-150 and 30-50 kDa), similar to those described by Hodgkinson *et al* (1989*b*) in the plasma of adult and fetal sheep. The properties of the 250 kDa material, including competition of labelled IGF-II binding by unlabelled IGF-II but not by IGF-I, were comparable to those obtained with the soluble type-II/mannose 6-phosphate receptor in fetal sheep plasma (Gelato *et al;* 1989). In comparison with the ligand binding achieved at this region in plasma, the relatively minor association occurring in tissues drained by the prescapular and popliteal lymph nodes would suggest that its access to or production from these tissues is greatly restricted, while the much greater association in intestinal lymph implies that these restrictions are relatively minor in the intestine, or that the gut may be a source of this receptor-like material.

Distribution of labelled IGF-II between the 130-150 and 30-50 kDa molecular mass regions following Superose-6 chromatography of plasma and lymph varied according to the fluid type. The progressive decrease in binding of IGF-II at the 130-150 kDa region from intestinal lymph through the prescapular and popliteal lymphs was accompanied by a corresponding rise in association of radioligand in the 30-50 kDa region. An explanation for this progression between lymphs may be the different relative abundances of IGF-II-preferring IGFBPs at each size region. That this is indeed the case is revealed by the ligand blots; IGF-II-preferring IGFBPs were more abundant in prescapular and popliteal lymphs than in intestinal lymph or in plasma, and eluted predominantly in the 30-50 kDa region. Further support for this is gained from the binding profiles of radiolabelled IGF-II in the native fluids when excess unlabelled IGF-I is also included in the incubation. Accordingly, association of radiolabelled IGF-II was inhibited by IGF-I more effectively at 130-150 kDa than at 30-50 kDa in the prescapular and popliteal lymphs but not so in plasma or intestinal lymph. Another factor that may contribute to the trend towards reduced binding of IGF-II at 130-150 kDa across the lymphs could be a lower abundance of the IGFBP-3 subunits of this

high molecular mass complex (Baxter & Martin; 1989). This appears to be true for popliteal lymph, in which the 52 and 46 kDa IGFBPs, proteins now identified by sequencing as ovine IGFBP-3 (Walton *et al;* 1989*a*), were less abundant than in prescapular and intestinal lymphs.

The trend towards decreased radioligand binding at 130-150 kDa, from intestinal lymph through prescapular to popliteal lymph, was not observed when labelled IGF-I was substituted for labelled IGF-II; binding of IGF-I at this region was greater in prescapular than in intestinal lymph. This may be explained, as above, by differences in the relative abundances of IGF-I and IGF-II-preferring IGFBPs between fluids. Indeed, the results show that the abundance of IGF-I-preferring IGFBPs at 30-50 kDa is higher in intestinal lymph than in prescapular lymph (Fig. 3.4). Thus the competition for binding of IGF-I would be greater at this region in intestinal lymph than in prescapular lymph.

The results of *in vitro* ligand-binding experiments using labelled des(1-3)IGF-I show that formation of the 130-150 kDa complex was similar in all lymphs. Because this ligand was present in excess of the binding capacity of the fluids, and because the 52 and 46 kDa doublet was more abundant in intestinal and prescapular lymph than in popliteal lymph, the similar binding of des(1-3)IGF-I in all lymphs would suggest that the α-subunit is the limiting factor in the formation of high molecular mass IGF-IGFBP complexes in intestinal and prescapular lymphs. Perhaps IGF-I and IGF-II would have bound in similar amounts at 130-150 kDa in all lymphs if radioligand had been included in the incubations in excess of binding capacity.

The ligand blots show that des(1-3)IGF-I is able to bind the IGFBP-3 doublet and the 28 kDa IGF-binding protein. However, the results of the competitive binding studies in native plasma, lymph and CSF suggest that the affinity of des(1-3)IGF-I for these IGFBPs is lower than that of IGF-I. This is demonstrated most clearly in the *in vitro* incubations, where binding of labelled des(1-3)IGF-I, unlike that of labelled IGF-I or IGF-II, was incomplete in all fluids tested. Furthermore, excess unlabelled IGF-I more effectively inhibited binding of labelled des(1-3)IGF-I than it did labelled IGF-I or IGF-II. The lower affinity of the IGF-I

analog for IGFBPs reflects the poorer affinity of des(1-3)IGF-I than of IGF-I or IGF-II for purified human IGFBP-3 (Forbes *et al;* 1988).

The apparent absence from sheep CSF of IGFBP-3, a binding protein that forms part of the circulating high molecular mass IGF complex (Baxter & Martin; 1989), would suggest that unless it is rapidly degraded in the brain, this complex and its ligand-binding subunits do not cross into CSF and are not synthesised locally. The apparent absence of high molecular mass IGFBPs from ovine CSF appears to be similar to CSF of the rat, a fluid in which the major IGF-binding species is a 30 kDa IGFBP and the larger IGFBPs are not detectable (Gargosky *et al;* 1990). These species may differ to the human in which higher molecular mass IGFBPs have been detected, albeit at very low levels (Hossenlopp *et al;* 1986; Rosenfeld *et al;* 1989, Roghani *et al;* 1991). Nevertheless, it is not clear whether IGFBP-3 is normally expressed in human CSF since Lamson *et al* (1989) failed to detect it in this fluid.

Indirect evidence has been presented for tissue-specificity in IGFBP production. Thus the 35 kDa IGFBP was much more abundant in CSF than could be expected if it were derived only from the plasma, as the levels of this binding activity that were detected by ligand blot of only 10 µl of CSF were comparable to those in 2 µl of plasma, while the protein content of CSF was less than 5% of plasma. Since the vascular endothelium in central tissues, unlike that in most other tissues, is characterised by the presence of tight intravascular junctions as well as a virtual absence of fenestrations (Betz & Goldstein; 1984), the apparent lack of transfer of most plasma IGFBPs into CSF is not surprising. Perhaps much of the IGFBP-2-immunoreactivity in CSF is synthesised within the brain.

The similar levels of albumin in all lymphs would suggest that there are only minor differences in the transfer of total plasma proteins to the extravascular spaces of tissues drained by these lymphs. Therefore differences in the relative amounts of each IGFBP, between lymphs, may reflect tissue specific synthesis. Accordingly, the 35 kDa binding protein was more prominent in prescapular lymph than in intestinal lymph, while in popliteal lymph it was the most abundant binding protein detected by ligand blots with labelled IGF-II.

Additional evidence of tissue specificity for binding proteins was obtained from the distribution of the 28 and 22 kDa binding proteins between the three lymphs. Thus the 28 kDa IGFBP was most abundant in intestinal lymph and least so in popliteal lymph, while the 22 kDa IGFBP was absent from intestinal lymph and also from plasma. IGFBP-3, on the other hand, may be released by tissues that contribute to the prescapular and intestinal lymphs. Clearly, these lymphs do not simply reflect ultrafiltrates of plasma.

The results show that the 35 kDa IGFBP detected in CSF, lymph and plasma cross-reacted strongly with anti-bovine IGFBP-2, suggesting that this protein is the ovine equivalent of bovine IGFBP-2. That IGFBP-2 is the major IGFBP in CSF is consistent with data in the rat in which the major CSF IGFBP species is immunoprecipitable by antiserum raised against rat IGFBP-2 (Lamson *et al;* 1989).

The cross-reactivity of the anti-bovine IGFBP-2 with the 18-25 kDa bands suggests that these proteins may be structurally related to the 35 kDa IGFBP. Although the 23.5 kDa IGFBP has since been purified and identified as a structurally distinct IGFBP (IGFBP-4: Walton *et al;* 1990), its crossreactivity with antisera against IGFBP-2 would suggest that some epitopes are shared. Perhaps the other bands are IGFBP-2-degradation products. That this may be the case is supported by their failure, and that of the 35 kDa IGFBP-2-immunoreactive protein, to bind radiolabelled des(1-3)IGF-I, a ligand which lacks the three N-terminal amino acids required for binding to IGFBP-2 (Szabo *et al;* 1988). Since IGFBP-2-immunoreactive proteins were the only IGF-binding species present in ovine CSF and because they did not appear to bind des(1-3)IGF-I, this potent analog of IGF-I (Ballard *et al;* 1987) is unlikely to be subject to regulation by IGFBPs in central tissues.

3.5 SUMMARY

The work presented in this chapter has demonstrated several important points:

1) The distribution of IGFBPs in lymph from small intestine differs to that of IGFBPs in lymph derived from peripheral tissues, and to CSF. Plasma is more similar to intestinal

lymph than it is to the peripherally-derived lymphs or to CSF, with respect to its IGF-binding characteristics and the relative abundances of its IGFBPs.

2) Because of the greater similarities in relative abundances of individual IGFBPs, and the IGF-binding characteristics of, plasma and intestinal lymph than of plasma and peripheral lymphs or CSF, it is likely that regulation of IGF activity by IGFBPs in the vasculature would be more like that in intestinal tissue. The differences in the relative abundances of IGFBPs combined with their different ligand-binding characteristics suggest that IGF activity may be regulated in a tissue-specific manner.

3) The greater proportion of IGFBP-3 in intestinal and prescapular lymphs than in popliteal lymph suggests that the tissues drained by the intestinal and prescapular nodes may be sites of synthesis for this binding protein. Nonetheless, the greater abundance of the smaller molecular mass IGFBPs in the prescapular and popliteal lymphs compared with intestinal lymph suggests that peripheral tissues may be sources of these IGFBPs.

4) The 35 kDa IGFBP, a protein which appears to be present at greater concentrations in prescapular and popliteal lymphs than in intestinal lymph, which also is the major IGF-binding species in CSF, is IGFBP-2-immunoreactive and would appear to have a greater affinity for IGF-II than for IGF-I. The central nervous system may be a site of synthesis for IGFBP-2. It is also noteworthy that the 22 kDa IGFBP, present only in the peripherally-derived lymphs and in CSF, is also IGF-II-preferring. Combined, these results suggest that the activity of IGF-II in tissues drained by these fluids would be subject to greater regulation than in the vasculature or in intestine.

5) While labelled IGF-I and IGF-II each bound to five IGFBP bands in both plasma and intestinal lymph, the binding of labelled des(1-3)IGF-I was limited to only three IGFBPs. These data, combined with the poorer affinity of des(1-3)IGF-I than of IGF-I or IGF-II for IGFBPs, suggest that des(1-3)IGF-I will be a useful tool in the investigation of IGFBP roles in the transfer of circulating IGF to tissues.

3.6 ACKNOWLEDGMENTS

The iodination reaction and purification of labelled IGFs were performed by Dr P.C. Owens and Mr S.E. Knowles and Mr M.A. Conlon. All surgical and cannulation procedures in this study were performed by myself in conjunction with Dr A.A. Martin. Drs L.C. Read, G. McIntosh and T. Smeaton are kindly acknowledged for their demonstrations, in lambs, of cannulation techniques for the intestinal, popliteal and prescapular= lymph nodes, respectively. Dr T. Kuchel is thanked for demonstration of CSF collection from the sub-arachnoid space of the lower spine, in sheep.

Chapter Four

Transfer of ^{125}I-Labelled IGF-I and its Analogs, Des(1-3)IGF-I and LR^{3}IGF-I, from Blood to Small Intestine.

CHAPTER FOUR: TRANSFER OF ^{125}I-LABELLED IGF-I AND ITS ANALOGS, DES(1-3)IGF-I AND LR^{3}IGF-I, FROM BLOOD TO SMALL INTESTINE.

4.1 INTRODUCTION

IGF-I is produced by the liver at a rate that can account for its circulating concentrations (Schwander *et al;* 1983). However, because this organ is virtually devoid of the type-I receptor (see Section **1.3**), hepatic IGF is unlikely to act in an autocrine or paracrine manner. Nevertheless, because IGF receptors are otherwise widespread, IGF secreted by the liver is presumed to serve an endocrine role, circulating from its site of synthesis to bind receptors in other target tissues.

For circulating IGF to act at tissue receptor sites, transfer from blood to tissue is a pre-requisite. That IGFs are indeed transferred from the circulation is supported by a range of studies: when IGF-I is chronically infused, growth effects are apparent in a variety of tissues and organs, including gut (Schoenle *et al;* 1982, 1985, Phillips *et al;* 1988*b*, Read *et al;* 1992); radiolabelled IGFs have been detected in extravascular tissue after acute infusion into the isolated heart or into the whole animal (Bar *et al;* 1988, Hodgkinson *et al;* 1991); radiolabelled IGFs are recovered in milk and in lymph after infusion into the circulation (Hodgkinson *et al;* 1991, Prosser *et al;* 1991, Prosser *et al;* 1992). These experiments provide qualitative evidence that IGF is transferred from the circulation. However, no study has yet quantitated IGF transfer to tissue.

Most circulating IGF is complexed with IGFBPs, mainly in the 130-150 kDa complex (Baxter; 1988*a*, Baxter & Martin; 1986). The roles of the IGFBPs in regulation of IGF transfer are controversial. Using a heart perfusion model, Bar *et al* (1990*a*) showed that transfer of IGF-I to tissue was not altered by association with an endothelial cell-derived IGFBP, now known to be IGFBP-4 (Boes *et al;* 1992). However, a role for binding proteins such as IGFBP-1 and IGFBP-2 in tissue targeting of IGF is suggested by the existence of an RGD sequence within their primary structures (Brewer *et al;* 1988, Brown *et al;* 1989). In fibronectin, for example, this sequence allows cellular attachment via the RGD

receptor (Pierschbacher *et al;* 1982, Ruoslahti & Pierschbacher; 1987), and the possibility that the RGD sequence supports a similar capacity in IGFBPs is strengthened by the demonstration that synthetic RGD peptide competitively inhibits binding of IGFBP to cells (Brewer *et al;* 1988). Which tissues are actually targeted by IGFBPs via the RGD site, if any, will depend upon tissue expression of the RGD receptor.

A more general role for IGFBPs as inhibitors of IGF transfer is inferred from the results of IGF bio-potency trials and clearance studies in which IGF-I has been compared with analogs that bind IGFBPs poorly. In the rat, des(1-3)IGF-I, an analog which binds less well to IGFBPs than IGF-I but which bears a similar affinity for the type-I receptor as IGF-I (Bagley *et al;* 1991), causes greater growth effects than IGF-I (Lemmey *et al;* 1991). LR^3IGF-I, an analog of IGF-I that binds even more poorly to IGFBPs than des(1-3)IGF-I, is more potent than either IGF-I or des(1-3)IGF-I despite having a lower affinity for the type-I receptor (Francis *et al;* 1992). Clearance of analogs that bind poorly to IGFBPs is more rapid than that of IGF-I in the rat (Walton *et al;* 1991). In the sheep, association of IGFs with circulating IGFBPs is associated with lower rates of clearance, particularly for IGFs in the 130-150 kDa complex (Davis *et al;* 1989, Francis *et al;* 1988*a*). These data are consistent with a general inhibitory role for IGFBPs in tissue-transfer of IGFs.

In this chapter I aimed to compare IGF-I transfer with that of the analogs des(1-3)IGF-I and LR^3IGF-I (LR^3IGF-I was not employed for experiments in previous chapters as it was not available when those studies were performed), using a model in which IGF transfer could be quantitated. I hypothesised that greater proportions of the analogs than of IGF-I would be transferred from the circulation to tissue. Based upon the relative biological potencies of IGF-I, des(1-3)IGF-I and LR^3IGF-I, I predicted transfer in the order LR^3IGF-I > des(1-3)IGF-I > IGF-I.

Specific aims of this chapter were:

1) Development of a vascular perfusion system with which to study IGF transfer from the vascular space to the tissue;.

2) Use of the model to compare transfer of IGF-I, des(1-3)IGF-I and LR^3IGF-I when infused at trace concentrations over a period during which the concentration of infusate attains equilibrium in blood.

4.2 MATERIALS AND METHODS

4.2.1 The Vascular Perfusion Model

Preliminary Procedures:

Perfusions were performed in 6-week old Merino-Dorset crossbred lambs, of either sex, which were not weaned at the time of experimentation but also had access to lucerne chaff and pasture fodder as part of the mother's diet. Anaesthesia was induced by halothane anaesthetic delivered via a face mask, after which lambs were intubated for continued delivery of anaesthetic via an endotracheal tube (Section **2.6**) for the duration of the surgical procedures and vascular perfusion. To enable monitoring of blood pressure and heart rate, and for the infusion of donor blood, catheters were installed in the cranial tibial artery and saphenous vein, respectively (Section **2.4.1**). Blood pressure and heart rate were monitored using a Statham pressure transducer (model 13119) which had been calibrated for pressures in the range of 0-150 mm Hg at 25 mm Hg intervals. A trace was recorded (Rickadenki B-140; Kogyo Co. Ltd.) at intervals of 15-30 min as a permanent record.

The lamb was laid on its back and lateral support was provided by sand-bags. The lower abdomen was shaved and a midline incision, approximately 15 cm in length, was made in the peritoneal wall. Bleeding from transected blood vessels was controlled by application of surgical clamps. Polyethylene sheeting was stapled around the perimeter of the peritoneal opening and was spread over the abdomen to provide a passive surface onto which

the intestine could be exteriorised. The small intestine was gently manipulated through the opening and was covered with polyethylene sheeting to minimise evaporative cooling. The tissue was also warmed by contact with a coil of plastic tubing through which heated water (37°C) was pumped: in this manner, intestinal temperature was maintained within 1-2°C of internal temperature. Intestinal temperature was monitored using a digital temperature probe while rectal temperature was measured using a standard glass thermometer.

Cannulations in Small Intestine:

A preliminary requirement for the development of the vascular perfusion model was the selection of a suitable region of intestine: 1) the intestinal vasculature had to be amenable to isolation from blood vessels that served adjacent intestinal regions, 2) both the artery and vein serving the area had to be accessible for installation of catheters, and 3) the lymphatic drainage of the region had to be accessible for cannulation. All criteria were met by a region of distal intestinal ileum, located about 1 m proximal to the caecum (Fig. 4.1). This portion of gut was served by several co-lateral branches (*1*) from the superior mesenteric artery (*2*) and vein(*3*), the major blood vessels serving the intestine. Lymph from the tissues was drained into a single lymph node (*4*) from which it flowed via a single efferent lymphatic. Generally most of the vascular branches in the distal ileum were well-suited to cannulation and perfusion so that should problems arise in one attempt, the opportunity would exist for perfusion of an alternative segment. In contrast to the ileum, the duodenal and jejunal vasculature were relatively ill-suited to cannulation because the often short lengths of the blood vessels in these regions made them difficult to cannulate.

A segment of distal small intestine was prepared for vascular perfusion with the final preparation represented schematically in Fig. 4.2. This preparation was achieved as follows:

1) The blood supply of a 10 cm segment of distal ileum, served by a single vascular branch from the superior mesenteric artery and one from the vein, was isolated from the blood supply of adjacent intestinal tissues by the application of silk ties. Ligatures (4-0) were secured around vascular anastomoses between the vessels serving the 10 cm length of intestine and those serving adjacent intestinal tissue, so that all blood entering the segment

Fig. 4.1 *The distal ileal intestine of the lamb.* The ileum is served by co-lateral branches (*B*) from the superior mesenteric artery (*A*) and vein (*V*), with lymph collected from a single lymph node (*LN*).

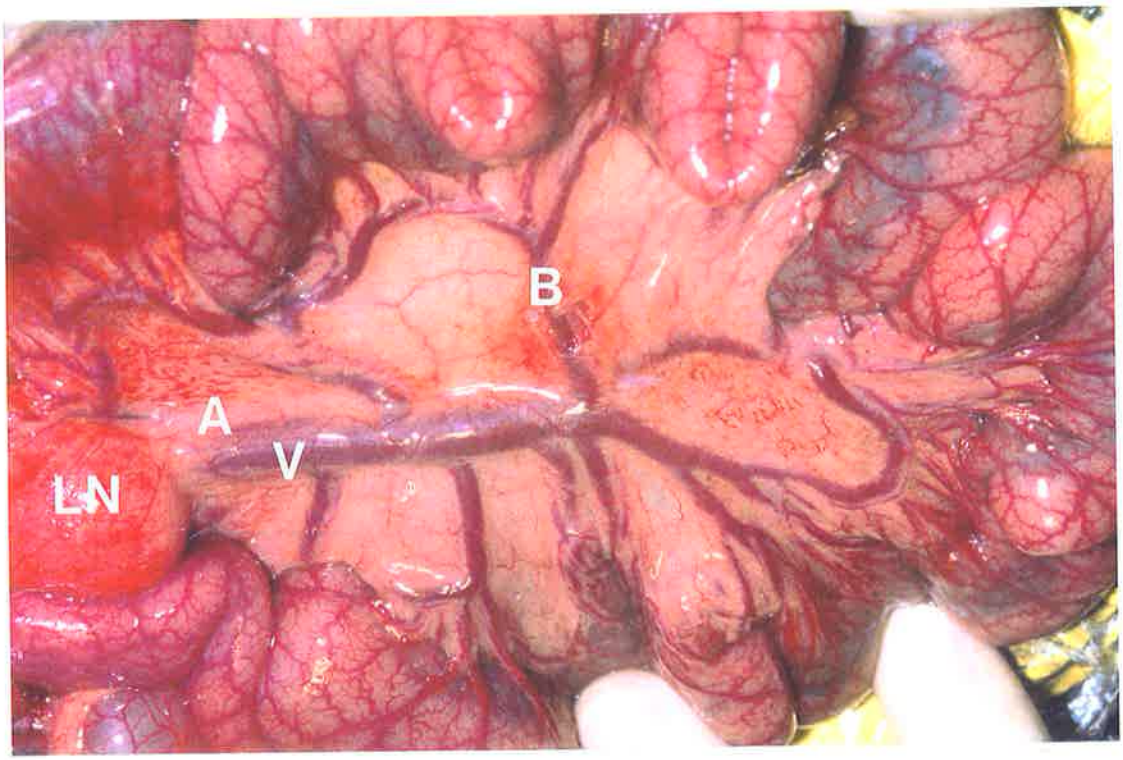
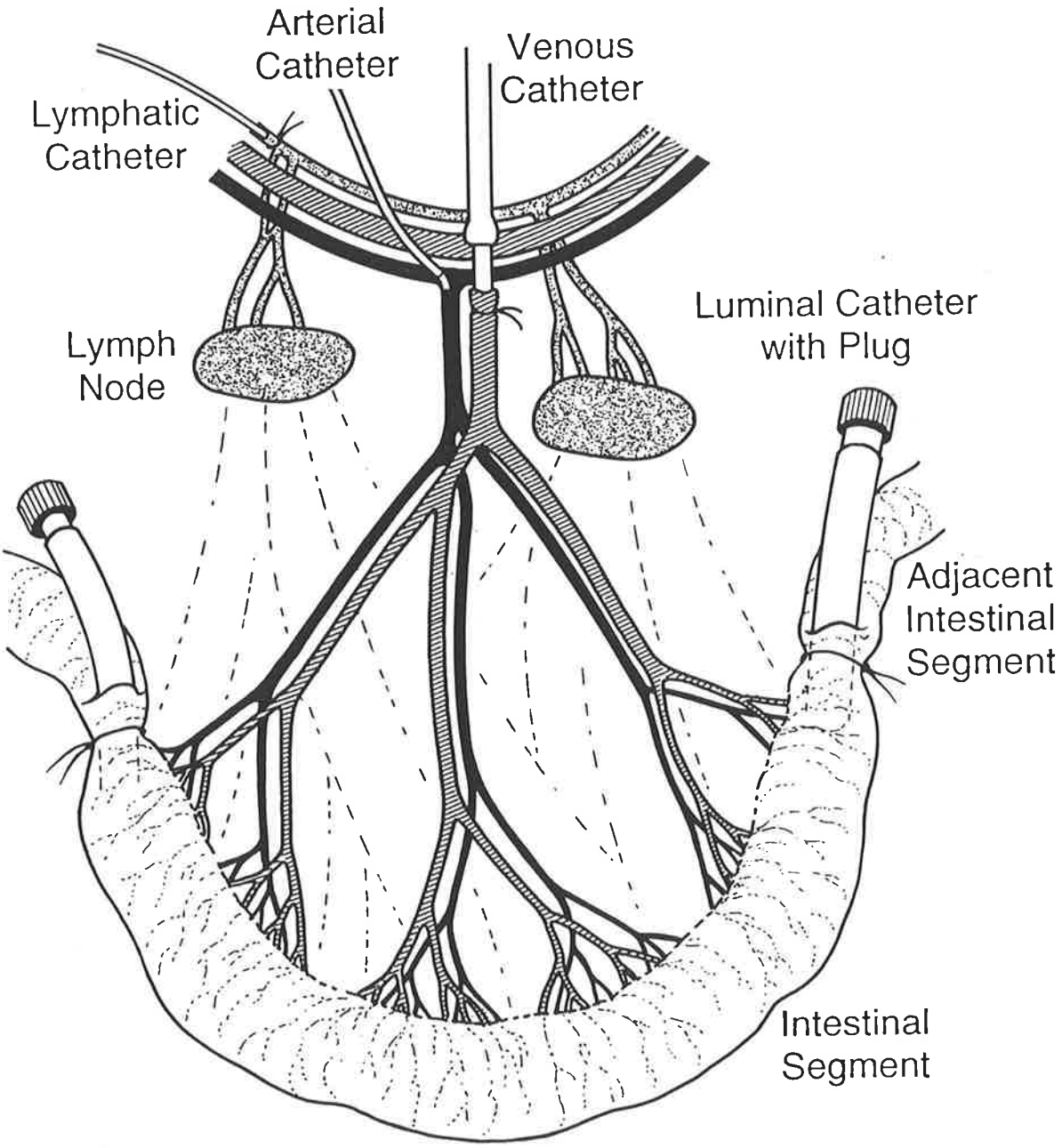

B
A
V
LN

Fig 4.2 *Schematic diagram of the vascularly perfused intestinal segment, with cannulae in place.*


Arterial
Catheter
Venous
Catheter
Lymphatic
Catheter
Lymph
Node
Luminal Catheter
with Plug
Adjacent
Intestinal
Segment
Intestinal
Segment

was supplied by the single arterial branch and was returned to the superior mesenteric vein via a single vessel.

2) For the luminal cannulation, silastic catheter tubing (4.8 mm ID, 7.9 mm OD) was positioned in the lumen of the segment, from both the proximal and distal directions, through small incisions in the adjacent intestine. Silk ligatures (2-0) were tightened around the intestine at the extremities of the segment in order to secure the luminal cannulae and prevent blood flow along capillary anastomoses with adjacent intestinal tissue. The lumen was carefully flushed with physiological saline (20 ml of 150 mM NaCl at 37°C) to remove intestinal secretions and dietary contents, and then was gently purged with 20 ml of air. The luminal catheters were plugged to prevent leakage of any luminally transferred IGFs during perfusion.

3) In order to determine whether significant amounts of vascularly infused IGFs left the intestinal segment via lymphatic pathways and to confirm that vascularly infused material had in fact reached the extravascular spaces, the efferent lymphatic of the ileal lymph node was catheterised using bevelled polyethylene tubing (0.8 mm ID, 1.2 mm OD) (see Section **3.2.2**.). Lymph was collected over timed intervals and tubes re-weighed following collection for calculation of lymph flow rate (1 g = 1 ml). In those samples collected during the vascular perfusion experiment, radioactivity was measured in a gamma counter.

4) Because the experimental model for IGF transfer entails a single-pass vascular perfusion, the entire venous outflow from the segment was collected to prevent return of radiolabelled IGFs to the general circulation and their consequent reperfusion through the intestinal segment. To achieve this, catheterisation of the venous branch of the intestinal segment was required. Blunt dissection was used to separate the vein from the artery over a length of 5 mm and two silk ligatures (4-0) were tied loosely around the vein in preparation for cannulation. In order to prevent blood coagulation and consequent cannula blockage, a bolus of heparin anticoagulant in physiological saline, at a dose calculated to achieve a circulating concentration of 10 IU/ml of blood (800 IU/kg body wt), was infused through the saphenous cannula and allowed to equilibrate in the circulation for 2-3 min. The ligature

closest to the superior mesenteric vessels was then tightened to cause the venous branch to swell and the vein was cannulated as rapidly as possible using a 14 G intravenous catheter (Surflo, Terumo Corp.). Both ligatures were tightened around the catheter to anchor it in position and a 20 cm length of flexible Silastic tubing (1.0 mm ID, 2.2 mm OD) was attached to allow direction of the efferent blood to pre-weighed collection tubes. Tubes were changed at timed intervals and reweighed for estimation of sample volume (1 g = 1 ml) and calculation of blood flow (ml/min). To compensate for blood loss, the lamb was transfused with donor sheep blood. For this purpose adult sheep blood, collected less than two weeks prior to the experiment, was pumped into the saphenous vein (Minipulse II, Gilson Medical Electronics) at a rate equal to that of blood flow from the segment. The donor blood was mixed with citrate anticoagulant (TUTA Blood Donor Pack, Tuta Laboratories [Aust.] Pty. Ltd.) at the time of collection but was heparinised (10 IU/ml) immediately prior to infusion into the lamb. Donor blood was maintained on a rocker for the duration of the experiment to prevent settling of red blood cells. Blood was passed through an in-line filter, bubble trap and 37°C water-jacketted heat exchanger to remove blood clots and air bubbles, and to adjust its temperature prior to infusion into the lamb.

5) In order to minimise disruption to the intestinal blood supply as well as irritation to the arterial wall, a very fine cannula was used to catheterise this vessel. The cannula consisted of a 27 G needle tip, bent to an angle of about 150° so that it would not project into and irritate the inner arterial wall, attached to a length of polyethylene catheter tubing (0.21 mm ID, 0.68 mm OD) and primed with 150 mM NaCl. Arterial cannulation was performed by careful insertion of the needle into the arterial lumen in the direction of blood flow and did not require interruption of the arterial blood supply. Arterial cannulation was performed as the final step in the preparation of the intestinal segment because the arterial catheter was sensitive to disturbance and further manipulation of the intestine was, therefore, precluded.

Selection of IGF Vehicle:

In order to maintain the state of the intestinal tissue close to the physiological during vascular perfusion, maintenance of a normal blood supply was essential. A preliminary

study was therefore performed in which the effects of various infusion solutions (vehicles) on efferent blood flow from the segment were compared. The aim was to select the vehicle that changed blood flow the least; this vehicle would be used as a carrier for radiolabelled IGFs in subsequent vascular perfusions. Common vehicles used in the literature include saline (150 mM NaCl), Krebs-Ringer buffer (137 mM NaCl, 5.36 mM KCl, 15.8 mM Na_2HPO_4, 0.41 mM $MgSO_4$, pH 7.4) and Hanks Balanced Salts (137 mM NaCl, 5.36 mM KCl, 0.35 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.55 mM D-Glucose, 4.17 mM $NaHCO_3$, 0.81 mM $MgSO_4$ and 1.25 mM $CaCl_2$, pH 7.4); all were compared for possible effects on blood flow from the intestinal segment. Furthermore, because IGFs would subsequently be infused in the presence of BSA to minimise non-specific adherence of labelled IGFs in the perfusate container and infusion catheter, blood flow was also compared during infusion of the three vehicles containing 1% (w/v) BSA.

A lamb was anaesthetised and instrumented with cranial tibial arterial and saphenous vein cannulae for respective monitoring of blood pressure and infusion of donor blood. A segment of intestine was then prepared for perfusion (Section **4.2.1**). Prior to vascular infusion of vehicle solutions, blood flow from the intestinal segment was measured over a 5 min period and an infusion pump was adjusted for delivery of vehicle at 5% (v/v) of blood flow (Gilson silastic catheter tubing [0.10 ml/m internal volume], Minipulse III; Gilson Medical Electronics). Infusion of saline into the segment artery was then commenced. Heparinised donor sheep blood was infused peripherally to replace blood lost from the intestine. Vehicles were infused, each for a period of 5 min, in the following order: saline, Krebs-Ringer, Hanks Balanced Salts, saline + 1% BSA, Krebs-Ringer + 1% BSA, Hanks Balanced Salts + 1% BSA. Vehicle infusion was briefly arrested (5 sec) at each change of the infusion medium, to prevent entry of air into the bloodstream and possible blockage of fine capillaries by air bubbles. For each 5 min infusion, blood was collected into a single pre-weighed tube and flow rate (ml/min) calculated from the increase in tube weight (1 g = 1 ml) per unit time. At the completion of this experiment and whilst under halothane anaesthesia, the lamb was euthanased by injection of saturated KCl (20 ml) into the saphenous vein.

Blood flow from the intestinal segment was 8 ml/min initially but was about 10% lower during saline infusion and fell another 5% with the substitution of Krebs-Ringer for saline. During infusion of Hanks Balanced Salts, flow rate was similar to that measured with saline infusion. Blood flow did not change appreciably when saline + BSA was substituted for Hanks Balanced Salts but fell by about 5% during infusion of Krebs-Ringer + BSA as it had during infusion of Krebs-Ringer without BSA. Infusion of Hanks Balanced Salts + BSA was associated with a return of blood flow to that measured during saline infusion. Thus, a similar pattern of blood flow was observed during infusion of vehicles alone as in the presence of 1% (w/v) BSA. These results are evidence for a minor reduction in blood flow as a result of vascular perfusion per se, with only small differences between vehicles. Erring on the side of caution, Krebs-Ringer ± 1% BSA was not considered as an IGF infusion vehicle because blood flow was lowest during perfusion with this vehicle. While flow rates using Hanks Balanced Salts and saline were similar to one another, Hanks Balanced Salts was selected because it is more physiological.

4.2.2 Vascular Perfusion using ^{125}I-labelled IGF-I, Des(1-3)IGF-I and LR^{3}IGF-I

IGFs:

Recombinant human IGF-I, des(1-3)IGF-I and LR^{3}IGF-I were purchased from GroPep Pty. Ltd. IGFs were iodinated with Na^{125}I (Nordion International Inc.) to a specific activity of 40 - 75 Ci/g, as previously described (Section **2.1**).

Vascular Perfusion:

After catheterization of the cranial tibial artery and saphenous vein (Section **2.4.1**), a 10 cm segment of distal ileum was prepared for perfusion as described in Section **4.2.1** except that the arterial infusion catheter was primed with Hanks Balanced Salts (+ BSA) containing radiolabelled IGF peptide.

Prior to infusion of radiolabelled IGFs, the rate of blood flow from the segment was measured over a 5 min period and the infusion pump adjusted for delivery of infusate at 2.5% (v/v) of blood flow; infusion rate was reduced from the 5% (v/v) used in *Selection of IGF Vehicle* (Section **4.2.1**) in an attempt to further minimise the effect of infusion per se, on blood flow. Radiolabelled IGF peptide (IGF-I, des[1-3]IGF-I or LR^3IGF-I) was then continuously infused into the intestinal segment for exactly 45 min so that the average concentration of radioligand achieved in plasma was about 25000 cpm/ml of blood (0.5 - 1 ng of radioligand per ml of plasma). 45 min was selected because, depending upon blood flow, 1-2 min of infusion was required for radioactivity in efferent blood to approach the concentration at which it was infused (changes in blood flow rate aside); a 45 min perfusion period would ensure that the intestinal tissue was exposed to more or less constant peptide concentrations for at least 95% of the infusion period. During perfusion, all blood leaving the intestinal segment was collected into pre-weighed tubes that were changed at 1 or 5 min intervals. Timed collections of lymph into pre-weighed tubes were also made during perfusion. Blood and lymph samples were maintained on ice at all times following collection.

To verify that no infused radioligand reached the general circulation, blood was sampled from the cranial tibial artery at intervals of approximately 15 min and counted for gamma radioactivity. Several animals were eliminated from the study on the basis of radioligand detected in systemic blood or because less than 95% of the infused tracer could be accounted for, in all recovered tissues and fluids, at the end of perfusion.

Post-perfusion:

At the end of the perfusion period, peptide infusion was stopped and blood flow through the intestinal segment was arrested by application of a surgical clamp to the blood vessels. The segment was immediately flushed using 10 ml of 0.9% NaCl containing 0.25% (w/v) BSA and was then purged with air; all luminal washings were collected during these procedures. The intestinal segment, lymph node, intestinal mesentery (containing blood vessels that served the segment) and the adjacent intestinal tissue were excised and

maintained on ice with all blood and lymph samples. All samples were weighed prior to measurements of radioactivity and TCA precipitability.

4.2.3 Sample Analysis

Size-exclusion Chromatography:

An aliquot of a mid-perfusion blood sample was centrifuged (2000 x *g* for 20 min at 4°C) for preparation of plasma, which was stored at -20°C for subsequent analysis by size-exclusion chromatography using a Superose-12 column (Section **2.3**). An aliquot of remnant infusate was similarly stored for size-exclusion chromatography and comparison with radioactivity recovered in plasma.

