# STUDIES OF COMPLEXES FORMED IN BLOOD *IN VIVO* BETWEEN AN INSULIN-LIKE GROWTH FACTOR ANALOG AND BINDING PROTEINS

by

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# ABSTRACT

Insulin-like growth factor-I (IGF-I) is a polypeptide growth factor with an insulin-like chemical structure and biological properties. IGF-I stimulates proliferation and differentiation of many cultured cells and also regulates prenatal and postnatal growth of animals. LR$^3$IGF-I is a synthetic analog of IGF-I that is biologically active *in vitro* and *in vivo*. Compared to IGF-I, LR$^3$IGF-I has much lower affinity for IGF-binding proteins and slightly lower affinity for the type-I IGF receptor. Treatment with LR$^3$IGF-I promotes liveweight gain in rats but not in non-rodents, e.g. pigs and sheep. Lord *et al.* (1994) showed that although [$^{125}$I]-LR$^3$IGF-I exhibits extremely weak binding to binding proteins in rat plasma, it binds significantly to proteins in plasma from sheep, pigs, humans and chickens. The *in vivo* formation of complexes between the different IGF-binding proteins and IGFs and the physiological functions of different types of complexes in blood are not well understood.

The aims of this study were to examine the molecular forms of circulating LR$^3$IGF-I during treatment of animals with biologically active doses of this analog and to identify factors which affect the *in vivo* formation of circulating complexes between LR$^3$IGF-I and IGF-binding protein(s).

In order to detect LR$^3$IGF-I in specimens obtained from animals treated with this analog, a simple assay for LR$^3$IGF-I was developed. Mouse monoclonal antibodies and rabbit polyclonal antisera against LR$^3$IGF-I were produced, their binding affinities and specificities were characterised and their binding complementarities were evaluated. Two antibodies were found to be suitable as the basis of an ELISA. The ELISA system was able to detect as little as 50 pg of LR$^3$IGF-I and the native peptides IGF-I and IGF-II have less than 0.01% cross

reactivity. Due to the high sensitivity of the ELISA, blood plasma from animals treated with pharmacologically active doses of this growth factor analog could be diluted between 20- and 100-fold before assay, at which concentrations plasma had no effect on the assay response to $LR^3IGF$-I. There was a positive correlation between results obtained by $LR^3IGF$-I ELISA of unextracted plasma and those obtained by $LR^3IGF$-I RIA. The advantages of the $LR^3IGF$-I ELISA compared to the $LR^3IGF$-I RIA include improved sensitivity, elimination of the need for radioiodination of $LR^3IGF$-I and elimination of the requirement for extraction of plasma before assay.

The molecular size classes of $LR^3IGF$-I in blood from animals treated with biologically active doses of this IGF analog were characterised by size exclusion high performance liquid chromatography of plasma at pH 7.4. Assays of $LR^3IGF$-I in the eluent by both ELISA and RIA showed that $LR^3IGF$-I was present in free (unbound) form in plasma from fasted cows intravenously infused with $LR^3IGF$-I for 7 hours at 12 $\mu g.kg^{-1}.h^{-1}$. This $LR^3IGF$-I treatment reduced blood levels of glucose and amino-acids in these heifers (Owens *et al.* 1994). However, in plasma from well-fed guinea pigs infused subcutaneously with this analog for 7 days at 14 $\mu g.kg^{-1}.h^{-1}$, the $LR^3IGF$-I activity detected by both RIA and ELISA was present in high molecular weight forms. $LR^3IGF$-I was also biologically active in this study, because treatment increased growth of several organs in these guinea pigs (Conlon *et al.* 1995*b*). In both of these *in vivo* experiments, treatment with $LR^3IGF$-I also reduced blood levels of IGF-I and IGF-II. *In vitro* addition of IGF-I and IGF-II to plasma from guinea pigs treated with $LR^3IGF$-I converted the high molecular weight $LR^3IGF$-I activity into the free form whereas addition of insulin did not. Therefore $LR^3IGF$-I was associated with IGF-binding protein(s) in blood during treatment of guinea pigs. The column fractions that contained the high molecular weight form of $LR^3IGF$-I also contained IGF-binding proteins of approximately

48, 43, 31 and 25 kDa. Complexes between LR$^3$IGF-I and guinea pig IGF-binding proteins could not be generated by *in vitro* addition of LR$^3$IGF-I to blood plasma from normal untreated guinea pigs. The mechanism responsible for the formation of IGF-binding protein/LR$^3$IGF-I complexes in guinea pigs is not clear.

Age and nutrition, which regulate the concentrations in blood of insulin-like growth factors and their binding proteins, were evaluated as determinants of the *in vivo* formation of circulating complexes between LR$^3$IGF-I and IGF-binding proteins. Male hooded Wistar rats aged either 5 weeks or 12 weeks were fed for 7 days either *ad libitum* or at one of three restricted rates, reduced either by 22%, by 44% or by 67%, compared to the *ad libitum* rate of feed intake. Rats were infused throughout this period either with LR$^3$IGF-I, at an average rate of 38 $\mu g.kg^{-1}.h^{-1}$, or with placebo (vehicle).

Undernutrition progressively decreased plasma concentrations of rat IGF-I in vehicle treated younger rats. Reduction of feed intake to 67% less than *ad libitum* in younger rats decreased plasma concentrations of IGF-binding proteins of ~40-50 kDa and ~24 kDa but increased concentrations of ~29 kDa IGF-binding protein. Reduction of feed intake to 44% less than *ad libitum* in younger rats decreased plasma concentrations of IGFBP of ~40-50 kDa but had no effect on other binding proteins. Treatment with LR$^3$IGF-I further reduced concentrations of rat IGF-I in plasma of undernourished younger rats by ~40-50% compared to vehicle treated animals. Treatment with LR$^3$IGF-I increased the concentrations of ~29 kDa IGF-binding protein in all feed-restricted groups of younger rats but not in *ad libitum* fed rats.

In older rats, only extreme undernutrition (reducing feed intake to 67% less than *ad libitum*) significantly decreased plasma levels of rat IGF-I in vehicle treated animals. Food

intake had no significant effect on plasma concentrations of IGF-binding proteins in older rats. Treatment with LR$^3$IGF-I did not affect plasma concentrations of rat IGF-I and IGF-binding proteins at any level of feed intake in older rats.

The concentrations of LR$^3$IGF-I in plasma after 7 days treatment were considerably lower than those of rat IGF-I in all nutrition groups at both ages. Plasma LR$^3$IGF-I was affected by the level of feed in younger but not older rats.

Size exclusion chromatography of plasma under non-dissociating conditions followed by LR$^3$IGF-I ELISA of the eluted fractions showed that all of the circulating LR$^3$IGF-I was in the free form in all groups of older rats and in the better fed groups of younger rats. However, about 30% of the LR$^3$IGF-I in blood plasma from the 44% underfed younger rats and ~100% in 67% underfed younger animals was present in plasma in a high molecular weight form(s). The high molecular weight form(s) of LR$^3$IGF-I in LR$^3$IGF-I treated 67% underfed young rats could be converted to free LR$^3$IGF-I by acidification to pH 2.5 indicating that LR$^3$IGF-I in rat plasma is associated with an IGF-binding protein under certain conditions. Moreover, fractions containing high molecular weight form(s) of LR$^3$IGF-I also contained ~29 kDa IGFBP.

Complex formation between LR$^3$IGF-I and a ~29 kDa IGFBP in undernourished young rats (44% and 67% underfed) may be promoted by the very low plasma concentrations of rat IGF-I and the high concentrations of ~29 kDa IGFBP which may result in high levels of unsaturated ~29 kDa IGFBP *in vivo*. Indirect evidence indicates that LR$^3$IGF-I forms complexes with a ~29 kDa IGF-binding protein and that this protein is IGFBP-1.

There were positive and negative associations between plasma concentrations of free $LR^3IGF$-I with several organ weights in young rats that were consistently stronger than those seen with total $LR^3IGF$-I levels. The association between levels of free $LR^3IGF$-I and kidney weight is an example of a negative association whereas the association between levels of free $LR^3IGF$-I and thymus weight is an example of a positive association. In all groups of animals in which circulating $LR^3IGF$-I was exclusively in the free form, treatment with this IGF-I analog increased thymus weight. In contrast, free $LR^3IGF$-I was associated with reduced growth response of the kidney.

In conclusion, this study shows that when $LR^3IGF$-I is administered to animals in pharmacologically active doses, it may be present in either the free form or bound to IGF-binding protein(s) in the circulation. Age and nutrition which are factors that regulate synthesis of endogenous IGF-I and IGF-binding proteins, affect the *in vivo* formation of complexes between the analog and IGFBP(s). This study also suggests that IGFBP-1 inhibits the pharmacological activity of circulating $LR^3IGF$-I on thymus whereas it appears to stimulate the pharmacological activity of $LR^3IGF$-I in kidneys.

## STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution. To the best of my knowledge and belief it contains no material that has previously been published or written by any other person, except where appropriate reference has been made in the text. I consent to this thesis being made available for photocopying and loan.

Oraprapai Gajanandana

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## PUBLICATION ARISING FROM THIS THESIS RESEARCH

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## ABSTRACTS OF PAPERS PRESENTED AT SCIENTIFIC MEETINGS

Gajanandana O, Francis GL, Wallace JC & Owens PC 1994 Characterization of mouse monoclonal antibodies to LR$^3$IGF-I. *2nd Annual International Scientific Meeting of Diagnostic System Laboratories, Inc.*, abstract 13.

Gajanandana O, Francis GL, Wallace JC & Owens PC 1995 An enzyme-linked immunosorbent assay (ELISA) of an analog of insulin-like growth factor I. *Proceedings of the Australian Society for Medical Research (South Australian Division)*, abstract 39p.

Gajanandana O, Conlon MA, Wallace JC & Owens PC 1995 Concentrations and molecular forms of an analog of insulin-like growth factor I (IGF-I) in blood plasma. *Endocrine Society of Australia, Proceedings 1995*, abstract 33.

Gajanandana O, Wallace JC & Owens PC 1995 Measurement of an analog of insulin-like growth factor-I in either free form or bound to IGF-binding protein in blood plasma using a new sandwich ELISA. *Australian Society for Medical Research, National Scientific Meeting, Proceedings 1995*, abstract P-77.

Gajanandana O, Wallace JC, Tomas FM, Quinn KJ & Owens PC 1997 Age and nutrition affect in vivo formation of complexes between an insulin-like growth factor and a binding protein. *Endocrine Society of Australia, Proceedings 1997*, abstract 103.

# ABBREVIATIONS

| | |
|---|---|
| A | absorbance |
| ANOVA | analysis of variance |
| Arg | Arginine |
| BSA | bovine serum albumin |
| cpm | counts per minute |
| CSIRO | Commonwealth Scientific and Industrial Research Organisation |
| CV | co-efficient of variation |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked immunosorbent assay |
| FCS | fetal calf serum |
| GH | growth hormone |
| g | gram |
| x g | times the force of gravity |
| hplc | high performance liquid chromatography |
| h | hour |
| Ig | immunoglobulin |
| IGF | insulin-like growth factor |
| IGF-I | insulin-like growth factor-I |
| IGF-II | insulin-like growth factor-II |
| IGFBP | insulin-like growth factor binding protein |
| i.v. | intravenously |
| kDa | kilodalton |

| | |
|---|---|
| l | litre |
| $LR^3IGF\text{-}I$ | long $R^3$ insulin-like growth factor-I |
| mg | milligram |
| min | minute |
| ml | millilitre |
| mRNA | messenger ribonucleic acid. |
| nm | nanometre |
| °C | degree Celsius |
| *P* | probability |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| $R^3$ | Arginine at position 3 |
| RGD | Arginine-Glycine-Aspartate |
| rh | recombinant human |
| RIA | radioimmunoassay |
| s.c. | subcutaneously |
| SDS | sodium dodecyl sulphate |
| sem | standard error of the mean |
| µg | microgram |
| µl | microlitre |
| µm | micrometre |
| v/v | volume per volume |
| w/v | weight per volume |

# CHAPTER 1

# General Introduction

## 1.1 INSULIN-LIKE GROWTH FACTORS

### 1.1.1 General Introduction

Insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are homologous single chain polypeptides of approximately 7.5 kDa molecular mass that have insulin-like structures and biological properties. They stimulate proliferation and differentiation of many cultured cells and are important regulators of prenatal and postnatal growth of mammals. IGFs are most commonly found *in vivo* associated with soluble binding proteins in non-covalent complexes. The IGF-binding proteins (IGFBPs) can exert both stimulatory and inhibitory effects on the *in vitro* action of IGFs. In adults, liver appears to be the primary source of IGF-I circulating in blood which represents a major pool of potential insulin-like and mitogenic activity. Circulating IGF-I distributes throughout the body to multiple tissue sites of action. In addition to endocrine functions, IGFs are synthesized by some cells in almost all tissues and thus may produce effects on neighbouring cells by paracrine mechanisms or on the source cells through autocrine mechanisms.