Distribution of Radioactivity:

The intestinal segment was weighed and homogenised (Ultra-Turrax T25; Janke & Kunkel) in 25 ml of ice cold 0.9% (w/v) NaCl containing 0.25% (w/v) BSA. Homogenisation was performed for 1 min at 9500 revolutions/min and 2 x 30 sec at 20500 revolutions/min, using both coarse (S 25 N-18 G) and fine (S 25 N-8 G) homogeniser heads. 200 µl aliquots of infusate, luminal washings, segment homogenate and of each whole blood and lymph sample were weighed into tubes and gamma radioactivity counted for estimation of tracer recovery in each fluid. Samples were weighed for an accurate determination of size because the high viscosity of blood and intestinal homogenate precluded accurate pipetting. Other tissues collected following the perfusion were dissected into small pieces for measurement of gamma radioactivity.

TCA Precipitability:

Quadruplicates of infusate, intestinal homogenate and luminal washings as well as duplicates of each blood sample were aliquoted for measurement of radioactivity TCA precipitability as an estimate of the proportion of tracer representing intact IGF peptide (Section **2.2**). Aliquots of 200 µl were pipetted into tubes and mixed with 700 µl of ice-

cold 0.25% (w/v) BSA before addition of 100 µl ice-cold 100% (w/v) TCA. Because infusate, homogenate and luminal contents contained lower amounts of protein than samples of whole blood, 200 µl of whole blood (collected from the intestine prior to vascular perfusion with labelled IGFs) was substituted for a similar volume of 0.25% (w/v) BSA TCA precipitability measurements in these samples. TCA insoluble radioactivity was precipitated during a 20-30 min incubation on ice and was pelleted by centrifugation (2000 x *g* for 20 min at 4°C). After separation of the supernatant from the pellet and measurement of radioactivity in both, percentage intact radioligand was calculated as the pellet radioactivity divided by the sum of pellet and supernatant radioactivities and multiplied by 100.

Haematocrits:

Haematocrits were performed on blood samples to enable calculation of the plasma concentration of infused IGF. Samples were drawn into microhaematocrit tubes which were then flame-sealed; the upper portion of the tube was cooled on a moist towel to prevent heat-destruction of red blood cells. After cooling to room temperature, the microhaematocrit tubes were centrifuged for 2 min (Micro-Haematocrit Centrifuge; Hawksley) and the fractional red blood cell content determined (Micro-Haematocrit Reader; Hawksley). Haematocrit values of samples collected at each 5 min interval during the perfusion were averaged to produce a mean value for the perfusion period.

4.2.4 Calculations and Statistical Analysis

Total Counts Infused and Recovered:

Total counts infused were calculated as the product of the weight (g) infused and the average counts/g measured in the infusate. Total recovery of counts was the sum of counts detected in the intestinal segment, luminal contents, mesentery, lymph node, adjacent intestinal tissue, and efferent blood and lymph.

Average Plasma Concentration of Infused IGF (C_{ave}):

C_{ave} was calculated for individual perfusions, using the following equation:

$$C_{ave} = I \times [i / b] \times [1 / (1 - h)],$$

where *I* is the concentration (ng/ml) of IGF peptide in the infusate, *i* is the volume of infusate (ml) perfused during the experiment, *b* is the total blood volume (ml) collected from the segment during vascular perfusion and *h* is the average fractional haematocrit of efferent intestinal blood during the perfusion. The individual C_{ave} values were averaged, according to IGF peptide infusion group, to yield group averages.

Transfer of Radioligand to Intestine:

Net transfer of radioactivity to intestinal tissue was calculated as the total counts transferred to lymph, lymph node, luminal contents and segment divided by total counts recovered in lymph, lymph node, luminal contents, segment, mesentery and efferent blood.

In order to calculate transfer of radioligand to intestinal tissue, it was necessary to adjust the total counts detected in the intestinal segment for the contribution made by radioactivity in blood trapped by the tissue vasculature. To estimate the volume of trapped blood, a portion of the intestinal homogenate was centrifuged (10000 x *g* for 30 min at 4°C) and the absorbance of haem (414 nm) in the supernatant (Ballard & Hanson; 1969) compared against that of lysed lamb blood. Serial two fold dilutions of lysed blood were made in water for use as standards. 100 µl aliquots of the supernatant (quadruplicates) and of lysed blood standards (quadruplicates) were pipetted into a microtitre plate and absorbance was measured using a microtitre plate-reader (Titertek Multiskan MCC; Joint venture of Labsystems and Flow Laboratories).

The value for blood dilution in the homogenate supernatant (*d*) was used in the following calculation:

$$\text{Blood Volume} = H \times (s / h) \times d$$

Thus the weight of a portion of this homogenate (*h*) and of the supernatant obtained after its centrifugation (*s*) were used to calculate the fraction of homogenate composed of supernatant (*s/h*) and this value was multiplied by the homogenate weight (*H*; combined weight of segment and added saline) to determine the total volume of supernatant. Multiplication of this value by the dilution of blood in the supernatant (*d*) yielded the estimated volume of blood retained in the intestinal segment. The number of counts (cpm) attributable to capillary-entrapped blood was calculated as the retained-blood volume multiplied by the concentration of radioligand (cpm/ml) in the final sample of efferent blood collected during vascular perfusion.

In experiments in which bleeding into the intestinal lumen was evident, blood content of the luminal wash was calculated for subtraction from the value for luminally recovered IGF to derive the estimate of luminally transferred IGF. Absorbance of the wash was compared against that of lysed blood to calculate the value for blood dilution, as described above. This was then multiplied by the luminal wash volume to calculate total blood content. Radioactivity attributable to blood was determined after multiplication by the average concentration of tracer in blood during perfusion (cpm/g).

Statistical Analyses:

Data was analysed by one-way analysis of variance. Values are expressed as mean ± SEM.

4.3 RESULTS

4.3.1 The Vascularly Perfused Intestinal Segment

Success Rate:

A total of 19 vascular perfusion experiments were attempted, of which 9 (47%) contributed data to the present chapter. Twelve perfusions were completed but three of these (16%) were unsuccessful because the infused radioligand was not recovered quantitatively.

Of those experiments not completed, two were aborted due to failed cannulations of intestinal blood vessels (11%) and five (26%) died under anaesthesia, apparently as a result of respiratory failure, before completion of the experiment.

Physiological Parameters:

The perfused intestine was used as a model for tissue-transfer of blood-borne IGFs because the physiological condition of the intestinal segment was disturbed as little as possible, with maintenance of vascular and nervous connections. Mean arterial blood pressure, which would otherwise fall with continued blood loss during the collections, was maintained in a physiological range by infusion of donor blood through the saphenous vein, as shown for a representative animal in Fig. 4.3. The visual appearance of the intestinal segment remained normal in all perfusions although minor luminal bleeding was detected in some experiments, due perhaps to overtightening of the ligatures around the intestine. Such bleeding accounted for less than 0.5% of total recovered radioactivity in all experiments.

Blood and Lymph Flow:

Average blood and lymph flow rates for individual lambs and each group, at both the pre- and peri-perfusion periods, are shown in Table 4.1. There was no net effect of infusion on blood or lymph flow, so that the average blood and lymph flows were similar in the pre- and peri-perfusion periods for the respective IGF-I, des(1-3)IGF-I and LR^3IGF-I groups. However, blood and lymph flow did vary considerably before and during the collections (Fig. 4.3), but in general such changes did not appear to be directly related to altered blood pressure, heart rate or the onset of anaesthesia. As a consequence of the changes in blood flow and the fact that the labelled IGFs were infused at a constant rate for the entire perfusion, the concentration of radioligand in the vascular supply was subject to some variation. Notwithstanding this, the average radioligand concentrations achieved over the infusion period were (mean ± SEM, n = 3/group) 0.7 ± 0.1 ng/ml for IGF-I, 0.6 ± 0.1 ng/ml for des(1-3)IGF-I and 0.9 ± 0.1 ng/ml for LR^3IGF-I.

Fig. 4.3. *Time-course of mean arterial blood pressure, heart rate, lymph flow and blood flow of a representative vascularly perfused lamb intestine.* Data are plotted relative to the start of infusion of radiolabelled IGF peptide (0 min). Heart rate and blood pressure were monitored from the time of cannulation of the cranial tibial artery (-120 min) and lymph and blood flow from the segment were measured from their respective cannulation times of -95 min and -30 min.

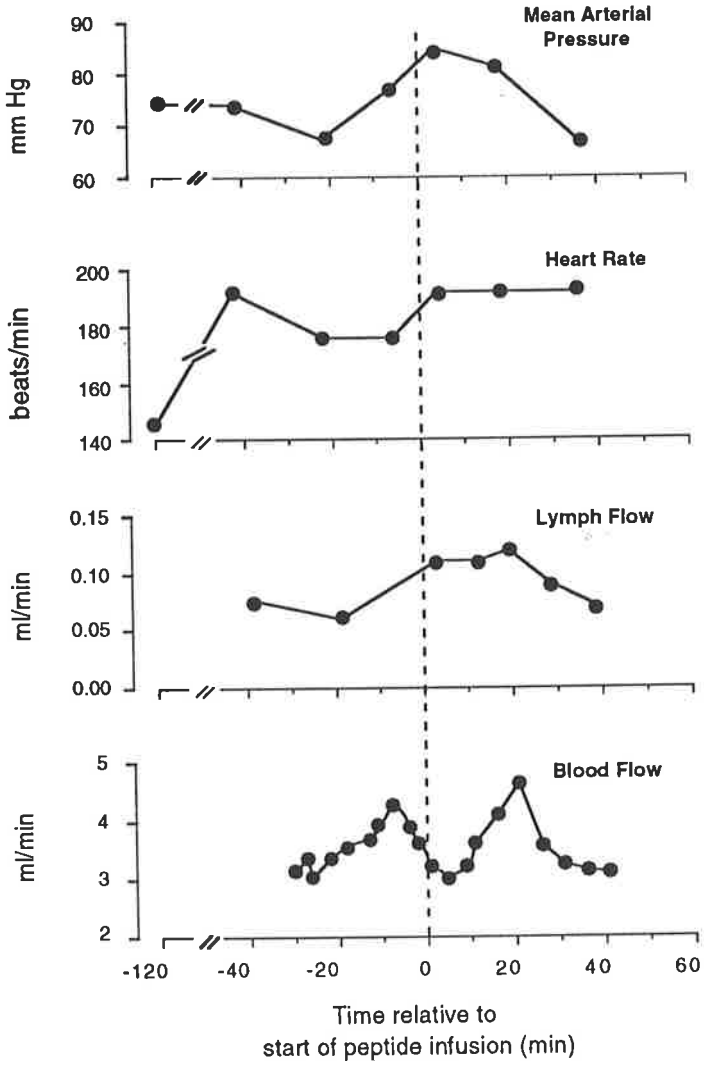

Mean Arterial Pressure
mm Hg
90
80
70
60
Heart Rate
beats/min
200
180
160
140
Lymph Flow
ml/min
0.15
0.10
0.05
0.00
Blood Flow
ml/min
5
4
3
2
-120
-40
-20
0
20
40
60
Time relative to
start of peptide infusion (min)

Table 4.1. *Average blood and lymph flow before and during infusion of radiolabelled IGFs at less than 1 ng/ml*

	IGF-I	des(1-3)IGF-I	LR^{3}IGF-I
Blood flow (ml/min)			
Pre-perfusion	7.5	4.1	4.6
	3.6	3.4	4.7
	3.5	6.3	8.6
Average	*4.9 ± 1.3*	*4.6 ± 0.9*	*6.0 ± 1.3*
Perfusion	7.3	5.2	4.8
	3.5	4.2	5.4
	9.5	8.4	4.8
Average	*6.9 ± 1.7*	*5.9 ± 1.3*	*5.0 ± 0.2*
Lymph flow (ml/min)			
Pre-perfusion	0.04	0.12	0.07
	0.07	0.10	0.16
	0.16	0.04	0.08
Average	*0.16 ± 0.04*	*0.08 ± 0.02*	*0.10 ± 0.03*
Perfusion	0.02	0.12	0.06
	0.03	0.13	0.10
	0.14	0.05	0.12
Average	*0.14 ± 0.02*	*0.10 ± 0.03*	*0.09 ± 0.02*

Average flow rates for each animal are shown for both the pre-perfusion and peri-perfusion periods, with means ± SEM for 3 lambs/group shown in *italics*. No significant differences were apparent between peptides.

4.3.2 Recovery and Distribution of Radioactivity

In successful experiments, tracer recovery ranged from 96 - 106% of the amount infused, radioactivity measured in the peripheral blood was insignificant and less than 0.15% of total radioactivity leaked from the perfused segment to adjacent intestinal tissue. Radioactivity detected in peripheral blood and adjacent intestine was not included in this calculation. In all experiments, tracer appeared rapidly in the venous outflow of the segment, so that the concentration of radioactivity in efferent blood approached that at which it was infused after only 1-2 min.

The distribution of radiolabelled IGF in efferent blood and lymph, as well as in mesentery, intestinal tissue, luminal contents and lymph node was expressed as a percentage of the total radioactivity recovered in them after the 45 min perfusion. In the IGF-I perfusions, a mean of 96.4% of the infused radioactivity was recovered in the efferent blood from the segment plus a minor amount (0.15%) in the mesentery of the intestinal segment which, because it contained the vascular network that supplied the segment, presumably reflected radioactivity in capillary-entrapped blood (Table 4.2). For this reason, mesenteric radioactivity was excluded from the calculation of amount transferred to tissue but was instead included in the value for efferent blood. In contrast to the large recovery in blood, a very small proportion of IGF-I tracer left the segment via the lymphatics (average of 0.088%, determined as the combined radioactivity in lymph and the lymph node), and less than 1% (average of 0.51%) was secreted into the intestinal lumen. The remainder of the infused IGF-I tracer was retained in the intestinal tissue (2.86%). Thus, the net transfer of tracer from blood to the intestine, estimated as the sum of the proportions recovered in lymph, the lymph node, luminal contents and intestinal tissue, was $3.46 \pm 0.22\%$ (Table 4.2).

The distribution profiles of the two analogs were very similar to that of IGF-I (Table 4.2). No significant differences were apparent between the percentages of labelled IGF-I, des(1-3)IGF-I or LR^3IGF-I recovered in venous blood, luminal contents or lymph, and although there was a trend towards increased des(1-3)IGF-I tracer in the intestinal tissue as

Table 4.2. *Recovery of radiolabelled IGFs, infused at less than 1 ng/ml*

	Percentage of Radioligand Recovered		
	IGF-I	des(1-3)IGF-I	LR^3IGF-I
Blood	96.4 ± 0.3	95.5 ± 0.6	96.2 ± 0.6
Mesentery	0.15 ± 0.06	0.06 ± 0.01	0.08 ± 0.02
Intestinal Tissue	2.86 ± 0.17	4.15 ± 0.80	3.00 ± 0.59
Luminal Contents	0.51 ± 0.13	0.29 ± 0.22	0.75 ± 0.15
Lymph Node	0.08 ± 0.08	0.01 ± 0.00	0.00
Lymph	0.008 ± 0.005	0.002 ± 0.001	0.000
Net Intestinal Transfer	3.46 ± 0.22	4.45 ± 0.63	3.75 ± 0.63

Data show the percentage of infused tracer recovered at the different sites. *Net Intestinal Transfer* is the sum of the percentage recovered in the intestinal tissue (adjusted for the contribution of radioactivity by capillary-entrapped blood), luminal contents, lymph and lymph node. Values are means ± SEM (3 lambs/group). No significant differences were apparent between peptides.

well as a higher net transfer compared with the other peptides, there were no statistically significant differences in intestinal retention of the three IGF peptides.

4.3.3 TCA Precipitability of Radioactivity

In addition to measuring the recovery and distribution of radioactivity at the end of the perfusion, the TCA precipitability of radioactivity in the intestinal homogenate and in the infusate and blood collected during perfusion were measured; precipitability was used as an estimate of intact radioligand. TCA precipitability of infusate radioactivity was at least 98% in all experiments, and that of radioactivity in efferent blood differed by less than 0.4%. Compared with the infusate, there was a small reduction in the TCA precipitability of IGF analogs retained in the intestinal tissue, so that the percentage of intact peptide was estimated as $96.8 \pm 1.5\%$, $94.8 \pm 1.2\%$ and $95.4 \pm 0.5\%$ for IGF-I, des(1-3)IGF-I and LR^{3}IGF-I, respectively, with no significant differences apparent between peptides. Measurements of TCA precipitability of radioactivity in lymph, lymph node and most luminal contents were not feasible because of the low concentrations of radioactivity in these samples. However, in samples of luminal contents in which measurement of TCA precipitability was possible, it ranged from 70-90%. The combined data from all precipitability measurements suggest that radiolabelled IGF peptides remain largely intact during passage through the vasculature of the perfused intestine, with some degradation occurring in the intestinal tissue and luminal contents.

4.3.4 Association of Radioligands with Plasma IGFBPs *In Vivo*

Size-exclusion chromatography was carried out on plasma samples collected at 20-25 min after the start of perfusion to confirm differential binding of IGF-I and the analogs to IGFBPs in plasma. Plasma from two of the three experiments in each of the IGF-I, des(1-3)IGF-I and LR^{3}IGF-I perfusion groups was analysed and the elution profiles are shown in Fig. 4.4. Chromatographic analyses were not performed on plasma from the first

animal in each group. Radiolabelled IGFs eluted in three peaks, with molecular masses of approximately 130-150 kDa, 30-50 kDa and 8 kDa. The 130-150 kDa region corresponded to the size of the ternary complex of IGF-I with IGFBP-3 and the α-subunit (Baxter; 1988, Baxter & Martin; 1989), whereas the 30-50 kDa peak presumably represented binary IGF-IGFBP complexes and the 8 kDa peak corresponded to the elution position of free radioligand.

The relative abundance of ^{125}I-labelled IGFs at each of the three size regions was estimated from the proportion of total eluted counts contained in each peak. For this purpose, radioactivity eluted at 9-12.5 ml, 13-15 ml or 15.5-18.5 ml was divided by that eluted between 9 and 18.5 ml. For IGF-I incubations in which no distinct peak of uncomplexed ligand was demonstrated, the tail portion of the 30-50 kDa peak (15.5-18.5 ml) was assumed to represent free ligand. Essentially all IGF-I was bound to IGFBPs since only 4-5% eluted at 8 kDa (Table 4.3). Most binding occurred at 30-50 kDa but lower association was evident at 130-150 kDa. Surprisingly, both des(1-3)IGF-I and LR^{3}IGF-I also were largely bound to IGFBPs although 12-13% of des(1-3)IGF-I and 13-14% of LR^{3}IGF-I eluted at the position of free IGF. Greater proportions of the analogs and especially LR^{3}IGF-I, than of IGF-I, were associated at 130-150 kDa (Table 4.3). As a proportion of the total radioactivity recovered from the column, 1.90 & 2.22%, 2.78 & 2.21%, and 1.43 & 0.69% of IGF-I, des(1-3)IGF-I and LR^{3}IGF-I tracer, respectively, eluted after 18.5 ml at the position of free radioiodide. The proportion of IGF infusate measured at the elution position of free iodide was similar (2.37 & 3.07%, 3.34 & 2.48% and 2.08 & 1.97% for IGF-I, des(1-3)IGF-I and LR^{3}IGF-I, respectively), suggesting that very little degradation of IGF peptides occurred in the vascular compartment of the perfused intestine. This data confirms the conclusion drawn from the TCA precipitability data, that IGFs recovered in blood from the segment are not degraded substantially during a single pass through the small intestinal vasculature. While it would have been desirable to use size-exclusion chromatography as a confirmation of the TCA estimate of intactness for radioligand in the intestinal segment, this was not feasible because of the low concentration of radioactivity in the homogenate.

Fig. 4.4. *Chromatography elution profiles of efferent venous plasma from perfusions with radiolabelled IGF-I (a, d), des(1-3)IGF-I (b, e) or LR^{3}IGF-I (c, f), at 0.5-1 ng/ml plasma.* Plasma samples were prepared from venous blood collected at 20-25 min from the start of peptide infusion, and chromatographed as described in Section **2.1** using a Superose-12 size exclusion column. The profiles show the recovery of radioactivity in 0.5 ml fractions as a percentage of total radioactivity eluted. The column was calibrated with protein markers; human alcohol dehydrogenase (150 kDa), BSA (69 kDa) and ^{125}I-IGFs (8 kDa).

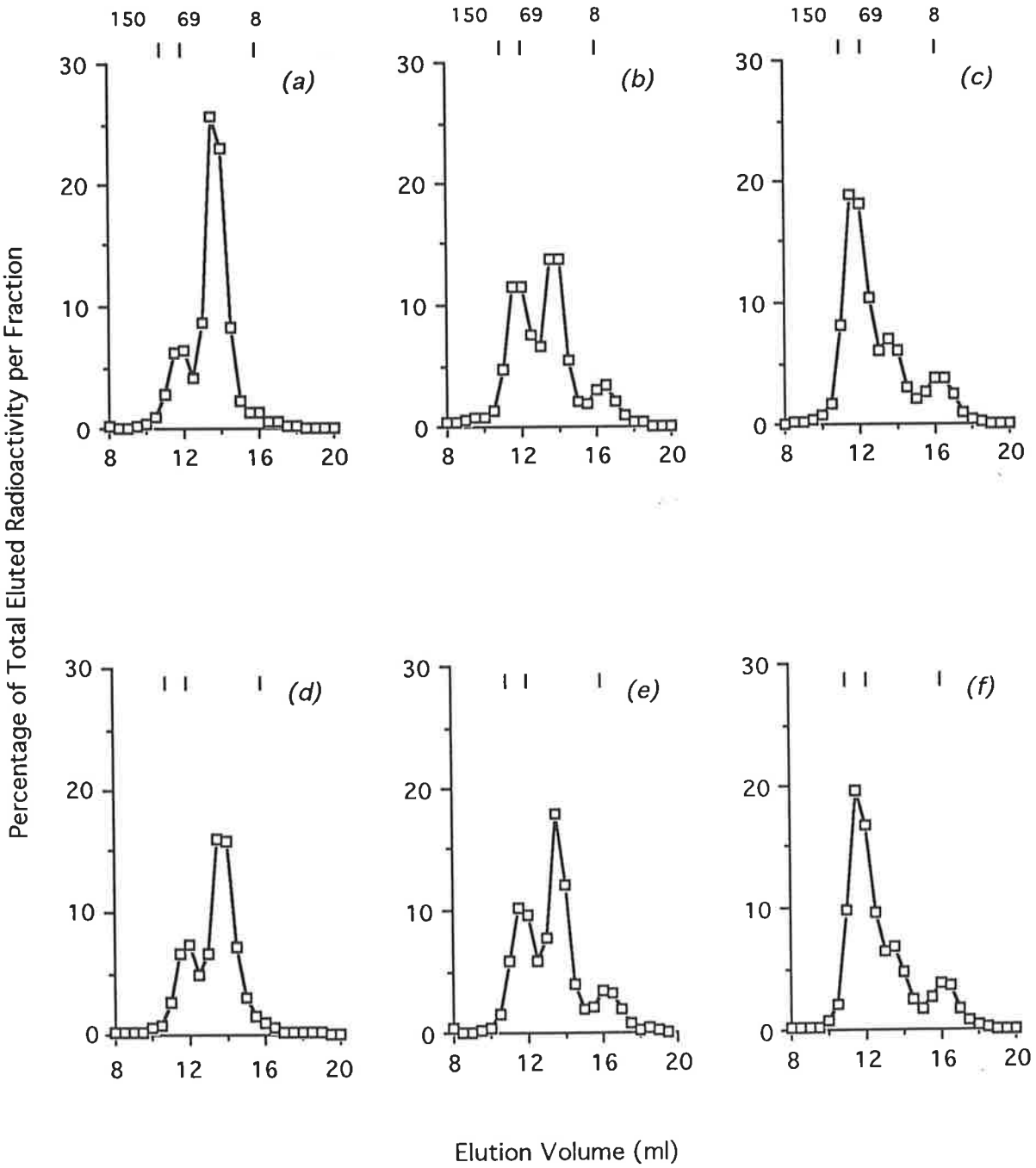

150
69
8
(a)
(b)
(c)
(d)
(e)
(f)
30
20
10
0
8
12
16
20
Percentage of Total Eluted Radioactivity per Fraction
Elution Volume (ml)

Table 4.3. *Recovery of radioactivity as a percentage of total radioligand following pH 7.2 size-exclusion chromatography of venous plasma from ovine intestine perfused with radiolabelled IGFs at less than 1 ng/ml*

	IGF-I	des(1-3)IGF-I	LR^3IGF-I
130-150 kDa	23-51	39-42	61-63
30-50 kDa	64-73	45-49	24-25
Total bound	95-96	87-88	86-87
8 kDa (% free peptide)	4-5	12-13	13-14

Data are the percentage of total radioactivity that eluted from the column between 9.0 and 18.5 ml, at 9.0-12.5 ml (130-150 kDa), 13.0-15.0 ml (30-50 kDa), and 15.5-18.5 ml (8 kDa) following pH 7.2 size-exclusion chromatography of efferent venous plasma collected from the vascular perfusion at 20-25 min after the start of infusion of radiolabelled peptides. Values show the range for two animals.

4.4 DISCUSSION

Use of the vascularly perfused lamb intestine provided the opportunity to measure net transfer of IGF-I peptides from blood to intestine under conditions that were close to the physiological. Thus blood supply and nervous connections were preserved during infusion of radiolabelled IGFs for 45 min, with blood pressure maintained during sampling by infusion of donor blood. While significant variation in blood flow was evident both before and during perfusion, as represented by one preparation in Fig. 4.3, no net effect of perfusion was apparent (Table 4.1). Nevertheless, because substantial variability in blood flow was observed, it appears likely that the slight decrease in blood flow indicated in the preliminary comparison of perfusion vehicles may have been the result of normal variation in intestinal blood flow within an animal. One aspect of the model that was of concern was the evidence of luminal bleeding, possibly a consequence of blood vessel rupture due to overtightening of intestinal ligatures. However, in the occasional experiments in which bleeding occurred, the value for luminally transferred IGF was adjusted after estimation of luminal blood volume. A disappointing aspect of the model was its low success rate; of the experiments commenced, 47% were successfully carried through to completion (at least 95% of infused tracer recovered in the perfused tissue and all collected fluids). Nevertheless, since 50% of the failures were due to premature death of the lamb during anaesthesia, apparently as a result of respiratory failure, it was reasoned that success rate could likely be improved by employing artificial ventilation during anaesthesia. Artificial ventilation was, therefore, employed for all subsequent experiments that involved general anaesthesia.

Net transfer of radiolabelled IGF peptides from blood to the intestinal segment was measured as the combined radioactivity transferred to lymph, lymph node, luminal contents and intestinal tissue at the end of a 45 min, single-pass perfusion. The contribution to this radioactivity made by capillary-entrapped blood is likely to be negligible because this was accounted for in the calculations. Almost all the IGF radioligand which left the vascular compartment was recovered in the intestinal segment but, as the present study was not designed to identify the distribution of IGF tracer within intestinal tissue, extravascular

radioactivity could not be distinguished from that associated with the vascular endothelium. Nevertheless, since lymph accounts for only a small percentage of transferred IGF, and because lymph is derived from the fluids draining the extravascular spaces of the tissue bed, it is likely that the proportion of transferred peptide remaining in the extravascular spaces is also small. Therefore, most transferred radioactivity is probably associated with tissue.

Because the flow rate of lymph was very low compared with that of blood (see Table 4.1), it was not surprising that total recovery of radioactivity in lymph was a small component of the total infused. Nevertheless, the concentration of counts in lymph averages less than 2% that of counts in the blood. This contrasts the finding of Hodgkinson *et al* (1991) who showed that immunoreactive (N-Met)IGF-I was present in (mammary) lymph at 50% of circulating concentrations, following intravenous infusion into sheep. The lower concentration detected in the present study may be explained by the fact that only a limited proportion of the intestine served by the distal ileal lymph node, was actually perfused. Thus, lymph collected during perfusion represented a mixture of that draining perfused and non-perfused intestine, with the result being that the concentration of radioactivity in lymph from the segment was effectively diluted. While it is plausible that IGFs may be transferred and utilised in an organ-specific manner, no data is available to substantiate such differences between mammary and intestine.

The vascular perfusion model appears to be useful for the estimation of IGF transfer. It is also apparent, however, that the perfusion conditions were not appropriate for the goal of determining the effect of reduced IGFBP interaction on IGF transfer because the differences in proportions of free IGF-I and analogs were much smaller than expected from the data in rats (Francis *et al;* 1986, Ballard *et al;* 1987, Walton *et al;* 1991). Nonetheless, the rank order of binding of IGFs to plasma IGFBPs was LR^3IGF-I < des(1-3)IGF-I < IGF-I and there was a trend towards greater transfer of des(1-3)IGF-I than of IGF-I. The lack of such a trend between LR^3IGF-I and IGF-I, despite the greater proportion of the analog free of IGFBPs, suggests that the reduced affinity of LR^3IGF-I for the type-I receptor (Francis *et al;* 1992) may affect its transfer in this experimental model.

The small extent of the differences in binding between IGF-I and the analogs was not expected, since LR^3IGF-I associates even more poorly with IGFBPs than does des(1-3)IGF-I (Francis *et al;* 1992), while the *in vitro* incubations demonstrated greater proportions of des(1-3)IGF-I in the free condition (Figs. 2.1 & 3.6). Perhaps the *in vitro* incubations (15 h at 4°C) were not representative of the vascular perfusion conditions in which peptides were exposed to plasma IGFBPs briefly at 37°C (*in vivo*) and afterwards on ice for 2-3 h. However, a more likely explanation for the low proportions of analogs eluting free after vascular perfusion is that the tracer concentration used was too low. Indeed, the concentration of labelled IGF used in the present study was 20% of that employed in Chapter **3**, and 2.5% of that used in Chapter **2** where the highest proportion of free peptide was evident. Thus higher tracer concentrations appear to favour greater distribution of IGF to the free peak. In the vascular perfusion model, greater differences in the proportions of free and binding protein-bound IGFs could probably be achieved by infusion of more tracer of lower specific activity. This is pursued in the next chapter.

The greater distribution of IGF-I to 30-50 kDa than to 130-150 kDa was surprising in view of the higher affinity of IGF-I for IGFBP-3, the binding subunit of the 130-150 kDa complex, than for binding proteins such as IGFBP-1 that are found in the smaller molecular mass region (Martin & Baxter; 1986, Baxter *et al;* 1987). This may relate to differences in the relative abundance of unoccupied binding sites at the two binding regions. Indeed, since the high molecular mass region contains IGFBP-3 associated with equimolar amounts of endogenous IGF and the α-subunit (Baxter & Martin; 1986, Baxter; 1988*a*), exogenous radioligand must compete to bind at this size. Smaller proportions of endogenous radioligand are found at 30-50 kDa than at 130-150 kDa (Daughaday *et al;* 1982) and, while the degree to which IGFBPs in the smaller molecular mass IGFBP region are saturated with endogenous IGFs is not known, a low saturation of IGF-binding sites at this region would plausibly result in greater binding of radiolabelled IGF-I. The greater binding at 30-50 kDa may also be accounted for if the constituent IGFBPs in this region were more plentiful than those in the high molecular mass complex. However since the relative abundancies of the

individual ovine IGFBPs have not been quantitated, the above explanations are largely speculative.

The extent to which des(1-3)IGF-I and particularly LR^3IGF-I bound to IGFBPs in the 130-150 kDa peak was also surprising (Fig. 4.4); the greatest ratio of radioligand binding at 130-150 kDa : 30-50 kDa was apparent using labelled LR^3IGF-I but was smallest using labelled IGF-I, despite the lower affinity of des(1-3)IGF-I and especially LR^3IGF-I for the ligand-binding subunit of the 130-150 kDa complex (Lord *et al;* 1993). However, Fig. 4.4 reveals that the analogs, particularly LR^3IGF-I, are unable to bind as effectively as IGF-I at 30-50 kDa. Furthermore, about 15% of the analogs remained uncomplexed with IGFBPs. Perhaps binding at 130-150 kDa was greater for the analogs than for IGF-I because greater proportions of analogs than of IGF-I were free to compete for binding sites in the complex. The data is consistent with this explanation but confirmation would require further experimentation.

An alternative explanation for the greater binding of analogs than of IGF-I at 130-150 kDa can be based upon evidence for differential affinity of various ligand-IGFBP-3 complexes for the α-subunit. Indeed Baxter *et al* (1992) have shown that the affinity of the α-subunit for IGF-IGFBP-3 complexes depends upon the structure of IGF in the complex. Since the ternary 130-150 kDa complex between IGF, IGFBP-3 and the α-subunit forms after ligand first binds to IGFBP-3 (Baxter; 1988*a*, Baxter & Martin; 1989), the data presented here suggest that interaction of des(1-3)IGF-I and LR^3IGF-I with IGFBP-3 may facilitate association of the duplex with the α-subunit in sheep plasma. In support of this argument, a case in point relates to differences between IGF-I and IGF-II in the formation of the 130-150 kDa complex; while IGFBP-3 binds IGF-I at lower affinity than it does IGF-II (Martin & Baxter; 1986), the binary complex between IGF-I and IGFBP-3 has a greater affinity for the α-subunit than does that between IGF-II and IGFBP-3 (Baxter, *et al;* 1989). Perhaps the poor affinity of des(1-3)IGF-I and LR^3IGF-I for IGFBP-3 is outweighed by a greater affinity of α-subunit for the binary complex with analogs than with IGF-I. Further experiments will be required to confirm the reasons for differences in the distribution of IGFs between IGFBPs.

4.5 SUMMARY

1) A single-pass vascular perfusion model was developed in the distal ileal intestine of the lamb. Blood pressure was maintained by infusion of donor blood during blood sampling.

2) Vascular perfusion at 5% (v/v) of blood flow appeared to cause some reduction in blood flow through the tissue. This reduction was not evident in perfusions at 2.5% (v/v) of blood flow.

3) Approximately 95% of labelled IGFs infused into the intestinal vasculature were accounted for in efferent blood from the segment. 3-4% of IGF was retained in the intestinal tissue, and the remainder was distributed between the segment mesentery, luminal contents, lymph and lymph node.

4) There were no statistically significant differences in the net transfer of IGF-I or the analogs to intestine, although a trend towards greater transfer of des(1-3)IGF-I than for IGF-I or LR^3IGF-I was apparent.

5) The TCA precipitability of radioligand recovered in efferent blood was essentially identical to that of the infusate, suggesting that no significant degradation of IGFs occurred in the vasculature of the intestinal segment. This was corroborated by chromatographic analysis of tracer and radioactivity in efferent plasma. Nevertheless, there did appear to be some degradation of intestinally-transferred IGFs.

6) Chromatography of efferent plasma from the perfused segments revealed that radiolabelled IGF-I was essentially all bound to IGFBPs and that, contrary to expectations, only small proportions of the analogs remained uncomplexed to IGFBPs.

4.6 ACKNOWLEDGMENTS

Technical assistance in the anaesthetisation of lambs, and in the preparation and vascular perfusion of intestinal segments, was kindly provided by Ms K.J Edson. Dr. L.C.

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Chapter Five

Transfer of ^{125}I-Labelled IGFs from Blood to Intestinal Tissue: Infusion at Low Specific Activity

CHAPTER FIVE: TRANSFER OF ^{125}I-LABELLED IGFS FROM BLOOD TO INTESTINAL TISSUE: INFUSION AT LOW SPECIFIC ACTIVITY.

5.1 INTRODUCTION

In the previous Chapter, the vascular perfusion model was successfully applied to the estimation of IGF transfer to intestinal tissue. The study aimed to address the hypothesis that plasma IGFBPs inhibited transfer of vascular IGF to tissue, by comparing transfer of radiolabelled IGF-I with that of radiolabelled IGF-I analogs that bound less well to IGFBPs. In comparing the net transfer of IGF-I, des(1-3)IGF-I and LR^{3}IGF-I, there were, however, no statistically significant differences apparent between peptides. Furthermore, chromatographic analysis of plasma collected during IGF perfusion revealed unexpectedly small proportions of labelled analogs free of IGFBPs. Perhaps a greater difference in the proportion of free versus bound IGF is required before increased transfer is evident.

In the present study, it was reasoned that the proportions of free labelled analogs in plasma of the vascularly perfused intestine could be elevated by infusion of higher amounts of labelled IGF peptide, or infusion of unlabelled peptide mixed with the same amounts of radioligand as used in Chapter 4. ie. Low Specific Activity radioligand. If under such conditions labelled IGF-I remained complexed to IGFBPs and greater proportions of the labelled analogs were free, greater differences in transfer would be anticipated. Such a strategy (Low Specific Activity radioligand) has been adopted in the present study because smaller amounts of radioactivity could be used. Therefore, prior to performing a vascular perfusion study, *in vitro* ligand binding comparisons were performed using plasma mixed with radiolabelled IGF-I or analogs, plus unlabelled peptide at a range of concentrations, to select an appropriate combination of ligand and radioligand for perfusion. The distribution of radioactivity between the free peak and IGFBP regions in each incubation was compared using size-exclusion chromatography. Subsequently, using the modified infusion conditions such that increased proportions of the labelled analogs, but not labelled IGF-I, eluted free, another set of vascular perfusions was performed.

Specific aims of this chapter were to:

1) Select a concentration of unlabelled ligand in plasma at which large proportions of the labelled analogs des(1-3)IGF-I and LR^{3}IGF-I would remain uncomplexed but at which labelled IGF-I would be mostly bound to IGFBPs;

2) Compare the transfer of radiolabelled IGF peptides in the vascular perfusion model, using the infusion conditions selected from the *in vitro* comparisons.

5.2 MATERIALS AND METHODS

5.2.1 Selection of Infusate Mix for Vascular Perfusion

IGFs:

Recombinant human IGF-I, des(1-3)IGF-I and LR^{3}IGF-I were purchased from GroPep Pty. Ltd. IGFs were iodinated using Na^{125}I from Nordion International Inc., as described in Section **2.1**. Specific activity of iodinated peptides ranged between 27 and 75 Ci/g.

Competitive Binding Study:

The aim of this preliminary experiment was to determine a concentration of unlabelled ligand at which increased proportions of labelled des(1-3)IGF-I and LR^{3}IGF-I remained free, in plasma, but at which labelled IGF-I remained bound to IGFBPs. The elution profiles of labelled des(1-3)IGF-I and LR^{3}IGF-I shown in Chapter **4** (Fig. 4.4) revealed apparent saturation of analog-binding sites, since over 10% of both peptides eluted as free ligand. On the other hand, binding sites for labelled IGF-I appeared to be relatively saturated, since no peak of free IGF-I was evident, and incubation of plasma with labelled IGF-I at up to 40 fold greater concentrations of labelled IGF-I was shown not to result in free IGF (Fig. 2.1). Accordingly, incubation of plasma with ligand at increased concentrations should produce greater proportions of analogs in the free peak, while

maintaining all IGF-I bound, up to a point. To determine this perfusion mixture, plasma was incubated with a fixed amount of radiolabelled IGF-I, des(1-3)IGF-I or LR^{3}IGF-I, mixed with its unlabelled homolog at a range of concentrations, under conditions that mimicked those of the perfusion as closely as possible. Thus, while specific activity of radioligand in the incubations decreased, the total concentration of IGF peptide increased. The plasma was then chromatographed and fractionated to determine the distribution of radioligand between the free peak and the IGFBP regions and to select a Low Specific Activity ligand/radioligand mixture for vascular perfusion.

As a source of plasma for the incubations, a 6-week old lamb was anaesthetised with halothane (Section **2.6**) for approximately 20 min, prior to collection of blood from the saphenous vein (Section **2.4.1**). The blood was immediately heparinised (10 IU/ml) and centrifuged for 20 min at 2000 x *g* and 4°C, after which the plasma was aspirated, aliquoted and stored at -20°C. For use in the incubations, unlabelled IGF peptides were diluted in heparinised PBS (Section **2.3**) containing 1% (w/v) BSA, to achieve concentrations of 0, 0.04, 0.36, 2, 10, 20 and 40 µg/ml and were mixed with an equal volume of homologous radiolabelled ligand (0.04 µg/ml). A volume of 20 µl was added to 400 µl of plasma at 37°C so that the radioligand concentration was 1 ng/ml of plasma in each incubation but unlabelled IGF-peptide was present at 0, 1, 10, 100, 250, 500 or 1000 ng/ml of plasma. The tubes were incubated at 37°C for a period of 1 min, as an approximation of the time between vascular infusion of IGF and collection from the venous branch of the perfused intestinal segment, and were then transferred to ice for 3 h. The latter period represented the maximum time that perfusion blood samples were kept on ice prior to plasma preparation. Samples were then frozen in liquid nitrogen and stored at -20°C until analysis by size-exclusion chromatography.

For chromatographic analysis, plasma samples were thawed and extracted with an equal volume of freon to remove lipid (Francis *et al;* 1990), after which a 200 µl aliquot was applied to a Superose-12 size-exclusion column. Chromatography at pH 7.2 was performed as previously described (Section **2.3**) and 0.5 ml fractions were collected for measurement of radioactivity in a gamma counter. The column was calibrated using protein markers of

known molecular mass: human alcohol dehydrogenase (150 kDa), BSA (69 kDa) and radiolabelled IGFs (8 kDa).

5.2.2 Vascular Perfusion of Intestine with Radiolabelled IGF-I, Des(1-3)IGF-I and LR^{3}IGF-I at Low Specific Activity

Artificial Ventilation:

In the previous vascular perfusion study, 50% of the experiments that failed did so because the lamb died prematurely as the result of respiratory failure during deep surgical anaesthesia. To address this problem, the current study was performed in lambs which were artificially ventilated (Mark 8 Respirator; Bird Corp., 100% oxygen). Arterial blood oxygen, carbon dioxide and pH were measured (288 Blood Gas System, Ciba Corning Diagnostics Corp.) to determine whether ventilation rate was adequate; thus ventilation rate was raised in response to rising PCO_2 and declining pH, to increase clearance of circulating CO_2, and was decreased in response to changes in the opposite directions. Blood samples from the cranial tibial artery were collected for gas and pH analysis as often as required to establish a ventilation rate at which blood pH and PCO_2 remained stable; 2-4 samples were collected for comparison with the first set of measurements. In initial experiments, this first sample was obtained immediately after induction of anaesthesia and installation of the arterial catheter, but in later experiments was collected prior to induction of anaesthesia after installation of the arterial catheter using local anaesthetic only (Lignocaine; Apex Laboratories).

Vascular Perfusion:

A 10 cm segment of distal ileum was prepared for vascular perfusion as described in Section **4.2.1**, except that intestinal ligatures were tightened firmly but not so much as to crush the intestinal tissue.

Vascular perfusion was performed as described in Section **4.2.2**, but with the following modifications. Firstly, instead of basing the IGF infusion rate at 2.5% (v/v) of the

pre-perfusion blood flow, for the entire experiment, it was maintained close to 2.5% of immediate flow rate throughout the perfusion; thus, after the weighing of each timed blood sample immediately following collection and calculation of blood flow, infusion rate was readjusted to 2.5% of this value. The advantage of such regulation was that the concentration of infused peptide in blood could be accurately controlled for the duration of the perfusion, irrespective of variations in blood flow during the experiment. Secondly, IGFs were infused into blood at 62 ng/ml/min; based on the average haematocrit of blood collected during the first series of vascular perfusions, infusion at this concentration would yield a plasma concentration of added IGFs of approximately 100 ng/ml, instead of the less than 1 ng/ml previously infused (Chapter **4**).

Post-perfusion and Sample Analysis:

All post-perfusion collections and sample analyses were performed exactly as described in Section **4.2.2.**

5.2.4 Calculations and Statistical Analysis

Calculations for total infused versus recovered radioactivity, average plasma concentration of infused IGF, and transfer of radioligand to intestine were performed as described in Section **4.2.4.** Statistical analyses were performed by one-way analysis of variance, followed by Fisher's PLSD post-hoc test where significant F-values were identified. Values are expressed as mean ± SEM.

5.3 RESULTS

5.3.1 Selection of Infusate Mix for Vascular Perfusion

Because there were only small differences in the proportions of labelled IGF-I and analogs that were free in plasma after vascular perfusion at less than 1 ng/ml (perfusions at

High Specific Activity), a series of *in vitro* incubations was performed to select a concentration of unlabelled ligand at which these differences could be increased, for use in the vascular perfusion model. Chromatographic analysis revealed radioligand distributed between three regions in all samples, with estimated molecular masses of 130-150 kDa, 30-50 kDa and 8 kDa. The relative abundance of ^{125}I-labelled IGFs at the three size regions was calculated as described in Section **4.3.4** and is shown in Fig 5.1 (*a-c*) for all three IGF peptides.

The proportion of radioligand bound at the two higher molecular mass peaks varied with the amount of unlabelled ligand included in the incubation. For incubations in which IGF-I was employed, increasingly large proportions of the radiolabelled ligand eluted in the 30-50 kDa peak as the concentration of unlabelled ligand was increased up to 250 ng/ml. This increase was at the expense of binding at 130-150 kDa, but there was no increase in the proportion of free radioligand (Fig. 5.1*a*). At 500 ng/ml, however, the proportion of radioligand at 30-50 kDa declined, with an accompanying increase in free IGF-I. This presumably reflected saturation of unoccupied ligand-binding sites. In incubations containing labelled analogs, as for those with IGF-I peptide, increased concentrations of the unlabelled homologs resulted in lower relative binding of the radioligand at 130-150 kDa (Fig. 5.1*b, c*). Nevertheless, the changes in distribution of the labelled analogs between the 30-50 kDa and 8 kDa peaks differed to that observed in the incubations with IGF-I. Thus, decreased binding of labelled des(1-3)IGF-I at 130-150 kDa was accompanied by concurrent increases in both binding at 30-50 kDa and in uncomplexed radioligand at 8 kDa when unlabelled des(1-3)IGF-I was added at up to 250 ng/ml, although binding at 30-50 kDa decreased as concentration of unlabelled ligand was increased further. In incubations containing LR^{3}IGF-I, free radioligand increased with log of peptide concentration while binding at 30-50 kDa remained virtually unchanged at concentrations up to 100 ng/ml, after which it declined.

With inclusion of radioligand only, at a 1 ng/ml concentration approximating that used in the High Specific Activity vascular perfusions (Chapter **4**), less than 5% of IGF-I eluted at the position of free ligand (Fig. 5.2*a*). In contrast to IGF-I, approximately 20% of

Fig. 5.1. *Molecular mass distribution of radiolabelled IGF-I (a), des(1-3)IGF-I (b) and LR^3IGF-I (c), estimated after incubation with lamb plasma and homologous unlabelled IGF peptide at a range of concentrations.* Plasma from an anaesthetised lamb was incubated with radiolabelled IGFs (1 ng/ml), plus unlabelled ligand at concentrations of 1-1000 ng/ml, under conditions similar to those employed in the vascular perfusion study (Chapter **4**), prior to chromatography using a Superose-12 size-exclusion column (Section **2.1**). Radioactivity eluting at 130-150 kDa (*squares*), 30-50 kDa (*circles*) and 8 kDa (*triangles*) was estimated as a proportion of the total radioligand at 9-12.5 ml, 13-15 ml and 15.5-18.5 ml, respectively. Total bound radioactivity is also shown (*crosses*).

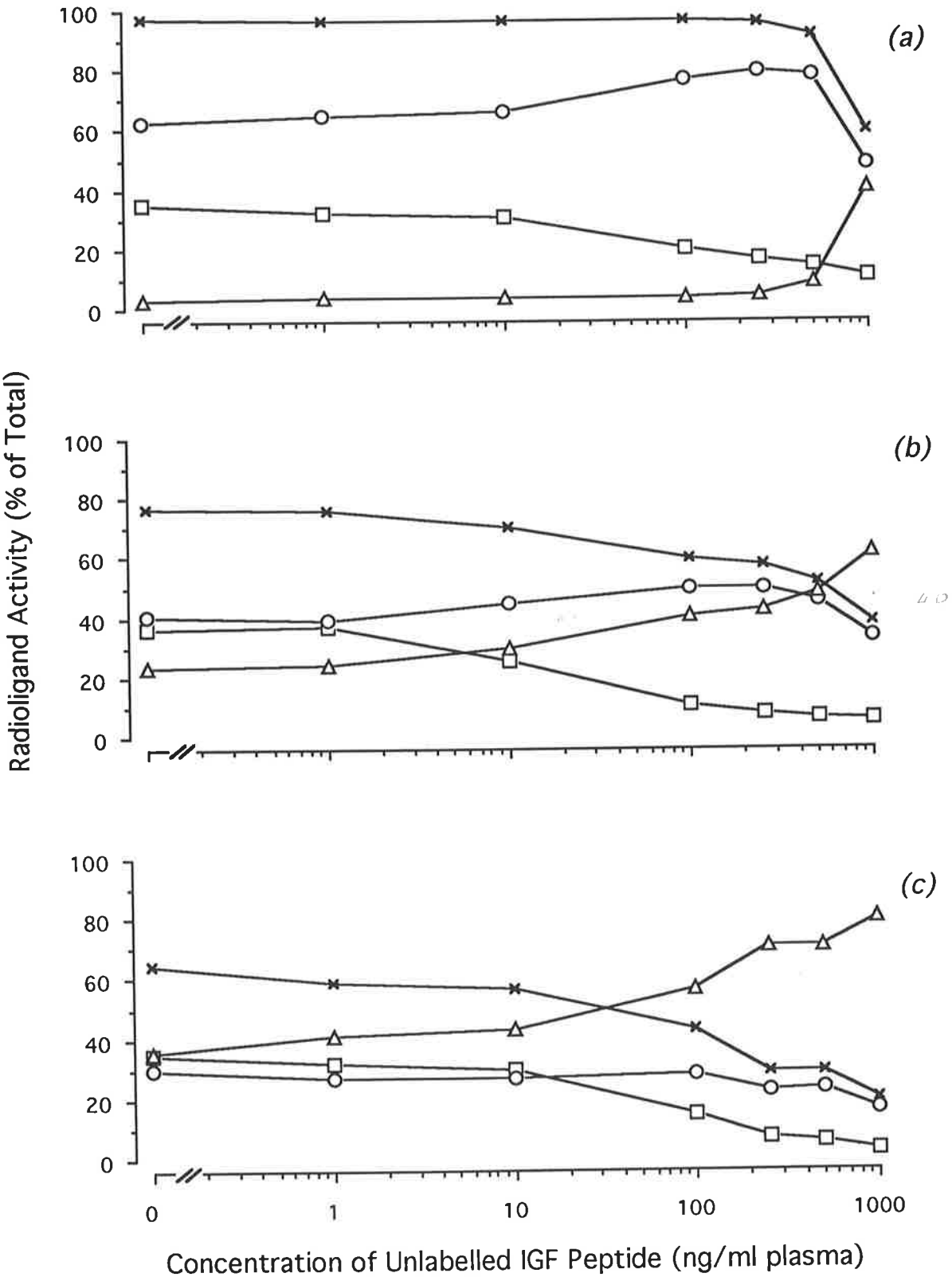
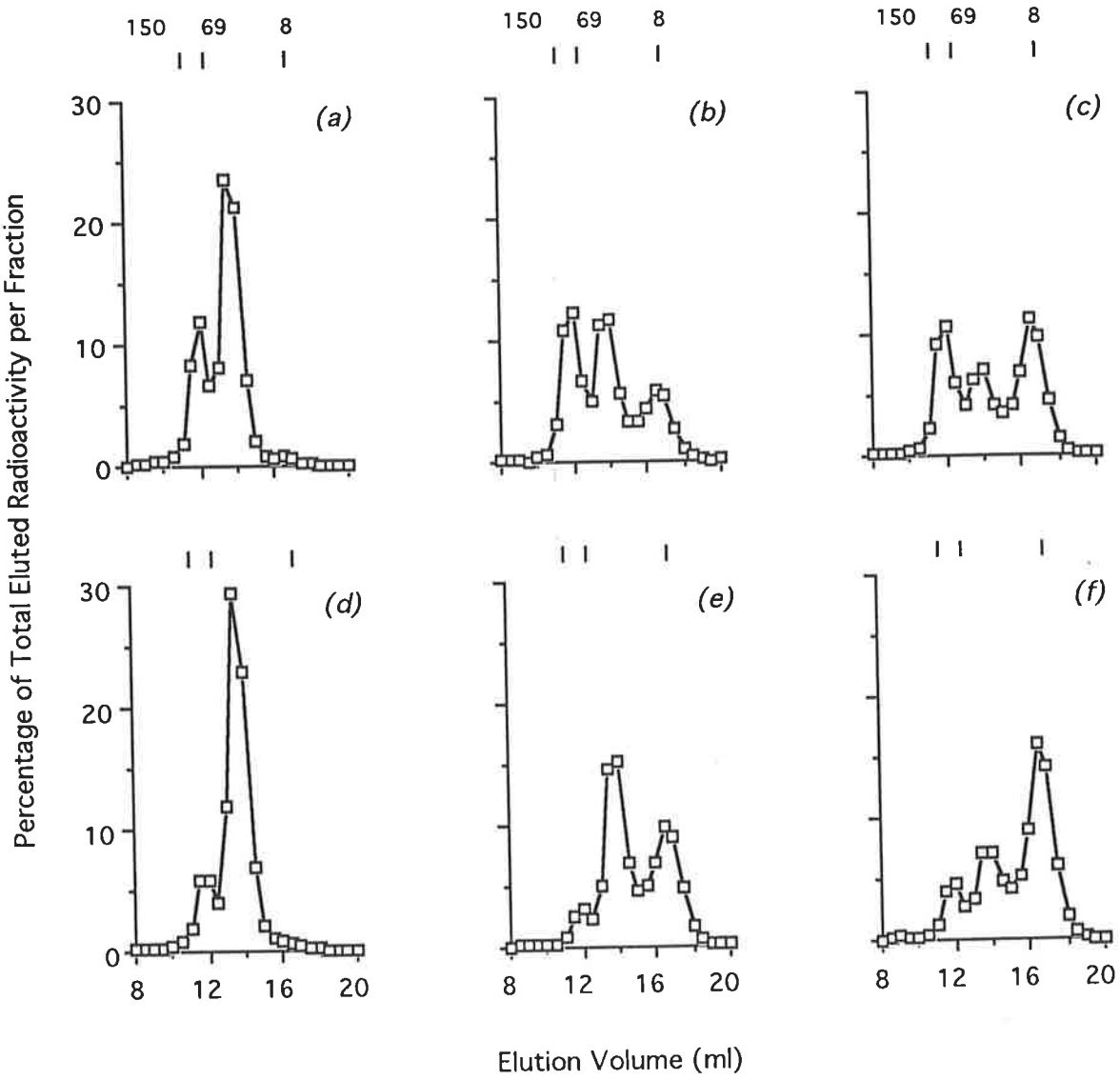


Fig. 5.2 *Size-exclusion chromatography of lamb plasma incubated in vitro with radiolabelled IGF-I (a, d), des(1-3)IGF-I (b, e) or LR^{3}IGF-I (c, f) at 1 ng/ml and without (a, b, c) or with (d, e, f) inclusion of unlabelled ligand at 100 ng/ml.* The profiles show recovery of radioactivity in 0.5 ml fractions as a percentage of total radioactivity eluted. The elution positions of human alcohol dehydrogenase (150 kDa), BSA (69 kDa) and radiolabelled IGFs (8 kDa) are indicated.


150
69
8
(a)
(b)
(c)
(d)
(e)
(f)
30
20
10
0
8
12
16
20
Percentage of Total Eluted Radioactivity per Fraction
Elution Volume (ml)

des(1-3)IGF-I and 35% of LR^{3}IGF-I remained uncomplexed in the absence of competing ligand (Fig. 5.2*b*, *c*). For use in the subsequent vascular perfusion study, however, a combination of 1 ng/ml radioligand and 100 ng/ml unlabelled peptide was selected since at this concentration the proportion of uncomplexed radioligand remained unchanged for IGF-I (Fig. 5*d*) but was doubled for des(1-3)IGF-I (40% free; Fig. 5.2*e*) and was increased by over 60% for LR^{3}IGF-I (57% free; Fig. 5.2*f*). The differences in these binding profiles, particularly with inclusion of a 100 fold excess of unlabelled IGF, are consistent with the lower relative affinity of des(1-3)IGF-I and especially LR^{3}IGF-I, compared with IGF-I, for IGF-binding proteins (Bagley *et al;* 1991, Francis *et al;* 1992).

5.3.2 The Vascularly Perfused Intestinal Segment

Success Rate:

From a total of 26 perfusions attempted, data from 15 (58%) are included in the present study. While 17 perfusions were completed, two (8%) were rejected because infused radioactivity was not recovered quantitatively (ie. less than 95% of infusate accounted for in all tissues and fluids). Of the remaining abortive attempts, three (12%) failed due to unsuccessful cannulation of intestinal blood vessels, while six (23%) died under anaesthesia before completion of the experiment.

The 58% success rate of the present experiment represents an improvement from the 47% attained for experiments in Chapter **4** and may have been the result of the refinements made to the basic perfusion model employed in that chapter. Thus, the use of looser ligatures around the intestine was successful in its aim of reducing bleeding into the intestinal lumen while, at the same time, the recovery of vascularly infused radioligand was not compromised; only 8% of the present experiments were rejected because infused radioactivity was not recovered quantitatively. The other refinement employed in the present experiments was the use of artificial ventilation. While as a proportion of all attempted experiments there was only a small reduction in lamb deaths during anaesthesia, the ratio of successful perfusions to

anaesthesia deaths was reduced nearly 30%, from 1:0.56 to 1:0.40. Those deaths that did occur during anaesthesia were perhaps the result of medullary depression and failure of the circulation, a consequence of oversupply of anaesthetic (Booth; 1982). Given closer monitoring for depth of anaesthesia, the incidence of such losses could probably be further reduced.

Physiological Parameters:

Arterial pH and blood gas partial pressures (P) for CO_2 and O_2 were monitored at intervals throughout anaesthesia. Means ± SEMs for the pre-anaesthesia pH and blood gas values were calculated from a single sample collected prior to anaesthesia from each of 10 animals, while the anaesthesia means ± SEMs were calculated from average values for the 2-4 blood samples collected from each of these lambs during surgery. Thus arterial pH and PCO_2 during anaesthesia were 7.46 ± 0.02 and 35.94 ± 0.95 MPa, respectively, almost identical to the pre-anaesthesia values of 7.44 ± 0.00 and 36.63 ± 2.51 MPa. Nevertheless, PO_2 during anaesthesia (375.1 ± 34.7 MPa) was approximately five times that of the pre-anaesthesia value (68.81 ± 2.21 MPa), probably due to the use of 100% oxygen for ventilation. In combination with a maximum inspiration pressure of 25 mm Hg, a ventilation rate of 36-42 breaths/min was found to be an effective range for maintenance of normal blood pH and PCO_2 values.

Blood and Lymph Flow:

The perfusion characteristics of the intestinal segment during infusion of labelled IGF at Low Specific Activity were similar to those described for perfusions at High Specific Activity (Section **4.3.1**). Thus, there was no net change in average efferent blood or lymph flow during vascular perfusion (Tables 5.1, 5.2), although flow rates fluctuated both before and during perfusion. Mean arterial blood pressure was maintained in a physiological range by infusion of donor blood; the representative blood pressure and heart rate data, and blood and lymph flow characteristics shown in Chapter **4** (Fig. 4.3) are typical also of the profiles obtained in the present experiments. Appearance of the segment remained normal during perfusion and no blood was recovered from the lumen after the perfusion.

TABLE 5.1. *Average blood flow before and during infusion of radiolabelled IGFs at 1 ng/ml with unlabelled ligand at approximately 100 ng/ml.*

	IGF-I	des(1-3)IGF-I	LR^3IGF-I
Blood flow (ml/min)			
Pre-perfusion	11.74	5.64	3.11
	3.88	1.88	3.69
	3.10	5.42	4.37
	3.84	2.09	3.16
	2.23	10.81	2.84
Average	*5.0 ± 1.7*	*5.2 ± 1.6*	*3.4 ± 0.3*
Perfusion	10.9	5.6	3.8
	3.3	1.6	2.3
	4.3	5.3	4.1
	3.3	1.7	2.5
	2.0	12.4	2.2
Average	*4.8 ± 1.6*	*5.3 ± 2.0*	*3.0 ± 0.4*

Values are means ± SEM for 5 lambs/group. No significant differences were apparent between peptides.

TABLE 5.2. *Average lymph flow before and during infusion of radiolabelled IGFs at 1 ng/ml with unlabelled ligand at approximately 100 ng/ml.*

	IGF-I	des(1-3)IGF-I	LR^3IGF-I
Lymph Flow (ml/min)			
Pre-perfusion	0.15	0.09	*Failed Cannulation*
	0.11	0.10	0.16
	0.14	0.16	0.09
	0.01	0.12	0.16
	0.08	0.05	0.08
Average	*0.10 ± 0.03*	*0.11 ± 0.02*	*0.12 ± 0.02*
Perfusion	0.15	0.17	*Failed Cannulation*
	0.15	0.05	0.10
	0.11	0.10	0.05
	0.02	0.11	0.11
	0.07	0.03	0.07
Average	*0.10 ± 0.02*	*0.09 ± 0.02*	*0.08 ± 0.01*

Values are means ± SEM for 5 lambs/group, except for perfusions using LR^3IGF-I where n = 4 due to a failed cannulation. No significant differences were apparent between peptides.

5.3.3 Recovery and Distribution of Radioactivity

The recovery of vascularly infused radioactivity in the current experiment was very similar to that of the previous perfusions, so that from 95 - 105% of the amount infused was collected. Of the infused radioactivity, only a minor proportion (generally less than 0.06%) was recovered in adjacent intestinal tissues and insignificant amounts of radioactivity were detected in the general circulation. Tracer recovered in adjacent intestine and general circulation was excluded from calculations of IGF distribution in tissues and fluids from the perfused segment. Radioactivities recovered in the perfused segment, luminal contents, lymph, lymph node and efferent blood were expressed as percentages of the total activity detected in these tissues and fluids. That which was attributable to intestinally trapped blood was subtracted from the total activity retained in the intestinal segment, and added to the counts recovered in efferent intestinal blood. This portion was not more than 0.26% of the total infused radioligand. As found in the previous vascular perfusion study, radioactivity appeared rapidly in efferent blood from the intestinal segment, reaching maximal concentration within about 2 min of infusion.

Most labelled IGF-I was not extracted from blood in a single pass through intestinal tissue but was collected in the efferent blood (96.4%). Some counts were recovered in the intestinal mesentery (0.13%) but this radioactivity presumably reflected blood content, since this tissue contained the vascular network through which radiolabelled IGFs were perfused. The remainder of the radioactivity was transferred to the intestinal tissues and fluids, with the majority retained in intestine (3.38%) and smaller proportions recovered in luminal contents (0.10%), lymph node (0.01%) and lymph (0.004%). Thus, the total proportion of IGF-I transferred to intestinal tissue was $3.49 \pm 0.93\%$ (Table 5.3).

Net transfer of vascularly infused analogs and their distributions between the intestinal tissue, luminal contents, lymph node and lymph were similar to that described for labelled IGF-I (Table 5.3). No statistically significant differences in net transfer, or transfer to any particular fluid or tissue compartment, were apparent between IGF-I, des(1-3)IGF-I

TABLE 5.3. *Recovery of radiolabelled IGFs infused at 1 ng/ml with unlabelled ligand at 100 ng/ml plasma*

	Percentage of Radioligand Recovered		
	IGF-I	des(1-3)IGF-I	LR^3IGF-I
Blood	96.4 ± 0.9	95.6 ± 0.9	96.9 ± 0.5
Mesentery	0.13 ± 0.06	0.21 ± 0.06	0.26 ± 0.03
Intestinal Tissue	3.38 ± 0.91	4.02 ± 0.90	2.64 ± 0.51
Luminal Contents	0.10 ± 0.03	0.15 ± 0.05	0.15 ± 0.05
Lymph Node	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01
Lymph	0.004 ± 0.003	0.013 ± 0.012	0.004 ± 0.002
Net Intestinal Transfer	3.49 ± 0.93	4.20 ± 0.93	2.81 ± 0.51

Data show the percentage of infused tracer recovered at the different sites. Net Intestinal Transfer is the sum of the percentage recovered in the intestinal tissue, luminal contents, lymph and lymph node. Values are means ± SEM for 5 lambs/group. No significant differences were apparent between peptides.

and LR^{3}IGF-I, although there was a trend towards greater retention and net transfer of des(1-3)IGF-I than of IGF-I or LR^{3}IGF-I.

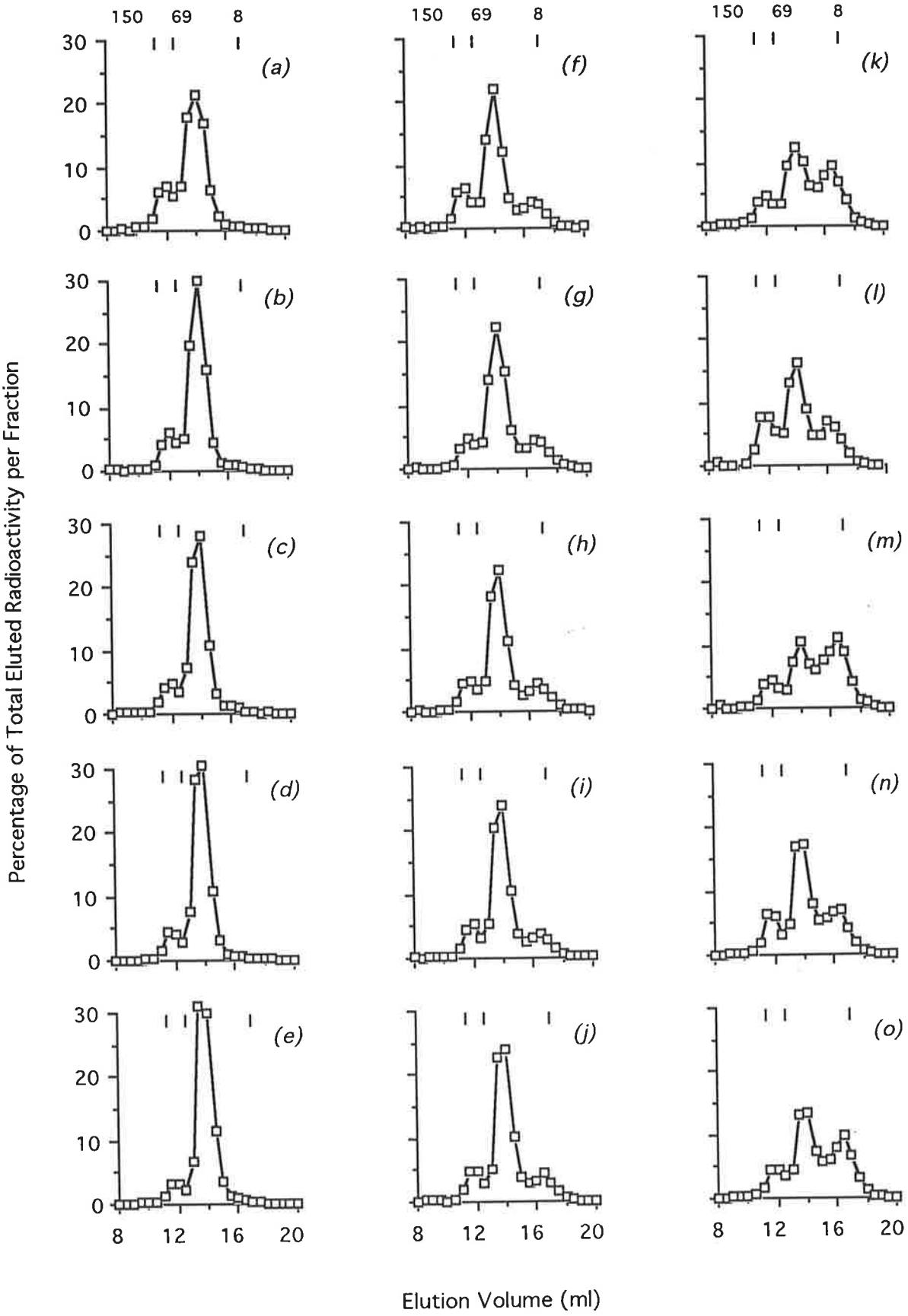
5.3.4 TCA Precipitability

Intact and degraded radioligand in efferent blood and in the intestinal segment were compared with those in the infusate by TCA precipitation. Intact radioligand was estimated as the TCA-precipitable fraction of this radioactivity, and degraded IGFs as the TCA-soluble component. Radioactivity in the infusate was at least 97% TCA-precipitable in all experiments and differed to that recovered in efferent blood by less than 0.2%, implying that blood-borne radioligand was not degraded in a single pass through distal ileal tissue. The precipitability of intestinally retained radioactivity was, however, somewhat lower than that in the infusate or blood; the percentages of intact radioligands in intestinal tissues were estimated at 90.5 ± 1.4%, 88.8 ± 0.3% and 93.2% ± 0.9% for IGF-I, des(1-3)IGF-I and LR^{3}IGF-I, respectively. No statistically significant differences were apparent between peptides. However, the reduced precipitability of intestinal radioactivity compared with that of infusate implies some radioligand degradation in the intestine. Measurements of TCA precipitability in lymph and lymph node and in most samples of luminal contents were not performed because of the low concentrations of radioactivity in these samples.

5.3.5 Association of Radioligands with Plasma IGFBPs *in vivo*

In order to confirm that the differences in *in vitro* binding of IGF-I, des(1-3)IGF-I and LR^{3}IGF-I to plasma IGFBPs were reflected in the vascular perfusion model, the distribution of radioligand in a mid-perfusion efferent plasma sample from each experiment (20-25 min) was analysed by size-exclusion chromatography. Elution profiles of radioligands in each experiment are shown in Fig. 5.3 and the relative distributions of the radioligands between the IGF and IGFBP regions, estimated as described in Section **4.3.4,** are summarised in Table 5.4. Radioligands bound to IGFBPs at 130-150 kDa and

Fig. 5.3. *Chromatographic elution profiles of radioactivity in venous plasma after vascular perfusion of radioligand, plus unlabelled ligand at 100 ng/ml plasma.* Peptides infused were IGF-I *(a-e)*, des(1-3)IGF-I *(f-j)* and LR^3IGF-I *(k-o)*. Plasma samples were prepared from venous blood collected at 20-25 min from the start of peptide infusion and were chromatographed on a Superose-12 size-exclusion column as described in Section **2.3**. The plots show the recovery of radioactivity in 0.5 ml fractions as a percentage of total radioactivity eluted. Human alcohol dehydrogenase (150 kDa), BSA (69 kDa) and radiolabelled IGFs (8 kDa) were used for molecular mass calibration of the column.


150 69 8
(a)
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(l)
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(n)
(o)
30
20
10
0
8 12 16 20
Percentage of Total Eluted Radioactivity per Fraction
Elution Volume (ml)

Table 5.4. *Recovery of radioactivity as a percentage of total radioligand following pH 7.2 size-exclusion chromatography of venous plasma from ovine intestine perfused with radiolabelled IGFs (1 ng/ml) with unlabelled ligand at 100 ng/ml*

	IGF-I	des(1-3)IGF-I	LR^3IGF-I
130-150 kDa	17 ± 2	17 ± 1	18 ± 2
30-50 kDa	79 ± 2	65 ± 1 *	47 ± 3 *
Total bound	96 ± 1	82 ± 1 *	65 ± 4 *
8 kDa (% free peptide)	4 ± 1	18 ± 1 †	35 ± 4 †

Data are the percentage of total radioactivity that eluted from the column between 9.0 and 18.5 ml, at 9.0-12.5 ml (130-150 kDa), 13.0-15.0 ml (30-50 kDa), and 15.5-18.5 ml (8 kDa) following pH 7.2 size-exclusion chromatography of efferent venous plasma collected from the vascular perfusion at 20-25 min after the start of infusion of radiolabelled peptides. Values shown are mean ± SEM of 5 lambs per group. † Significantly greater than IGF-I ($p<0.001$), and * Significantly less than IGF-I ($p<0.001$). LR^3IGF-I perfusions also had significantly higher % free peptide and lower % bound (total and 30-50 kDa) compared with des(1-3)IGF-I perfusions.

30-50 kDa, as had been demonstrated when radiolabelled IGFs were infused in the absence of unlabelled ligand (Fig. 4.4). In plasma from perfusions in which IGF-I analogs were employed, distinct peaks of uncomplexed radioligand (8 kDa) were evident. Also as previously found (Chapter **4**), most (96%) radiolabelled IGF-I eluted in association with IGFBPs so that only 4% was free (Fig. 5.3*a-e*). While the radiolabelled analogs des(1-3)IGF-I and LR^{3}IGF-I (Fig. 5.3*f-j* and *k-o*, respectively) were also mainly bound to IGFBPs, significantly greater proportions of the analogs than of IGF-I eluted free of IGFBPs (18% and 35% for des(1-3)IGF-I and LR^{3}IGF-I, vs 4% for IGF-I; $p<0.001$) (Table 5.4). In addition, significantly smaller proportions of analogs than of IGF-I eluted in the 30-50 kDa region ($p<0.001$). However, a similar proportion of each peptide eluted at 130-150 kDa.