The studies described in this thesis are an investigation of factors that affect formation of complexes between insulin-like growth factors and their binding proteins in blood *in vivo*.

### 1.1.2 Historical background

The IGFs were first identified as three different *in vitro* biological activities in blood serum: "sulphation factor activity" for cartilage, "nonsuppressible insulin-like activity" (NSILA) for adipose tissue explants and "multiplication-stimulating activity" (MSA) for cultured chick embryo fibroblasts.

#### 1.1.2.1 SULPHATION FACTOR

Incorporation of [$^{35}$S]-sulphate into chondroitin sulphate by cartilage *in vitro* is markedly suppressed in rats that have been made deficient in growth hormone (GH) by hypophysectomy and can be restored to normal by giving GH *in vivo*. However, Salmon and Daughaday (1957) discovered that the incorporation of [$^{35}$S]-sulphate into cartilage *in vitro* could not be improved by addition of GH to media containing cartilage segments. Nevertheless, addition of serum from hypophysectomised rats treated with GH had greater ability than serum from untreated hypophysectomised rats to stimulate chondroitin sulphate synthesis by cartilage *in vitro*, whereas addition of GH to serum from hypophysectomised rats had no effect. This lead to the concept that the action of growth hormone (also known as somatotropin) on the skeletal growth of animals required production of an intermediary substance in circulating blood. This material was originally named sulfation factor and later somatomedin (Daughaday *et al.* 1972).

### 1.1.2.2 NONSUPPRESSIBLE INSULIN-LIKE ACTIVITY (NSILA)

Subsequent to the development of the first antibodies to insulin, it was observed that only a small fraction of the bioactive insulin in serum and plasma was due to insulin itself. The concentration of insulin in serum and plasma as determined by specific radioimmunoassay was found to be only one-tenth that determined by *in vitro* bioassays. Also, there were substances in blood similar to insulin but their insulin-like activity *in vitro* could not be suppressed by antibodies specific to insulin. These substances were known as nonsuppressible insulin-like activity (Froesch *et al.* 1963).

### 1.1.2.3 MULTIPLICATION-STIMULATING ACTIVITY (MSA)

Pierson & Temin (1972) purified a peptide from calf serum which stimulated the replication of fibroblasts *in vitro*. Dulak & Temin (1973) identified this substance in conditioned medium of a rat hepatocyte cell line and termed it multiplication-stimulating activity (MSA).

### 1.1.2.4 INSULIN-LIKE GROWTH FACTOR-I AND -II

Rinderknecht and Humbel (1978*a*, 1978*b*) isolated and sequenced two forms of NSILA and showed that these proteins were closely related to each other and to pro-insulin in structure. They named them insulin-like growth factors I and II (IGF-I and IGF-II). Purification and structural determination has revealed that somatomedin-C, somatomedin-A and IGF-I are the same peptide (Klapper *et al.* 1983, Enberg *et al.* 1984) while MSA is rat IGF-II (Marquardt *et al.* 1981).

### 1.1.3 IGF protein and gene structure

The complete amino-acid sequences of IGF-I and IGF-II were first described for the human IGFs (Rinderknecht & Humbel 1978*a*, 1978*b*). IGF-I and IGF-II are single chain polypeptides of 70 and 67 amino acids and have molecular weights of 7649 and 7471, respectively. These peptides belong to the insulin family which includes insulin and relaxin (Blundell and Humbel 1980). IGF-I and IGF-II have over 60% identity to each other and also share 47-49% homology with the A and B chains of insulin. Their domains which correspond to the A and B chains of insulin are joined by a connecting peptide corresponding to the proinsulin C-peptide. Unlike the mature bioactive form of insulin, the C-peptide of the IGFs is not cleaved away. The IGFs also contain a D-region extension peptide at the carboxy terminus that is not found in insulin. The precursor forms of IGF-I and IGF-II contain signal peptides of about 25 amino acids at the amino-terminus of the B-region and a further extension at the carboxy terminus known as the E domain (reviewed by Baxter 1988). IGFs are highly conserved across animal species. Bovine (Honegger & Humbel 1986), porcine (Francis *et al.* 1989*b*), human (Rinderknecht & Humbel 1978*a*) and guinea pig IGF-I (Bell *et al.* 1990) are identical, whereas ovine (Francis *et al.* 1989*a*), rat (Shimatsu & Rotwein 1987) and mouse IGF-I (Bell *et al.* 1986) differ from human by one, three and four amino acid residues, respectively. Porcine (Francis *et al.* 1989*b*), bovine (Francis *et al.* 1988*b*), ovine IGF-II (Francis *et al.* 1989*a*) differ from human IGF-II by one, three and four amino acids, respectively. Rat and mouse IGF-II differ by two amino acids, and they have four and six amino acid residue differences, respectively, compared with human IGF-II (Soares *et al.* 1986, Stempien *et al.* 1986).

Each IGF is the product of separate single, large complex genes of ~95 kb and 35 kb for IGF-I and IGF-II, respectively. The human IGF-I gene is on the long arm of chromosome 12 and is composed of six exons, the last two of which are used alternatively to derive different precursors (IGF-IA and IGF-IB). Transcription of the IGF-I gene results in multiple messenger RNAs ranging in size from ~1 to 7.5 kb. The lengths of the 3′ untranslated ends of the transcripts account for most of the size variation. Control of the processing of these mRNAs and their translation are significant steps in the regulation of their expression. The human IGF-II gene is on the the short arm of chromosome 11 immediately downstream of the insulin gene. It also is composed of multiple exons, and its transcription results in mRNAs of multiple sizes that are due to alternate exon usage, including 5′ untranslated exons and 3′ untranslated regions of varying sizes. IGF genes do not contain classical 5′ promotors, enhancers, and regulatory elements (reviewed by D′Ercole 1996).

### 1.1.4 IGF receptors

IGFs bind to three types of cell membrane receptors, namely the type 1 IGF receptor, the type 2 IGF receptor and the insulin receptor. The type 1 IGF receptor and the insulin receptor are homologous membrane glycoproteins of apparent molecular weight of 300-350 kDa, belonging to the family of tyrosine kinase receptors (Ullrich *et ai.* 1986). They have similar heterotetrameric structures consisting of two extracellular ligand-binding alpha subunits, linked by disulphide bonds to the extra-cellular domain of the transmembrane beta subunit. The cytoplasmic portion of the β-subunit contains the tyrosine kinase domain. The signalling pathways for type 1 IGF receptors are complex involving activation of inherent tyrosine kinase activity of the receptor, that leads to phosphorylation of other important tyrosines on the receptor as well as on other intracellular substrates (reviewed by Jones and

Clemmons 1995, LeRoith 1996). This activates a cascade of protein kinases leading to mitogenic and metabolic responses. The type 1 IGF receptor appears to mediate most of the biological actions of IGF-I and IGF-II and is present in a wide variety of tissues and cell lines (Cohick & Clemmons 1993). The type 1 IGF receptor binds both IGF-I and IGF-II with high affinity. Insulin binds to the type 1 IGF receptor with 100- to 500- fold lower affinity than the IGFs (Cohick & Clemmons 1993). Insulin receptors demonstrate similar high affinity for insulin and 100 fold lower affinity for IGF-I (Jones & Clemmons 1995).

Type 1 IGF receptors have been found in liver, brain, stomach, muscle, heart, kidney, lung (Werner *et al.* 1989), thymus (Verland & Gammeltoft 1989) in rats. Werner *et al.* (1989) also reported a decline in IGF-I receptor mRNA levels in liver, brain, stomach, muscle, heart, kidney and lung during postnatal development but the extent of the developmental decrease differed among the tissues studied. Lowe *et al.* (1989) reported that fasting increased specific [$^{125}$I]-IGF-I binding in lung, testes, stomach, kidney and heart but not in brain.

The type 2 IGF receptor is structurally distinct from the type 1 and insulin receptors, being a 260 kDa monomer. This receptor is identical to the cation-independent, mannose-6-phosphate receptor, a protein involved in the intracellular targetting of lysosomal enzymes (Morgan *et al.* 1987, MacDonald *et al.* 1988). The IGF-II/M-6-P receptor consists of 15 contiguous extracellular repeating domains. The cytoplasmic domain is relatively short and does not contain tyrosine kinase activity (reviewed by LeRoith 1996). Cell surface IGF-II/M-6-P receptors bind IGF-II with high affinity (reported $K_d$ range 0.017-0.7 nmol/l). Affinity for IGF-I is more than 500-fold lower and the receptor does not significantly bind insulin (reviewed by Jones & Clemmons 1995). The IGF-II/M-6-P receptor binds M-6-P residues in

the carbohydrate portion of lysosomal enzymes and participates in the targetting of enzymes to lysosomes, either from the trans-Golgi or from the surface (reviewed by Nissley & Lopaczynski 1991, Jones & Clemmons 1995). A range of other mannose-6-phosphate glycoproteins are recognized by these receptors including renin, proliferin, thyroglobulin and transforming growth factor-β (Jones & Clemmons 1995). It is not clear which, if any, of the biological actions of IGF-II are mediated by this receptor. There are studies suggesting that some of the biological actions of IGF-II are mediated by this receptor, including stimulation of $Ca^{++}$ influx in BALB/c 3T3 cells (Nishimoto *et al.* 1987), activation of G proteins (Murayama *et al.* 1990) and migration of rhabdomyosarcoma *in vitro* (Minniti *et al.* 1992). On the other hand, antibodies directed against the IGF-II/M-6-P receptor are reported to have no effect on biological responses to IGF-II in H-35 hepatoma cells, normal hepatocytes, L6 myoblasts, and ovarian granulosa cells (reviewed by Jones & Clemmons 1995).

### 1.1.5 Regulation of IGFs

The major regulatory influences on IGF-I synthesis and secretion are growth hormone, nutrition and developmental status. From birth the concentrations of IGF-I in blood progressively increase during childhood, rise more rapidly during pubertal growth and thereafter decrease slightly throughout adulthood. In rats, hepatic expression of IGF-I increases progressively with increasing postnatal age (Lund *et al.* 1986), while expression in lung, muscle and stomach decreases postnatally (Adamo *et al.* 1989). In the fetal rat, IGF-II mRNA is abundant in liver and many other tissues but decreases markedly at birth in all tissues except brain (reviewed by Cohick & Clemmons 1993). The high expression of IGF-II mRNA in the fetus compared with adult suggests a role in fetal growth (Lund *et al.* 1986). The decrease in IGF-II mRNA levels corresponds to the decrease in circulating levels of

IGF-II postnatally. Although the circulating IGF-II levels are low to undetectable in the adult rat, they are very high in adult human (Baxter 1988).

Growth hormone enhances IGF-I gene transcription and increases IGF-I mRNA abundance in most rodent tissues (reviewed by Daughaday & Rotwein 1989). In some IGF-responsive tissues such as epiphyseal growth plate, ovary and kidney, local IGF-I synthesis is partly regulated by GH (Jones and Clemmons 1995). However, the expression of IGF-I in the uterus where estrogen appears to control its synthesis, is independent of GH (Murphy *et al.* 1987). Hepatic IGF-I mRNA and blood IGF-I are decreased in severe GH deficiency and both are elevated in states of GH excess (D'Ercole 1996). Regulation of IGF-II expression is GH-independent (Jones and Clemmons 1995). IGF-I and IGF-II gene expression are independent of GH during fetal development (Jones & Clemmons 1995).

Nutrition is an important regulator of blood levels of IGF-I (Clemmons & Underwood 1991, Thissen *et al.* 1994). Plasma IGF-I peptide concentrations and hepatic IGF-I mRNA abundance are decreased by restriction of dietary energy or protein in rats (Takahashi *et al.* 1990, Thissen *et al.* 1991, VandeHaar *et al.* 1991, Miura *et al.* 1992). Treatment with GH does not normalize the attenuation of growth or restore IGF-I expression during undernutrition (Clemmons & Underwood 1991).

Infusion of IGF-I into protein-restricted rats fails to normalize tail length, weight or tibial epiphyseal width despite normalization of serum IGF-I levels and enhancement of spleen and kidney growth (Thissen *et al.* 1991). Nutritional deprivation decreases hepatic IGF-I production by diminishing IGF-I gene expression and also increases the clearance and

degradation of serum IGF-I through changes in the levels of circulating IGFBPs (Thissen *et al.* 1994).

IGFs are also regulated by other factors, generally those that are trophic for the tissue concerned. For example, follicle-stimulating hormone (FSH) increases IGF-I mRNA in ovarian granulosa and testicular Sertoli cells, parathyroid hormone (PTH) does the same in cartilage, thyroid-stimulating hormone (TSH) in the thyroid gland and estrogens in the uterus (D'Ercole 1996). Thyroid hormones potentiate the effect of growth hormone in inducing IGF-I mRNA in liver (Wolf *et al.* 1989, Tollet *et al.* 1990). Parathyroid hormone increases IGF-I mRNA and IGF-I secretion in osteoblast-enriched cultures from fetal rat bone (McCarthy *et al.* 1989). Epidermal growth factor also stimulates IGF-I expression in isolated rat renal collecting ducts (Rogers *et al.* 1991). Treatment with estradiol enhances IGF-I mRNA abundance in the uterus whereas chronic estrogen treatment inhibits GH-induced hepatic IGF-I mRNA and serum levels (Murphy & Friesen 1988). Cortisol and other glucocorticoids decrease hepatic expression of IGF-I (reviewed by Rotwein 1991). The other important regulators of IGFs are IGF-binding proteins which affect the availability of IGFs to their receptors and thus modulate the biological actions of IGFs (see section 1.2).

### 1.1.6 Biological actions of IGFs

#### 1.1.6.1 IGF ACTION *IN VITRO*

In general, the effects of the IGFs *in vitro* include acute effects on protein and carbohydrate metabolism and longer term effects on cell replication and differentiation. The IGFs exert classical insulin effects *in vitro* on all target tissues of insulin although at

significantly higher concentrations than insulin. These include stimulation of glucose and amino acid uptake, stimulation of lipid, glycogen and protein synthesis and inhibition of lipolysis (Froesch *et al.* 1985). In addition to their weak insulin-like actions, IGFs also stimulate the synthesis of collagen and proteoglycan in endothelial cells, fibroblasts and chondrocytes (D'Ercole 1996). The IGFs have also been shown to be growth promoting factors for many different cultured cells *in vitro*, stimulating mitosis and differentiation, biosynthesis of DNA, RNA and protein and inhibiting protein breakdown (Jones & Clemmons 1995). For example, IGFs increase protein synthesis and decrease protein breakdown in rat myoblasts (Ballard *et al.* 1986). IGFs have been shown to induce proliferation of many cell types *in vitro* including fibroblasts, smooth and skeletal muscle cells, keratinocytes, neuronal cells, chondrocytes, osteoblasts, hematopoietic cells, a variety of epithelial cells including mammary, thymic and bronchial and numerous cancer cell lines (D'Ercole 1996). IGF-I is usually more potent than IGF-II in enhancing cell proliferation. Studies in Balb/c 3T3 fibroblasts showed that other growth factors such as fibroblast growth factor and platelet-derived growth factor make these cells competent to respond to IGFs. When these cells are competent to respond to IGF in G1 of the cell cycle, IGF stimulates their progression into S phase (reviewed by Jones & Clemmons 1995). IGFs have been shown to stimulate myoblast differentiation, neuritic outgrowth in cultured neurons and induce cartilage, osteoblast and adipocyte replication (reviewed by D'Ercole 1996). The effects of the IGFs on the pituitary illustrate the roles of IGF-I and IGF-II in inhibiting growth hormone synthesis and secretion as a component of a negative feedback mechanism (Morita *et al.* 1987).

### 1.1.6.2 IGF ACTION *IN VIVO*

Administration of IGF-I into animals causes hypoglycaemia (Zapf *et al.* 1986, Jacob *et al.* 1989, Douglas *et al.* 1991). IGF-I stimulates the growth of hypophysectomized rats (Schoenle *et al.* 1982) and partially protects against the growth retardation and nitrogen losses associated with streptozotocin-induced diabetes (Tomas *et al.* 1991*a*), dietary protein deficiency (Tomas *et al.* 1991*b*), gut resection (Lemmey *et al.* 1991), dexamethasone treatment (Tomas *et al.* 1992) and impaired kidney function (Martin *et al.* 1991, Miller *et al.* 1992, Martin *et al.* 1994). IGF-I can also stimulate growth of normal rats (Hizuka *et al.* 1986, Tomas *et al.* 1993*a*). IGF-II can also promote the growth of rats but less potently than IGF-I (Conlon *et al.* 1995*a*). Administration of IGF-I stimulates wound healing in rats treated with corticosteroids (Suh *et al.* 1992). IGF-I infusion increased glomerular blood flow and filtration rate in both normal and fasting rats (Hirschberg & Kopple 1989, Hirschberg *et al.* 1991). Treatment with IGFs does not generally promote growth of non-rodents. Long term infusion of IGF-I does not stimulate growth in young mini-poodles (Guler *et al.* 1989) or statural growth in chickens (Tixier-Boichard *et al.* 1992). Continuous subcutaneous infusion of IGF-I into well-fed lambs for two weeks did not affect growth rate although it significantly increased plasma IGF-I and reduced blood urea (Moritz *et al.* 1996). Similar treatment of well-fed guinea pigs for 7 days with IGF-I or IGF-II did not improve liveweight gain although the weights of several visceral organs were increased (Conlon *et al.* 1995*b*). Furthermore, infusion of IGF-I to well fed adolescent pigs did not promote growth (Walton *et al.* 1996). However, IGF-I can acutely inhibit degradation and improve retention of protein in hindlimb muscle of lambs (Oddy & Owens 1996).

## 1.2 INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

### 1.2.1 Overview

The majority of IGFs are not in free form in blood and tissue fluids but rather are noncovalently associated with soluble binding proteins. The IGF-binding proteins appear to have a number of actions. They prolong the half-life of IGFs in blood, transport IGFs in the vasculature and across capillary membranes, localize the IGFs to specific tissues and cell types, control IGF interaction with cell surface receptors and extracellular matrices and modulate the biological actions of IGFs (Jones and Clemmons 1995). In addition, recent evidence indicates that IGF-binding proteins not only regulate IGFs bioavailability but also seem to have their own receptors that can have direct, IGF independent effects on cellular activity. Several studies indicate that IGFBP-3 might inhibit cell proliferation independent of IGFs and type 1 IGF receptors by a mechanism that does not involve sequestering of IGF-I (Liu *et al.* 1992, Oh *et al.* 1993, Cohen *et al.* 1993). Zadeh and Binoux (1997) recently reported that a 16-kDa IGFBP-3 fragment inhibited the stimulation of DNA synthesis by basic fibroblast growth factor in mouse fibroblasts with a targeted disruption of the type 1 IGF receptor gene.

Six types of insulin-like growth factor binding proteins have been isolated and well characterised. They bind IGF-I and IGF-II with variable affinities and do not significantly bind insulin. The amino acid sequences at the amino and carboxy terminus are highly conserved. These include 18 cysteine residues that are conserved in IGF-binding proteins -1 to -5, twelve in the amino terminal region and six in the carboxy terminal region (Rechler 1993). These form intra-molecular disulphide bridges. Human and rat IGFBP-6 lack 2 and 4

of the amino terminal cysteines, respectively (Shimasaki *et al.* 1991). IGFBP-1 and -2 are not glycosylated. IGFBP-1 and -2 also contain an arg-gly-asp sequence near their carboxyl terminus. This sequence has been shown to mediate cellular attachment to extracellular matrix proteins (Ruoslahti & Pierschbacher 1988). IGFBP-3, -4, -5 and -6 have been shown to be glycosylated. IGFBP-3 and -4 are N-glycosylated whereas IGFBP-5 and -6 are O-glycosylated (Rechler 1993, Jones & Clemmons 1995).

In blood serum and plasma, most of the IGF-I and IGF-II is found in ternary complexes (~140 kDa), formed by association between IGFs, IGFBP-3 and an acid-labile subunit (ALS) (Baxter & Martin 1989). Only small amounts of IGFs are carried by the other IGFBPs, and less than 1% of IGF-I circulates in the free form. The ternary complex does not cross the capillary barrier (Binoux & Hossenlopp 1988). The formation of the ternary complex protects IGF-I and IGFBP-3 from proteolysis and prolongs the half-life of both IGFBP-3 and IGF-I. The half-life of unbound IGFBP-3 is between 30 and 90 minutes and the half-life of free IGF-I is less than 10 minutes whereas the ~140 kDa ternary complex has a circulating half life of 12-15 h (Jones & Clemmons 1995).

### 1.2.2 IGFBP-1

Human IGFBP-1 has been purified from human amniotic fluid, placental endometrium and Hep G2 cell conditioned medium (reviewed by Rechler 1993). Human IGFBP-1 consists of 234 amino acids and has a molecular mass of 25.3 kDa (Lee *et al.* 1993). The N-terminal and C-terminal regions are highly homologous among rat, human and cow and contain 18 conserved cysteines which are postulated to provide a framework for ligand binding. The 65 residue midregion is less conserved between species and does not contain

cysteines, but does include a Pro-Glu-Ser-Thr (PEST) domain that is typical of rapidly metabolized proteins (Lee *et al.* 1993). IGFBP-1 can associate with cells through the RGD (Arg-Gly-Asp) sequence which enables binding to the $\alpha 5\beta 1$ integrin (fibronectin receptor) on the cell surface (Jones *et al.* 1993*c*).

IGFBP-1 is found in several phosphorylated states that affect its affinity for IGFs (Clemmons 1994). IGFBP-1 binds IGF-I and -II with approximately equal affinity and its affinity constant at pH 7.0 for IGF-I is fivefold greater than that of the type 1 IGF receptor (Cohick & Clemmons 1993). Serum IGFBP-1 levels are regulated mainly by nutrition and hormone status (Lewitt & Baxter 1991). Baxter & Cowell (1987) initially demonstrated a diurnal variation in plasma IGFBP-1 level in humans. IGFBP-1 levels are decreased after feeding and increased during fasting in humans. Insulin inhibits the synthesis of IGFBP-1 whereas cortisol and glucagon stimulate IGFBP-1 production (Lee *et al.* 1993). Increased plasma IGFBP-1 has been observed in fasting, insulin-dependent diabetes mellitus (Suikkari *et al.* 1988), non-insulin-dependent diabetes mellitus (Brismar *et al.* 1988), hypopituitarism (Busby *et al.* 1988) and after prolonged exercise (Suikkari *et al.* 1989). Plasma IGFBP-1 was rapidly decreased after refeeding (Yeoh & Baxter 1988) and after insulin infusion during a glucose clamp in IDDM patients (Brismar *et al.* 1988). Lewitt *et al.* 1991 suggested that IGFBP-1 might counter-regulate the availability of the IGFs for glucose homeostasis since infusion of human IGFBP-1 into rats blocks the hypoglycemic effect of IGF-I and increases blood glucose levels. Lewitt *et al* (1994) reported that IGFBP-1 concentrations decrease with increasing developmental age: highest in fetal rat serum and decreased in adult animals. During fasting, females have higher levels of IGFBP-1 concentrations than male rats. IGFBP-1 concentrations were suppressed in genetically obese Zucker (fa/fa) rats and Wistar rats which were fed a diet high in fat content. IGFBP-1 levels increased in streptozotocin

induced diabetes and were suppressed to normal with insulin treatment of those animals. Exercise stimulated IGFBP-1 concentrations in fasting animals (Lewitt *et al.* 1994). IGFBP-1 levels were increased in intrauterine growth retardation, chronic renal failure, fasting and growth hormone deficiency but decreased in obesity (reviewed by Lee *et al.* 1993).