As a proportion of the total radioactivity recovered from the column, $3.08 \pm 0.57\%$, $3.49 \pm 0.59\%$ and $3.42 \pm 0.58\%$ of radiolabelled IGF-I, des(1-3)IGF-I and LR^{3}IGF-I, respectively, eluted later than the free IGF at the position of free iodide. The recovery of radioactivity at the position of free iodide was similar when the infusate was analysed by size-exclusion chromatography ($3.20 \pm 0.87\%$, $3.60 \pm 0.72\%$ and $4.32 \pm 0.58\%$ for IGF-I, des(1-3)IGF-I and LR^{3}IGF-I, respectively), suggesting that degradation of IGF peptides in the vascular compartment of the perfused intestine was negligible. Because of the low concentration of radioactivity in the intestinal homogenate, size-exclusion chromatography was not used as a confirmation of the TCA estimate of intactness for radioligand in this tissue.

5.4 DISCUSSION

In vitro ligand-binding studies were performed under conditions that mimicked those of the vascular perfusion to select conditions under which reduced proportions of labelled analogs were complexed to IGFBPs. The results show that binding of labelled IGF peptides to IGFBPs *in vitro* was in the order IGF-I > des(1-3)IGF-I > LR^{3}IGF-I, at all concentrations of unlabelled IGF, with net binding of analogs reduced with log of unlabelled ligand

concentration. At a concentration of 100 ng/ml, the proportion of des(1-3)IGF-I tracer eluting free was increased to 40% from the 20% achieved with tracer alone, representing a doubling in the proportion of free des(1-3)IGF-I. Free LR^3IGF-I tracer, on the other hand, was increased from 35% to 57% with inclusion of unlabelled ligand, representing a 62% increase in the free component, while the proportion of labelled IGF-I eluting at the position of free peptide did not change with inclusion of 100 ng/ml unlabelled IGF-I. Thus, the proportions of analogs eluting free of IGFBPs were substantially greater at Low Specific Activity than at High Specific Activity. It was anticipated, therefore, that vascular perfusion with labelled IGFs plus unlabelled ligand would be accompanied by increased differences in the proportions of free versus bound analogs and greater differences in tissue-transfer between IGF-I and the analogs.

Size exclusion chromatography of plasma from the vascularly perfused intestine revealed that radiolabelled IGF-I was essentially fully bound to IGFBPs when infused at Low Specific Activity (100 ng/ml unlabelled peptide), as previously found when infused without unlabelled ligand (Chapter 4). Increased proportions of labelled analogs eluted at the molecular mass of free IGF but the extent of these increases differed to those predicted from the *in vitro* incubations. Thus, the proportion of non-complexed LR^3IGF-I in the present experiment was about 35% compared with 13-14% when infused at High Specific Activity, an increase that was well in excess of the 62% rise predicted from the *in vitro* perfusions. Surprisingly, however, the proportion of free des(1-3)IGF-I was only 18% in the present perfusion experiment compared with 12-13% when infused without unlabelled ligand. This represented a 23% increase in uncomplexed ligand, falling well short of the predicted doubling in free des(1-3)IGF-I. The reasons for the discrepancies between predicted and attained results are not clear, but the most likely explanation is that the *in vitro* conditions did not exactly reflect those in the perfused intestine. In particular, anaesthesia as well as surgical trauma may have modified the IGF-binding capacity in plasma by either changing binding protein levels or endogenous IGF concentrations. The effect of halothane anaesthesia on plasma IGFs and IGFBPs is investigated in a subsequent chapter and the results of that study used in the 'General Discussion' chapter to explain the differences in

extents of analog binding that were apparent between the *in vitro* and *in vivo* experiments (compare Figs. 4.4, 5.2 and 5.3).

Because the proportion of free des(1-3)IGF-I that was achieved in the current series of perfusions was only a small increase over that attained previously (Chapter **4**), a large difference in net transfer between it and IGF-I would perhaps have been unexpected. Indeed, in this series of vascular perfusions as in those of Chapter **4**, there was merely a trend towards greater transfer of des(1-3)IGF-I than of IGF-I. While interpretations of such trends must be viewed cautiously when considered in isolation, the fact that this trend was evident in both perfusion experiments lends weight to the hypothesis that IGFBP interaction effects a net reduction in transfer. However, when we consider the lack of difference in transfer between LR^3IGF-I and IGF-I, especially in the present experiment where 35% of LR^3IGF-I was free, the present data provide little evidence to support a generalised inhibitory role for IGFBPs in regulation of IGF transfer. Nevertheless, indirect evidence has been presented by others to suggest an inhibitory role for IGFBPs in the transfer of IGFs from the vasculature. Thus, when radiolabelled IGF-I is infused into the vascular supply of the mammary gland, transfer to milk of the perfused gland is greater than transfer to the adjacent non-perfused gland (Prosser *et al;* 1991). Presumably the lower transfer to the non-perfused gland was the result of greater IGF binding to IGFBPs by the time the ligand had recirculated to this gland.

It is plausible that the lack of significant differences between net transfer of labelled IGF-I, des(1-3)IGF-I and LR^3IGF-I in the present experiments may reflect limitations of the experimental model employed. If available receptor sites had become saturated with ligand before completion of the 45 min perfusions, the value for net transfer of IGF peptides may merely reflect the maximum ligand-binding capacity of the intestinal segment; in this case no differences in net transfer would be evident between IGF peptides. Differences may be evident with a shorter perfusion period. However, because the kinetics of transfer were not addressed in these studies, these possibilities remain entirely speculative.

The results show that infusion of IGF peptides at 100 ng/ml in plasma did not cause a significant change in blood or lymph flow. This contrasts with an earlier finding (Prosser

et al; 1990) in which infusion of IGF-I into the pudic artery of the goat mammary gland, at a concentration similar to that used in the present experiment, resulted in a 30% increase in mammary blood flow over 5 h of peptide infusion. The lack of net changes in intestinal blood flow during infusion of IGFs may perhaps be attributed to the reduced period of perfusion employed; effects on blood flow may have been observed had the IGF-infusion period been extended.

The tissue distribution of transferred IGF was not addressed in the present study. Nevertheless, it is likely that most intestinal radioactivity was associated with tissues for, while some radioactivity was detected in lymph, the total collected via this pathway was less than 1% of that recovered in intestinal tissue. Since lymph is derived from the extravascular spaces, the amount remaining in the extravascular spaces is likely to be only small.

5.5 SUMMARY

1) The vascular perfusion model was improved in a number of ways, including the use of looser ties around the intestine to prevent damage to blood vessels and reduce bleeding into the lumen. Success rate was also improved, partly through use of artificial ventilation during anaesthesia.

2) Size-exclusion chromatography of plasma, following *in vitro* incubation with radiolabelled IGF-I, des(1-3)IGF-I or LR^{3}IGF-I, plus unlabelled homologous ligand at a range of concentrations, was performed to determine an appropriate mixture of labelled and unlabelled ligand for vascular perfusion. A 100 ng/ml concentration of unlabelled ligand was selected, at which 1) labelled IGF-I should have remained mainly complexed; 2) free labelled des(1-3)IGF-I should have increased from the 13% obtained during infusion of radioligand, to about 26%; 3) free LR^{3}IGF-I should have increased from the 15% obtained with infusion of radioligand only, to about 24%.

3) The distribution of radioligand between blood, lymph and the intestinal tissues and fluids was similar to that determined in the previous vascular perfusion study (Chapter 4), such

that about 96% of vascularly infused IGF was recovered in the efferent blood from the segment, 3-4% was retained in the perfused intestinal segment, with the remainder distributed between the segment mesentery, luminal contents, lymph and lymph node.

4) As obtained for vascular perfusions at 1 ng/ml of plasma, perfusion at 100 ng/ml did not results in statistically significant differences, in transfer of IGF-I and the analogs to intestinal tissue or in net transfer to intestine (radioactivity in the intestinal segment, luminal contents, lymph and lymph node). There was a trend towards increased transfer of des(1-3)IGF-I compared with IGF-I, although no such trend was apparent for LR^3IGF-I compared with IGF-I.

5) The TCA precipitability of radioligand collected in efferent blood from the segment was essentially the same as that of the infusate, suggesting that IGFs are not significantly degraded in a single pass through the intestine. However, precipitability of radioactivity recovered in the intestinal tissue was somewhat lower, suggesting that some degradation of peptide occurred in the intestinal tissue.

6) Size-exclusion chromatography of plasma from the vascular perfusions revealed that when infused with unlabelled ligand at 100 ng/ml, labelled IGF-I remained essentially fully bound to plasma IGFBPs, while approximately 16% and 35% of labelled des(1-3)IGF-I and LR^3IGF-I were free. This compared with 13% and 15% for analogs perfused at less than 1 ng/ml. Substantial increases in the proportions of free ligands had been predicted from the results of the *in vitro* incubations for both des(1-3)IGF-I and LR^3IGF-I, but were only realised in perfusions using LR^3IGF-I. The lack of such an increase for des(1-3)IGF-I cannot be explained by the present study but may relate to effects of extended anaesthesia or surgery.

5.6 ACKNOWLEDGMENTS

Ms K. Edson is gratefully acknowledged for general surgical assistance during the preparation and perfusion of intestinal tissue segments, for cooperation in the weighing of all

samples and sample aliquots, and for the preparation of plasma from blood samples. Measurements of blood pH and blood gas partial pressures were shared by Ms Edson and Ms C. Mardell. Occasional surgical assistance was provided by Ms K. Penning. Mr A. Weatherly and Ms J Goodall are thanked for assistance with the collection of donor blood. Ms K. Edson, Ms. K Penning and Ms C. Mardell also assisted with this collection, on occasion.

Chapter Six

Effect of Anaesthesia on Plasma IGF-Binding Characteristics and Concentrations of Endogenous IGFs and IGFBPs

CHAPTER SIX: EFFECT OF ANAESTHESIA ON PLASMA IGF-BINDING CHARACTERISTICS AND CONCENTRATIONS OF ENDOGENOUS IGFS AND IGFBPS.

6.1 INTRODUCTION

A general observation made in Chapters **4** and **5** was that the binding of analogs, especially that of des(1-3)IGF-I, to plasma IGFBPs was much greater following vascular perfusion than at equivalent concentrations in the *in vitro* incubations. This suggests that the *in vitro* conditions of association did not accurately reflect the *in vivo* conditions. However, the incubation time and temperatures employed *in vitro* were similar to those *in vivo*, and while it could be suggested that exposure of radioligand to tissue and blood cells accounts for the greater binding to IGFBPs *in vivo*, a mechanism by which this could occur is unclear. A plausible explanation for the more extensive binding *in vivo* arises from the difference in time, relative to the start of anaesthesia and surgery, at which blood was collected for *in vitro* analyses (20 min) compared with that collected from the vascularly perfused intestine (2-3 h); perhaps the greater binding of analogs *in vivo* was the result of an increase in plasma IGF-binding capacity over time. In particular, extended periods of surgery, anaesthesia, high blood PO_2 and food deprivation may all have contributed to such changes.

An increase in IGF-binding capacity could result from decreased circulating IGF concentrations, increased circulating IGFBP, or a combination of the two. While food deprivation has been shown to result in reduced plasma IGF concentrations (Underwood *et al;* 1991), significant changes have not previously been demonstrated over intervals as short as 2-3 h and it seems unlikely, therefore, that food deprivation would account for the increased binding capacity of plasma in the perfusion experiment. Blood oxygen partial pressure (PO_2) may be an important factor in the regulation of plasma IGF-binding capacity. Indeed low blood PO_2 has been shown to result in increased plasma IGFBP-1 over a period of hours (McLellan *et al;* 1992) and it appears also to cause a reduction in circulating IGF concentrations (Iwamoto *et al;* 1992). While high blood PO_2, as measured in the perfusion experiment (Chapter **5**), would be unlikely to induce the same IGFBP as induced during

hypoxaemia, the possibility that other IGFBPs may be induced under conditions of high PO_2 cannot be excluded. The response of plasma IGFs and IGFBPs to surgery and/or anaesthesia has not previously been described. However, the results of some studies suggest that halothane may alter both utilisation and synthesis of glucose (Biebuyck *et al;* 1972, Romero *et al;* 1987, Cronau *et al;* 1988, Crosby and Atlas; 1988), which together with insulin is a key regulator of circulating IGFBP-1 (Suikkari *et al;* 1988, Snyder & Clemmons; 1990). Should halothane cause a net decrease in plasma glucose concentration, an increase in IGF-binding capacity could be effected through elevated circulating IGFBP.

The hypothesis I addressed in the present study was that the substantial difference in ligand-binding between the *in vitro* incubations and vascular perfusion studies (Chapters **4** & **5**), resulted from an increase in IGF-binding capacity during halothane anaesthesia. Blood samples were collected from food deprived lambs (controls), from hyperoxic anaesthetised lambs and from normoxic anaesthetised lambs. Plasma was incubated with radiolabelled IGF and analysed by size-exclusion chromatography to establish that anaesthesia increased the binding capacity of plasma. Following these studies I sought to further characterise the factors that might contribute to such a change. The study was therefore extended to measurements of endogenous IGF concentrations, plasma insulin and glucose, as well as IGFBP characterisation by ligand blotting, immunoblotting and purification studies.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Recombinant human IGF-I, IGF-II and des(1-3)IGF-I were purchased from GroPep Pty. Ltd., while recombinant ovine insulin was obtained from Lilly Research Laboratories. For radioiodination, IGF-I, IGF-II, des(1-3)IGF-I and insulin were dissolved in 10 mM HCl and were reacted with $Na^{125}I$ (Nordion International Inc.) to specific activities in the

range 27-73 Ci/g, as described in Section **2.1.** Radioligands were diluted in heparinised PBS (Section **2.3**) containing 2.5 mg/ml BSA.

Anaesthetics used were halothane and Lignocaine. ^{14}C-Molecular mass standards were used for the estimation of IGFBP molecular masses in ligand blotting and immunoblotting experiments. Immunoblotting experiments utilised antibodies raised in rabbits against human IGFBP-1 (Upstate Biotechnology Inc.), bovine IGFBP-2 (provided by Dr. B. Forbes), ovine IGFBP-3 and IGFBP-4 (both provided by Dr. P.E. Walton), and antibody against rabbit gamma-globulin raised in goats and conjugated to horse radish peroxidase (Dakopatts). The conjugated antibody was detected using a chemiluminescence detection reagent (RPN 2106; Amersham). Assay of plasma insulin concentrations was performed using antiserum against ovine insulin (GP-2; kindly provided by Dr P. Wynn) and a donkey anti-guinea pig solid phase immunoglobulin-coated cellulose suspension (IDS). Assay of IGF-I in acid-gel chromatographed plasma was performed using an antibody raised in rabbits against human IGF-I (MAC 89/1; Dr. P.C. Owens) and a donkey anti-rabbit solid phase immunoglobulin-coated cellulose suspension (IDS).

A range of biologicals were used in the purification of IGFBPs from plasma. Phenylmethyl-sulphonyl fluoride (PMSF), bacitracin, protamine sulphate, activated charcoal and Coomassie brilliant blue R-250 were all purchased from Sigma Chemical Co. Solvents including acetonitrile (BDH Ltd.), trifluoracetic acid (TFA; Fluka), heptafluorobutyric acid (HFBA [anhydrous]; Beckman Instruments Inc.) and propan-1-ol (Mallinkrodt Australia) were also utilised.

6.2.2 Blood Collections

Male 6-7 week old Merino-Dorset crossbred lambs were used in all experiments. Lambs were not weaned at the time of experimentation and had access to lucerne chaff or pasture fodder until within 2-4 h of experimentation. The jugular vein was catheterised on the day before the experiment, as described in Section **2.4.1.** Immediately prior to the

experiment, a catheter (1.0 mm ID, 1.5 mm OD) was inserted into the cranial tibial artery (Section **2.4.1**) using topical application of anaesthetic (Lignocaine). Arterial blood P_{O_2} and P_{CO_2} as well as pH were monitored before and during anaesthesia, while blood samples were collected from the venous catheter for measurement of IGFs, IGFBPs, glucose and insulin.

Pre-anaesthesia blood samples were withdrawn from both catheters, after which lambs were masked with halothane (5%) to enable intubation with an endotracheal tube (Section **2.6**). Because plasma concentrations of some IGFBPs appear to be responsive to changes in arterial P_{O_2} (McLellan *et al;* 1992), and since P_{O_2} was very high in the vascular perfusion studies, it was possible that hyperoxia may have contributed to increased IGF-binding capacity during anaesthesia, rather than such changes being purely an effect of halothane anaesthetic. Hence two experimental groups of halothane-anaesthetised (1-3%) lambs were included in this study, one ventilated with halothane in oxygen (n=2) to produce hyperoxia as for the perfusion studies of Chapter **5**, and the other ventilated with halothane in air (n = 2) to maintain normoxia. Jugular and arterial blood were sampled at 30 min intervals during 4 h of anaesthesia. All blood samples were heparinised (10 IU/ml) immediately upon collection and were then kept on ice. As an extension of the anaesthesia study, additional venous blood samples were collected over a period of 3-4 h following extubation of the air-ventilated anaesthetised animals, to monitor IGFs, IGFBPs, glucose and insulin during recovery from anaesthesia. Lambs were not given access to food during the recovery period. Venous blood samples were centrifuged at 10000 x *g* for 5 min at 4°C, following which the plasma fraction was divided into aliquots and rapidly frozen in liquid nitrogen. Plasma was stored at -20°C.

Because fasting has previously been associated with altered circulating concentrations of both IGFs and IGFBPs (Busby *et al;* 1988*b*, Underwood *et al;* 1991), it was necessary to control for the contribution of food deprivation to any such changes that may arise during anaesthesia. Therefore, jugular vein blood samples were collected from two control lambs that were deprived of food over a similar period to that experienced by anaesthetised animals. Plasma was prepared and stored as described above.

6.2.3 *In vitro* Radioligand-Binding

Des(1-3)IGF-I was selected over LR^3IGF-I for the ligand-binding studies because the larger disparity between the *in vitro* and *in vivo* binding of des(1-3)IGF-I (Chapter **5**) suggested this ligand would be the most sensitive tool for detection of changes in IGF-binding capacity. IGF-I was not appropriate for use in the competitive binding study as it was essentially fully bound to IGFBPs both *in vitro* and *in vivo,* allowing no scope for detection of increased IGF-binding capacity. 250 µl of defatted plasma (Francis *et al;* 1990) was preheated to 37°C and incubated for 1 min with 12.5 µl of heparinised PBS (Section **2.3**) containing 1% (w/v) BSA, 0.25 ng of radiolabelled des(1-3)IGF-I plus 25 ng of unlabelled ligand. The final concentration of exogenous IGF peptide in this incubation was therefore essentially identical to that used in the Low Specific Activity vascular perfusion study. A 200 µl portion was immediately applied to a Superose-12 size-exclusion column and eluted in 0.5 ml fractions as described in Section **2.3**. Radioactivity contained in the fractions was measured in a gamma counter and the molecular masses of peaks of radioactivity were estimated by comparison with the elution positions of protein markers: alcohol dehydrogenase (150 kDa), BSA (69 kDa) and radiolabelled IGFs (8 kDa). Absorbance of the eluate was monitored at 280 nm.

6.2.4 Ligand Blots

Western ligand blotting was used to detect changes in the relative abundances of plasma IGFBPs during anaesthesia. Dissociated samples and standards (15 µl containing the equivalent of 1 µl plasma) were electrophoresed and transferred to nitrocellulose as previously described (Section **2.5**). Nitrocellulose sheets were probed under modified incubation conditions (sheets were each probed overnight at room temperature with 10 ng of radioligand) but all other aspects of the procedure were as described in Section **2.5**. Samples from all animals were blotted using IGF-I, but samples from some animals were run on a second gel and following transfer to nitrocellulose were incubated with des(1-3)IGF-I instead of IGF-I; this would confirm that an increase in the capacity of plasma to bind the analog

during anaesthesia, as determined in the *in vitro* radioligand-binding studies, was a direct result of increased analog-binding to IGFBP. IGF-I was used for most ligand blots as it bound a greater range of IGFBPs than did des(1-3)IGF-I (see Chapter **3**) and may therefore have revealed changes in the levels of IGFBPs not detectable using des(1-3)IGF-I. Excess ligand was removed in two washing steps and the nitrocellulose was dried prior to detection of bound radioligand by autoradiography on X-ray film. Molecular masses of IGFBPs were estimated against the positions of the ^{14}C-labelled markers. Exposure times for autoradiographs are indicated in the figure legends.

6.2.5 IGFBP Purification

As the most direct method of identifying the anaesthesia-induced IGFBP, I aimed to purify it from plasma and, following identification by N-terminal amino acid analysis, to compare it with IGFBPs in plasma from anaesthetised lambs by ligand blotting and immunoblotting techniques. IGFBP purification was performed using affinity gel chromatography, and several reverse-phase high pressure liquid chromatography (HPLC) procedures, based on techniques published by Walton *et al* (1989*a*) and described briefly in Walton *et al* (1990).

Blood Collection:

Blood was collected from an anaesthetised ewe as a source of plasma that was enriched for the anaesthesia-induced IGFBP. Following cannulation of the cranial tibial artery and the jugular vein (Section **2.4.1**), the sheep was ventilated with halothane in air to maintain arterial PO_2, PCO_2 and pH near pre-anaesthesia values (Section **5.2.2**). Blood was sampled from the jugular catheter before induction of anaesthesia and at intervals of 2 h during anaesthesia for ligand blot analysis (^{125}I-IGF-I, as described above) to confirm the anaesthesia-induction of IGFBP. Because a time-dependent increase in plasma IGFBP had been demonstrated during anaesthesia in the lambs, the total period of anaesthesia for the sheep was extended from the 4 h period used for lambs to 8 h to promote a further increase

in concentration of circulating IGFBP. After 8 h of anaesthesia, both carotid arteries were catheterised for blood collection: the neck was shaved and an incision made directly over the trachea; the skin was reflected away from the wound to gain access to the carotid arteries, located to either side of the trachea; blunt dissection was used to clear connective tissue from the vessels over a length of approximately 2 cm and two silk ligatures (2-0) were placed loosely around each; the distal ligatures, with respect to the direction of blood flow, were tightened and surgical clamps applied caudally to temporarily arrest blood supply from the heart; incisions were made between the two ligatures around each vessel and a polyethylene cannula (5.5 mm ID, 7.5 mm OD) was inserted towards the heart; the ligatures were tightened to secure the catheter in the blood vessel, and the clamps released for collection of as much blood as possible. Blood was collected in 500 ml bottles and heparinised to 10 IU/ml. After centrifugation for 20 min at 2000 x *g* and 4°C, plasma was prepared from the blood and stored at -20°C.

Affinity Extraction of IGFBPs from Ovine Plasma:

A pool of IGFBPs was extracted from 0.75 litres of plasma, based upon established methods (Walton *et al;* 1989*a*). Plasma was defatted as previously described (Section **2.3**) and was then treated to inhibit bacterial growth (bacitracin; 700 mg/l) and protease activity (PMSF; 17.4 mg/l). It was then acidified by addition of an equal volume of acetic acid (2 M) and adjusted to pH 3.0 with glacial acetic acid, after which endogenous IGFs were extracted by mixing with a cation exchange resin (SP-Sephadex C25; Pharmacia-LKB Biotechnology) overnight at 4°C; for this procedure, mixing was performed with a propeller-type stirrer to avoid the grinding and fracture of gel beads that could occur with extended use of a magnetic stirrer. The resin was then removed by filtration (Whatman N° 1; Whatman International Ltd.) and the filtrate adjusted to pH 6.5 by addition of NaOH (10 M). Precipitated proteins were removed by centrifugation (4000 x *g* for 30 min at 4°C) and bacitracin and PMSF were added to the clarified supernatant in the same net quantities as used above.

For extraction of IGFBPs from the clarified supernatant, an IGF-I affinity column (Walton *et al;* 1989*a*) was provided by Dr P.E. Walton. The column was equilibrated in a phosphate-saline buffer (0.5 M NaCl, 50 mM Na_2HPO_4, pH 6.5) for 1 h at 1 ml/min, after which the plasma preparation was pumped (1 ml/min) through the gel; IGFBPs were extracted in three sequential passes. The column was then washed with phosphate-saline buffer to remove non-specifically adsorbed proteins (14 h at 0.4 ml/min) and IGFBPs were eluted using acetic acid (0.5 M, 1.3 ml/min). The eluate was collected in 3 min fractions.

Charcoal-Binding Assay:

A charcoal-binding assay (Scott *et al;* 1985) was used for the detection of IGF-binding activity in fractions. The principle of this method of detection is that IGFBPs compete with a suspension of activated charcoal for binding of radiolabelled IGF; the method relies upon free ^{125}I-IGF binding to charcoal and ^{125}I-IGF-IGFBP remaining in solution so that following centrifugation to pellet the charcoal, IGF-binding activity is detected as an increase in radioactivity in the supernatant. The method was employed for its speed and convenience, and because its usefulness in the purification of IGFBPs has already been proven (Walton *et al;* 1989*a*). A 10 µl aliquot of each fraction was diluted 100 fold in Assay buffer (50 mM NaH_2PO_4, 0.02% [w/v] NaN_3, pH 6.5, with 0.25% [w/v] BSA) and duplicate 10 µl subsamples mixed with 300 µl of Assay buffer containing 15000 cpm ^{125}I-IGF-II for a 1 h incubation at room temperature. Tubes were transferred to ice and 1 ml of an ice-cold activated charcoal suspension (0.05% [w/v], 0.02% [w/v] protamine sulphate in Assay buffer) was added for a further incubation of 30 min. Charcoal-bound radioactivity was then pelleted by centrifugation (2000 x *g* for 15 min at 4°C) and 50% of the supernatant (650 µl) was placed in a separate tube for measurement of radioactivity in a gamma-counter. Additional tubes were included in the assay for measurement of non-specific radioligand-binding (charcoal replaced by Assay buffer), maximum radioligand-binding (sample replaced by Assay buffer) and total added radioligand.

HPLC-Purification of IGFBPs:

Affinity column fractions that contained ligand-binding activity were combined and filtered (0.22 μm nylon filter unit; Activon Scientific Products) and acetonitrile containing 0.1% (v/v) TFA was added to the filtered IGFBP pool to 15% (v/v). The preparation was applied (1 ml/min) to a Brownlee Aquapore OD-300 reverse-phase HPLC column (4.6 mm x 220 mm BLG18-224; Applied Biosystems Inc.) which had been pre-equilibrated with an aqueous solution containing 0.1% (v/v) TFA and 15% (v/v) acetonitrile and was subsequently eluted into siliconised tubes by application of an acetonitrile gradient (15-60% [v/v] over 45 min at 1 ml/min). Subsamples of each 1 min fraction were then diluted in Assay buffer for measurement of IGFBP activity using the charcoal binding assay described above. The assay described two main regions of IGF-binding activity. Fractions corresponding to the main peak of activity were combined and the concentration of acetonitrile in the pool was reduced to about 15% by a 1:3 dilution in 0.1% (v/v) TFA. The column was reequilibrated in 0.1% (v/v) TFA and 15% (v/v) acetonitrile and the diluted IGFBP pool was applied as described above. Adsorbed material was eluted using three acetonitrile gradients (by volume: 15 - 24% over 5 min, 24 - 36% over 125 min, 36 - 80% over 55 min, all at 1 ml/min) and collected in 1 min fractions. Subsamples of 10 μl were diluted 100 fold and assayed for IGF-binding activity as described above, after which fractions containing ligand-binding activity were combined and diluted 1:3 in 0.13% (v/v) aqueous HFBA. The pool was re-applied to the HPLC column, after equilibration in aqueous HFBA (0.13% [v/v]), and four gradients of propan-1-ol in 0.13% (v/v) HFBA (propan-1-ol by volume: 0 - 27% over 27 min, 27.0 - 31.5% over 45 min, 31.5 - 32.0% over 100 min and finally 32.0 - 80% over 30 min, all at 1 ml/min) were used to elute adsorbed proteins. The eluate was collected in 1 min fractions which were assayed for IGF-binding activity as described but without prior 100 fold dilution.

The charcoal-binding assay of fractions from this final HPLC purification step revealed several peaks of IGF-binding activity. Alternate fractions from across these peaks were subsampled for analysis of IGFBP content by ligand blotting. Small portions were incubated with an equal volume of Dissociating Buffer, before electrophoresis and transfer to

nitrocellulose (Section **2.5**). The nitrocellulose sheets were ligand blotted as described in Section **6.2.4** but using radiolabelled IGF-II, and autoradiographed using X-ray film. IGF-II was the only radiolabelled IGF species available at the time this procedure was performed; use of this ligand in place of IGF-I was justified by the fact that IGF-II binds at least the same number of IGFBP species as IGF-I (Fig. 3.2) and would therefore be expected to detect all IGFBPs extracted from plasma on the IGF-I-affinity column. Fractions which by ligand blotting contained IGFBPs of molecular mass 29 kDa and 31 kDa were combined in separate pools and then dispensed into siliconised plastic tubes for lyophilisation using a Speed Vac evaporator (Savant Inc.).

Electrophoretic Purification and Sequencing of IGFBPs:

Because the 29 and 31 kDa IGFBP pools contained a mixture of IGFBP and/or protein peaks, further purification was necessary before sequence analysis could be performed. For this purpose, I chose to use sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), with transfer of separated proteins to polyvinylidine-difluoride (PVDF) membrane for sequence analysis. However, in order to gain a signal strong enough for accurate sequence analysis, a protein load of about 10 µg was required for electrophoresis in each sample lane. For this reason, the protein contents of IGFBP pools were measured before SDS-PAGE.

The contents of a single tube from each lyophilised IGFBP pool, were reconstituted in 1 ml of Diluent (10 mM acetic acid, 0.05% [v/v] Tween 20). Protein standards were prepared in triplicate (1 ml; BSA at 1, 2.5, 5, 7.5, 10, 15 and 20 µg/ml in Diluent) and together with the IGFBP samples, were incubated (60 min at 60°C) with an equal volume of reagent solution (Micro BCA Protein Assay Kit [Pierce], [v/v]; 50% reagent MA, 48% reagent MB, 2% reagent MC). After cooling to room temperature, absorbances were measured at 540 nm in an Ultra Violet Spectrophotometer (UV-1202-02; Shimadzu Corporation). Protein content of IGFBP pools was calculated from a plot of absorbance versus protein content. In order to conserve the IGFBPs for subsequent electrophoretic and sequencing procedures, protein was assayed in only single aliquots of each IGFBP pool.

However, because of the high consistency of absorbance values for standards (coefficient of variation < 2%), strong linearity of the plot for absorbance versus protein concentration ($r^2 = 0.997$) and the mid-range absorbances of the samples, the probability of significant error in the values for unknowns is likely to be very low.

After assay for protein content, about 10 µg of both the 29 kDa IGFBP pool (two tubes) and the 31 kDa IGFBP pool (three tubes) were dissolved in 10 mM HCl (100 µl/tube) and combined into separate tubes. The contents of each tube were again concentrated by evaporation (described above) after which they were solubilised in 20 µl of 25% (v/v) Dissociating Buffer. The samples, together with C^{14}-Rainbow molecular mass markers in an adjacent lane, were electrophoresed on a 4%/10% discontinuous polyacrylamide gel, as previously described (Section **2.5**) except that the gel thickness (0.75 mm) was half that used for earlier electrophoretic separations. The transfer procedure was also modified to accommodate the conditions required for sequence analysis; samples and markers were transferred to a pre-wetted PVDF membrane (Millipore Corp.) instead of nitrocellulose; an amino acid-free transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulphonic acid, 10% [v/v] methanol, pH 11) was substituted for the glycine buffer (glycine would otherwise contribute a high background during sequencing of PVDF-adsorbed proteins); transfer at 300 mA was required for only 15 min (instead of 45 min) because of the altered buffer conditions and thinner gel used.

The positions of protein bands on the PVDF membrane were located using a protein stain. PVDF was soaked in water for 5 min before staining for 5 min (0.5% [w/v] Coomassie blue-R-250, 50% [v/v] methanol, 5% [v/v] acetic acid). Excess stain was removed in two washing steps (5 min each, using 50% [v/v] methanol, 10% [v/v] acetic acid) to reveal blue-dyed protein bands. The 29 kDa protein was easily visible and was cut from the membrane using a clean scalpel blade and then stored in a siliconised vial at -20°C. However, no band was visible in the lane corresponding to the 31 kDa IGFBP band so the 31 kDa protein could not be sequenced.

The purified 29 kDa protein band was sequenced, under contract, by Mrs D. Turner from the Department of Biochemistry, The University of Adelaide. 25 pmol of peptide was analysed by automatic Edman degradation on an Applied Biosystems Inc. 475A protein sequencer, as described by Baxter *et al* (1986).

6.2.6 Immunoblots

Since the 29 kDa IGFBP was of similar molecular mass to the IGFBP(s) induced during anaesthesia in the lamb and because sequence analysis had established its identity, the 29 kDa IGFBP was used in direct comparisons with plasma from anaesthetised lambs. To determine whether the purified and anaesthesia-induced IGFBPs were indeed of the same size, or bore immunological similarities, they were compared in ligand blotting and immunoblotting experiments. Purified IGFBP was analysed alongside plasma (2 µl/lane) collected prior to anaesthesia, or after 4 h of anaesthesia; samples were electrophoresed through 4%/10% (w/v) discontinuous polyacrylamide gels and transferred to nitrocellulose as described in Section **2.5**. Comparisons were made using samples from one air-ventilated and one oxygen-ventilated anaesthetised lamb. To confirm that the rate of migration of the purified protein did not vary according to the protein load on the gel, lanes that contained purified IGFBP mixed with pre-anaesthesia plasma were also included.

Prior to immunoblotting the transferred proteins, the nitrocellulose sheets were ligand blotted using ^{125}I-IGF-I (Section **6.2.4**), so that alignment of the radioligand-binding proteins with immunoreactive proteins could be used to identify those bands that bound both antibody and ligand. For immunoblotting, non-specific binding sites were blocked during an incubation (overnight at 4°C) with 3% (w/v) BSA in TBS (150 mM NaCl, 10 mM Tris, pH 7.4). All remaining steps were performed at room temperature. Filters were washed for 30 min in three changes of TBS containing 0.1% (v/v) Tween 20, and were then incubated for 1 h in polyclonal antisera (diluted 1/1000 in TBS containing 1% [w/v] BSA) raised in rabbits against either human IGFBP-1, bovine IGFBP-2, ovine IGFBP-3 or ovine IGFBP-4. These filters were again washed in four changes of TBS over 40 min, before incubation for

1 h with a horse radish peroxidase-conjugated goat anti-rabbit gammaglobulin (1/5000 in TBS with 1% [w/v] BSA). Four further washing steps were performed over 40 min and the nitrocellulose blotted dry. They were then bathed in chemiluminescence substrate-detection reagent for 1 min (RPN 2106; Amersham International plc), after which excess reagent was removed by blotting. Chemiluminescence was visualised by a 1-3 min exposure to X-ray film.

6.2.7 Glucose Assay

Circulating concentrations of glucose were measured because glucose is a potent regulator of IGFBPs in other species (Suikkari *et al;* 1988) and the documented effects of halothane on glucose synthesis and metabolism (Biebuyck *et al;* 1972, Romero *et al;* 1987, Cronau *et al;* 1988, Crosby and Atlas; 1988) suggested that its concentration may have changed during anaesthesia. Glucose was assayed by a combined hexokinase and glucose 6-phosphate dehydrogenase method (Slein, Cori & Cori; 1950) modified for automated analysis by a Technicon RA-XT Systems automatic analyser (Technicon Instruments Corporation; Method N° SM4-013K86). Assay of glucose was performed as a service by the Department of Chemical Pathology (Adelaide Women's and Children's Hospital, Adelaide, SA, Australia).

6.2.8 Insulin Assay

Insulin was measured because, like glucose, it appears to be involved in the regulation of IGFBP concentrations (Snyder & Clemmons; 1990) and, as the counter-regulator of glucose, its concentration may vary if glucose level changes during anaesthesia. Ovine insulin was assayed using a double antibody radioimmunoassay (RIA), with plasma stripped of insulin added to insulin standards to control for non-specific interference in the assay.

Charcoal-Stripped Plasma:

10 g of activated charcoal was mixed with 1 litre of H_2O until wetted and was then allowed to settle overnight at 4°C. The supernatant was aspirated for removal of fines, and excess water removed from the pellet after centrifugation (2000 x *g* at 4°C for 20 min). The pellet was reconstituted in 100 ml of ovine plasma (stirred for 8 h at room temperature) and then allowed to settle overnight. The mixture was then centrifuged at 6000 rpm for 30 min (Sorvall RC2-B) at room temperature, after which the supernatant was decanted and recentrifuged at 15000 rpm for a further 30 min. The supernatant was decanted and filtered through 3 sheets of Whatman 540 Hardened Ashless Filter Paper. Removal of insulin was confirmed by lack of interference in an assay for ovine insulin (personal communication; Dr P.C. Owens).