IGFBP-1 is phosphorylated *in vivo* as well as in cell culture (reviewed by Jones & Clemmons 1995). Nonphosphorylated IGFBP-1 has lower affinity for IGF-I compared with phosphorylated IGFBP-1 (Jones *et al.* 1991). Jones *et al.* (1993*a*) demonstrated that IGFBP-1 is phosphorylated on serine residues at position 101, 119 and 169. Substitution of alanine for serine 101 can prevent phosphorylation at this site resulting in reduction in affinity for IGF-I (Jones *et al.* 1993*a*). Some studies suggest that the isoforms which inhibit IGF action are phosphorylated, while the isoform that stimulates IGF action is nonphosphorylated (Frost & Tseng 1991, Jyung *et al.* 1994).

IGFBP-1 inhibits IGF-I stimulated aminoisobutyric acid uptake in JEG3 choriocarcinoma cells, IGF-I stimulated thymidine incorporation in porcine aortic smooth muscle cells and fibroblasts and IGF-I stimulation of JMGE-3 human osteosarcoma cells (reviewed by Jones & Clemmons 1995). The inhibitory effects of IGFBP-1 upon binding of IGF-I to cell surface receptors on human endometrial membranes was reported by Rutanen *et al.* (1988). IGFBP-1 also inhibits glucose incorporation into BALB/c 3T3-fibroblasts (Okajima *et al.* 1993). Lewitt *et al.* (1991) showed that administration of IGFBP-1 into rats increased blood glucose levels, suppressing the hypoglycaemic effect of IGF-I by decreasing the levels of free IGF-I.

Elgin *et al.* (1987) have shown that IGFBP-1 (purified from human amniotic fluid) markedly enhanced DNA synthesis in response to IGF-I of several cell types including human, mouse, and chicken embryo fibroblasts as well as porcine aortic smooth muscle cells. Clemmons *et al.* (1990) showed that an IGF-I mutant, in which residues B1 through 17 were substituted with insulin residues and which had a reduction in affinity for IGFBP-1, did not optimally stimulate DNA synthesis in porcine aortic smooth muscle cells.

### 1.2.3 IGFBP-2

IGFBP-2 was purified from rat BRL-3A cells and MDBK bovine kidney cells (reviewed by Rechler 1993). IGFBP-2 also has an RGD sequence, but it is neither phosphorylated nor glycosylated. In rats, liver is the prominent site of IGFBP-2 expression in the fetus, although kidney, stomach, lung, and brain also express moderate levels (Cohick & Clemmons 1993). Expression is very high in rat brain and higher in fetal than adult tissues (Ooi *et al.* 1990, Orlowski *et al.* 1990).

Clemmons *et al.* (1991) determined factors that regulated plasma concentrations of IGFBP-2 in humans. They reported that mean IGFBP-2 concentrations in infants are greater than those of normal adults. The mean IGFBP-2 concentrations of hypopituitary adults was increased two-fold compared to normal. However, there was no reduction of IGFBP-2 concentrations in acromegaly. IGFBP-2 did not change significantly post-prandially or after a glucose infusion. However, fasting for 9 days increased plasma IGFBP-2 in humans. In rats, IGFBP-2 hepatic mRNA abundance is increased in fasting and diabetes, and administration of insulin lowers its abundance (Clemmons *et al.* 1993). IGFBP-2 expression

was enhanced in hypothyroidism in young rats and this change was reversible with thyroxine administration (Salomen *et al.* 1991).

IGFBP-2 inhibits thymidine incorporation in chick embryo fibroblasts and rat astroglial cells (reviewed by Rechler & Nissley 1990). IGFBP-2 purified from bovine kidney cells inhibits the ability of IGF-I and IGF-II to stimulate DNA synthesis or protein accumulation in chick embryo fibroblasts (Ross *et al.* 1989). IGFBP-2 inhibited the effect of IGF-I and IGF-II on stimulation of smooth muscle cell migration after wounding (Gockerman *et al.* 1995).

### 1.2.4 IGFBP-3

IGFBP-3 has been purified from human plasma, rat serum, porcine serum, bovine serum, porcine follicular fluid and murine (Swiss 3T3) fibroblast culture medium (Rechler 1993). IGFBP-3 in human serum is a glycoprotein which appears as a doublet of 40-43 kDa on SDS-PAGE gels under reducing conditions (Martin & Baxter 1986).

Serum levels of IGFBP-3 are age dependent in humans, being lowest at birth, increasing during childhood to reach a peak during puberty, and decrease thereafter (Baxter & Martin 1986). IGFBP-3 concentrations in blood are elevated in acromegalics, and plasma IGFBP-3 is lower than normal in hypopituitary patients (Baxter & Martin 1986, Hardouin *et al.* 1989). Patients with GH deficiency have low serum levels of IGFBP-3 that are normalized with GH therapy. IGF-I infused into hypophysectomized rats has been reported to increase serum IGFBP-3 levels (Zapf *et al.* 1989).

Interactions between the IGFBPs and glycosaminoglycans such as heparin may be involved in the regulatory control of IGF exerted by the IGFBPs at the level of the extracellular matrix and capillary endothelium. Several observations suggest that IGFBP-3 might bind to heparan sulfate proteoglycans on cell surfaces. IGFBP-3 contains sequences rich in basic amino acids that represent potential heparin-binding domains (Arai *et al.* 1994). IGFBP-3 also binds to immobilized heparin (Arai *et al.* 1994, Booth *et al.* 1995). Addition of heparin increased medium concentrations of IGFBP-3 and decreased IGFBP-3 binding to human neonatal skin fibroblasts (Martin *et al.* 1992). Heparin also inhibited the clearance of exogenous IGFBP-3 from medium by rat Sertoli cells (Smith *et al.* 1994). Pretreatment of Sertoli cells with sodium chlorate, an inhibitor of cell surface proteoglycan sulphation decreased IGFBP-3 clearance suggesting there is a mechanism which influences the concentrations of IGFBP-3 in this cell medium involving the interaction between IGFBP-3 and cell surface proteoglycans (Smith *et al.* 1994).

Clemmons *et al.* (1993) reviewed that IGFBP-3 and -5 have a sequence of 18 amino acids in the carboxy terminus that contains 13 basic amino acids. These basic amino acids could form a charge interaction with cell surface and matrix associated components such as heparan sulphate. When IGFBP-3 is in solution, it has approximately 12-fold higher affinity for IGF-I than when it associates with cell surfaces (McCusker *et al.* 1990). Following cell surface association, the affinity constant decreases into a range where IGF bound to this protein can interact with cell surface receptors. Glycosylation does not appear to mediate cell surface association or biologic activity (Conover 1991).

### 1.2.5 IGFBP-4

IGFBP-4 has been purified from the TE 89 human osteosarcoma, U-2 human osteosarcoma, PC3 human prostatic carcinoma, HT29 human colon carcinoma cell line, T98G human glioblastoma, rat B104 neuroblastoma cell line, rat serum, human serum and ovine plasma (reviewed by Rechler 1993).

1, 25-Dihydroxyvitamin D3 increases secretion of IGFBP-4 by human osteoblast-like cells *in vitro* (Scharla *et al.* 1993). Treatment of osteoblast-like cells with parathyroid hormone also caused an increase in IGFBP-4 mRNA levels (Kudo *et al.* 1997).

IGFBP-4 appears to inhibit IGF actions under most, if not all, experimental conditions (Jones & Clemmons 1995). IGFBP-4 inhibits the effect of IGF-I on stimulation of cell proliferation in chicken bone cells (Mohan *et al.* 1989) and B104 neuroblastoma cells (Cheung *et al.* 1991).

### 1.2.6 IGFBP-5

IGFBP-5 has been purified from adult rat serum, human bone, T98G human glioblastoma cells and human CSF (Rechler 1993).

The molecular mass of IGFBP-5 is approximately 28,000 (Rechler 1993). Unlike the other IGFBPs, IGFBP-5 binds strongly to bone cells because of its high affinity for hydroxyapatite (Bautista *et al.* 1991, Kanzaki *et al.* 1994).

IGFBP-5 could inhibit IGF-I stimulated DNA and glycogen synthesis in human osteosarcoma cells when a molar excess was used (Kiefer *et al.* 1992). Ling *et al.* (1993) showed inhibition of steroidogenesis in granulosa cells stimulated with IGF-I, suggesting that IGFBP-5 had an inhibitory function.

IGFBP-5 has the unique property of adhering tightly to fibroblast ECM. When IGFBP-5 was incubated with ECM, it potentiated the effect of IGF-I on cell growth (Jones *et al.* 1993*b*). The affinity of IGFBP-5 for IGF-I was lowered when it was associated with ECM suggesting that matrix-associated IGF-I would be more capable of being released to cell surface receptors (Jones & Clemmons 1995). These authors suggest that the lowering of IGF binding affinity by ECM association may be an important factor in regulating the ability of IGFBP-5 to potentiate IGF actions.

### 1.2.7 IGFBP-6

IGFBP-6 has been purified from SV40 transformed human lung fibroblasts, nontransformed human lung fibroblast, cerebrospinal fluid from human adults and children human serum, rat serum, porcine follicular fluid and the U-2 human osteosarcoma cell line (reviewed by Rechler 1993).

IGFBP-6 is an O-glycosylated protein with a 10- to 100-fold higher affinity for IGF-II than for IGF-I (Cohick & Clemmons 1993).

Recombinant human IGFBP-6 inhibited IGF-II-induced differentiation of L6A1 myoblasts but it had no effect on IGF-I-induced differentiation (Bach *et al.* 1994). IGFBP-6

was also involved in growth inhibition in SH-SY5Y human neuroblast cells (Babajko *et al.* 1997).

### 1.2.8 IGFBP-7

Mac25 cDNA was originally cloned from leptomeningial cells and subsequently reisolated through differential display in human senescent mammary epithelial cells (Murphy *et al.* 1993, Swisshelm *et al.* 1995). Mac-25 has 40% to 45% similarity and 20 to 25% identity with the six known IGFBPs, especially at the N-terminus, where 11 out of the customary 12 conserved cysteines were identified. Oh *et al.* (1996) have shown that baculovirus generated mac25 protein binds both IGF-I and IGF-II in a specific manner, and thus has been speculated to be IGFBP-7. In comparison with IGFBP-3, rh-mac25 has at least a 5-6 fold lower affinity for IGF-I and 20-25 fold lower affinity for IGF-II (Oh *et al.* 1996). Mac25 mRNA was found in a wide range of normal human tissues, with decreased expression in breast, prostate, colon and lung cancer cell line (Oh *et al.* 1996). Wilson *et al.* (1997) identified 31 kDa mac25 in the conditioned media of Hs578T breast cancer cells, as well as in normal human urine, cerebrospinal fluid and amniotic fluid.

### 1.2.9 Acid labile subunit of the ternary complex in blood

The majority of serum IGFs are found in a high molecular weight (~150 kDa) ternary complex comprising IGF-I or IGF-II (γ-subunit), IGFBP-3 (β-subunit) and an acid-labile subunit (ALS, α-subunit) (Baxter & Martin 1989). cDNA sequences for human and rat ALS have been isolated and characterized (Leong *et al.* 1992, Dai & Baxter 1992). Native ALS isolated from human serum appears on SDS-PAGE as a doublet of 84-86 kDa which can be

converted to a single 70 kDa protein by *N*-glycanase treatment (Baxter *et al.* 1989). Neither IGFBP-3 nor IGFs alone bind ALS but IGF-I or IGF-II bind IGFBP-3 first and this binary complex then binds with the acid-labile subunit (Baxter & Martin 1989, Baxter *et al.* 1989). Temperature, ionic strength and pH also affect the formation of binary and ternary complexes (Holman & Baxter 1996). The acid -labile subunit circulates in 2-3 fold molar excess over IGFBP-3 and the IGFs (Baxter 1990)

### 1.2.10 IGFBP proteases

When levels of IGFBP-3 were monitored by western ligand blotting after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), serum IGFBP-3 became undetectable after the seventh to eight week of pregnancy (Gargosky *et al.* 1990*a*, Giudice *et al.* 1990, Hossenlopp *et al.* 1990). However, IGFBP-3 levels as assessed by RIA were elevated in human pregnancy serum (Baxter & Martin 1986). Levels of immunoreactive α-subunit were also moderately elevated in term pregnancy (Baxter 1990). After preincubation of pregnancy serum with an equal volume of nonpregnancy serum before analysis by SDS-PAGE and ligand blotting, there was a reduction in the intensity of IGFBP-3 bands resulting from proteolytic activity (Giudice *et al.* 1990, Hossenlopp *et al.* 1990). This reduction was inhibited by EDTA and aprotinin, suggesting a cation-dependent serine protease was involved (Hossenlopp *et al.* 1990). The disappearance of IGFBP-3 in pregnancy serum measured by western ligand blotting was also demonstrated in rats (Gargosky *et al.* 1990*b*).

IGFBP-3 proteolysis has subsequently been detected in normal adults (Gargosky *et al.* 1992, Lalou & Binoux 1993) and in some pathological conditions, including in severe illness (Davies *et al.* 1991) and in patients after elective surgery (Davenport *et al.* 1992). In

addition, IGFBP-3 proteolysis was also found in different biological fluids such as peritoneal, follicular, amniotic, seminal and cerebrospinal fluids (Gargosky *et al.* 1992).

The functional significance of circulating proteolytic activity is unclear. Despite the presence of pregnancy-associated proteases, most of the circulating insulin-like growth factor-I and -II are present in the 150 kDa complex during human pregnancy (Gargosky *et al.* 1991). IGFBP-3 can also form ternary complexes in the presence of radiolabeled α-subunit after acidification and neutralization of pregnancy serum suggesting that IGFBP-3 may be functionally normal in pregnancy (Suikkari & Baxter 1992). However, Lassarre & Binoux (1994) have shown the effect of structural alteration of IGFBP-3 by proteases, resulting in reduced affinity for IGFs and accelerated kinetics of dissociation, leads to an increase in measurable IGF-I in the free form in the circulation. Blat *et al.* (1994) have shown that DNA synthesis in cultured chick embryo fibroblasts was stimulated to a greater extent by pregnancy serum than nonpregnancy serum, despite the fact that they have similar levels of measurable immunoreactive IGF-I and IGF-II.

Binoux *et al.* (1994) suggested an inverse relationship between GH/IGF-I status and the extent of IGFBP-3 proteolysis. The proportions of proteolysed IGFBP-3 were estimated to be 37% in normal subjects, more than 50% in GH-deficient patients and 15% in acromegalic patients (Binoux *et al.* 1994). IGFBP-3 proteolysis was increased in tall children (Binoux *et al.* 1994). It was found that greater proportions of IGFBP-3 were proteolysed in lymph than in serum (Lalou & Binoux 1993). Binoux *et al.* (1994) observed the proteolysis of IGFBP-1 in amniotic fluid and the proteolysis of IGFBP-2 in cerebrospinal fluid. Proteases may promote the release of IGF from complexes with binding proteins and increase their bioactivity with tissues.

## 1.3 ANALOGS OF INSULIN-LIKE GROWTH FACTORS

Des(1-3)-IGF-I, a variant of IGF-I lacking the first three N-terminal residues (Gly-Pro-Glu), was found in human fetal and adult brain extracts (Sara *et al.* 1986), adult human brain (Carlsson-Skwirut *et al.* 1986), bovine colostrum (Francis *et al.* 1986) and porcine uterus (Ogasawara *et al.* 1989). Des(1-3)-IGF-I is approximately 5-10 times more potent than native IGF-I in its ability to stimulate protein synthesis, stimulate DNA synthesis and inhibit protein breakdown in cultured myoblasts (Francis *et al.* 1986, 1988*b*, Ballard *et al.* 1987). In addition, it is also more potent than IGF-I in stimulating growth *in vivo* in rodents (Gillespie *et al.* 1990, Tomas *et al.* 1991*a*, Ballard *et al.* 1991*b*, Tomas *et al.* 1993*a*). The increase in potency was not associated with a comparable increase in affinity for type-1 IGF receptor binding (Ballard *et al.* 1987) but is due to reduction in affinity for IGF-binding proteins. $Glu^3$ of IGF-I appears to be critical for the binding of IGF-I to IGF-binding proteins (Bagley *et al.* 1989). Des(1-3)-IGF-I had a markedly reduced affinity compared to IGF-I for a binding protein present in the serum-free conditioned medium of bovine kidney (MDBK) cells (Szabo *et al.* 1988). Ross *et al.* (1989) showed that this binding protein did not influence the biological activities of des(1-3)-IGF-I. Forbes *et al.* (1988) showed that the three amino-terminal residues of IGF-I are important for IGF-I binding to small binding proteins, but have only minor importance in the binding of IGF-I to IGFBP-3. They showed that des(1-3)-IGF-I competes well for the binding to IGFBP-3 (BP 53) of either labelled IGF-I or IGF-II but poorly with IGFBP-1 and not at all with the IGFBP-2. Des(1-3)-IGF-I is cleared more rapidly from the blood than IGF-I in rats (Ballard *et al.* 1991*a*) and lambs (Francis *et al.* 1988*a*) but not in lactating goats (Prosser *et al.* 1995).

Various synthetic analogs that have different binding properties for either the IGFBPs or receptors have been produced (Cascieri & Bayne 1994). Substitution of the first 16 residues of IGF-I with the first 17 amino acids of the B-chain of insulin markedly reduced the affinity for IGFBP-1, -2, -3, -4, and -5 (Cascieri & Bayne 1994). [$Tyr^{15}$, $Leu^{16}$] IGF-I has normal affinity for IGFBP-1, -2 and -3 but reduced affinity for IGFBP-4 and IGFBP-5 by 10-fold and 100-fold, respectively. [$Gln^{3}$, $Ala^{4}$] IGF-I has reduced affinity for IGFBP-3 by 10-fold whereas its affinity for IGFBP-1, -2, -4 and -5 is more greatly reduced. [$Gln^{3}$, $Ala^{4}$, $Tyr^{15}$, $Leu^{16}$] IGF-I has 20-fold reduced affinity for IGFBP-1 and -2, and > 100-fold reduced affinity for IGFBP-3, -4 and -5. The B-chain mutant and [$Gln^{3}$, $Ala^{4}$, $Tyr^{15}$, $Leu^{16}$] are more potent than IGF-I in stimulating DNA synthesis in BALB C/3T3 cells and in stimulating $^{14}C$-glucose incorporation into muscle glycogen *in vivo*. These results support the view that IGF-binding proteins inhibit the actions of IGFs. Analogs with mutations in residues 49-51 of the A chain have normal affinity for IGFBP-3 but dramatically reduced affinity for IGFBP-1 and IGFBP-2. The A-chain mutant has 13-fold and 7-fold reduced affinity for IGFBP-4 and -5 while [$Thr^{49}$, $Ser^{50}$, $Ile^{51}$] IGF-I has 60-fold and >100 fold reduced affinity for IGFBP-4 and -5, respectively (Cascieri & Bayne 1994, Cascieri *et al.* 1994).

Analogs with reduced affinity for the type 1 IGF receptor have also been developed. For example, analogs in which serine or leucine were substituted for tyrosine at position 24 have reduced affinity for the type 1 IGF receptor whereas they maintained normal binding to the IGFBPs in acid-treated human serum. Substitution of tyrosine at position 31 with alanine also results in a 6-fold loss in affinity for this receptor. The double mutation [$Leu^{24}$, $Ala^{31}$] IGF-I results in a dramatic loss (200-fold) in affinity for type 1 IGF receptor (Cascieri & Bayne 1994, Cascieri *et al.* 1994).

Replacement of the A region of IGF-I or residues 49-51 with the homologous residues in the A chain of insulin results in reduced affinity for type 2 IGF receptor, while normal affinity for the type 1 IGF receptor and for the IGF-binding proteins is retained (Cascieri & Bayne 1994, Cascieri *et al.* 1994).

### 1.3.1 LR$^3$IGF-I

Long-Arg$^3$-insulin-like growth factor-I (LR$^3$IGF-I) is an 83 amino acid analog of human IGF-I comprising the complete IGF-I sequence except for the substitution of arginine for glutamate at position 3 in the wild type IGF-I sequence and for the addition of a 13 amino acid peptide extension at the amino terminus (Francis *et al.* 1992). Ballard *et al.* (1993) compared the potencies of IGF-I and LR$^3$IGF-I in terms of their binding to IGF-binding proteins and to the type 1 IGF receptor as well as their biological activities (Table 1.1). LR$^3$IGF-I binds mitogenic type-1 IGF receptors with 4 times lower affinity than authentic IGF-I. It binds very poorly to all preparations of binding proteins including ovine IGFBP-3, rat IGFBP-3, total rat plasma IGFBPs, ovine IGFBP-4 and the binding proteins released from rat L6 myoblast cells (Table 1.1). The affinity of LR$^3$IGF-I for these IGF-binding proteins is approximately 1000-fold lower than that of IGF-I (Ballard *et al.* 1993). In spite of its 4-fold lower affinity for type 1 IGF receptor binding, LR$^3$IGF-I is significantly more potent than IGF-I under many conditions *in vitro* and *in vivo*. LR$^3$IGF-I is more potent than native IGF-I in its ability to stimulate synthesis of protein and DNA and inhibit protein breakdown in cultured rat L6 myoblasts (Francis *et al.* 1992). It is more potent than IGF-I in promoting liveweight gain in rats, including normally growing animals and animals in catabolic states (Ballard *et al.* 1991*b*, Tomas *et al.* 1992, 1993*a*, 1993*b*). For example, LR$^3$IGF-I is approximately 2.5 fold more potent than IGF-I at improving weight gain and nitrogen

Table 1.1

Relative potencies of IGF-I and $LR^3$IGF-I *in vitro*.

Concentrations that produce 50% of maximal responses have been compared

| | Potency ratio $LR^3$IGF-I:IGF-I |
|---|---|
| Binding to ovine IGFBP-3 | 0.002 |
| Binding to rat IGFBP-3 | 0.001 |
| Binding to total rat plasma IGFBPs | 0.0002 |
| Binding to L6 myoblast BP | 0.001 |
| Binding to ovine IGFBP-4 | 0.0005 |
| Binding to type 1 IGF receptor | 0.23 |
| Stimulation of protein synthesis in L6 cells | 5.8 |
| Stimulation of DNA synthesis in L6 cells | 4.5 |
| Inhibition of protein breakdown in L6 cells | 16.0 |
| Inhibition of protein breakdown in H35 cells | 6.0 |

From Ballard *et al.* (1993) *Growth Regulation* **3** 40-44.

retention in dexamethasone-treated rats (Tomas *et al.* 1992). Administration of LR$^3$IGF-I over 14 days to growing female rats led to an increase in body weight gain, nitrogen retention and fractional weights of the gut, spleen, kidney and thymus (Tomas et al. 1993*a*). LR$^3$IGF-I also increased growth rate and nitrogen balance in diabetic rats but did not overcome the glucosuria (Tomas *et al.* 1993*b*). In addition, LR$^3$IGF-I also promotes gut growth in dexamethasone-treated and normal rats (Read *et al.* 1992, Steeb *et al.* 1994).