Radioimmunoassay of Insulin:

Plasma from anaesthetised and control lambs (80 µl; duplicates) was mixed with 200 µl of RIA Buffer (50 mM NaH_2PO_4, 0.1% [w/v] NaN_3, 10 mg/ml BSA, pH 7.4). A similar volume of plasma that had been stripped of insulin by extraction with activated charcoal, was added to insulin standards (0-2000 pg in 200 µl RIA Buffer, triplicates). After addition of a guinea pig antibody raised against ovine insulin (50 µl; final concentration of 1/150000), tubes were incubated for 3 days at 4°C. Subsequently, ^{125}I-ovine insulin (20000 cpm in 50 µl buffer) was added to all tubes for an overnight incubation at 4°C. A donkey anti-guinea pig solid-phase immunoglobulin-coated cellulose suspension (100 µl) was added and incubated for 30 min at room temperature, with subsequent addition of water (1 ml) and pelleting of immune complexes by centrifugation (2000 x *g* for 10 min at 4°C). Supernatants were aspirated and radioactivity in the pellets was measured in a gamma counter. Insulin concentrations in unknowns were measured by comparison with the standards, using Riacalc LM (version 2.57; Wallac Oy). Maximum radioligand binding in the assay was 12% and the intra-assay coefficient of variation was 3.9%. Samples in which insulin concentrations fell below the sensitivity of the standard curve were assumed to contain insulin at the minimum detectable concentration of the assay; 50 pg/ml.

6.2.9 Assay of IGFs

IGF-I and IGF-II were assayed in plasma samples from all lambs to determine whether IGF concentrations changed during anaesthesia or food deprivation. Because IGFBPs have previously been shown to interfere in the measurement of IGFs (Daughaday *et al;* 1980, Zapf *et al;* 1981, Daughaday *et al;* 1987), IGFs were separated from IGFBPs before assay. The technique employed to effect this separation involved the reduction of sample pH to dissociate IGF-IGFBP complexes and was followed by HPLC size-exclusion chromatography under acidic conditions to separate the dissociated components. Separation techniques such as acid-ethanol extraction and acid-ethanol cryoprecipitation result in incomplete separation of IGF-IGFBP complexes (Crawford *et al;* 1992) and may compromise the accuracy of IGF measurements in sheep plasma. Furthermore, measurement of IGF-II is usually underestimated, compared with the acid column method, when separation is effected by the acid-ethanol methods (personal communication; Dr. P.C. Owens).

Separation of IGFs from IGFBPs:

Plasma IGFs were dissociated from IGFBPs by mixing with an equal volume of 4 x Mobile Phase (0.8 M acetic acid, 0.2 M trimethylamine [Scott & Baxter; 1986] adjusted to pH 2.8 using HCl and containing 0.4% [v/v] Tween 20) and were diluted 1:1 in H_2O. The mixture was centrifuged (2 min at 1000 x *g*) through an 0.22 µm cellulose acetate filter membrane (Alltech Associates [Aust.] Pty. Ltd.) to remove particulates and 200 µl of the filtrate was applied to a Protein Pak 125 HPLC column (Waters Associates Inc) via a manual injector (U6K; Waters Associates Inc.). Samples were eluted at 0.5 ml/min (K25D; ETP-Kortec Pty. Ltd.) in 1 x Mobile Phase which had been filtered (0.45 µm GV filter membrane; Millipore Corp.) and degassed overnight.

The eluate was collected in four pools, in order: IGFBP, intermediate, IGF and tail pools; all IGF and IGFBP pools were assayed, with some intermediate and tail pools also included to confirm that IGF and IGFBP elution positions remained constant between chromatographic runs. The timing for collection of these pools was determined as follows.

Six dissociated plasma samples were chromatographed and collected in 0.5 ml fractions that were each assayed in the IGF-I RIA. Fractions containing IGF-I were detected through reduced precipitability of radioactivity due to competitive inhibition of radioligand binding to the primary antibody; this also applied to fractions containing IGFBPs since IGFBPs sequestered the ^{125}I-IGF-I, thereby reducing the amount of radioligand available for antibody binding. Thus two peaks of activity were detected, with base-line separation attained between them. ^{125}I-IGF-I was chromatographed separately to confirm that the second peak corresponded to IGF. The elution ranges of the binding protein and IGF peaks in the six chromatographic runs were averaged to determine the timepoints for collection of IGFBP, intermediate, IGF and tail pools for assay.

Radioimmunoassay of IGF-I:

IGF-I immunoreactivity in the IGF pool from acid column-chromatographed plasma was determined using a double antibody RIA. Triplicate sample portions of 100 µl were neutralised by addition of 60 µl of 0.4 M Tris Base and were then mixed with 200 µl of IGF-RIA Buffer (30 mM NaH_2PO_4, 10 mM EDTA, 0.02% [w/v] sodium azide, 0.05% [v/v] Tween 20 at pH 7.5). To allow measurement of IGF-I concentrations in samples, tubes that contained IGF-I at known concentrations (2.5-1300 pg in 200 µl of IGF-RIA Buffer) were mixed with 60 µl of 0.4 M Tris Base and 100 µl of the acid column Mobile Phase (see above). A rabbit polyclonal antiserum prepared against hIGF-I (MAC 89/1, courtesy Dr P. C. Owens) was added in a volume of 50 µl of IGF-RIA Buffer to yield a final concentration of 1/40000 and additional tubes that contained no unlabelled IGF-I were included as: a) 'blanks' to assess non-specific binding (IGF-RIA buffer was substituted for antibody), and b) 'references' to determine the value for maximum uncompeted radioligand binding in the assay (IGF-RIA buffer was substituted for IGF standards). Radiolabelled IGF-I was then added to the tubes (20000 cpm in 50 µl of IGF-RIA buffer) for an overnight incubation at 4°C. Primary immune complexes were precipitated by a 30 min incubation at room temperature with donkey anti-rabbit immunoglobulin-coated cellulose suspension (100 µl; IDS) followed by addition of H_2O (1.0 ml) and pelleting by centrifugation for 10 min at 2000 x *g* and 4°C. Following aspiration of the supernatant, pellets were counted

in a gamma counter. Concentration of IGF-I in unknowns was measured by comparison with the standards, using Riacalc LM (version 2.57; Wallac Oy). Crossreactivity of IGF-II in the assay was 1% (personal communication; Dr P.C. Owens). Maximum radioligand binding in the assay was 41%, while the intra- and inter-assay coefficients of variation were 4.7% and 9.7%, respectively. The RIA for IGF-I, developed by Dr. P.C. Owens, was modified from that published by Owens *et al* (1990) by the use of a substitute primary antiserum in the first phase, while in the second phase a donkey anti-rabbit immunoglobulin-coated cellulose suspension was used in place of a solubilised antibody preparation.

Radioreceptor-Assay of IGF-II:

IGF-II receptor-binding activity in the IGF pool from acid-column chromatographed plasma was measured by radioreceptor assay using an ovine placental microsome preparation and radiolabelled hIGF-II. Ovine placental membranes were prepared as described by Baxter & De Mellow (1986).

Prior to assay of IGF-II activity, 100 µl sub samples (triplicates) were neutralised with 60 µl of 0.4 M Tris Base. The first incubation was performed following a protocol similar to that described for the IGF-I RIA (above), but with the following modifications; 1) a 100 mM Tris, 10 mM $CaCl_2$, pH 7.4 buffer containing 5% (w/v) BSA was used in place of IGF-RIA buffer; 2) standards contained hIGF-II (19-1000 pg in 200 µl); 3) 100 µl of an ovine placental microsome preparation was added in place of 50 µl of antibody; 4) radiolabelled IGF-II was used instead of radiolabelled IGF-I. Following an overnight incubation at 4°C, 1 ml of 100 mM Tris, 100 mM $CaCl_2$, pH 7.4 buffer containing 1% (w/v) BSA was added to precipitate the placental membranes and after a thorough vortexing, the precipitate was pelleted by centrifugation (2000 x *g* for 30 min at 4°C). The supernatant was aspirated and counts contained in the pellets were measured in a gamma counter. Concentration of IGF-II in unknowns was calculated by comparison with the standards, using Riacalc LM (version 2.57; Wallac Oy). The IGF-II assay method was developed by Dr. P.C. Owens, modified from that of Owens *et al* (1990) such that all solutions contained Tris-HCl at 0.1 M instead of at 0.01 M, a refinement which significantly enhanced assay

sensitivity (personal communication; Dr P.C. Owens). Ovine IGF-II has previously been shown to be equipotent to human IGF-II in this system (personal communication; Dr P.C. Owens) and the crossreactivity of ovine IGF-I is less than 0.2% (Francis *et al;* 1989*a*). IGF-II was measured in a single assay in which the maximum radioligand binding was 28%, and the internal coefficient of variation was 10%.

6.3 RESULTS

6.3.1 Blood Gases and pH

The present study was designed to determine whether the IGF-binding capacity of plasma changed during the imposition of anaesthesia and ventilation conditions similar to those used in the vascular perfusion study (Chapter **5**), and if so, whether such changes were due to food deprivation, effects of halothane, or the combined effects of halothane and high blood P_{O_2}. For this reason, two groups of anaesthetised lambs were included in the study, with one maintained in a hyperoxic state by ventilation with oxygen and the other in a normoxic state by ventilation with air. The combined data from all anaesthetised animals showed that P_{O_2} before anaesthesia ranged from 66-92 MPa, while P_{CO_2} ranged from 33-38 MPa. In air-ventilated lambs, P_{O_2} varied from pre-anaesthesia values by less than 20%, while in oxygen-ventilated animals, P_{O_2} was elevated by 360-370%. Average P_{CO_2} during anaesthesia was elevated by 10-35% and blood pH values were maintained within 0.1 pH unit of the pre-anaesthesia value.

6.3.2 *In Vitro* Radioligand-Binding

To determine the effect of the experimental protocols on IGF-binding to plasma IGFBPs, radiolabelled des(1-3)IGF-I was incubated with plasma collected prior to anaesthesia as well as with plasma sampled at intervals during 4 h of anaesthesia and recovery from anaesthesia; radioligand binding was then analysed by size exclusion

chromatography. Estimates of radioligand distribution between the free and IGFBP-bound states were made as previously described (Section **5.3.1**). In plasma collected before anaesthesia, 54-86% of radiolabelled des(1-3)IGF-I eluted at the molecular mass of uncomplexed ligand (8 kDa), as illustrated for an oxygen- and an air-ventilated lamb in Fig. 6.1*a* and *f*, respectively, and for the second animal from each group in Fig. 6.2*a* and *f*. The remaining radioactivity eluted bound at higher molecular mass regions, mostly at 30-50 kDa and a smaller amount at 130-150 kDa.

Ligand-binding capacity of plasma, collected during halothane-anaesthesia of both oxygen-ventilated lambs (Fig. 6.1*b-e* & 6.2*b-e*) and air-ventilated lambs (*g-j*) increased significantly within 1-2 h of induction of anaesthesia and continued to rise as anaesthesia was continued. The reduced proportion of free ligand evident during anaesthesia was offset mainly by increased radioactivity at 30-50 kDa but with smaller increases also apparent at 130-150 kDa. In animals whose plasma exhibited relatively high ligand binding before anaesthesia, maximum binding was achieved sooner into anaesthesia than in those whose pre-anaesthesia plasma bound smaller proportions of the radioligand. Nevertheless, the changes in IGF-binding characteristics of plasma from the oxygen- and air-ventilated lambs were qualitatively similar. In plasma collected during recovery from air-ventilated anaesthesia (*k, l*), the elution profile of radioligand returned towards that of pre-anaesthesia plasma. However, radioligand-binding capacity remained substantially greater at 3 or 4 h after withdrawal of anaesthesia than before the onset of anaesthesia .

Because the experimental lambs were deprived of food during anaesthesia, so too were the non-anaesthetised controls. Radioligand association in plasma from two lambs that had been deprived of food for 8 h, a period similar to that experienced by anaesthetised animals, was therefore analysed (Fig. 6.3). Between 2 and 8 h of food deprivation, there was a minor increase in the proportion of free ligand in one animal (6%) (*a-d*), while in the other a small decrease (4%) was evident (*e-h*). Changes in the proportions of free ligand were balanced by equivalent but opposite changes in the proportions of tracer eluting in higher molecular mass regions. Thus food deprivation during anaesthesia could not account for the marked increases in radiolabelled des(1-3)IGF-I association with plasma IGFBPs.

Fig. 6.1 *Superose-12 size-exclusion chromatography of plasma (200 µl) from individual anaesthetised lambs ventilated with halothane in oxygen (a-e) or in air (f-l), following incubation with 125I-des(1-3)IGF-I, as described (Section* ***6.2.3****).* Radioactivity in each 0.5 ml fraction is expressed as a percentage of the total radioactivity recovered from the column. The radioligand-binding profile of plasma before anaesthesia (*a, f*) is compared with that of plasma sampled after 1, 2, 3 and 4 h of halothane-anaesthesia and oxygen- (*b-e*) or air-ventilation (*g-j*), and during recovery in samples collected at 2 and 4 h after withdrawal of anaesthesia and air-ventilation (*k, l*). The elution positions for the protein markers alcohol dehydrogenase (150 kDa) and BSA (69 kDa), and for radiolabelled des(1-3)IGF-I (8 kDa) are indicated by arrows.

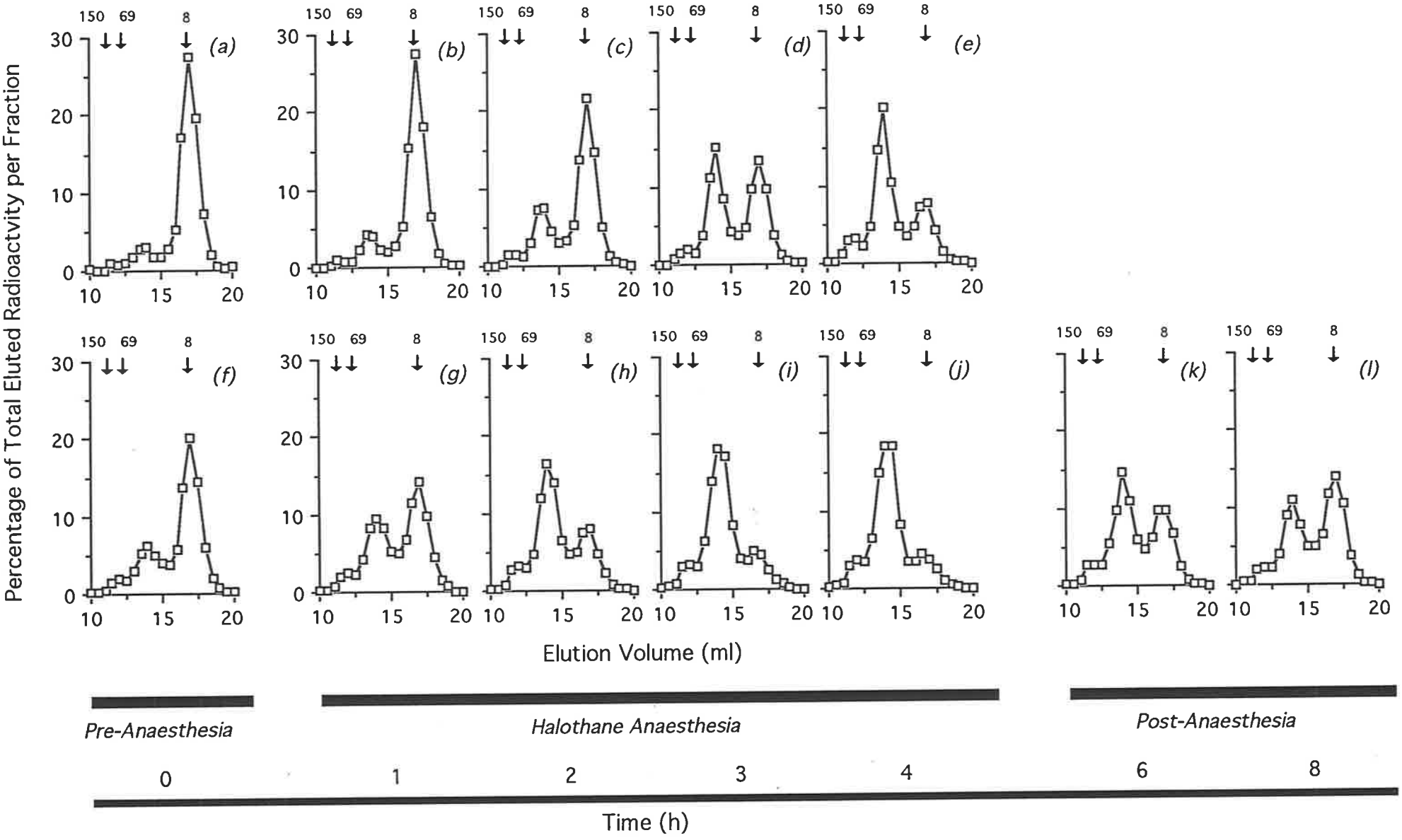

Percentage of Total Eluted Radioactvity per Fraction
150 69 8
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
30
20
10
0
10 15 20
Elution Volume (ml)
Pre-Anaesthesia
Halothane Anaesthesia
Post-Anaesthesia
0 1 2 3 4 6 8
Time (h)

Fig. 6.2 *Superose-12 size-exclusion chromatography of plasma (200 µl) from additional individual anaesthetised lambs ventilated with halothane in oxygen (a-e) or in air (f-l), following incubation with 125I-des(1-3)IGF-I, as described (Section* ***6.2.3****).* Radioactivity in each 0.5 ml fraction is expressed as a percentage of the total radioactivity recovered from the column. The radioligand-binding profile of plasma before anaesthesia (*a, f*) is compared with that of plasma sampled after 1, 2, 3 and 4 h of halothane-anaesthesia and oxygen- (*b-e*) or air-ventilation (*g-j*), and during recovery in samples collected at 1.5 and 3 h after withdrawal of anaesthesia and air-ventilation (*k, l*). The elution positions for the protein markers alcohol dehydrogenase (150 kDa) and BSA (69 kDa), and for radiolabelled des(1-3)IGF-I (8 kDa) are indicated by arrows.

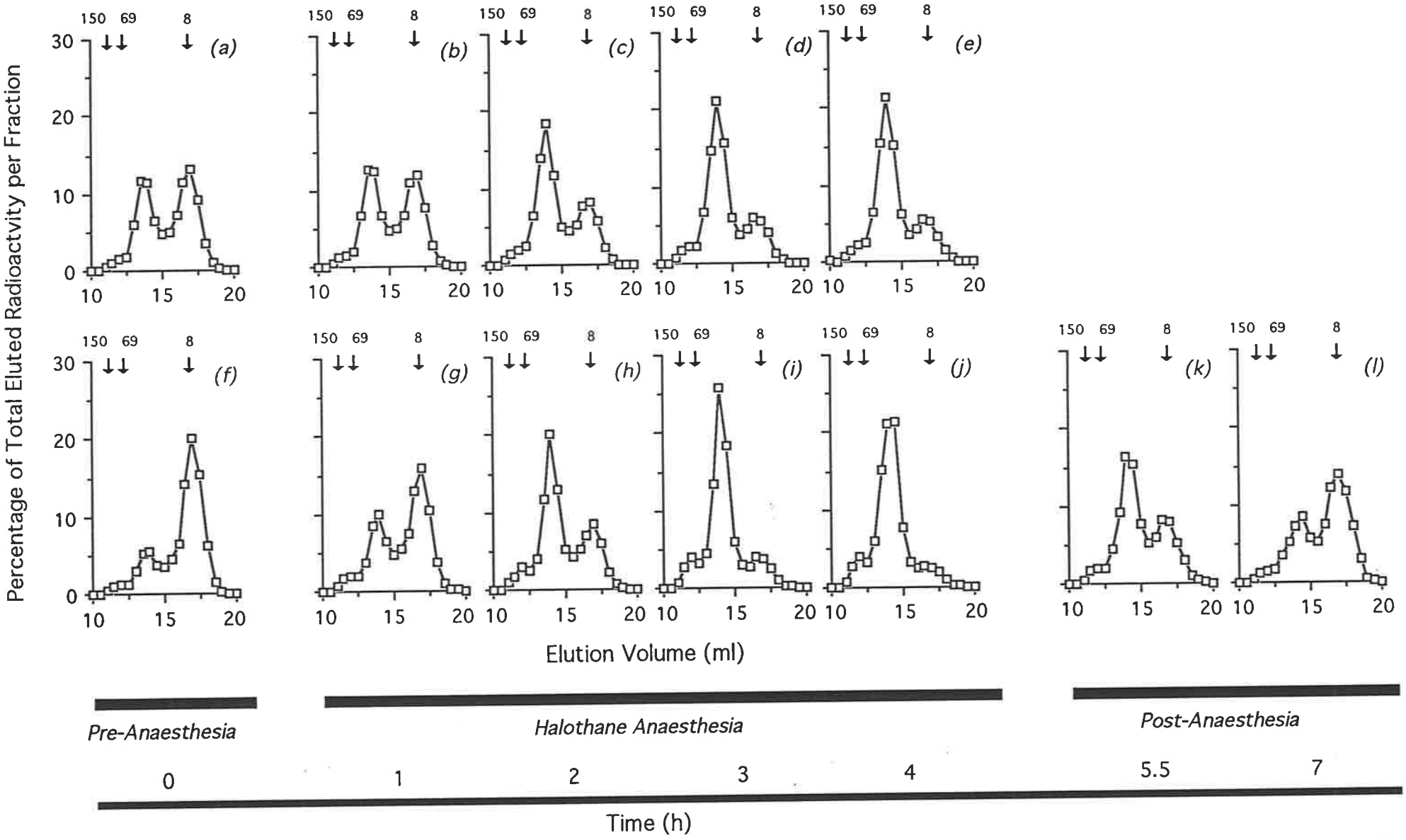
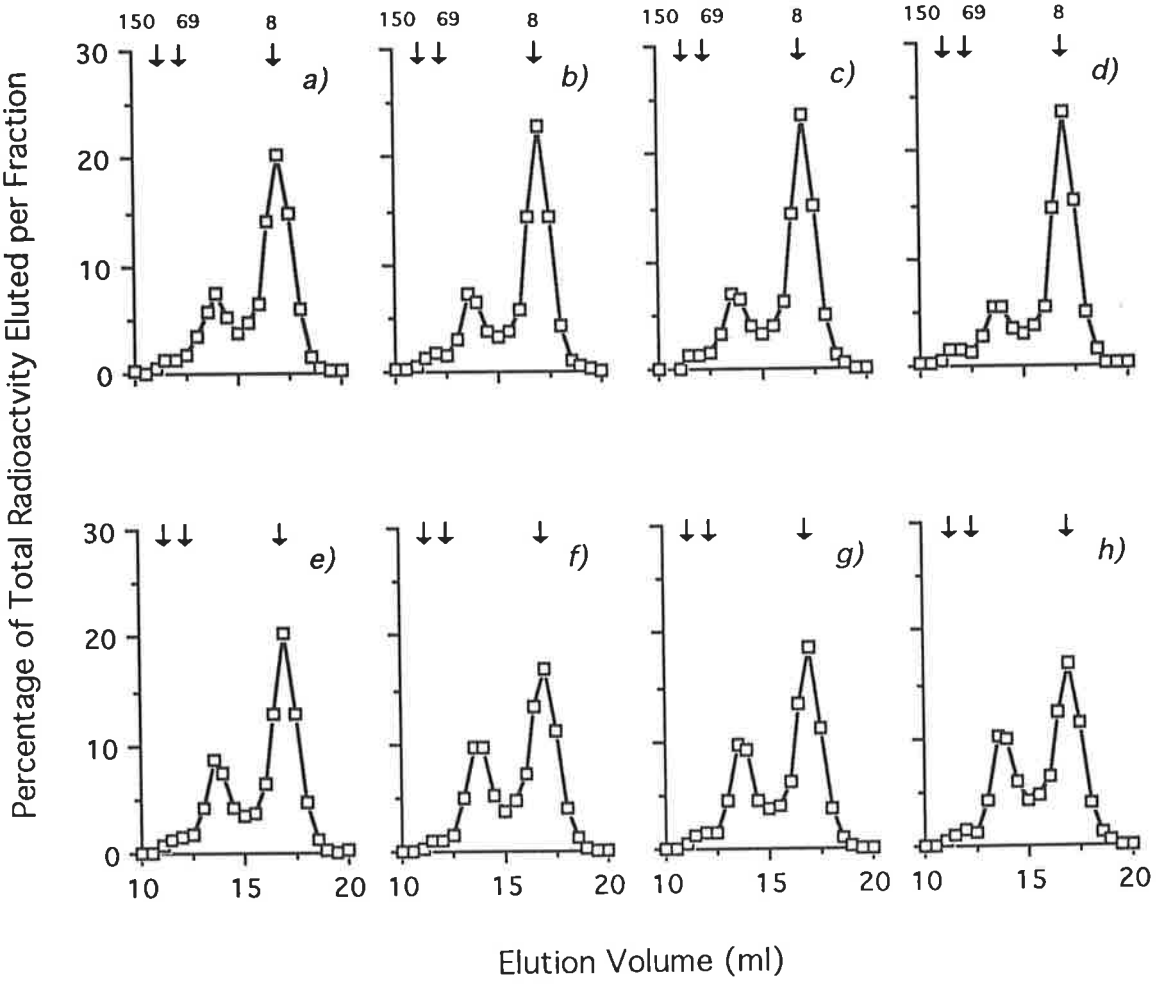

150 69 8
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
30
20
10
0
10 15 20
Percentage of Total Eluted Radioactvity per Fraction
Elution Volume (ml)
Pre-Anaesthesia
Halothane Anaesthesia
Post-Anaesthesia
0 1 2 3 4 5.5 7
Time (h)

Fig. 6.3 *Superose-12 size-exclusion chromatography of plasma (200 µl) from two food-deprived lambs (a-d and e-h), following incubation with ^{125}I-des(1-3)IGF-I as described (Section* ***6.2.3****).* Radioactivity in each 0.5 ml fraction is expressed as a percentage of the total radioactivity recovered from the column. The radioligand-binding profiles of plasma collected after 2 (*a, e*), 4 (*b, f*), 6 (*c, g*) and 8 h (*d, h*) of food deprivation are shown (*a-d*). Elution positions for protein markers alcohol dehydrogenase (150 kDa) and BSA (69 kDa), and for radiolabelled des(1-3)IGF-I (8 kDa) are indicated by *arrows*.


150 69 8
a)
b)
c)
d)
e)
f)
g)
h)
30
20
10
0
10 15 20
Percentage of Total Radioactvity Eluted per Fraction
Elution Volume (ml)

6.3.3 Ligand Blots

Ligand blotting with radiolabelled IGF-I and des(1-3)IGF-I was used to characterise the IGF-binding proteins in plasma from anaesthetised and non-anaesthetised lambs. In all animals six ligand-binding bands were revealed using ^{125}I-IGF-I, shown for one lamb from each group in Fig. 6.4 (*a-c*) and for the other of each group in Fig. 6.5 (*a-c*). The most intense ligand-binding generally occurred at 48 and 43 kDa, with additional bands detected at 35, 31, 29 and 24 kDa. Intensity of the respective IGFBP bands did not change during food deprivation alone (*a*). In contrast, halothane-anaesthesia in both oxygen- and air-ventilated animals induced an increase in IGF-I binding at 31 kDa (*b*, *c*), which was detectable after 90 min to 2 h, and was markedly increased by 4 h. A smaller increase in the 35 kDa IGFBP band occurred in some animals, but no appreciable change in binding activity at 48 or 43 kDa was detected in any experiment. Withdrawal from anaesthesia was followed by a decrease in the relative abundance of previously elevated binding proteins, such that within 4 h the plasma IGFBP profile had returned approximately to that of blood sampled before anaesthesia (*c*).

When labelled des(1-3)IGF-I was used instead of IGF-I radioligand (Fig. 6.6*a, b*), fewer IGFBP bands were detected. Des(1-3)IGF-I bound only at 48 and 43 kDa in samples from food-deprived lambs (Fig. 6.6*a*) and in plasma collected prior to anaesthesia or early in anaesthesia. However in samples taken later in the anaesthesia period, ligand binding was also detected at 31 kDa and increased in intensity with duration of anaesthesia (Fig. 6.6*b*). Nevertheless, the relative intensity of this band compared with that of the 48 or 43 kDa bands was much lower when des(1-3)IGF-I was used as ligand than it was using IGF-I, indicating the greater relative affinity of the halothane-induced IGFBP for IGF-I.

6.3.4 IGFBP Purification and Sequence Analysis

Purification and sequence analysis were combined as the most direct methods for identification of the anaesthesia-induced IGFBP. However, prior to extraction of IGFBPs,

Fig. 6.4 *Ligand blotting of IGFBPs in plasma from a control lamb (a) and from individual halothane-anaesthetised lambs ventilated with oxygen (b) or with air (c).* Following incubation with a Dissociating Buffer, plasma IGFBPs were separated on 10% (w/v) polyacrylamide gels, transferred to nitrocellulose and autoradiographed after probing with radiolabelled IGF-I as described (Section **6.2.4**). The lanes each contain 1 µl of plasma; calculated molecular masses of bands (kDa) are indicated on the *left*. Blots compare the plasma IGFBP profile during 2, 3, 4, 6 and 8 h of food deprivation (*a*) with those of plasma collected after 0, 0.5, 1, 1.5, 2, 3 and 4 h of halothane-anaesthesia and oxygen-ventilation (*b*) or air-ventilation (*c*). Recovery from anaesthesia at 0.5, 1, 2 and 4 h is also shown (*c*). Blots were exposed to x-ray film for between 4 and 14 days.

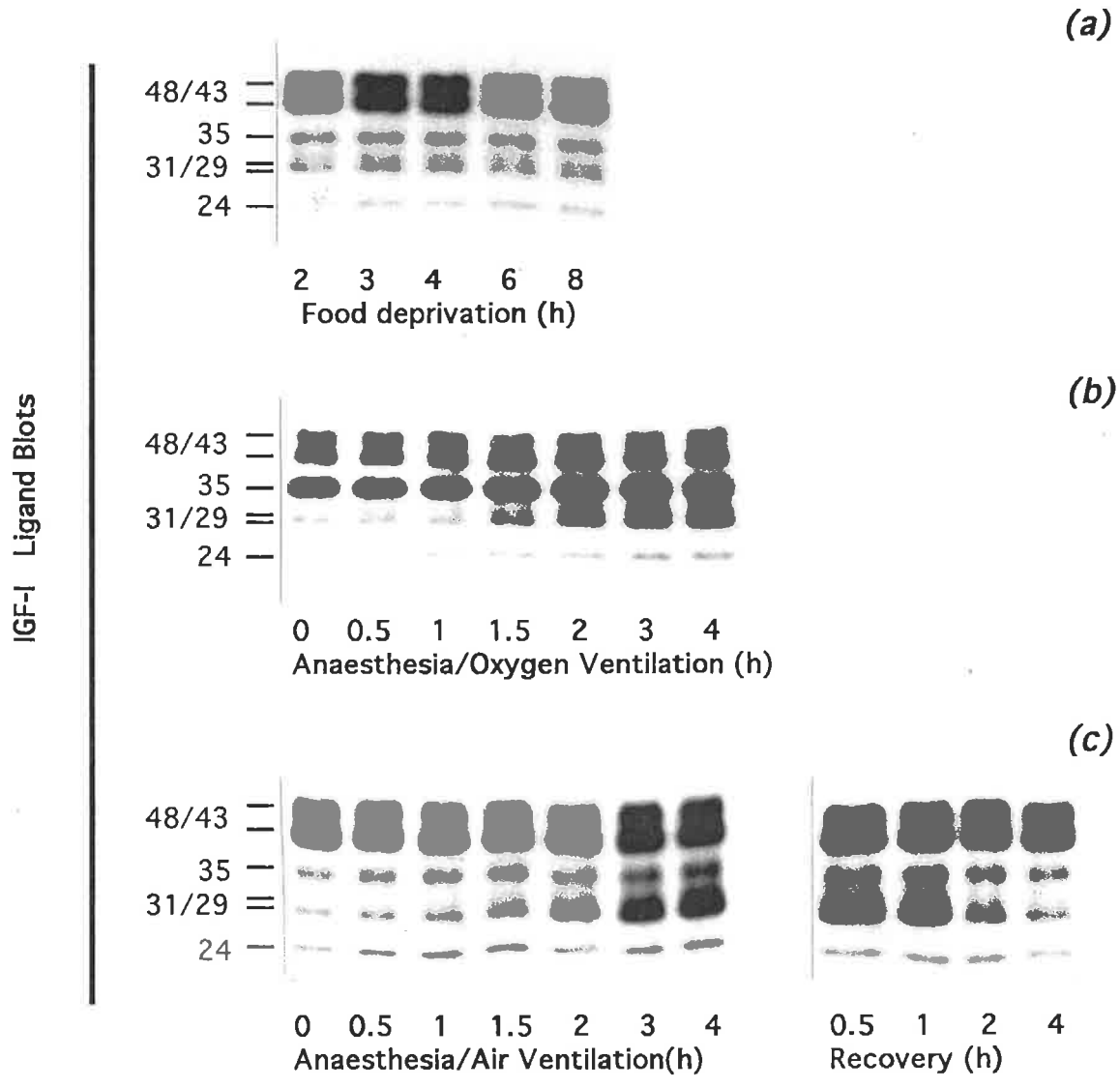

IGF-I Ligand Blots
(a)
48/43
35
31/29
24
2 3 4 6 8
Food deprivation (h)
(b)
48/43
35
31/29
24
0 0.5 1 1.5 2 3 4
Anaesthesia/Oxygen Ventilation (h)
(c)
48/43
35
31/29
24
0 0.5 1 1.5 2 3 4
Anaesthesia/Air Ventilation(h)
0.5 1 2 4
Recovery (h)

Fig. 6.5 *Ligand blotting of IGFBPs in plasma from a second control lamb (a) and from additional individual halothane-anaesthetised lambs ventilated with oxygen (b) or with air (c) using radiolabelled IGF-I.* Blots were performed as described in Fig. 6.4. Lanes each contain 1 µl of plasma and calculated molecular masses of bands (kDa) are indicated on the *left*. Blots compare the plasma IGFBP profile during 2, 3, 4, 6 and 8 h of food deprivation (*a*) with those of plasma collected after 0, 0.5, 1, 1.5, 2, 3 and 4 h of halothane-anaesthesia and oxygen-ventilation (*b*) or air-ventilation (*c*). Recovery from anaesthesia at 1.5 and 3 h is also shown (*c*). Blots were exposed to x-ray film for between 10 and 14 days.