The higher potency in rats of LR$^3$IGF-I compared to wild type IGF-I and IGF-II appears to be due to its much lower affinity for IGF-binding proteins, despite the slightly lower affinity of LR$^3$IGF-I for the type-1 IGF receptor. [$^{125}$I]-LR$^3$IGF-I is also cleared from blood more rapidly than [$^{125}$I]-IGF-I (Ballard *et al.* 1993, Bastian *et al.* 1993).

## 1.4 ROLE OF CIRCULATING BINDING PROTEINS IN ENDOCRINE ACTIONS OF INSULIN-LIKE GROWTH FACTORS

Several studies have examined the *in vivo* and *in vitro* formation of complexes between IGFs and their binding proteins using radio-iodinated IGF-I, IGF-II or IGF analogs. For example, Cascieri *et al.* (1988) studied the B-chain mutant and [Gln$^3$, Ala$^4$, Tyr$^{15}$, Leu$^{16}$]IGF-I which have 1000 and 500 times lower affinity than IGF-I for the 150 kDa binding protein in adult rat serum. They demonstrated that [$^{125}$I]-IGF-I, [$^{125}$I]-B chain mutant and [$^{125}$I]-[Gln$^3$, Ala$^4$, Tyr$^{15}$, Leu$^{16}$]IGF-I have *in vivo* serum half-lives of 100, 27.5 and 26.9 min, respectively, after iv injection. They concluded that serum binding protein-bound peptide is cleared from the serum more slowly than free IGF peptide. The tissue distribution of [$^{125}$I]-IGF-I and [$^{125}$I]-B chain mutant are similar 10 min after dosing, with more than 80% of the tissue-sequestered peptide in the kidney (Cascieri *et al.* 1988). Drakenberg *et al.*

(1990) examined the molecular forms of radiolabelled IGF-I and des(1-3)IGF-I after iv administration into adult or neonatal rats. They also demonstrated that [$^{125}$I]-des(1-3)IGF-I, which displayed weaker affinity of binding to IGF binding proteins than [$^{125}$I]-IGF-I, was more rapidly degraded after iv administration into both adult and neonatal rats. The uptake of [$^{125}$I]-des(1-3)IGF-I into kidney, liver and brain of neonatal rats was significantly higher than for [$^{125}$I]-IGF-I 15 min after iv administration. Many other *in vivo* studies have shown that the clearance rate of IGF peptides from blood is faster when the growth factor is not complexed with binding proteins (Cohen & Nissley 1976, Zapf *et al.* 1986, Cascieri *et al.* 1988, Francis *et al.* 1988*a*, Davis *et al.* 1989, Ballard *et al.* 1991*a*, Bastian *et al.* 1993). Administration of radioiodinated IGF-I, IGF-II or des(1-3)IGF-I into rats showed that the amount of radioactivity found in the adrenals, brain, skin, stomach, duodenum, ileum, jejunum and colon was in rank order, des(1-3)IGF-I>IGF-I>IGF-II (Ballard *et al.* 1991*a*). They concluded that the plasma binding proteins inhibit the transfer of the growth factors from blood to tissues.

One limitation of using radioiodinated peptides to study complex formation between IGFs and IGF-binding protein is that [$^{125}$I]-iodinated IGFs do not have the same affinities for IGF receptors and binding proteins as the native growth factors (Ballard *et al.* 1987, Francis *et al.* 1989*a*, Gelato *et al.* 1989, Suikkari & Baxter 1991). For example, iodination of IGF-I has been shown to reduce the affinity of IGF-I for binding to IGFBP-3 from pregnant human serum (Suikkari & Baxter 1991) and radio-iodination of IGF-II abolishes its ability to bind to type 1 IGF receptors on cultured myoblasts (Francis *et al.* 1989*a*). Moreover, administration of trace amounts of [$^{125}$I]-IGFs *in vivo* does not produce a metabolic or growth response, so the biological activities of different complexes between IGFs and binding proteins cannot be examined by this approach. However, Hodgkinson *et al.* (1991) studied the distribution of

unlabelled circulating IGF-I into tissues after iv infusion of N-Met IGF-I into lactating sheep and analysis with an antiserum specific for the analog. They found that N-Met IGF-I infusion increased total plasma and lymph IGF-I immunoreactivity but not that of cerebrospinal fluid. N-Met IGF-I was associated with 150 kDa binding protein complexes in plasma whereas it bound to the 40-50 kDa pool of binding proteins in lymph. N-Met IGF-I was distributed into extra-vascular tissue accounting for 36% (kidney) to 62% (spleen) of total tissue IGF-I immunoreactivity after infusion for 20 h. They suggested this data indicated that circulating IGF-I was distributed outside the vascular space into peripheral tissues and occurred on a tissue-specific basis, and thus may fill specific endocrine functions in selected tissues (Hodgkinson *et al.* 1991). Lewitt *et al.* (1993*b*) determined the effect of human IGFBP-1 on the circulating half-life of human IGF-I and on hIGF-I stimulated 2-deoxy glucose uptake in tissues of rat. They reported that the circulating half-life of IGF-I was prolonged by IGFBP-1 and that IGF-I stimulated hexose uptake into heart, soleus and red quadriceps muscles was inhibited by IGFBP-1 *in vivo*.

## 1.5 AIMS OF THIS STUDY

The *in vivo* formation of complexes between the different IGF-binding proteins and IGFs and the physiological functions of different types of complexes are not completely understood. Difficulties in discriminating between endogenous and exogenous IGF-I in animals treated with IGFs at bioactive doses have hindered studies of complex formation.

The aims of this study were:

1) To investigate the molecular forms of circulating $LR^3IGF$-I during treatment of animals with biologically active doses of this analog.

2) To identify factors which affect the *in vivo* formation of circulating complexes between $LR^3IGF$-I and IGF-binding proteins

3) To investigate the association between free $LR^3IGF$-I and the biological activity of this analog *in vivo*.

# CHAPTER 2

# General Materials and Methods

## 2.1 GENERAL MATERIALS

### 2.1.1 Animal Ethics

All animal procedures were carried out in accordance with the guidelines of the National Health and Medical Research Council of Australia. Antibody production in mice was approved by the Animal Ethics Committee of the University of Adelaide. Antibody production in rabbits and treatment of fed and underfed rats with $LR^3IGF$-I were approved by the Animal Experimentation and Ethics Committee of the CSIRO Division of Human Nutrition. Treatment of cows with $LR^3IGF$-I was approved by the Animal Care and Ethics Committee of the Tropical Cattle Research Centre, Rockhampton, Qld, Australia. Treatment of guinea pigs with $LR^3IGF$-I was approved by the Animal Ethics Committee of the Women's and Children's Hospital, North Adelaide, SA, Australia.

### 2.1.2 Reagents

Recombinant human IGF-I, IGF-II, $R^3IGF$-I, long-IGF-I, long-$R^3IGF$-I and rabbit anti-$LR^3IGF$-I serum were obtained from GroPep Pty. Ltd., Adelaide, SA, Australia. IGF-I, IGF-II and $LR^3IGF$-I were radioiodinated with $Na^{125}I$ from Amersham International plc, (Amersham, Bucks, U.K.) to specific activities between 70 and 85 Ci/μg using chloramine T (Owens *et al* 1990). Freund's complete and incomplete adjuvant, Dulbecco's modified Eagle's minimum

essential medium (D-MEM) and F-12 Nutrient Mixture (HAM) were obtained from GIBCO BRL (Gaithersburg, MD, USA). Polyethylene glycol (PEG) 1500 was from Cell Biology Boehringer Mannheim, Germany (Prod. No. 783641, Lot No. 14764600). Ovalbumin (Grade III A5378), bovine serum albumin (BSA), oxalacetic acid, insulin, hypoxanthine, azaserine, *ortho*-phenylene diamine dihydrochloride and dimethylsulphoxide were from Sigma Chemical (St Louis, MO, U.S.A.). Fetal calf serum (FCS) was from CSL Limited (Melbourne, Vic., Australia). SP2/Ø-Ag14 mouse myeloma cells were from the American Type Culture Collection (Rockville, Maryland, U.S.A.). Vinyl assay plates (96-well) were from Costar (Cat. No. 2595, Cambridge, MA, U.S.A.). Rabbit immunoglobulin fraction (normal), peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (Code P260) and peroxidase-conjugated goat immunoglobulins to rabbit immunoglobulins (Code P0448) were from DAKOPATTS A/S, Denmark. Sheep antiserum to mouse immunoglobulin (Product Code DS) and sheep anti-rabbit immunoglobulin, IgG fraction (Product Code RC) were from Silenus (Hawthorn, Vic., Australia). All other reagents and solvents were analytical grade unless noted otherwise.

### 2.1.3 Standard equipment

#### 2.1.3.1 Gamma scintillation spectrometer (γ-counter)

Gamma radiation emitted by $^{125}I$ was measured in a γ-scintillation spectrometer (Wallac 1261 MultiGamma Gamma Counter, Wallac Oy, Turku, Finland). Data transformations and reductions, including calculation of RIA results were performed in-line using a benchtop computer TCI PC (Model D4035, Trident Computer Industries P/L, Hornsby NSW, Australia) running the Wallac RiaCalc/FiaCalc DM program.

2.1.3.2 Microtitre plate reader

Spectrophotometric absorbances in individual wells of microtitre plates were measured using an automated microplate reader (Model EL310, Bio-tek Instruments, Inc., Winooski VT, USA).

2.1.3.3 Beckman J6 Centrifuge

Precipitated antibodies and complexes with antibodies formed in solution phase procedures were pelleted in 12 x 75 mm tubes by centrifugation at the speeds and times specified using a Beckman J6-B centrifuge and a Beckman JS4.2 rotor (Beckman Instruments, Inc., Fullarton CA, USA) at 4°C.

2.1.3.4 Microfuge

Suspended cells and precipitating proteins in acid-extracted plasma were pelleted in 1.5 ml microtest tubes (Prod. No. 0030 102 002, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) by centrifugation at room temperature at the speeds and times indicated using a Biofuge Pico (Heraeus Instruments, Osterode, Germany).

## 2.2 GENERAL METHODS

### 2.2.1 Production of mouse monoclonal antibodies

#### 2.2.1.1 ANTIGEN PREPARATION

A mixture of IGF-I and LR$^3$IGF-I was conjugated to ovalbumin using glutaraldehyde as follows. IGF-I (2.5 mg), LR$^3$IGF-I (2.5 mg) and ovalbumin (10 mg) were dissolved in 5 ml of 0.1 mol/l Tris-HCl pH 8 containing 0.15 mol/l NaCl. A volume of 2 ml of 0.13 mol/l glutaraldehyde solution in Tris buffer was added slowly dropwise to the IGF/ovalbumin mixture with constant stirring. Crosslinking was allowed to proceed for 2 h at room temperature. The reaction mixture was then dialyzed overnight at 4 °C through a membrane (Spectra/Por MWCO 3500, Spectrum Medical Industries Ltd., Houston TX, USA) of nominal $M_r$ cut-off of 3,500 against 2 l of Tris buffer with 2 changes and the conjugate solution was stored as 1.4 ml aliquots at -20 °C.

#### 2.2.1.2 IMMUNIZATION OF MICE

Six week old female BALB/c mice were injected intraperitoneally with ovalbumin conjugated to a mixture of hIGF-I and LR$^3$IGF-I. In the first immunization, each mouse received 140 µl (300 µg) of the conjugate mixed with an equal volume of Freund's complete adjuvant. They were subsequently reimmunized at 2 to 3 week intervals with 300 µg of conjugate in Freund's incomplete adjuvant (1:1, vol:vol). After the 7$^{th}$ immunization, mice were left for 3 months and then reboosted twice with 300 µg of conjugate in incomplete

Freund's adjuvant over 3 weeks. Three weeks later, one animal was challenged with 300 µg of conjugated antigen in PBS pH 7.4 and sacrificed four days later for hybridoma preparation.

### 2.2.1.3 SOLUTION PHASE DETECTION OF ANTIBODIES IN PLASMA FROM IMMUNIZED MICE

Seven days after each immunization, mice were bled by cutting a tail vein. The blood was collected by pipetting with heparinized capillaries into a 1.5 ml "Eppendorf" tube. The plasma was obtained after 10 min centrifugation at 10,000 rpm in a Microfuge. The heparinized plasma was tested for anti-IGF-I titre (see below). The mouse showing the highest titre (1 in 33,000) in this test was selected for monoclonal antibody production.

The solution phase assay of anti-IGF-I immunoglobulin titre in mouse plasma was performed as follows. Test plasma was diluted to several concentrations between 1 in 100 and 1 in 30,000 in RIA buffer (30 mmol/l $NaH_2PO_4$ pH 7.5, 0.02% (w/v) protamine sulphate, 10 mmol/l disodium EDTA, 0.02% (w/v) $NaN_3$, 0.05% (v/v) Tween-20) and triplicate 50 µl aliquots of this diluted mouse plasma was added to 200 µl of RIA buffer. Fifty µl of [$^{125}$I]-IGF-I (~20,000 cpm) was added to each tube and these were incubated overnight at 4 °C. Fifty µl of sheep antiserum to mouse immunoglobulin (Silenus, Code DS, 2 mg/ml) diluted 1/10 in RIA buffer and 50 µl of normal mouse serum diluted 1/250 in RIA buffer were added to each tube. After vortex mixing, the tubes were incubated on ice for 30 min. [$^{125}$I]-IGF-I associated with mouse immunoglobulins bound to sheep antibodies was precipitated by the addition of 1 ml 6% (w/v) PEG in NaCl (150 mmol/l) and the tubes were centrifuged at 4,000 rpm for 20 min at 4 °C in a Beckman J6 centrifuge. Supernatants were aspirated and radioactivity in the precipitates was measured in a γ-scintillation spectrometer.

### 2.2.1.4 PRODUCTION OF CULTURES OF HYBRIDOMA CELLS

Monoclonal antibodies were obtained from hybridoma cells produced by fusion of mouse spleen lymphocytes with mouse myeloma cells (Kohler & Milstein 1975).

#### 2.2.1.4.1 PREPARATION OF MYELOMA CELLS

The SP2/Ø-Ag14 (SP2/Ø) myeloma cell line was chosen for fusion to mouse spleen lymphocytes. SP2/Ø cells lack the enzyme hypoxanthine phosphoribosyl transferase (HPRT) and are therefore 6-thioguanine resistant. These cells lack the 'salvage pathway' which requires HPRT and exogenous hypoxanthine for nucleotide synthesis. SP2/Ø cells therefore exclusively utilize the 'de novo' pathway for nucleotide synthesis. Azaserine inhibits this pathway. Therefore, SP2/Ø cells cannot replicate in medium containing azaserine. Hybrids between SP2/Ø myelmoma cells and spleen lymphocytes can therefore be selected in medium containing azaserine because they can utilise the 'salvage pathway' obtained from the normal mouse genome in the spleen lymphocytes if cell fusion is successful. Normal (non-fused) spleen lymphocytes cells generally die within two weeks in this selection medium because it does not contain the growth factors essential for replication of these nontransformed mammalian cells.

SP2/Ø cells were grown in DMEM/F12 containing 20% FCS in flasks at 37 °C in 5% $CO_2$ incubator. The myeloma cells were in the exponential phase of growth before fusion. On the day of fusion, the cells were counted and made to a total of $3.2 \times 10^7$ cells in 38 ml. These cells were washed twice with serum-free DMEM/F12 and centrifuged in a microfuge at

1200 rpm for 10 min after each wash. These myeloma cells were then fused to lymphocytes freshly isolated from mouse spleen.

#### 2.2.1.4.2 PREPARATION OF LYMPHOCYTES FROM MOUSE SPLEEN

Mouse #14.5 was selected for hybridoma preparation because its plasma had the highest titre against IGF-I (1 in 33,000). The animal was killed by cervical dislocation. The spleen was removed aseptically and placed in a sterile dish containing serum-free DMEM/F12 medium. Fat and other tissue was trimmed from the surface of the spleen. The organ was washed again with serum-free DMEM/F12 in a fresh sterile dish. The spleen was cut and gently disrupted between two sterile frosted slides in a Petri dish containing 10 ml of serum-free DMEM/F12 medium. Once the spleen was teased open, the cell suspension was transferred to a 50 ml conical tube with a Pasteur pipette. The lymphocyte suspension was washed twice with serum-free DMEM/F12 by centrifugation at 1200 rpm for 10 min. Approximately $1.6 \times 10^8$ spleen lymphocyte cells were obtained from mouse #14.5.

#### 2.2.1.4.3 FUSION OF MYELOMA CELLS WITH SPLEEN LYMPHOCYTES

The fusion protocol was a modification of that described by Oi and Herzenberg (1980). In this fusion, $1.6 \times 10^8$ spleen cells were mixed with $3.2 \times 10^7$ SP2/Ø myeloma cells in 10 ml of serum-free DMEM/F12 (ratio of 5:1 spleen cells to SP2/Ø) and centrifuged in a microfuge at 1200 rpm for 5 min at room temperature. The supernatant was carefully discarded. The cells were maintained at 37 °C in a water bath for further manipulations. One ml of prewarmed 50% (w/v) PEG 1500 in 75 mmol/l Hepes buffer pH 8.0 was added dropwise to the cell suspension over a one minute period with slight agitation. The

suspension was then gently stirred with a pipette for an additional minute. To stop the fusion, 2 ml of serum-free DMEM/F12 was added dropwise over a two minute interval with gentle stirring. A further 7 ml of the same medium was then added over 3 min. The cell suspension was then centrifuged in a microfuge at 1200 rpm for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 96 ml of 20% FCS in DMEM/F12 (hybridoma culture medium) containing 13.6 µg/ml hypoxanthine, 150 µg/ml oxalacetic acid, 20 µg/ml insulin as well as ~1.8 x $10^6$ normal spleen lymphocytes per ml (as feeder cells) prepared from a nonimmunized mouse as described in 2.2.1.4.2. One hundred µl of this mixed cell suspension (~2 x $10^5$ total cells) was added into each well of ten 96-well culture plates and maintained at 37 °C in 5% $CO_2$. The following day, 100 µl of the same medium to which azaserine (1 µg/ml) had been introduced was added to each well. Four days after fusion, half of the medium was aspirated from each well and replaced with fresh selection medium containing 0.5 µg/ml azaserine. Medium was changed in this manner every 3 to 5 days depending on the growth rates of cultures in different wells. Fourteen days after fusion most of the medium was removed and replaced with azaserine-free hybridoma culture medium containing 13.6 µg/ml hypoxanthine, 150 µg/ml oxalacetic acid, 20 µg/ml insulin.

Hybridoma cultures were screened for antibody production when the monolayer reached ≥50% confluence (see 2.2.1.5.1.1 for assay details). This occurred between nine and 20 days after fusion.

### 2.2.1.5 Characterisation of hybridoma cultures and isolation of clones

Viable hybridoma cultures were obtained in 471 of 960 culture wells. All viable hybridoma cultures produced mouse immunoglobulins. The cultures were expanded in

48-well plates containing hybridoma culture medium with 13.6 μg/ml hypoxanthine, 150 μg/ml oxalacetic acid, 20 μg/ml insulin (as above) between 10 and 21 days after fusion depending on growth rate.

Two to five days later (depending on when individual cultures reached ≥50% confluence) they were screened for production of anti-IGF immunoglobulins using both solid phase and solution phase assays (see 2.2.1.5.1.2 and 2.2.1.5.1.3). Conditioned media was collected on two to three occasions from each of these expanded cultures. Fifty of the 471 expanded cultures, which were producing mouse immunoglobulins, contained antibodies that reacted with either plates coated with IGF-I or with [$^{125}$I]-IGF-I in solution on at least one occasion. Based on the strength of their reactions in the above tests, the consistency of production of antibodies that react with IGF-I and the ability of the cultures to survive, 19 of these 50 cultures were selected for cloning using the limiting dilution technique (see 2.2.1.5.2). Eight stable hybridoma clones producing antibodies that reacted either with microtitre plates coated with IGF-I or with [$^{125}$I]-IGF-I in solution, or both, were established.

The specificities of antibodies in culture supernatants obtained from these stable hybridoma clones were initially assessed by comparing their abilities to bind to solid phase IGF-I, IGF-II and LR$^3$IGF-I (see 2.2.1.5.1.4). Three different groups of antibodies were identified (Table 2.1) by adsorption to solid phase IGFs: those that bound strongly to IGF-I and LR$^3$IGF-I but bound weakly to IGF-II and to the uncoated plate (from clones 1A7-F5-E5, 1A7-F5-E8 and 4C12-A2-B10), those that bound strongly to all IGF peptides and to the uncoated plate (from clones 6H3-D4-C4, 7E3-D2-F9, 8C11-E7-E3, 9A2-G11-F9), and one that bound strongly to all IGF peptides but bound weakly to the uncoated plate (from clone 6B10-D9-G5).

Table 2.1 Preliminary characterization of specificity of monoclonal antibodies in cloned hybridoma culture supernatants by ELISA

| Antibody | Ig Class/Type | Solid phase capture reaction | | | |
|---|---|---|---|---|---|
| | | Blank | IGF-I | $LR^3IGF-I$ | IGF-II |
| 1A7-F5-E5 | $IgG_1$, κ | - | ++++ | ++++ | - |
| 1A7-F5-E8 | $IgG_1$, κ | - | ++++ | ++++ | - |
| 4C12-A2-B10 | $IgG_{2b}$, κ | - | ++ | + | - |
| 6B10-D9-G5 | IgM, κ | - | ++ | ++++ | +++ |
| 6H3-D4-C4 | IgM, κ | + | ++++ | ++++ | ++++ |
| 7E3-D2-F9 | IgM, κ | + | ++++ | ++++ | ++++ |
| 8C11-E7-E3 | IgM, κ | + | ++++ | ++++ | ++++ |
| 9A2-G11-F9 | IgM, κ | ++ | ++++ | ++++ | +++ |

### 2.2.1.5.1 HYBRIDOMA SCREENING ASSAYS

#### 2.2.1.5.1.1 Detection of mouse immunoglobulins in conditioned media

Microtitre 96-well plates were coated with 100 µl of 1:3000 diluted sheep anti-mouse immunoglobulins (Silenus DS, 2 mg/ml stock) in 50 mmol/l carbonate buffer pH 9.6 (coating buffer) for 4 h at 37 °C. Plates were washed 4 times with 1.5 mmol/l $KH_2PO_4$, 6.5 mmol/l $Na_2HPO_4$ and 500 mmol/l NaCl containing 0.05% (v/v) Tween 20 (PBS-Tween 20). They were then "blocked" by addition of 100 µl of 10 mg/ml BSA in PBS-Tween 20 and incubated overnight at 4 °C. Plates were again washed as above. Hybridoma culture supernatants (100 µl) were added to each well and incubated for 3 h at 37 °C. After washing as above, captured mouse antibodies were detected after addition of 100 µl of peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKOPATTS, immunoglobulin concentration 1.3 g/l) diluted 1:2000 in 5 mg/ml BSA in PBS-Tween 20. The plates were incubated for 1.5 h at 37 °C and rewashed as above. Substrate for peroxidase was o-phenylene diamine (5.5 mmol/l in 100 mmol/l citrate/phosphate buffer pH 5.0, containing hydrogen peroxide at 10 mmol/l, 100 µl/well). The reaction was stopped after 30 min incubation at 37 °C by addition of 1 mol/l sulphuric acid (50 µl). The absorbance at 490 nm was measured using an automated microplate reader.