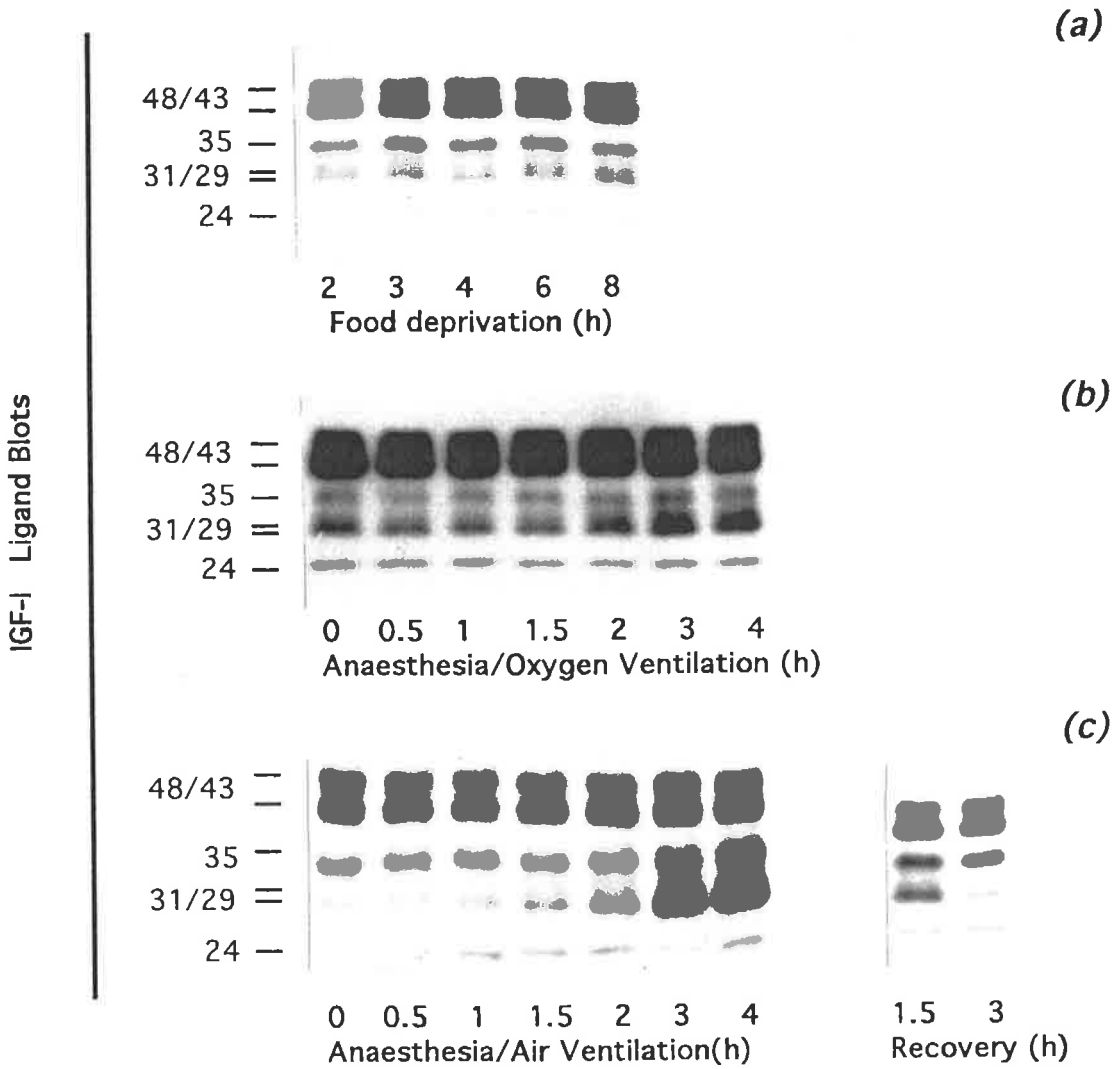
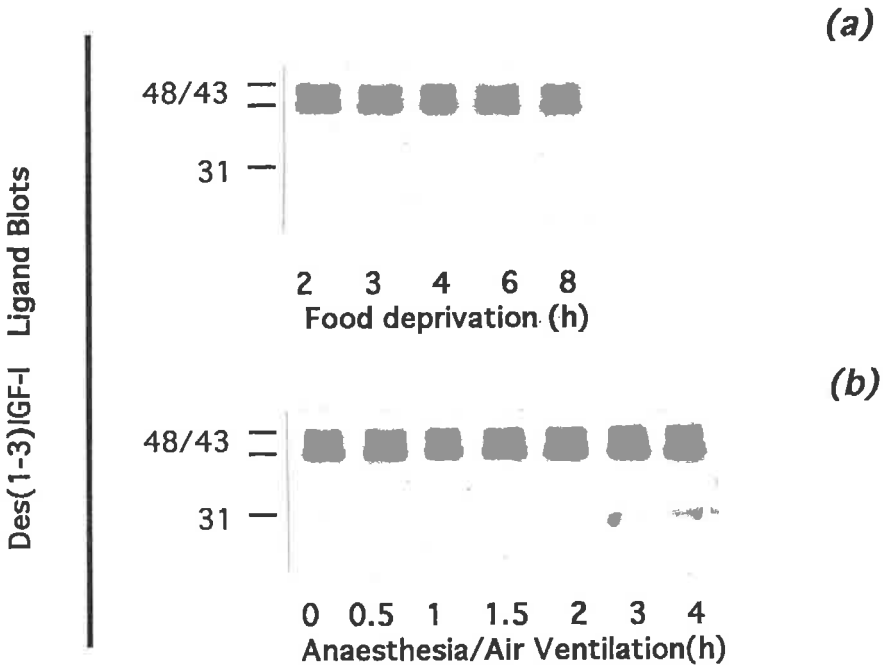

IGF-I Ligand Blots
(a)
48/43
35
31/29
24
2 3 4 6 8
Food deprivation (h)
(b)
48/43
35
31/29
24
0 0.5 1 1.5 2 3 4
Anaesthesia/Oxygen Ventilation (h)
(c)
48/43
35
31/29
24
0 0.5 1 1.5 2 3 4
Anaesthesia/Air Ventilation(h)
1.5 3
Recovery (h)

Fig. 6.6 *Ligand blotting of IGFBPs in plasma from a control lamb (a) and from an air-ventilated halothane-anaesthetised lamb (b) using radiolabelled des(1-3)IGF-I.* Blots were performed as described in Fig. 6.4. The lanes each contain 1 µl of plasma; calculated molecular masses of bands (kDa) are indicated on the *left*. Blots compare the plasma IGFBP profile during 2, 3, 4, 6 and 8 h of food deprivation (*a*) with those of plasma collected after 0, 0.5, 1, 1.5, 2, 3 and 4 h of halothane-anaesthesia and air-ventilation (*b*). Blots were exposed to x-ray film for 4 days.


(a)
Des(1-3)IGF-I Ligand Blots
48/43
31
2 3 4 6 8
Food deprivation (h)
(b)
48/43
31
0 0.5 1 1.5 2 3 4
Anaesthesia/Air Ventilation(h)

ligand blotting was used to confirm an increase in IGFBP(s) of similar molecular mass as that induced during anaesthesia in lambs. A time-dependent increase in intensity of a 31 kDa IGFBP, over a period of 8 h anaesthesia, is demonstrated in Fig. 6.7.

Having confirmed the induction of IGFBP during anaesthesia, and following removal of IGFs from the plasma, IGFBPs were extracted by affinity chromatography. The preparation was applied to the IGF-I affinity column and after washing to remove non-adsorbed proteins, adsorbed proteins were eluted using 0.5 M acetic acid. Fig. 6.8 shows the profile of IGF-binding activity contained in the equivalent of 0.1 µl from each 1.3 ml fraction.

Fractions 16-20 (61-67 ml) were combined for further purification by several stages of reverse phase chromatography. In the first of these procedures, proteins were applied to a reverse phase column and eluted using a 15-60% acetonitrile gradient. Fig. 6.9 shows the protein elution profile and the corresponding IGF-binding protein activity in aliquots from the 1 min fractions. Most protein and ligand-binding activity eluted in the 17 - 33 min peak and was followed by a smaller region of activity commencing at 33 min; fractions corresponding to 17 - 33 min were combined and reapplied to the column. Acetonitrile was brought up to 24% (v/v) over 5 min and was then slowly increased to 36% (v/v) over 125 min. This relatively shallow gradient was employed in an effort to achieve greater separation of IGFBP regions within the main peak. However, most protein and IGFBP activity eluted between 25 and 70 min and the resolution of IGF-binding regions within this range was poor (Fig. 6.10). Although proteins continued to elute beyond 70 min, the peaks did not contain substantial IGF-binding activity. Fractions corresponding to 25 - 70 min were combined and reapplied to the column for a final reverse phase purification, the aim of which, as with the previous step, was to resolve the IGFBP region into distinct peaks of activity. For this purpose, a series of very shallow propan 1-ol gradients was employed. Propan 1-ol was brought up to 27% (v/v) over 27 min, increased to 31.5% (v/v) over 45 min (27 - 72 min) and then to 32% (v/v) over 100 min (72 - 172 min) before a rapid rise to 80% (v/v) over 30 min (172 - 202 min). Six regions of IGF-binding activity were eluted between 27 min and 172 min, peaking at 42, 65, 75, 105, 130 and 150 min, and

Fig. 6.7 *Ligand blotting of plasma from an anaesthetised sheep.* A sheep was anaesthetised using halothane anaesthetic and blood samples collected at 0, 2, 4, 6 and 8 h for plasma preparation. Plasma was incubated in Dissociating Buffer prior to electrophoresis through a 10% polyacrylamide gel, transfer to nitrocellulose and probing with ^{125}I-IGF-I as previously described (Section **6.2.4**). The blot was autoradiographed for 3 days.

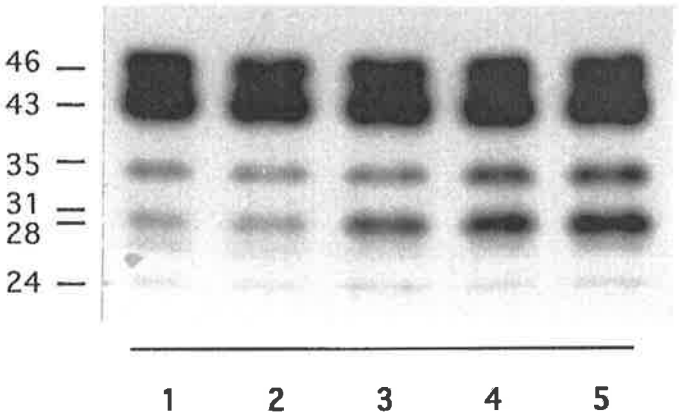

46
43
35
31
28
24
1
2
3
4
5

Fig. 6.8 *Elution of IGFBP from an IGF-I affinity column.* A charcoal-binding assay was used to detect IGF-binding activity in 0.1 µl equivalents of each 1.3 ml fraction (Section **6.2.5**). Values are percentage specific binding of ^{125}I-IGF-I in 0.1 µl from each fraction.

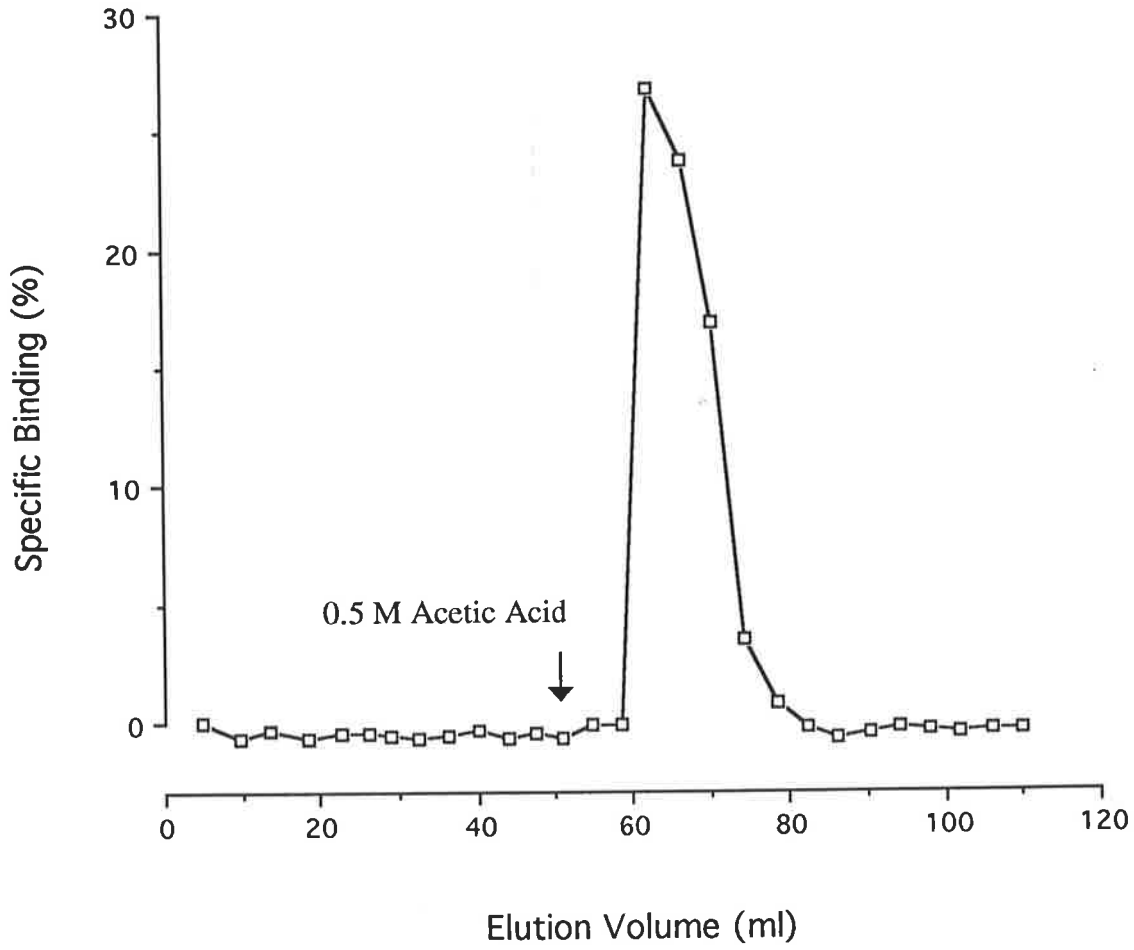

30
20
10
0
Specific Binding (%)
0.5 M Acetic Acid
0
20
40
60
80
100
120
Elution Volume (ml)

Fig. 6.9 *Reverse phase HPLC chromatography of IGF-I-affinity-extracted IGFBPs; Step-1.* An affinity-extracted IGFBP pool was loaded onto a Brownlee OD-300 aquapore reverse-phase HPLC column and eluted over 45 min using a 15-60% acetonitrile gradient, as described in Section **6.2.5**. The protein absorbance profile (280 nm) is shown in the *lower* panel (___) together with the corresponding percentage volume of acetonitrile (- - - -). IGF-binding activity in 0.1 µl of each fraction is shown in the *upper* panel (squares).

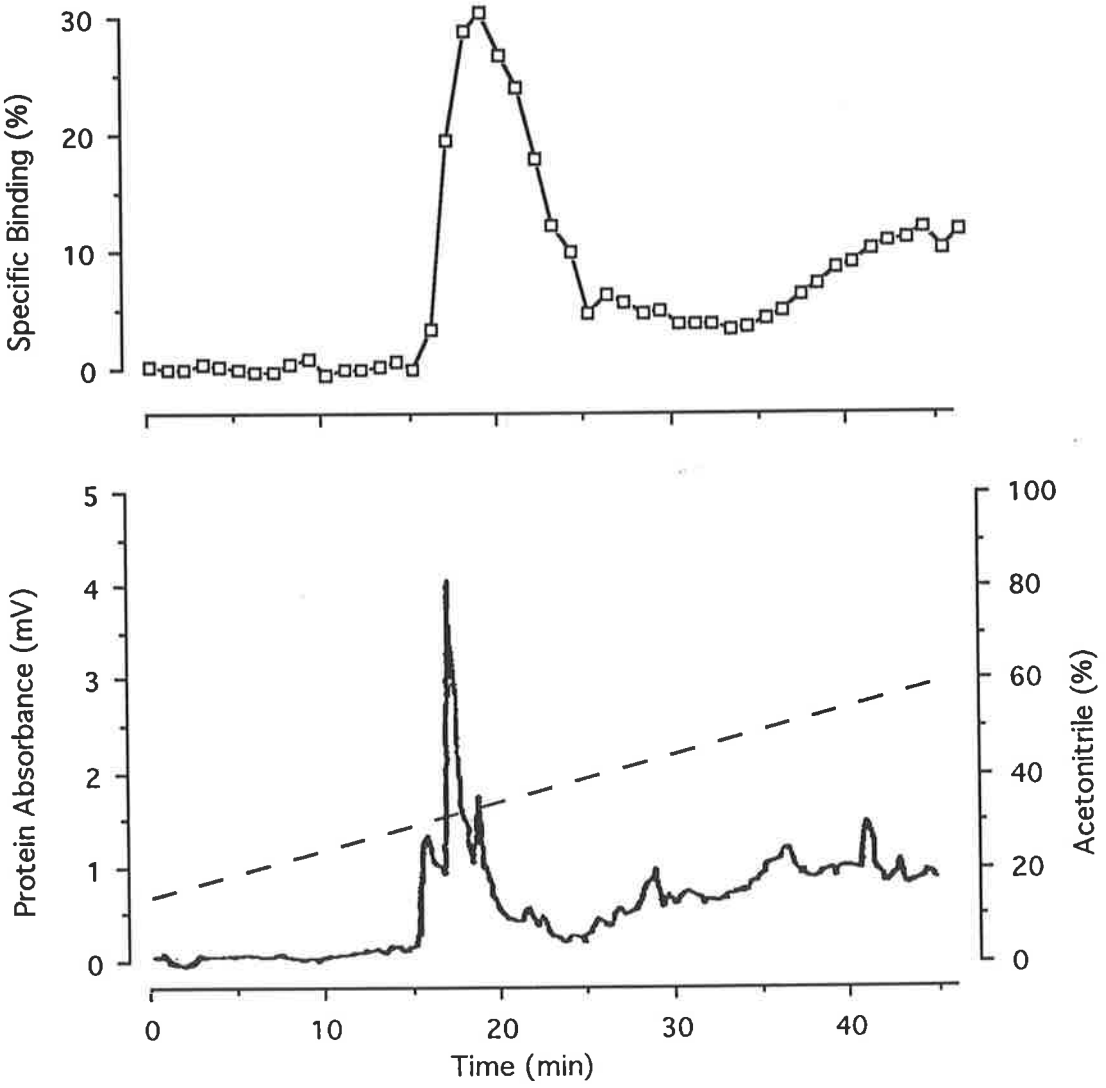
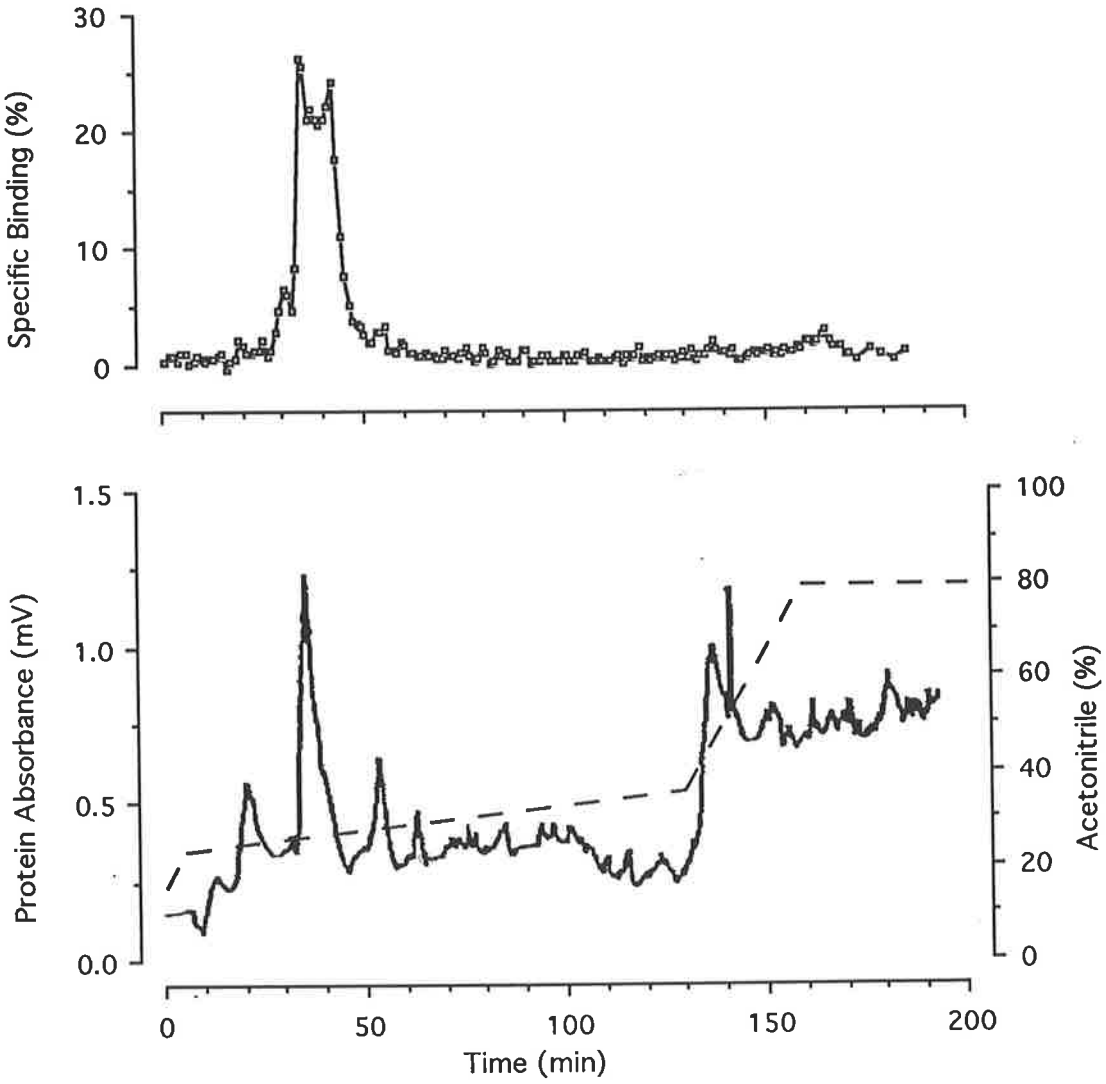

Specific Binding (%)
30
20
10
0
Protein Absorbance (mV)
5
4
3
2
1
0
100
80
60
40
20
0
Acetonitrile (%)
0
10
20
30
40
Time (min)

Fig. 6.10 *Reverse phase HPLC chromatography of IGF-I-affinity-extracted IGFBPs; Step-2.* Fractions 17-33 from the first reverse phase purification procedure were combined and reloaded onto the column. Elution was performed using three acetonitrile gradients; 0-24% over 5 min, 24-36% over 125 min, and 36-80% over 30 min, as described in Section **6.2.5**. The protein absorbance profile (280 nm) is shown in the *lower* panel (___) together with the corresponding percentage volume of acetonitrile (- - - -). IGF-binding activity in 0.1 µl of each fraction is shown in the *upper* panel (squares).

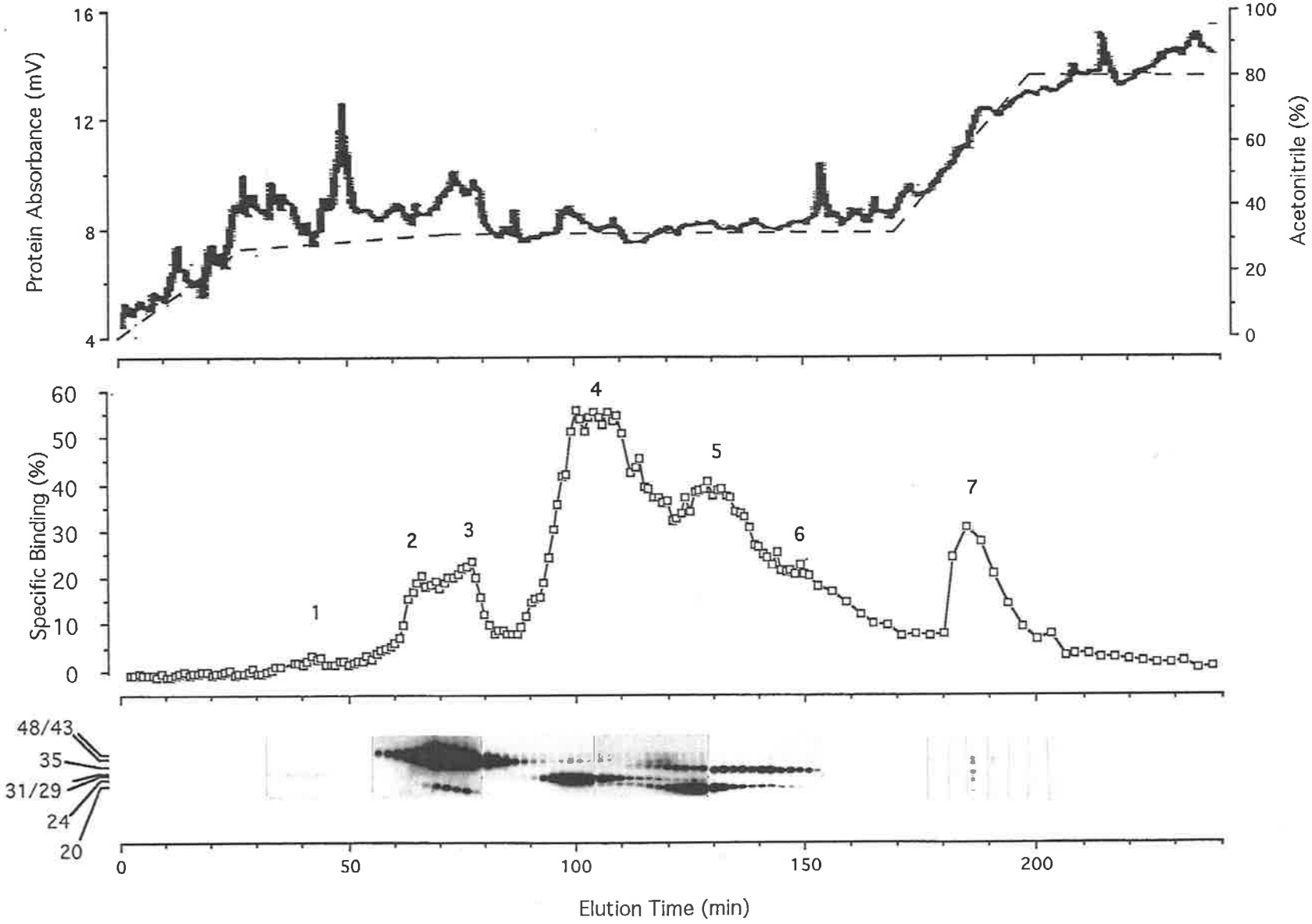

Specific Binding (%)
30
20
10
0
Protein Absorbance (mV)
1.5
1.0
0.5
0.0
100
80
60
40
20
0
Acetonitrile (%)
0
50
100
150
200
Time (min)

with an additional binding region at 185 min (Fig. 6.11). While the IGFBP-regions were better separated using the shallow propan 1-ol gradients than with acetonitrile gradients, clearly there remained considerable overlap between IGF-binding regions in this final reverse-phase stage.

Fractions corresponding to IGF-binding activity in the final reverse phase step were ligand blotted to determine the molecular masses of IGFBPs eluted in the various IGF-binding regions. Blotting revealed IGFBPs of 48, 43, 35, 31, 29, 24 and 20 kDa (Fig. 6.11). The first IGF-binding peak contained only the 31 kDa IGFBP but the intensity of this band was very low compared with that of bands in subsequent peaks. Stronger ligand-binding activity was evident in the second and third peaks at which the 48, 43 and 20 kDa IGFBPs eluted. While most of the 48 and 43 kDa bands eluted in the second and third peaks, small amounts continued to elute from the column across the remaining peaks of IGF-binding activity. IGFBP activity in the fourth peak corresponded mainly to the 29 kDa species although small amounts of this IGFBP trailed into the fifth and sixth peaks. The fifth and sixth peaks contained mainly the 24 and 35 kDa IGFBPs, with the 24 kDa species concentrated in the fifth region and the 35 kDa IGFBP distributed more or less evenly between both peaks. The seventh IGF-binding region contained a mixture of IGFBPs including the 48, 43, 35, 31, 29 and 24 kDa species but the bands in this peak were of very low intensity.

The relative abundance of IGFBP as indicated from charcoal binding was very different to that indicated by ligand blotting. For example, IGFBPs in peaks two and three were a relatively small proportion of the total IGF-binding activity when compared by charcoal binding, while by ligand blotting the IGFBPs at these regions bound more radioligand than IGFBPs in other regions. On the other hand, IGFBPs in the fourth peak appeared the most abundant by charcoal binding assay but not so by ligand blotting. Neither was the protein absorbance profile a particularly good indicator of the relative abundance of IGFBPs for while the second and third peaks of IGFBP activity appeared to correspond to protein peaks, no distinct protein peaks were apparent for other IGFBP regions. However, neither ligand blotting nor charcoal binding, as used in the present study, is a quantitative

Fig. 6.11 *Reverse phase HPLC chromatography of IGF-I-affinity-extracted IGFBPs; Step-3* Protein absorbance at 280 nm *(upper panel)* is shown during elution from a Brownlee OD-300 aquapore reverse phase HPLC column, at 1 ml/min, using four gradients of propan-1-ol in 0.13% HFBA (0-27% over 27 min, 27-31.5% over 45 min, 31.5-32.0% over 100 min and 32-80% over 30 min). A charcoal binding assay was performed for detection of IGF-binding activity (*middle panel*) using 10 µl subsamples from each 1 min fraction; IGF-binding regions are numbered 1-7. Alternate fractions corresponding to the elution of IGF-binding activity were ligand blotted *(lower panel)* for identification of IGFBPs on the basis of molecular mass, as described in Section **6.2.4**. Lanes each contain 7.5 µl of the fraction. Calculated molecular masses (kDa) of IGFBP bands are indicated on the *left*. Blots were autoradiographed for 3 days.


Protein Absorbance (mV)
16
12
8
4
Acetonitrile (%)
100
80
60
40
20
0
Specific Binding (%)
60
50
40
30
20
10
0
1
2
3
4
5
6
7
48/43
35
31/29
24
20
0
50
100
150
200
Elution Time (min)

method of detection for IGFBPs. Accordingly, the charcoal binding assay relies upon measurement of IGFBP-bound radioligand in solution, so that any IGFBP which binds to charcoal may appear less abundant in this assay. By ligand blotting, IGFBPs may differ in the efficiency with which they transfer to nitrocellulose. Therefore, without measurement of IGFBPs by specific assays, it cannot be determined which technique gives the more quantitative indication of relative IGFBP abundance.

Because IGFBPs contained in the first and fourth peaks were of approximately similar size to the IGFBP induced during anaesthesia, fractions that corresponded to these peaks were purified by SDS-PAGE for sequence analysis. Prior to this purification, however, the protein contents of these peaks (30-55 min pool; 31 kDa IGFBP, 96-112 min pool; mainly 29 kDa species) were measured. Protein contents were 1.04 µg/ml for the 30-55 min pool and 1.86 µg/ml for the 96-112 min pool. About 10 µg of protein from each pool was subjected to SDS-PAGE and transferred to PVDF membrane. After staining of the membrane for protein, the major band detected in the 96-112 min pool was of molecular mass 29 kDa, a size similar to that of the major IGFBP contained in this pool. Some relatively faint bands were evident at higher molecular masses but were a relatively minor component of the total protein. In the lane containing the 30-55 min pool, no protein was detectable by staining. This pool may therefore have contained a large mixture of proteins, but at concentrations below that detectable by protein staining. Since the 31 kDa protein could not be detected, further analysis involved only the 29 kDa protein. Sequence analysis revealed major amino acids in order: Asp-Glu-Ala-Ile-His-x-Pro-Pro-, consistent with the previously reported N-terminal sequence for ovine and human IGFBP-4 (Walton *et al;* 1990, La Tour *et al;* 1990). The major and minor amino acid yields are shown in Table 6.1.

6.3.5 Immunoblots

The combined approaches of ligand blotting and immunoblotting were used in side-by-side comparisons of purified ovine IGFBP-4 and plasma from anaesthetised lambs to

Table 6.1. *Sequence analysis of the 29 kDa IGFBP; yields for residues 1-8.*

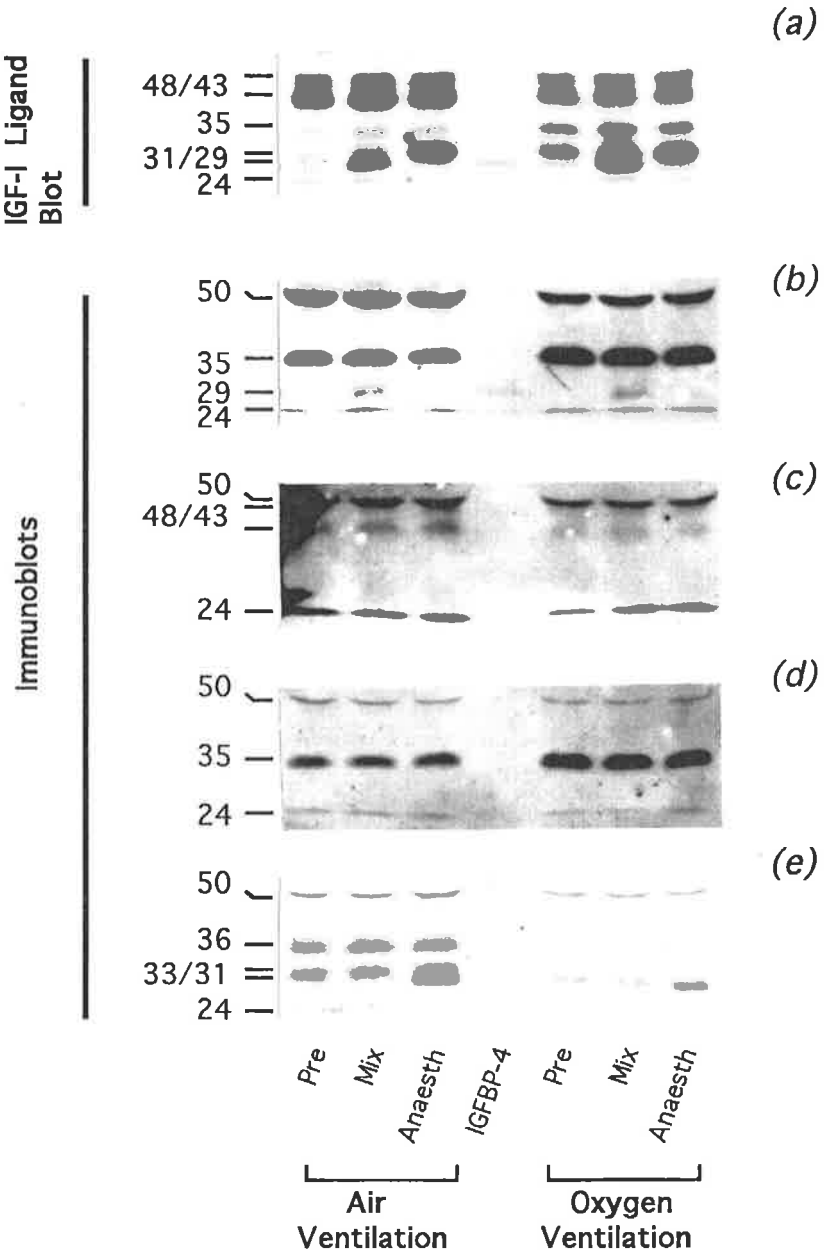
	Yields (pmol) for respective position numbers							
Amino Acid	1	2	3	4	5	6	7	8
Asp	**8.87**	0.83	0.27	0.08	0.31	0.20	0.31	0.08
Asn	0.09	0.17	0.08	0.05	0.06	0.06	0.13	0.10
Ser	0.92	0.17	0.19	0.25	0.28	0.20	0.10	0.26
Gln	2.95	0.09	0.08	0.18	0.10	0.15	-	0.10
Thr	0.77	-	0.06	0.13	-	0.10	-	0.09
Gly	1.10	0.58	0.53	0.64	0.43	0.67	0.53	0.90
Glu	0.18	**8.00**	1.22	0.22	0.06	0.16	0.17	0.14
Ala	0.61	0.31	**10.92**	1.62	0.55	0.48	0.55	0.46
His	0.31	0.22	0.16	0.18	**0.82**	0.25	0.34	0.58
Tyr	0.26	0.03	0.03	-	-	-	-	-
Arg	0.85	0.34	0.37	0.67	0.63	0.76	0.54	0.58
Pro	0.55	0.38	0.35	0.22	0.34	0.54	**4.46**	**6.06**
Met	0.16	0.04	-	-	-	0.03	-	0.07
Val	0.27	0.15	0.15	0.22	0.23	0.28	0.25	0.34
*Trp	10.83	13.57	15.23	10.08	13.88	14.72	16.79	20.75
Phe	0.20	0.17	0.53	0.88	0.77	-	1.37	-
Ile	-	-	0.06	**5.75**	1.26	-	-	-
Lys	0.34	-	-	-	-	-	-	-
Leu	0.20	0.15	0.24	-	0.09	0.34	0.35	0.43

Predominant amino acids are shown in **bold.**

* The high apparent values for tryptophan were due to extensive overlap of the Trp peak with diphenyl urea, a reaction byproduct formed during sequence analysis.

determine similarities in size and/or immunoreactivity of the purified IGFBP and anaesthesia-induced IGFBP. Antibodies, raised against ovine IGFBP-4, ovine IGFBP-3, bovine IGFBP-2 and human IGFBP-1, were used to probe ^{125}I-IGF-I-blotted nitrocellulose, the lanes of which contained pre-anaesthesia plasma, anaesthesia plasma, pre-anaesthesia plasma to which purified IGFBP-4 had been added, or IGFBP-4 alone. IGFBP-4 migrated at 29 kDa, whether analysed alone or mixed with plasma (Fig. 6.12*a*). However, the anaesthesia-induced protein migrated with an apparent molecular mass of 31 kDa. The clear distinction between the 31 and 29 kDa radioligand-binding bands, in this ligand blot, argues against IGFBP-4 as the anaesthesia-induced binding protein. This conclusion was strengthened by immunoblots using antiserum against IGFBP-4 (Fig. 6.12*b*) which revealed an IGFBP-4-immunoreactive band at 29 kDa in plasma to which the purified protein had been added but which did not otherwise demonstrate an increase in the intensity of 29 kDa signal after 4 h of anaesthesia. Thus, the antiserum detected purified 29 kDa ovine IGFBP-4 but showed no cross-reactivity with the 31 kDa band in plasma from anaesthetised lambs. Anti-ovine IGFBP-4 also detected a 24 kDa IGFBP, a size consistent with that of the 29 kDa ovine IGFBP-4 species after deglycosylation (personal communication; Dr. P.E. Walton), and a 35 kDa band presumably representing cross-reactivity with IGFBP-2. The high apparent crossreactivity of this IGFBP-4 antiserum with the 35 kDa protein, previously shown to be IGFBP-2-immunoreactive (Fig. 3.3), is not unexpected in view of the reported 50% crossreactivity of commercially available IGFBP-4 antiserum with IGFBP-2 (Upstate Biotechnology Inc.). The strong signal intensity that was evident at 50 kDa in all plasma samples, did not coincide with a radioligand-binding band. Nevertheless, this material also exhibited crossreactivity with antisera against IGFBP-3, -2, and -1 (compare Fig. 6.12*b-d*) and presumably represents an artefact of the immunoblotting technique. Immunoblots using anti-ovine IGFBP-3 revealed a doublet at 48 and 43 kDa (Fig. 6.12*c*), as expected from the known molecular mass of ovine IGFBP-3 (Walton *et al;* 1990), together with a strong signal at 24 kDa in all plasma samples. Intense binding of anti-bovine IGFBP-2 was coincident with the 35 kDa ligand-binding band (Fig. 6.12*d*), a size that is similar to that reported for IGFBP-2 in sheep (Walton *et al;* 1990). This antiserum also displayed crossreactivity with

Fig. 6.12 *Western ligand blot and immunoblots of plasma collected before and during anaesthesia.* Plasma samples from individual air-ventilated anaesthetised lambs and oxygen-ventilated anaesthetised lambs were ligand blotted using ^{125}I-IGF-I (*a*), and then immunoblotted using antisera against either ovine IGFBP-4 (*b*), ovine IGFBP-3 (*c*), bovine IGFBP-2 (*d*) or human IGFBP-1 (*e*). After incubation of the nitrocellulose with a peroxidase-conjugated second antibody, positions of immunoreactive proteins were revealed by incubation with a chemiluminescence-substrate and detection reagent and subsequent exposure to X-ray film (**6.2.6**). The ligand blot was exposed to x-ray film for 3 days and immunoblots were exposed for between 1 and 3 min. Samples are: pre-anaesthesia plasma (*'Pre'*), pre-anaesthesia plasma mixed with purified IGFBP-4 (*'Mix'*), 4 h anaesthesia plasma (*'Anaesth'*), and purified IGFBP-4 alone. Estimated molecular masses of bands (kDa) are shown at *left*.


(a)
IGF-I Ligand Blot
48/43
35
31/29
24
(b)
Immunoblots
50
35
29
24
(c)
50
48/43
24
(d)
50
35
24
(e)
50
36
33/31
24
Pre
Mix
Anaesth
IGFBP-4
Pre
Mix
Anaesth
Air Ventilation
Oxygen Ventilation

the 24 kDa IGFBP. However, neither the IGFBP-2 nor IGFBP-3 antiserum detected the 31 kDa anaesthesia-induced IGFBP nor the purified 29 kDa IGFBP-4.

Anti-human IGFBP-1, on the other hand, bound intensely to the 31 kDa band (Fig. 6.12*e*) and, consistent with the ligand blotting studies, the intensity of this immunoreactive signal was increased after 4 h of anaesthesia. The 29 kDa ovine IGFBP-4 was not detected using this antiserum but additional IGFBP-1-immunoreactive bands were detected in each plasma sample at approximately 33 and 36 kDa. These bands, like the 50 kDa material, neither coincided with ligand-binding activity nor changed in intensity as a result of anaesthesia but may represent antiserum-crossreactivity with binding proteins that failed to bind ligand in the conditions employed during ligand blotting.

6.3.6 Insulin and Glucose Measurements

In the food-deprived control lambs (Fig. 6.13*a*), plasma glucose concentrations declined by approximately 10% over the 6 h sampling period. Insulin concentrations were erratic. More definite changes in blood glucose and insulin were apparent during anaesthesia in both oxygen-ventilated (Fig. 6.13*b*) and air-ventilated lambs (Fig. 6.13*c*), so that glucose concentrations decreased by 10-40% within the first hour of anaesthesia, while insulin concentrations fell by 60-95% over the same period. In three of the four experimental animals, the concentrations of both insulin and glucose remained depressed until the conclusion of anaesthesia. Withdrawal of anaesthetic was followed by a re-bound in glucose and insulin levels towards the pre-anaesthesia concentrations. Thereafter, glucose stabilised close to the pre-anaesthesia value.

6.3.7 Assay of IGFs

Plasma IGF-I and IGF-II concentrations declined continuously during anaesthesia but changed very little in the non-anaesthetised lambs. IGF-I concentrations fell by 30-65%

Fig. 6.13 *Glucose (open symbols) and insulin concentrations (filled symbols) in food-deprived controls (a), halothane-anaesthetised lambs with oxygen-ventilation (b) and halothane-anaesthetised lambs with air-ventilation (c).* Values for two animals *(circles and squares)* are shown in each panel and the time span of anaesthesia is indicated. The minimum detectable concentration of insulin in the assay, 50 pg/ml, is indicated by the dashed line; samples in which detectable insulin fell below this limit were assumed to contain insulin at 50 pg/ml.

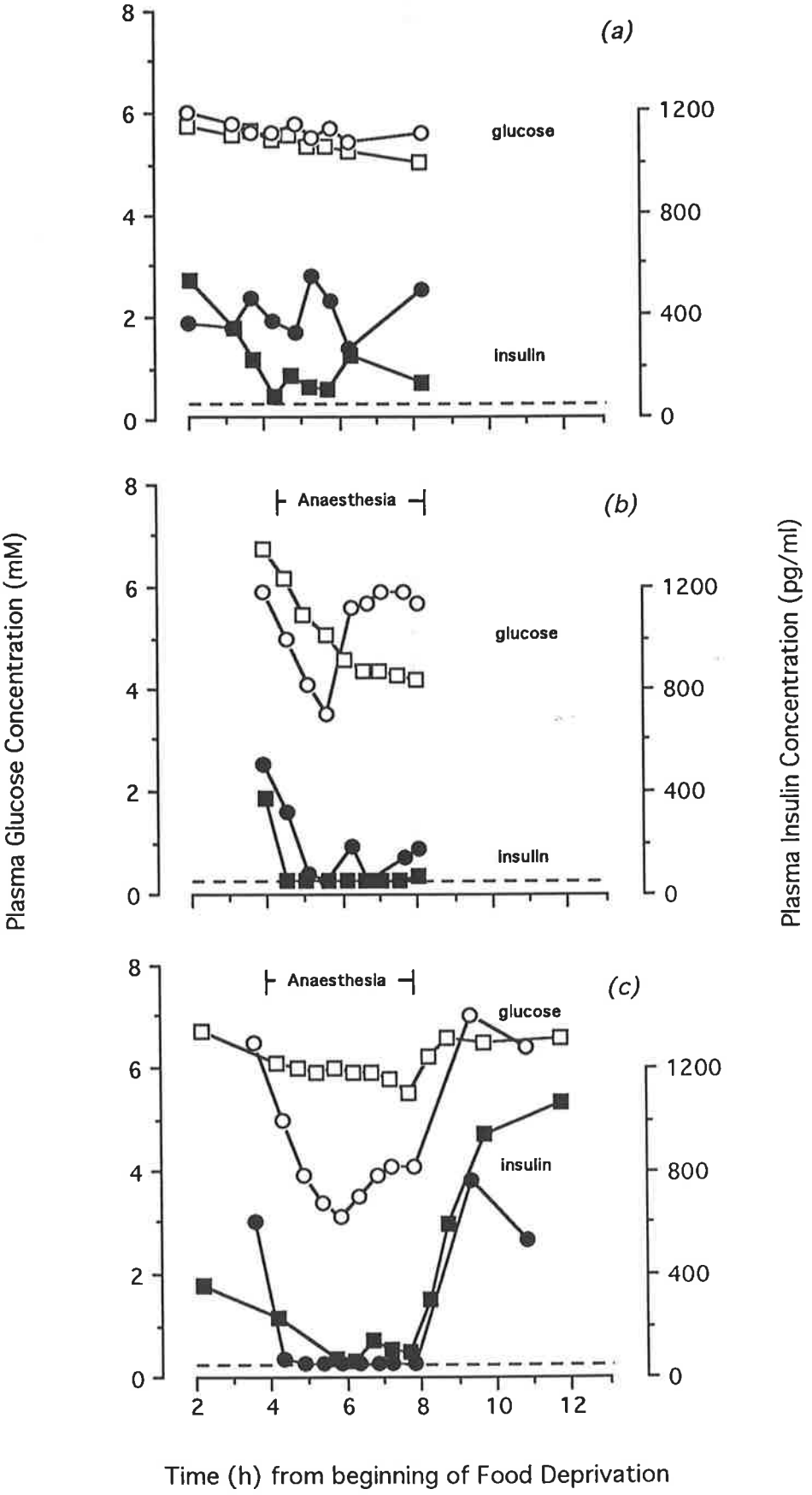
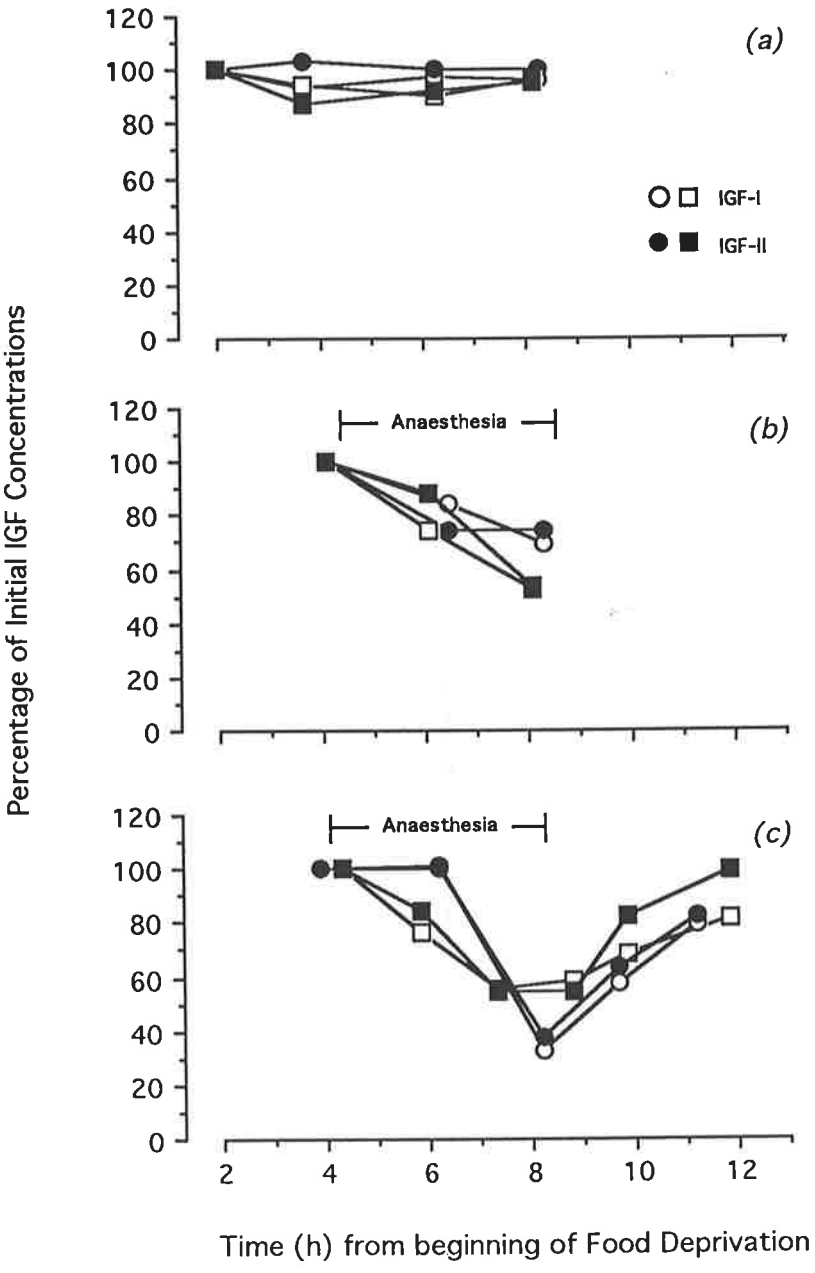

(a)
glucose
insulin
(b)
Anaesthesia
glucose
insulin
(c)
Anaesthesia
glucose
insulin
Plasma Glucose Concentration (mM)
Plasma Insulin Concentration (pg/ml)
Time (h) from beginning of Food Deprivation

Fig. 6.14 *Percentage changes in endogenous IGF-I (open symbols) and IGF-II concentrations (filled symbols) in food-deprived controls (a), halothane-anaesthetised lambs with oxygen-ventilation (b) and halothane-anaesthetised lambs with air-ventilation (c).* Values for two lambs *(circles and squares)* are shown in each panel and the time span of anaesthesia is indicated. The initial concentrations of IGF-I and IGF-II (means ± SEM for all six animals) were 181 ± 34 and 211 ± 10 ng/ml respectively.


Percentage of Initial IGF Concentrations
120
100
80
60
40
20
0
(a)
IGF-I
IGF-II
Anaesthesia
(b)
Anaesthesia
(c)
2
4
6
8
10
12
Time (h) from beginning of Food Deprivation

during 4 h of halothane-anaesthesia, with either oxygen-ventilation (Fig. 6.14*b*) or air-ventilation (Fig. 6.14*c*), while minimal decreases occurred in the food-deprived controls (Fig. 6.14*a*). IGF-II declined during anaesthesia in a similar manner to IGF-I and changed little during food deprivation alone. Withdrawal of anaesthesia was followed by a rise in plasma IGF-I and IGF-II concentrations towards initial values (Fig. 6.14*c*).

6.4 DISCUSSION

This study demonstrates a dramatic increase in plasma binding capacity for exogenous IGF during halothane-anaesthesia in both hyperoxic and normoxic lambs. This could reflect a reduction in the concentration of endogenous IGFs, an increase in amount of plasma IGFBPs, or combined changes in both variables. Indeed, the results show that the rise in radioligand binding capacity during anaesthesia coincided with a marked increase in concentration of a 31 kDa IGFBP, in addition to declines in IGF-I and IGF-II concentrations.

Changes in IGF and IGFBP concentrations may contribute differentially to the increased binding of des(1-3)IGF-I at 30-50 and 130-150 kDa demonstrated by size-exclusion chromatography. Specifically, because IGFBPs of molecular mass 24-35 kDa have previously been shown to elute at 30-50 kDa by size-exclusion chromatography (Fig. 3.4), the increased concentration of 31 kDa IGFBP probably contributes directly to the greater binding activity at 30-50 kDa during anaesthesia. The observed decreases in endogenous IGF concentrations may also contribute to the increased radioligand-binding at 30-50 kDa because while much endogenous IGF is contained in the 130-150 kDa complex (Baxter & Martin; 1986, Baxter; 1988*a*), approximately 30% could be associated with small molecular mass IGFBPs (Gargosky *et al;* 1991). Lower circulating IGF concentrations probably account for the increased radioligand-binding at 130-150 kDa whereas elevations in levels of IGFBPs are unlikely to explain the greater binding at this size because there was no increase in intensity of the 43 and 48 kDa IGFBPs of the high molecular mass complex. Thus the overall result of the changes in IGF and IGFBP concentrations was a net expansion

in the available ligand-binding sites, resulting in increased formation of labelled complexes at low and high molecular masses.

The available antibodies against IGFBPs unfortunately were not completely specific. However, the results show that antibodies against IGFBP-1 bind relatively strongly to the anaesthesia-induced 31 kDa IGFBP, while those raised against ovine IGFBP-4, a protein of similar size, failed to bind at this position. The immunoblot data also suggest that the anaesthesia-induced protein is not a breakdown product of the 48, 43 and 35 kDa IGFBPs because antibodies against IGFBP-2 and IGFBP-3, which bound primarily to these three bands, did not recognise the elevated activity at 31 kDa. In support of this, no decreases in the intensity of these higher molecular mass IGFBP bands were detected by ligand blotting. Hence, although the possibility that other binding proteins contributed to the 31 kDa IGFBP band cannot be excluded, the intense binding of antiserum against IGFBP-1 combined with the absence of detectable crossreactivities with antisera against IGFBPs -2, -3 and -4 strongly suggests that IGFBP-1 was the major binding protein induced.

The mechanism of the anaesthesia-induced changes in circulating IGFBP-1-like activity and IGFs has been addressed indirectly. Thus, contributions of food deprivation as well as oxygen supply have been excluded as potential mechanisms of induction. Nutrition is known to be a key regulator of IGFs (Underwood *et al;* 1991), yet withdrawal of food for up to 8 h did not mimic the responses of IGFs and IGFBPs observed in anaesthetised lambs deprived of food for a similar period. While increased plasma IGFBP-1 and decreased IGF-I may result from low arterial P_{O_2} (McLellan *et al;* 1992, Iwamoto *et al;* 1992), changes in IGF and IGFBP levels as a result of high P_{O_2} have not previously been described. Nevertheless, the potential contribution of P_{O_2} to changes in IGFs and IGFBP concentrations during anaesthesia can be excluded on the basis of the qualitively similar changes in hyperoxic and normoxic lambs. It is therefore clear that the results of Chapter **5**, in which radioligand-binding in the vascular perfusion study was greater than that attained *in vitro*, can be explained by anaesthesia-associated events and are not related to nutrition or blood P_{O_2}. The increase in IGFBP-1-like activity during halothane-anaesthesia in lambs is consistent with a previous report of elevated IGFBP-1 in the pentobarbital/ketamine-anaesthetised rat, but a

starvation response could not be eliminated in that investigation (Lewitt *et al;* 1992). It is possible that the halothane-anaesthesia induced increase in 31 kDa IGFBP resulted from impaired clearance of IGFBP or binding protein breakdown products, but further investigation would be required to confirm this hypothesis.

Measurement of insulin and glucose concentrations were performed to further characterise the factors that contribute to increased IGF-binding capacity during anaesthesia. Studies of IGFBP-1 regulation in other species suggest a complex relationship with both glucose and insulin such that increased circulating levels of IGFBP-1 are observed when concentration of glucose is depressed by insulin administration, but insulin depresses IGFBP-1 when glucose is maintained at a constant concentration (Suikkari *et al;* 1988, Jacob *et al;* 1989). Since the data show that anaesthesia is associated with decreases in both plasma insulin and glucose concentrations, it is possible that regulation by glucose and/or insulin is the mechanism by which IGFBP-1-like activity was induced in this study. Nevertheless, the possibility that rises in plasma cortisol, a factor known to induce IGFBP-1 (Conover *et al;* 1993), contributed to increased immunoreactive IGFBP-1 during anaesthesia, cannot be excluded.

It is unlikely that the decreases in insulin and glucose concentrations are directly responsible for the declines in IGF concentrations measured in the present experiment for while insulin concentrations correlate with those of IGF in the long term (Smith *et al*; 1989), evidence for acute regulation of IGF by insulin and glucose is lacking. Plasma IGF concentrations do not rise during insulin infusion into glucose-clamped rats (Jacob *et al;* 1989) or decline in lambs infused continuously with insulin over a 5 h period, despite a greater than 40% reduction in plasma glucose concentration (Douglas *et al;* 1991). Furthermore, IGF synthesis by cells from the liver, a major site of IGF production (Schwander *et al;* 1983), does not appear to be reduced in the absence of insulin over a 24 h period (Houston & O'Neill; 1991).

The decline in glucose may relate to direct effects of the anaesthetic agent. Halothane has been shown to alter glucose metabolism in a variety of ways, including reduced

gluconeogenesis (Biebuyck *et al;* 1972, Romero *et al;* 1987, Cronau *et al;* 1988) increased glycogenolysis (Biebuyck & Lund; 1974, Rosenberg *et al;* 1977) and decreased tissue utilisation (Romero *et al;* 1987, Crosby & Atlas; 1988). Accordingly, the observed net decrease in circulating glucose in lambs is consistent with a predominant inhibitory effect of halothane on gluconeogenesis, rather than a stimulation of glycogenolysis or reduction in glucose uptake. Halothane has also been shown to inhibit synthesis of proteins by the liver (Flaim *et al;* 1983, Malledant *et al;* 1990), a major site of IGF and IGFBP synthesis (Scott & Baxter; 1986). However, no such effect of volatile anaesthetics on IGF synthesis has yet been demonstrated, and the rapid increase in IGFBP concentration in plasma during halothane-anaesthesia suggests that a generalised suppression of protein synthesis is unlikely to contribute greatly to the declines in IGF-I and IGF-II concentrations during anaesthesia.

The continued depression of plasma glucose concentrations during anaesthesia, despite the declines in circulating insulin levels, suggests that sources of insulin-like activity besides insulin are present in the circulation during anaesthesia. Indeed, because IGFs circulate at high concentrations compared with those of insulin, and display an approximately 15% crossreactivity with the insulin receptor (Ballard *et al;* 1986), they have the potential to exert significant hypoglycemic effects. There is some evidence to suggest that the insulin-like effects of circulating IGFs may be buffered by IGFBP-1; for example, infusion of IGFBP-1 into the circulation of the rat results in hyperglycaemia (Lewitt *et al;* 1991). However, it is not clear whether such a role is served by IGFBP-1 in the sheep, for despite the increased concentrations of IGFBP-1-like protein and decreased concentrations of both IGF-I and IGF-II during anaesthesia, glucose remained depressed in most experiments.

A direct protective role for IGFBP-1 during hypoglycaemia has not been clearly elucidated, although it is possible that this protein contributes to the clearance of plasma IGFs and a reduction in the size of the circulating IGF pool. Indeed, the circulating half lives of radiolabelled IGFs when associated with the small molecular mass IGFBPs are less than a fifteenth of those of IGF in the high molecular mass complex (Hodgkinson *et al;* 1989*a*, Davis *et al;* 1989), the form in which most endogenous IGF circulates. Thus it is plausible that the anaesthesia-associated rise in low molecular mass IGF-binding sites could result in

some redistribution of endogenous IGFs from the high molecular mass complex to smaller IGFBPs, which in turn would be expected to increase the clearance rates of endogenous IGFs and contribute to lower circulating concentrations. While a recent study shows that IGFBP-1 may reduce the clearance of free IGF-I (Lewitt *et al;* 1993), that study may not reflect the effect of IGFBP-1 on clearance of endogenous IGFs because these IGFs circulate mainly as high molecular mass IGF-IGFBP complexes (Baxter & Martin; 1986, Baxter; 1988*a*). Thus the data are consistent with an argument for increased clearance of endogenous IGFs in a condition where IGFBP-1-like protein is elevated, and suggest that IGFBP-1 may play a role in the regulation of circulating IGF concentrations.

In conclusion, the decreased circulating IGF-I and IGF-II concentrations together with increased plasma IGFBP-1 do not appear to be the result of food deprivation or high blood P_{O_2} during anaesthesia, but rather a result of halothane anaesthesia itself. The data suggest that these changes may be effected through decreased plasma glucose and/or insulin. The net increase in IGF-binding capacity during anaesthesia would appear to explain the lower binding of des(1-3)IGF-I in the *in vitro* incubation study (Chapter **5**) than in the vascular perfusion studies, because blood for the *in vitro* study was collected relatively early in anaesthesia.

6.5 SUMMARY

1) The IGF-binding capacity of lamb plasma increased progressively throughout halothane-anaesthesia, but not during an equivalent period of food deprivation. The increase was demonstrated in lambs that were either hyperoxic or normoxic during anaesthesia. Withdrawal from anaesthesia was associated with the return of plasma IGF-binding capacity towards pre-anaesthesia values. These findings explain the extensive binding of des(1-3)IGF-I to IGFBPs in the vascular perfusion system and comparatively poor binding *in vitro* that was shown in Chapter **5**, since the vascular perfusion experiment was performed after an extended period of anaesthesia.

2) The increase in plasma IGF-binding capacity during anaesthesia was the result of progressive declines in endogenous IGF-I and IGF-II concentrations, and the general rise in abundance of an IGFBP species.

3) The anaesthesia-induced binding protein was IGFBP-1-immunoreactive and its plasma level was elevated in association with reduced concentrations of glucose and insulin. Since these factors are known regulators of IGFBP-1 in other species, the data suggest that the binding protein induced during anaesthesia was ovine IGFBP-1.

4) While halothane has been shown to inhibit gluconeogenesis, stimulate glycogenolysis and cause reduced glucose utilisation in other experimental models, the net decrease in plasma glucose during anaesthesia is consistent with a predominant inhibition of gluconeogenesis in the lamb.

5) It is suggested that IGFBP-1 may play a role in regulating circulating IGF-I and IGF-II levels, perhaps by altering the distribution of plasma IGFs between the circulating IGFBPs and thereby changing accessibility of this IGF to target tissues.

6.6 ACKNOWLEDGMENTS

Ms. S. Harrer and Ms C. Mardell are thanked for assistance during the cannulation of blood vessels, and were also responsible for the measurements of blood gas partial pressures and pH. Dr. S.J. Hazel is thanked for assistance in cannulating the sheep carotid artery. Iodination and purification of all radiolabelled peptides used in the present chapter was performed by Mr. S.E. Knowles. Charcoal-stripped plasma for use in the insulin assay was prepared by Mr. F. Carbone and ovine placental membranes for use in the IGF-II assay were prepared by Ms K. Moyse.

Chapter Seven

Species Comparisons of IGF Binding to Plasma IGFBPs

CHAPTER SEVEN: SPECIES COMPARISONS OF IGF BINDING TO PLASMA IGFBPS

7.1 INTRODUCTION

An unexpected aspect of the results obtained in the earlier experimental chapters was the extensive binding of the IGF-I analogs des(1-3)IGF-I and LR^3IGF-I to IGFBPs in sheep plasma, because published data reveals negligible binding in rat plasma (Ballard *et al;* 1991, Walton *et al;* 1991). While the experiments in Chapter **6** demonstrated that extended halothane anaesthesia contributed to the extensive binding in sheep plasma, this could not fully account for differences in binding to ovine and rat plasma IGFBPs since the IGF-I analog des(1-3)IGF-I was found to also bind substantially in pre-anaesthesia plasma. The existence of species specificities in the interactions between IGFs and IGFBPs is therefore implied. The major objective of the current study was to confirm the existence of such species specificities and to determine whether they could be explained by differences in IGF binding to the constituent IGFBPs in plasma. To explore these possibilities, plasma from lambs and rats was compared by a combination of *in vitro* ligand binding and ligand blotting methods, using radiolabelled IGF-I, des(1-3)IGF-I and LR^3IGF-I.

A secondary aim of this study was to extend the binding comparisons for IGF-I, des(1-3)IGF-I and LR^3IGF-I in rat and sheep plasma to agriculturally important species, including the pig and chicken, and also to man. In the context of the overall aims of this thesis, the results of comparisons between the animal species could be used to select an alternative animal to the sheep in which IGF transfer studies could be performed in the future (described in Chapters **4** & **5**); selection of a species would be based upon poor binding of analogs and extensive binding of IGF-I to plasma IGFBPs. The results of comparisons between the IGF peptides in animals and man could also be of predictive value in determining the relative potency of IGF-I and the analogs for future agricultural and medical applications, since IGFs which bind poorly to IGFBPs are more potent promoters of growth (Lemmey *et al;* 1991, Tomas *et al;* 1992, 1993*a*, 1993*b*).

7.2 MATERIALS AND METHODS

7.2.1 IGFs

Recombinant human IGF-I, des(1-3)IGF-I and LR^{3}IGF-I (GroPep Pty. Ltd.) were iodinated (Na^{125}I; Nordion International Inc.) to specific activities in the range 27-73 Ci/g, as described in Section **2.1**.

7.2.2 Plasma Collections

Blood from Merino-Dorset crossbred lambs (6 weeks), humans (9 and 26 years), pigs (3 months), chickens (42 days) and Sprague Dawley rats (3 and 6 months) was collected into chilled tubes containing heparin at a final concentration of 10 IU/ml. Samples were taken from two males of each species, except that one rat sample represented a pool of 10 animals. Collections from lambs, humans, pigs and chickens were performed by acute venipuncture of the jugular vein, a peripheral arm vein (median basilic), the external jugular vein and a wing vein (deep ulnar), respectively. Blood was collected from rats following decapitation. After centrifugation for 20 min at 2000 x *g* and 4°C, plasma was aspirated and stored at -70°C, until analysis.

7.2.3 *In Vitro* IGF-Binding and Size-Exclusion Chromatography of Plasma IGFBPs

Plasma (200 µl) was incubated for 15 h at 4°C with 0.8 ng of radiolabelled IGF-I, des(1-3)IGF-I or LR^{3}IGF-I in 10 µl of heparinised PBS (Section **2.3**) containing 1% (w/v) BSA, plus 8 µl of heparinised PBS with BSA. Similar incubations with radiolabelled IGFs but with the substitution of 0.8 µg (8 µl) unlabelled IGF-I for buffer, were included to demonstrate competitive binding of radioligand in plasma. The profiles of each radioligand were also compared in additional incubations using plasma from a second animal of each species. Unlabelled IGF-I was not included in these incubations. Following all incubations, mixtures were kept at -20°C pending analysis by size-exclusion chromatography.

Samples were thawed and defatted with an equal volume of freon (Section **2.3**), after which a 100 µl portion was applied to a Superose-12 size-exclusion column and chromatographed as described in Section **2.3**. Radioactivity contained in 0.5 ml fractions was measured in a gamma counter. The column was calibrated with protein markers for estimation of IGFBP molecular masses: human γ-globulin (150 kDa), BSA (69 kDa) and radiolabelled IGFs (8 kDa). Absorbance of the eluate was monitored at 280 nm.

7.2.4 Ligand Blots

Ligand blots were performed to characterise ligand-binding specificities of the various IGFBPs in each species. Plasma IGFBPs were compared in one sample from each species using labelled IGF-I, des(1-3)IGF-I and LR^3IGF-I. Dissociated standards (5 µl of ^{14}C-molecular mass markers in a volume of 15 µl) and samples (15 µl containing 1 µl of lamb, human, rat or pig plasma, or 4 µl of chicken plasma) were electrophoresed in parallel lanes through 4%/10% discontinuous polyacrylamide gels and transferred to nitrocellulose as described in Section **2.5**. Non-specific binding sites were blocked by incubation with 1% (w/v) BSA and the membrane was then probed with 10 ng of radiolabelled IGF-I, des(1-3)IGF-I or LR^3IGF-I, at similar specific activities, as described in Section **6.2.4**. Excess radioligand was removed in two washing steps and the membrane dried before visualisation of IGFBPs by autoradiography on X-ray film. IGFBP molecular masses were estimated against the positions of the ^{14}C-labelled markers. Exposure times for autoradiographs are indicated in the figure legends.

7.3 RESULTS

7.3.1 Chromatography of Labelled IGFs in Plasma

The association of ^{125}I-labelled IGF-I, des(1-3)IGF-I and LR^3IGF-I with binding proteins in native plasma from the five species was evaluated by first incubating each

radioligand with plasma *in vitro*, followed by size-exclusion chromatography at pH 7.2. Variability in this experiment was minimised by carrying out the incubations using the same volumes of plasma simultaneously with all three radioligands.

All plasma samples were shown to bind labelled growth factors, but the extent of the association varied according to the plasma species and IGF peptide used (Fig. 7.1*a-e* & Fig. 7.2*a-e*). Chromatography of rat plasma after incubation with radiolabelled IGF-I demonstrated most radioligand bound to binding proteins (*a*), with minor binding at 30-50 kDa but the predominant form having a molecular mass of 130-150 kDa. This is consistent with formation of the IGFBP-3 ternary complex (Baxter *et al;* 1989). Radiolabelled IGF-I associated at similar molecular masses also in plasma from lambs, humans and pigs (*b, c, d*) but in these incubations a substantial proportion also eluted at 30-50 kDa. This intermediate peak, expected to contain binary complexes of IGF-I with the various IGFBPs, was the only area of substantial ligand binding in chicken plasma (*e*); only a small shoulder of higher molecular mass IGF-binding activity was evident in this species. Total binding of labelled IGF-I ranged from 70% in the chicken to 90% in the lamb, with association in rat, human and pig plasma similar for the three species but in amounts that were intermediate between those in lamb and chicken plasma.

A parallel series of incubations in which labelled des(1-3)IGF-I was substituted for IGF-I radioligand demonstrated marked differences in binding between the five species (Fig. 7.1*f-j* & Fig. 7.2*f-j*). Association of des(1-3)IGF-I in rat plasma was negligible (*f*) but was 40 - 50% in lamb, human and pig plasma and less than 30% in chicken plasma (*g, h, i, j*). In all mammalian plasmas, the greatest reduction in ligand binding compared with that attained using labelled IGF-I, was apparent at 130-150 kDa; the decrease in binding at 30-50 kDa was comparatively small and binding at this size in human plasma was actually greater using des(1-3)IGF-I than using IGF-I. Nevertheless, the greatest formation of the ternary complex, with this analog, was evident in plasma from lambs.

In all incubations for which labelled LR^3IGF-I was employed, association of radioligand was poorer than obtained with either IGF-I or des(1-3)IGF-I radioligands

Fig. 7.1 *Chromatography on a Superose-12 column at pH 7.2 of plasma incubated previously with labelled IGF-I (a, b, c, d, e), des(1-3)IGF-I (f, g, h, i, j) or LR^3IGF-I (k, l, m, n, o) both with (open circles) and without (closed circles) the inclusion of 0.8 ng unlabelled IGF-I in each incubation.* Values are expressed as percentage of total eluted radioactivity contained in each 0.5 ml fraction. The elution positions of molecular mass standards (γ-globulin; 150 kDa, BSA; 69 kDa and IGFs; 8 kDa) are indicated by arrows. Plasma samples are from rat (*a, f, k*), sheep (*b, g, l*), human (*c, h, m*), pig (*d, i, n*) and chicken (*e, j, o*).

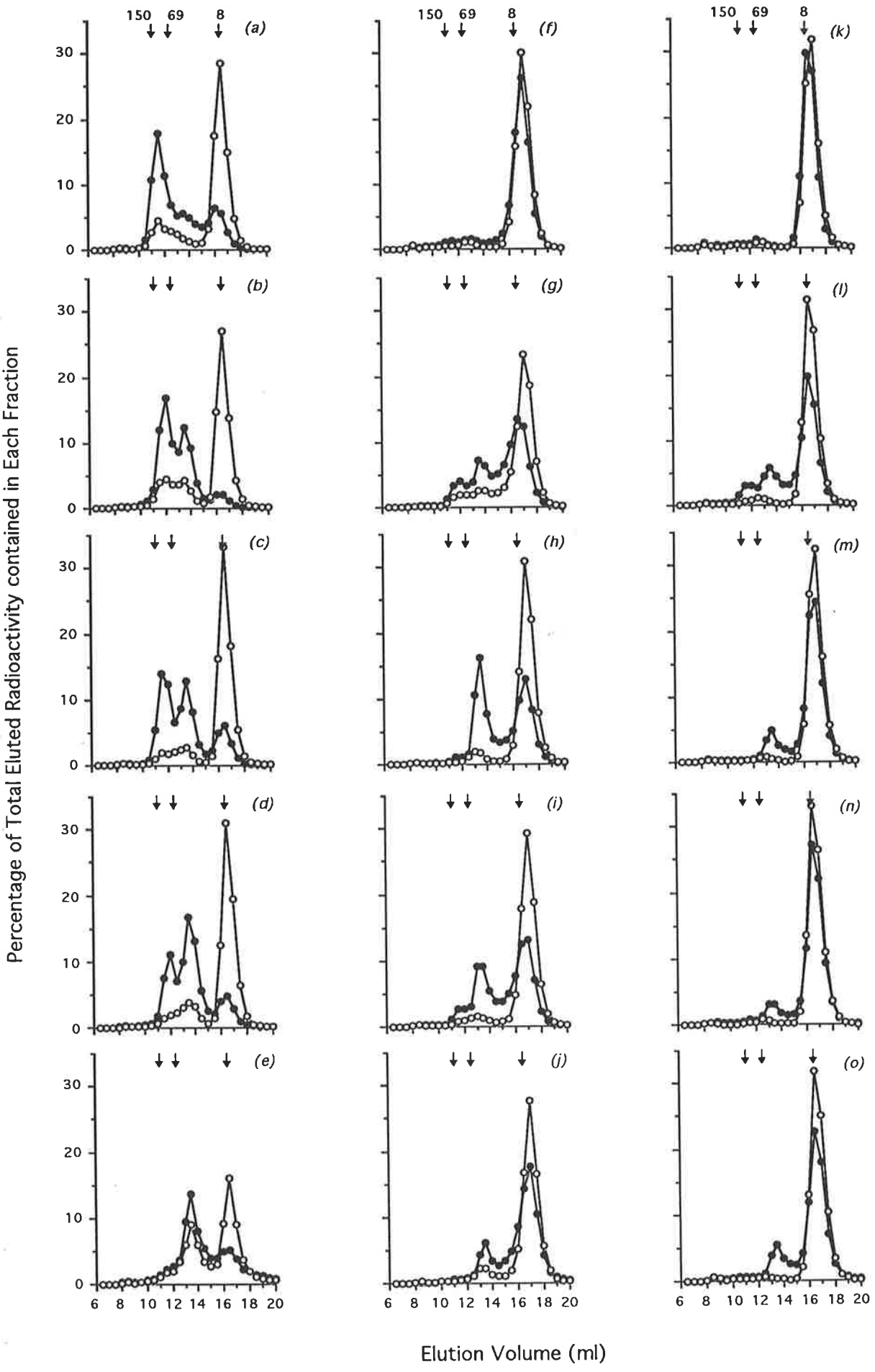
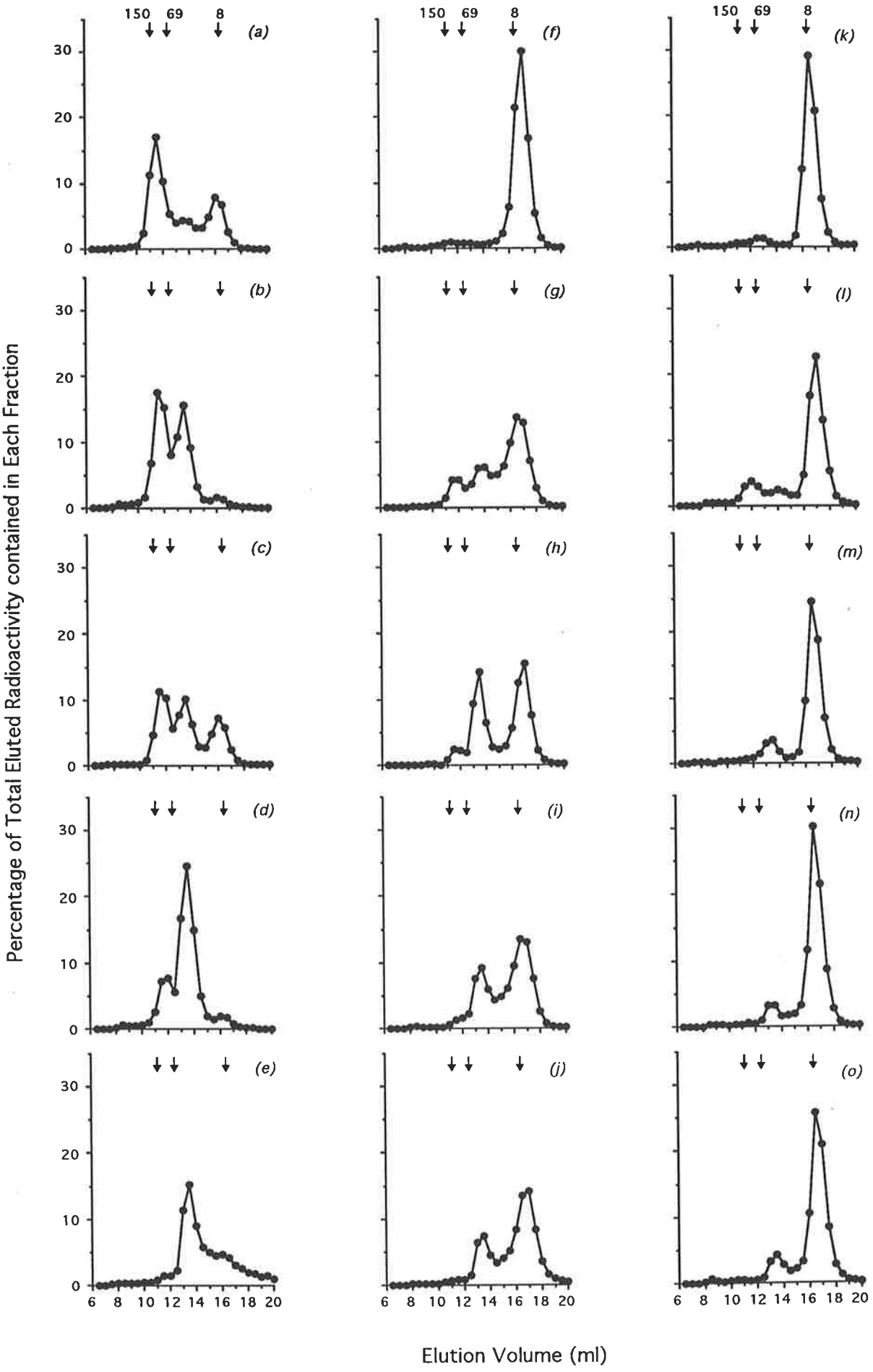

150 69 8
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
Percentage of Total Eluted Radioactivity contained in Each Fraction
Elution Volume (ml)
0
10
20
30
6 8 10 12 14 16 18 20

Fig. 7.2 *Chromatography at pH 7.2 of plasma samples from a second rat, sheep, human, pig and chicken, on a Superose-12 column, after incubation with radiolabelled IGF-I (a, b, c, d, e), des(1-3)IGF-I (f, g, h, i, j) or LR^{3}IGF-I (k, l, m, n, o), without inclusion of unlabelled IGF-I.* Values are expressed as the percentage of total eluted radioactivity contained in each 0.5 ml fraction. The elution positions of molecular mass standards (γ-globulin; 150 kDa, BSA; 69 kDa and IGFs; 8 kDa) are indicated by arrows. Plasma was from rat (*a, f, k*), sheep (*b, g, l*), human (*c, h, m*), pig (*d, i, n*) and chicken (*e, j, o*).


150 69 8
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
Percentage of Total Eluted Radioactivity contained in Each Fraction
Elution Volume (ml)
0
10
20
30
6 8 10 12 14 16 18 20

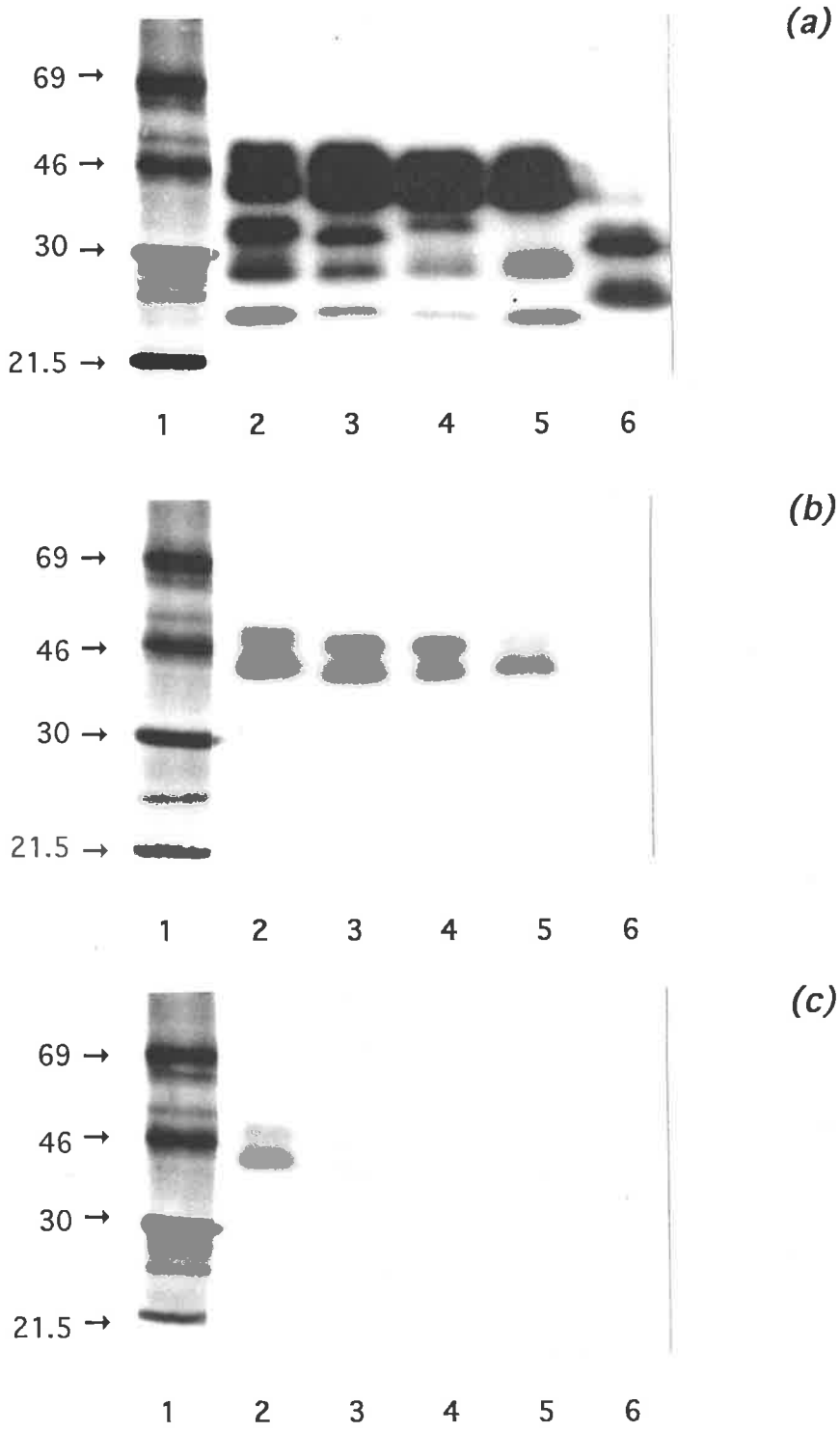
(Fig. 7.1*k-o* & Fig. 7.2*k-o*). No binding of this N-terminally extended IGF-I analog was evident in rat plasma (*k*) although that in the other species was measurable (*l, m, n, o*). Total binding of LR^{3}IGF-I ranged from about 10 to 30% in these species, with all ligand eluting at 30-50 kDa in human, pig and chicken plasmas, and significant association with the 130-150 kDa complex evident only in plasma from the lamb.

Specificity of radioligand binding was demonstrated in plasma from all species by paired incubations in which 0.8 μg unlabelled IGF-I was included with ^{125}I-labelled IGF-I, des(1-3)IGF-I or LR^{3}IGF-I (Fig. 7.1). The extent to which unlabelled IGF-I inhibited radioligand binding was dependent upon the labelled IGF peptide used, and also upon the species from which the plasma was obtained. The reduction in ^{125}I-IGF-I binding in the presence of unlabelled IGF-I was similar in all species (60-80%) except chicken in which it fell by only 30%. The extent to which binding of ^{125}I-des(1-3)IGF-I was inhibited was greater than that of labelled IGF-I in all species but the sheep (60% reduction), indicative of the greater relative affinity of IGFBPs from most species for IGF-I than for des(1-3)IGF-I. Addition of unlabelled IGF-I to incubations employing ^{125}I-LR^{3}IGF-I resulted in almost complete competition of radioligand binding in plasma from all species, so that the affinity of this radioligand for IGFBPs appeared to be poorest of the three.

7.3.2 Ligand Blots

Since the data shown in Figs. 7.1 and 7.2 indicated marked species differences in the binding of IGFs in native plasma, it was plausible that the IGFBPs themselves may have widely differing specificities for the radioligands. To reveal the IGFBP species contained in plasma and their relative affinities for IGF-I and the analogs, plasma from each species was ligand blotted using radiolabelled IGF-I, des(1-3)IGF-I and LR^{3}IGF-I (Fig. 7.3). The IGF-I ligand blot (Fig. 7.3*a*) demonstrated binding predominantly at a doublet in the 40-50 kDa region in plasma from the lamb, pig, human and rat. In chicken plasma, however, these bands were a relatively minor component of the total ligand-binding activity. Minor differences in molecular masses of doublet bands were apparent between species, such that

Fig. 7.3 *Ligand blots using (a)IGF-I, (b) des(1-3)IGF-I and (c) LR^{3}IGF-I as radioligand.* The lanes represent patterns obtained following electrophoresis of 1 µl sheep (*lane 2*), pig, (*lane 3*), human, (*lane 4*) or rat (*lane 5*) plasma and 4 µl of chicken plasma (*lane 6*). Molecular mass standards are shown in *lane 1* with their sizes (kDa) indicated at *left*. Blots were exposed to X-ray film for 14 days.


(a)
69
46
30
21.5
1 2 3 4 5 6
(b)
69
46
30
21.5
1 2 3 4 5 6
(c)
69
46
30
21.5
1 2 3 4 5 6

IGFBPs were of 48 and 43 kDa, 47 and 42 kDa, 46 and 41 kDa, 46 and 41 kDa, and 45 and 39 kDa in plasma from lamb, pig, human, rat and chicken, respectively. The lower molecular mass bands accounted for smaller proportions of the radioligand binding in all species except chicken, in which they predominated. The estimated sizes of these smaller IGFBPs also varied according to animal species: 35, 29 and 24 kDa in lamb, 33, 29 and 24 kDa in pig, 35, 29 and 24 kDa in human, 29 and 23 kDa in rat and 34, 32, 27 and 25 kDa in chicken.

The use of des(1-3)IGF-I in place of IGF-I for ligand blotting produced binding exclusively at the IGFBP-3 region bands but the intensities of the doublets were well below those observed with labelled IGF-I (Fig 7.3*b*). This was despite having used each radioligand in similar quantities and at similar specific activities, and with the same period of exposure to X-ray film, and implies that the IGFBP-3-sized proteins bear a lower affinity for des(1-3)IGF-I than for IGF-I. This poorer binding was evident in all species and particularly in chicken plasma where no bands were detected using des(1-3)IGF-I. Binding to mammalian plasma IGFBPs was similar for all species but the rat in which association of the analogs was particularly poor. The pattern of ligand binding obtained by blotting with LR^{3}IGF-I (Fig. 7.3*c*) was similar to that found with des(1-3)IGF-I except that the bands were even less intense and sheep plasma bound substantially more ligand than plasma from the other species. Again, no binding was detectable at this size in chicken plasma.

7.4 DISCUSSION

Two different approaches have been used here in order to compare the relative binding of radiolabelled IGF-I with that of two analogs, labelled des(1-3)IGF-I and LR^{3}IGF-I, to plasma IGFBPs of the rat, lamb, human, pig and chicken. One approach involved binding comparisons after the removal of endogenous ligand, as was the case in the ligand blots, and the other involved binding comparisons in native plasma that contained endogenous IGFs. This latter approach revealed that plasma from all five species exhibit

characteristic binding patterns. For example in rat plasma, the neutral size-exclusion chromatographic profiles show that binding sites are essentially unavailable for des(1-3)IGF-I or LR^{3}IGF-I. Surprisingly though, these results contrast with the data obtained by ligand blotting with the analogs, and are best explained by free IGFBP-3 not contributing significantly to the binding of radiolabelled IGFs in rat plasma. Nevertheless, incubation of labelled IGF-I with rat plasma results in an association predominantly at 130-150 kDa, which can only occur with the binding of an α-subunit to a binary complex of IGFBP-3 and IGF-I (Baxter *et al;* 1989). These two results seemingly can only be reconciled by involving unusual property differences in the relative capacities of the three IGFs to displace IGF-I from the IGFBP-3 complex, so that labelled IGF-I but not labelled des(1-3)IGF-I nor LR^{3}IGF-I may displace endogenous IGF-I from the complex. There is, however, no structural evidence for this proposal. It is also plausible that the virtual absence of IGF-II from plasma of adult rats (Glasscock *et al;* 1991) may contribute to the unusual binding properties of rat plasma compared with plasma from other species.

That a major portion of IGF-I binding occurred at 130-150 kDa in the other mammalian plasma samples was not surprising, since ligand blotting with IGF-I revealed that the high molecular mass IGFBP bands, characteristic in size of IGFBP-3 from the 130-150 kDa complex (Martin & Baxter; 1986, Walton *et al;* 1990, Walton *et al;* 1989*a*), were abundant in such plasma samples. The comparatively poor binding at 130-150 kDa in chicken plasma probably relates to the low abundance of the high molecular mass IGFBP subunits in plasma from this species, as evident from the ligand blot.

Substitution of radiolabelled des(1-3)IGF-I for IGF-I in the plasma incubations resulted in reduced binding at both IGFBP regions in plasma from all species but human, in which although overall binding of the analog was lower, binding of labelled des(1-3)IGF-I at 30-50 kDa was more than that of labelled IGF-I at the same position. Since the ligand blot data indicate that des(1-3)IGF-I only binds to IGFBP-3 in these plasma samples, association of des(1-3)IGF-I at 30-50 kDa is likely to be accounted for by IGFBP-3. However, the ligand blots show also that the analog binds more poorly than IGF-I to these IGFBPs. Since the ternary complex forms after association of IGF with the binding subunit (Baxter; 1988*a*),

the greater amount of des(1-3)IGF-I than of IGF-I at 30-50 kDa in human plasma can only be explained by a small decrease in net binding of IGF to IGFBP-3 resulting in a greater decrease in formation of the 130-150 kDa complex. The result of the substitution of IGF-I for des(1-3)IGF-I is a binary complex with a much lower affinity for the α-subunit. This is consistent with the greater proportion of radiolabelled des(1-3)IGF-I than of labelled IGF-I eluting at 30-50 kDa.

In view of the lack of detectable ligand-binding bands in chicken plasma when labelled des(1-3)IGF-I was used for blotting, the binding of this analog in native chicken plasma was surprising. However, given that plasma from the other species bound des(1-3)IGF-I at only the IGFBP-3-sized bands, it would appear likely that IGFBP-3 is responsible for this binding. The ligand blotting results do not exclude this possibility; blots of the mammalian plasma samples reveal that binding of des(1-3)IGF-I to IGFBP-3 is poor compared with that of IGF-I, so that a proportionally lower binding of des(1-3)IGF-I than of IGF-I to chicken IGFBP-3, which binds IGF-I only weakly, may simply be below the limit of detection by this method. The association of LR^3IGF-I that was apparent in chicken plasma, despite the failure to detect ligand-binding bands with this analog, is probably similarly accounted for by binding to IGFBP-3.

The data show that replacement of radiolabelled IGF-I with LR^3IGF-I in the *in vitro* incubations with mammalian plasma resulted in even lower radioligand binding than was observed using labelled des(1-3)IGF-I. This result was consistent with the generally poorer binding of LR^3IGF-I to IGFBP-3-sized proteins in the ligand blots and suggests that LR^3IGF-I has a lower affinity for IGFBP-3 than either IGF-I or des(1-3)IGF-I. The almost complete inhibition of labelled LR^3IGF-I binding but not of labelled IGF-I or des(1-3)IGF-I, by excess unlabelled IGF-I, is a further indication of the relatively poor affinity of LR^3IGF-I for IGFBPs. In comparisons between sheep, human and pig plasma samples, binding of this analog was very poor in the latter two species and highest in sheep, in accordance with its greater binding to IGFBP-3-sized proteins in sheep than in pig or human plasma. Nevertheless, the binding of LR^3IGF-I at 130-150 and 30-50 kDa regions in sheep plasma was lower, at each size than that of des(1-3)IGF-I in sheep plasma.

In conclusion, it is clear that substantial inter-species differences exist with respect to the binding of radiolabelled IGFs to IGFBPs. While binding of labelled des(1-3)IGF-I and LR^{3}IGF-I in ovine plasma was greater than in plasma from any other species, this was most starkly contrasted by their elution profiles in rat plasma. With regards to the vascular perfusion studies, it is unfortunate that the binding properties of the analogs in rat plasma were used as a basis for their use in perfusion studies with lambs, as binding in rat plasma is clearly unrepresentative of binding in ovine plasma. Nevertheless, these extensive differences were not expected. Since amino acid sequence data available at the beginning of the perfusion studies indicated a highly conserved structure for IGF-I in different species (Rinderknecht & Humbel; 1978*a*, Shimatsu & Rotwein; 1987, Dawe *et al;* 1988, Francis *et al;* 1989*a*, Francis *et al;* 1989*b*), the species specificities in the radioligand binding profiles are unlikely to reflect species differences in ligand structure and, therefore, presumably represent structural differences in IGFBPs between species These data point to the need for detailed structural studies on the interactions between IGF-I, IGF-I variants and the individual IGFBPs from each species.

7.5 SUMMARY

1) Marked species differences were demonstrated, notably that the N-terminally modified IGF-I variants which exhibited extremely weak binding in rat plasma, bound significantly in plasma from the other species.

2) Ligand blotting experiments with plasma from the five species showed a consistent pattern in that IGF-I binding was much greater than that of des(1-3)IGF-I, which in turn was greater than binding of LR^{3}IGF-I.

3) These experiments, firstly, that the IGF-binding properties of plasma following the removal of endogenous IGFs do not necessarily reflect the situation with untreated plasma. Secondly, the increased potencies of des(1-3)IGF-I and LR^{3}IGF-I in rat growth studies, that

have been ascribed to higher concentrations of these peptides in free form, cannot necessarily be extended to other species.

7.6 ACKNOWLEDGMENTS

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Chapter Eight

General Discussion and Future Directions

Chapter Eight: General Discussion

IGFs circulate in conjunction with a range of IGFBPs, mainly as a complex with IGFBP-3 and the α-subunit (Baxter & Martin; 1986, Baxter; 1988*a*). That binding proteins fulfil specific regulatory roles would appear certain, since a further five distinct IGFBP species, in addition to glycosylation and phosphorylation variants of some, have been identified (Shimasaki *et al;* 1991*a*, Shimasaki *et al;* 1991*b*, Frost & Tseng; 1991, Jones *et al;* 1991). However, these roles are not clearcut. Indeed, *in vitro* studies demonstrate that IGFBPs may both inhibit and enhance the effects of IGFBPs on IGF bioactivity (Ross *et al;* 1989, Koistinen *et al;* 1990, Feyen *et al;* 1991, Conover; 1992), while *in vivo* experiments demonstrate their capacities to prolong the circulating half lives of IGFs and to minimise their hypoglycaemic potential (Davis *et al;* 1989, Hodgkinson *et al;* 1989*a*, Lewitt *et al;* 1991). I chose to investigate the overall role of the circulating IGFBPs in regulating tissue-transfer of vascular IGFs by comparing transfer of IGFs that bound normally and poorly to IGFBPs. The hypothesis I addressed was that plasma IGFBPs served to inhibit IGF transfer to tissue.

Prior to investigating the tissue transfer of IGFs, per se, a preliminary study was undertaken to compare the IGFBPs in, and IGF-binding characteristics of, plasma with those of a range of extravascular fluids; IGF action at extravascular target sites will presumably be regulated by IGFBPs in the immediate vicinity of those sites. Thus plasma was compared with lymph from intestinal, prescapular and popliteal lymph nodes, and with CSF. A greater similarity of plasma to intestinal lymph than to popliteal or prescapular lymphs, or to CSF, was demonstrated by both ligand blotting and competitive binding experiments. The latter three fluids, especially CSF, contained greater proportions of IGF-II-preferring IGFBPs, with the relative abundances of the IGFBP species varying according to the fluid type. These results suggest that binding protein transfer from the vasculature, and/or binding protein synthesis, vary in a tissue-specific manner. Furthermore, the IGF regulatory environment of the vascular space appears to more closely reflect that of the intestinal interstitium than the extracellular spaces in other tissues.

For a model in which to test the hypothesis that IGFBPs generally serve to inhibit transfer to tissue, I developed an *in vivo* vascular perfusion technique in the ovine small intestine. This enabled tissue transfer of blood-borne IGFs to be studied under close to physiological conditions (Chapter **4**). The growth factors selected for these comparisons were IGF-I, in addition to two analogs, des(1-3)IGF-I and LR^3IGF-I. These analogs were chosen based upon their poor binding, compared with IGF-I, to IGFBPs in rat plasma (Bagley *et al;* 1991; Francis *et al;* 1992). Similar differences in binding between IGF-I and the analogs were anticipated in plasma from lambs but, as the size-exclusion chromatographic analyses of plasma from the perfusion studies revealed, the differences that were attained in this species were smaller than expected.

Notwithstanding the lower than expected proportions of uncomplexed analogs achieved in the vascular perfusion studies, the proportion of free radioligand in perfusions with labelled des(1-3)IGF-I was approximately 3 fold greater than that attained using IGF-I, in both the High Specific Activity and Low Specific Activity perfusion experiments (Chapters **4** & **5**). In perfusions using labelled LR^3IGF-I, free peptide was 3 fold higher and 8 fold higher in the High Specific Activity and the Low Specific Activity experiments, respectively, than attained using IGF-I. It follows that if tissue transfer of IGF was a function of the proportion of free ligand, differences in the percentage transfer of IGF-I and analogs would have been anticipated. Indeed, there was a trend towards greater transfer of labelled des(1-3)IGF-I than of labelled IGF-I in both experiments, consistent with an inhibitory role for IGFBPs in the regulation of IGF transfer. However, the absence of such a trend with respect to transfer of labelled LR^3IGF-I, particularly in the second vascular perfusion study where a much larger proportion of the labelled analog was free, suggests that IGF transfer may not simply be a function of the proportion of ligand that is free. Moreover, the lack of increased LR^3IGF-I transfer over that of IGF-I points to the involvement of receptor-mediated processes in IGF transfer, since in addition to binding IGFBPs poorly, LR^3IGF-I, unlike IGF-I and des(1-3)IGF-I, also binds less well to the type-I receptor (Bagley *et al;* 1991, Francis *et al;* 1992).

The distribution of IGF radioligand between the high and low molecular mass IGFBPs may be an important factor contributing to the regulation of IGF blood-tissue transfer, since the degree to which a circulating macromolecule is excluded from the extravascular space varies according to its molecular mass (Mayerson *et al;* 1960, Garlick & Renkin; 1970, Simionescu *et al;* 1972, Firrell *et al;* 1982). For example, in comparing the transfer from blood to lymph of ^{125}I-labelled dextran molecules of differing molecular radii, Simionescu *et al* (1972) showed that the lymph : plasma concentration ratio decreased with increasing molecular size of the vascularly infused dextran. This relationship appears also to be true for endogenous macromolecules, since Bill (1977) showed that the lymph : plasma concentration ratio for albumen (69 kDa) was closer to unity than was that for 150 kDa γ-globulin. The vascular wall therefore presents a size-selective barrier to tissue transfer of circulating macromolecules. Accordingly, transfer of IGFs within the 130-150 kDa complex would be lower than that of IGFs within the 30-50 kDa binary complexes. Since in the High Specific Activity vascular perfusions the binding of analogs in the 130-150 kDa peak was greater than that of labelled IGF-I, the lack of significant differences between transfer of IGF-I and analogs may be partly accounted for by greater size-related restriction to transfer of IGFBP-bound analogs than of IGFBP-bound IGF-I. The same reasoning cannot, however, be used to explain the lack of significant differences between transfer of IGF-I and analogs in the Low Specific Activity vascular perfusions, since a similar proportion of each labelled IGF species was bound at 130-150 kDa. The similarity in transfer of labelled IGF-I and analogs in that experiment could perhaps be accounted for by saturation of receptor mediated transfer, due to the higher concentrations of IGFs used in the perfusions.

The smaller proportion of IGFBP-bound IGFs eluting at 130-150 kDa in the Low Specific Activity than in the High Specific Activity perfusions is consistent with the results of the *in vitro* incubations (Fig. 5.1), in which a lower proportion of radioligand bound at this size in the presence of unlabelled ligand. Nevertheless, it was not anticipated that inclusion of unlabelled ligand would change the relative distribution of radioligand between 130-150 kDa and 30-50 kDa; proportional reductions in binding at both regions would be predicted if the unlabelled ligand competed for binding to IGFBPs in the exact same way as

its labelled homolog. The disproportionately large reduction in radioligand binding at 130-150 kDa occurring in the presence of unlabelled ligand, therefore, points to differences in the affinities of labelled and unlabelled IGFs for IGFBPs. Since the high molecular mass complex is composed of IGF, IGFBP-3 and α-subunit, these results indicate a higher affinity of the unlabelled IGFs than of the labelled IGFs, for IGFBP-3, or a higher affinity of the α-subunit for the non-labelled IGF-IGFBP-3 duplex than for the duplex with radiolabelled IGF-I. Others have previously described binding differences between labelled and unlabelled ligands, such that unlabelled IGF-I but not iodinated IGF-I is able to bind the IGFBP-3 purified from human pregnancy plasma (Suikkari & Baxter; 1991). Perhaps iodination of IGF results in structural modifications that alter its affinity for ovine IGFBPs.

The data obtained from the two vascular perfusion experiments would suggest that net transfer of IGFs to intestine was broadly concentration-dependent. Thus, the results show that transfer of radiolabelled IGF-I in the High Specific Activity and Low Specific Activity perfusions was 3.46% and 3.49%, respectively. Since the Low Specific Activity perfusate included an approximately 100 fold excess of unlabelled ligand, the total transfer of IGF in these perfusions must have been in the order of 100 fold greater than in the High Specific Activity experiment.

The similar percentage transfer of ^{125}I-IGFs when infused with or without a 100 fold excess of unlabelled peptide (Low Specific Activity and High Specific Activity perfusions, respectively) contrasts with the findings of Bar *et al* (1988), who described a reduction in transfer of radioiodinated IGF-II to heart tissue when unlabelled ligand was included in the perfusion medium. Specifically, radioligand transfer was reduced to 63% by inclusion of unlabelled ligand at 10 ng/ml and to 21% with unlabelled ligand at 500 ng/ml, compared with the transfer of radioligand infused in the absence of unlabelled ligand (1 ng/ml). Nevertheless, an important difference between that and the present study was the replacement of the coronary blood supply by a buffer solution; normal arterial blood supply was maintained in the intestinal perfusion model. Transfer of infused IGFs to cardiac tissue was, therefore, not subject to regulation by plasma IGFBPs as it was in the intestine. Since Bar *et al* (1988) showed that concentrations of unlabelled IGF as low as 10 ng/ml

competitively inhibited radioligand transfer in the heart perfusion model, the apparent lack of such inhibition in the intestine, using unlabelled ligand at concentrations as high as 100 ng/ml, would suggest that much lower proportions of radioligand were available for transfer when infused into blood than when infused in buffer alone. This is consistent with the results demonstrating binding of most infused radioligand to plasma IGFBPs and infers an inhibitory role for IGFBPs in the regulation of IGF transfer.

While the mechanisms by which vascular IGFs are transferred to the intestine have not been addressed directly, contributions by both specific and non-specific mechanisms are possible. Non-specific mechanisms may include simple diffusion and filtration through endothelial pores with transfer inversely proportional to molecular mass, as discussed above. Alternatively, IGFs may be transcytosed across capillary walls after binding to endothelial type-I receptors. Indeed, *in vitro* studies have demonstrated that endothelial cells endocytose receptor-bound radiolabelled IGFs and release them largely (80-90%) intact (Banskota *et al;* 1986). Another plausible mechanism of IGF transfer arising from receptor binding is transcytosis inside pinocytotic vesicles. Miyata *et al* (1988) demonstrated that both fluid phase endocytosis and exocytosis were stimulated as a result of ligand binding to the type-I receptor, suggesting a mechanism whereby IGF-I could stimulate the transfer of plasma proteins, including itself and IGFBPs, through the capillary wall. While this stimulation was apparent using epidermoid carcinoma KB cells, it remains to be determined whether IGF-I elicits a similar response from vascular endothelial cells.

As the vascular perfusion studies were not designed to identify the cellular distribution of IGF tracer within the intestinal tissue, no distinction was made between extracellular or intracellular radioactivity, nor between radioactivity outside the vascular space or that associated with the capillary endothelium. Nonetheless, the small proportion of transferred radioactivity that was recovered in lymph indicates that very little radioactivity was apportioned to the extravascular fluids. Most transferred IGF radioactivity was, therefore, likely to be inside cells and associated with receptors and/or IGF-binding proteins on their surfaces. Indeed, the type-I receptor is expressed throughout the gastrointestinal tract and particularly in the ileum (Heinz-Erian *et al;* 1991), the tissue used in the vascular perfusion

experiments. The possibility that a portion of the transferred IGF resided within intestinal cells cannot be excluded, since the largely intact state of radioligand in the intestinal tissue is consistent with the minor degradation, shown for IGF-I, following binding and internalisation via the type-I receptor (Banskota *et al;* 1986, Bar *et al;* 1988, Furlanetto; 1988). Nevertheless, much of the transferred radioactivity may have been associated with cell surface IGFBPs. Thus McCusker *et al* (1990) have shown that ^{125}I-(QAYL)-IGF-I, an IGF-I analog that binds IGFBPs very poorly but binds the type-I receptor with a similar efficiency to IGF-I, binds to fibroblast cell surfaces only 20% as well as IGF-I.

Although the differential between TCA-precipitability of infused radioligand and that recovered in the intestinal tissue suggests the partial degradation of radioligand by the intestine, this difference may also arise from a greater rate of transfer for TCA-soluble infusate-components than for the insoluble components. Due to its smaller size, TCA-soluble material such as iodide would be expected to equilibrate with the extravascular space more rapidly than the larger TCA-insoluble IGFs, according to the inverse relationship between molecular mass and transfer (Mayerson *et al;* 1960, Garlick & Renkin; 1970, Simionescu *et al;* 1972, Firrell *et al;* 1982).

The follow-up studies presented in this thesis were designed to determine the reasons for the lower than expected differences in binding of IGF-I and the analogs to ovine plasma IGFBPs. In comparisons between IGF-I and analog binding in rat and lamb plasma (Chapter 7), IGF-I was shown to bind well in plasma from both species but the binding of analogs in ovine plasma was much greater than in rat plasma. Significant binding of des(1-3)IGF-I and LR^{3}IGF-I also occurred in plasma from pigs, chickens and man, such that the poor binding in rat plasma appeared not to be generally applicable to all species. Hence, the greater potencies of des(1-3)IGF-I and LR^{3}IGF-I in rats, apparently due to increased proportions of ligand in the free state, may not necessarily be extended to other species.

While species differences contributed to the surprisingly extensive binding of analogs in the vascular perfusion experiments, unexpected effects associated with halothane anaesthesia also contributed to reduced binding differences between IGF-I and the analogs.

Indeed, halothane anaesthesia was found to cause an increase in the number of unoccupied IGF-binding sites in plasma, as the combined result of reduced endogenous IGF concentrations and an increase in immunoreactive-IGFBP-1 (Chapter **6**). The decrease in IGF concentrations may have contributed free binding sites on IGFBP-3 because most IGF circulates in a complex with IGFBP-3 and the α-subunit (Baxter & Martin; 1986, Baxter; 1988*a*). Since the data demonstrate that immunoreactive IGFBP-3 binds both des(1-3)IGF-I and LR^3IGF-I (Chapters **6** & **7**), it follows that the decrease in endogenous circulating IGFs would have contributed directly to the greater binding of either analog in the plasma of anaesthetised lambs. The results of Chapter **6** show, furthermore, that the anaesthesia-induced immunoreactive-IGFBP-1 was able to bind labelled des(1-3)IGF-I. The greater binding of des(1-3)IGF-I in plasma from anaesthetised lambs may, therefore, be partly explained by the increase in concentration of this IGFBP. Whether or not immunoreactive-IGFBP-1 also contributed to the extensive binding of LR^3IGF-I in the plasma of vascularly perfused lambs was not pursued in these studies, but could perhaps be determined by ligand blotting of plasma collected during anaesthesia.

Future transfer studies employing the vascularly perfused small intestine could include several modifications so that additional data can be obtained from the system. For example, if the amount of perfused radioactivity was increased substantially over the amounts used here, it is likely that TCA-precipitability measurements of the intestinal lymph would be feasible. Comparison of the value for lymph with that for the homogenised intestine may reveal any compartmentalisation of intact and degraded IGFs in/on cells or in the fluids bathing them. An increase in the amount of radioactivity used for perfusion would facilitate autoradiographic studies on intestinal tissue sections; such studies would enable the cellular distribution of transferred radioligand to be described. Nevertheless, in order to further pursue the overall role of plasma IGFBPs in regulating the tissue transfer of IGF, use of the vascular perfused intestine in the lamb would be contingent upon the design of novel IGF-I analogs with very poor or no affinity for ovine IGFBPs or, using the IGFs employed in this Thesis, modification of infusion conditions to produce greatly increased proportions of free labelled des(1-3)IGF-I and LR^3IGF-I but not of labelled IGF-I. Selection of IGF peptide

concentrations for vascular perfusion experiments in anaesthetised animals could perhaps be made after performing *in vitro* incubations in plasma collected at a similar time, following commencement of anaesthesia, as that of the perfusion experiment itself. Alternatively, the vascular perfusion model could be adapted by replacement of the arterial blood supply with a Krebs-Henselite oxygenated buffer. While such a modification may not be appropriate for determining the general role of circulating IGFBPs in regulating IGF transfer, it would enable comparisons to be made between transfer of free IGF-I and that of ligand complexed to specific IGFBPs. Indeed, ovine IGFBP -2, -3 and -4, in addition to the α-subunit, have been purified in recent years so that such experiments in sheep may now be feasible (Walton *et al;* 1990, Hodgkinson *et al;* 1993).

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