The positive control for the detection of mouse immunoglobulins captured by microtitre plates coated with sheep antibodies to mouse immunoglobulins was a mouse monoclonal antibody against IGF-I (3D1/1/2), which was a generous gift from Prof. R. C. Baxter (Sydney NSW), diluted 1:128,000 in 5 mg/ml BSA in PBS-Tween 20. The negative

controls were SP2/Ø conditioned medium, fresh hybridoma selection medium and PBS-Tween 20 containing 5 mg/ml BSA.

#### 2.2.1.5.1.2 Solid phase detection of anti-IGF-I immunoglobulins in conditioned medium

Media conditioned by viable hybridoma cultures (*i.e.* those that replicated and produced mouse immunoglobulins) were evaluated for their ability to bind to IGF-I in a solid phase assay. The assay system was identical to that described above for measurement of mouse immunoglobulins (see 2.2.1.5.1.1) except that hybridoma-conditioned media (100 µl) was added to each well of a 96-well plate previously coated for 4 h at 37 °C with 100 µl of 2.5 µg/ml IGF-I in coating buffer instead of sheep anti-mouse immunoglobulins. Blocking, washing, detection of mouse immunoglubulins and controls were as described above.

#### 2.2.1.5.1.3 Solution-phase detection of anti-IGF-I immunoglobulins in conditioned media

Supernatants from hybridoma cell cultures were also evaluated for their ability to bind [$^{125}$I]-IGF-I in a solution phase assay. The positive control was mouse monoclonal antibody 3D1/1/2 against IGF-I diluted 1:24,000 both in selection medium and in RIA buffer. The negative controls were selection medium and RIA buffer containing no added antibody. Hybridoma-conditioned medium (100 µl) was incubated with 50 µl of [$^{125}$I]-IGF-I in 250 µl of RIA buffer (see 2.2.1.3). After 16 h incubation at 4 °C, IGF-I tracer bound to mouse antibodies was precipitated by adding 20 µl of a suspension of cellulose coated with goat anti-mouse serum (Sac-Cel AA-SAC4, IDS, Boldon, U.K.) and incubating at room temperature for

30 min. Water (1 ml) was added and the tubes were centrifuged at 2,000 rpm for 10 min at 4 °C in a Beckman J6 centrifuge. Supernatants were aspirated and the radioactivity in the precipitates was measured in a γ-scintillation spectrometer.

#### 2.2.1.5.1.4 Solid-phase detection of anti-IGF-I immunoglobulins with different specificities

Supernatants from hybridoma cell cultures were evaluated for their ability to bind solid phase IGF-I, IGF-II and $LR^3$IGF-I. The assay system was the same as described above for measurement of mouse immunoglobulins (see 2.2.1.5.1.1) except that different plate wells were coated for 4 h at 37 °C either with 100 µl of coating buffer (blank) or with IGF-I, IGF-II or $LR^3$IGF-I at 2.5 µg/ml in coating buffer.

### 2.2.1.5.2 ISOLATION OF HYBRIDOMA CLONES

Only nineteen of the 50 expanded hybridoma cultures that were initially found to react with either solid phase IGF-I or solution phase [$^{125}$I]-iodo-IGF-I, or both, grew successfully and maintained consistent production of IGF-I antibodies. These 19 cultures were subcultured at limiting dilutions to produce monoclonal cultures of antibody producing hybridoma cells. The cells were suspended, counted on a hemocytometer and diluted to 10 cells/ml and 5 cells/ml in cloning medium (75% hybridoma medium containing 13.6 µg/ml hypoxanthine, 150 µg/ml oxalacetic acid, 20 µg/ml insulin and 25% SP2/Ø conditioned medium). One hundred µl aliquots of the 10 cells/ml and 5 cells/ml suspensions were added to culture wells. Thus the cells were theoretically dispersed to 1 and 0.5 cells/well.

Thirteen viable potential clones which produced antibodies that reacted positively with IGF-I coated microtitre plates were obtained. These cultures were expanded and cloned for a second time. Eight monoclonal cultures that produced antibodies to solid phase IGF-I were obtained from the second round of cloning. These were expanded further by culturing in 25 $cm^3$ and 75 $cm^3$ flasks in hybridoma culture medium. Cloned cells were also stored frozen in liquid nitrogen at -196 °C (see 2.2.1.7.1).

### 2.2.1.6 EXPANSION OF HYBRIDOMA CLONES *in vivo*

By growing hybridomas in the peritoneal cavity of mice, large quantities of monoclonal antibodies can be produced at relatively high concentrations (10-12 mg of antibody per ml of ascites fluid) without the need for large scale *in vitro* cell culture. BALB/c mice were primed by intraperitoneal injection of 0.5 ml pristane (2,6,10,14-tetramethyl pentadecane) 10 days before intraperitoneal inoculation with $10^6$ to $10^7$ hybridoma cells in 0.5 ml of sterile saline. After 5 to 11 days, when the mouse had swollen to approximately the proportions of a pregnant female near term, the mouse was sacrificed. The ascites fluid was removed from the peritoneal cavity with a syringe The ascites fluid was clarified by centrifugation and stored as aliquots at -70 °C.

### 2.2.1.7 FREEZING AND THAWING OF HYBRIDOMA CELLS

#### 2.2.1.7.1 FREEZING OF HYBRIDOMA CELLS

Only hybridoma cultures in log phase growth were used for freezing. The cells were suspended, centrifuged at 1200 rpm for 10 min in a microfuge and resuspended at $10^6$-$10^7$ cells/ml in freezing medium (9:1 of FCS to dimethyl sulphoxide). One ml aliquots of this suspension were pipetted into "cryotubes" (Nunc, Denmark) and sealed. The sealed cryotubes were placed immediately in a -70 °C freezer. After 24 h, the cells were removed and stored in a liquid nitrogen tank.

#### 2.2.1.7.2 THAWING OF HYBRIDOMA CELLS

The cryotubes containing frozen cells were thawed in a 37 °C water bath. The cells were transferred into a centrifuge tube diluted ten-fold with serum-free DMEM/F12 and centrifuged at 1200 rpm for 10 min at room temperature. The cell pellet was resuspended in serum-free DMEM/F12 and recentrifuged before resuspension in 1 ml of DMEM/F12 with 10% FCS. The cell suspension was then distributed into 96-well plates and cultured in hybridoma medium. The cell cultures were then expanded in a 24-well plate and grown in a 25 $cm^3$ tissue culture flask.

#### 2.2.1.8 DETERMINATION OF IMMUNOGLOBULIN ISOTYPES

Immunoglobulin isotypes were characterised with the mouse typer isotyping panel (BIO-RAD, California, U.S.A.). Sheep antiserum to mouse immunoglobulin (Silenus, DS, 2 mg/ml, diluted 1:3000 in coating buffer) was dispensed (100 µl/well) and plates were incubated at 37 °C for 4 h. After washing four times with washing buffer (PBS-Tween 20), wells were "blocked" at 4 °C overnight with 100 µl of 10 mg/ml BSA in PBS-Tween 20. Plates were washed another four times with PBS-Tween 20. Supernatant from each hybridoma culture was added to 9 wells (100 µl each) and incubated at 4 °C overnight. The plate was washed as above and 50 µl of purified rabbit anti-mouse subclass specific anti-serum (specific for either $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$, IgM, IgA, κ-chain or λ-chain) or 5 mg/ml BSA in PBS-Tween 20 as a negative control were added to the appropriate wells containing medium conditioned by each hybridoma culture. The plate was incubated for 1.5 h at 37 °C and rewashed with PBS-Tween 20. One hundred microlitres of peroxidase-conjugated goat anti-rabbit immunoglobulins (DAKOPATTS, Code No. P448, antibodies 0.25 g/l) diluted 1:2000 in 5 mg/ml BSA in PBS-Tween 20 was added to each well. After incubation for 1 h at 37 °C, the plate was washed and peroxidase activity measured as described above.

### 2.2.2 Rabbit polyclonal antibodies to IGF-I and $LR^3IGF$-I

Rabbit polyclonal antisera and immunoglobulins were either donated by Mr. S.E. Knowles (CSIRO Division of Human Nutrition) or obtained from GroPep Pty Ltd., Adelaide.

# CHAPTER 3

## Development, characterisation and application of an enzyme-linked immunosorbent assay for LR$^3$IGF-I

### 3.1 INTRODUCTION

The current procedure for measurement of LR$^3$IGF-I in biological fluids is radioimmunoassay (RIA) (Conlon *et al.* 1995*b*, Walton *et al.* 1996). Despite the low affinity of LR$^3$IGF-I for IGF-binding proteins, these binding proteins interfere in the LR$^3$IGF-I RIA in much the same way as they do in RIAs for IGF-I and IGF-II unless they are removed prior to assay (Scott & Baxter 1986, Daughaday *et al.* 1987, Owens *et al.* 1990, Breier *et al.* 1991). The preferred method is chromatographic extraction under acidic, dissociating conditions before RIA (Hintz & Liu 1977, Powell *et al.* 1986, Scott & Baxter 1986, Daughaday *et al.* 1987, Owens *et al.* 1990, Breier *et al.* 1991, Crawford *et al.* 1992). The determination of LR$^3$IGF-I in serum or plasma by RIA is therefore a tedious multi-step and time-consuming procedure since it requires chromatographic separation of IGFs from their binding proteins. This chapter describes the development, performance and characteristics of a sensitive and specific non-isotopic assay that can be used to measure LR$^3$IGF-I in blood plasma obtained from animals treated with this IGF-I analog.

## 3.2 EXPERIMENTAL

### 3.2.1 Complementary binding of LR$^3$IGF-I by two antibodies

Complementarity between antibodies, i.e. their abilities to simultaneously complex with LR$^3$IGF-I, was investigated in ELISA format. Eight different mouse monoclonal immunoglobulins (see 2.2.1.5) were used as solid phase antibodies and five different rabbit anti-LR$^3$IGF-I polyclonal immunoglobulins or sera and one rabbit anti-human IGF-I serum (see 2.2.2) were used in solution phase in these evaluations. Plate wells were firstly coated as described in section 2.2.1.5.1.1 with 100 µl of various mouse ascites fluids containing different monoclonal antibodies diluted to 1 in 500 or 1 in 1000 in coating buffer. These were used to capture LR$^3$IGF-I from 100 µl of 10 ng/ml LR$^3$IGF-I in PBS-Tween 20 containing BSA at 5 mg/ml during an incubation at 37 °C for 2 h. Various concentrations of different rabbit antisera (1 in 500, 1 in 1500 and 1 in 4500) diluted in 5 mg/ml BSA in PBS-Tween 20 including Nelson rabbit anti-LR$^3$IGF-I immunoglobulins and GroPep rabbit anti-LR$^3$IGF-I serum were then added in 100 µl and incubated for 2 h at 37 °C. Plates were washed and wells were then incubated with peroxidase-conjugated goat anti-rabbit immunoglobulins followed with substrate solution, as described in section 2.2.1.5.1.1.

### 3.2.2 Characterisation of antibodies

The complementary antibodies which were subsequently used for LR$^3$IGF-I ELISA, mouse IgG "1A7-F5-E5" and rabbit immunoglobulin "Nelson", as well as GroPep rabbit anti-LR$^3$IGF-I antiserum were further studied in solution phase to determine their affinity constants ($K_a$) and specificities. Antibody solutions (50 µl), diluted to bind 30-40% of

[$^{125}$I]-LR$^3$IGF-I, were incubated with 50 µl of [$^{125}$I]-LR$^3$IGF-I (20,000 cpm), 50 µl of unlabelled IGF peptides at concentrations ranging from 0.1 to 1000 nmol/l and 150 µl of RIA buffer overnight at 4 °C. [$^{125}$I]-LR$^3$IGF-I which bound to mouse antibodies was precipitated with goat anti-mouse Sac-Cel (see 2.2.1.5.1.3). [$^{125}$I]-LR$^3$IGF-I which bound to rabbit antibodies was precipitated by addition of 50 µl of 1/20 diluted sheep anti-rabbit IgG (Silenus, Code RC) and 10 µl of 1/200 diluted normal rabbit immunoglobulin fraction (DAKOPATTS, Code No. X903). After removal of the supernatants by aspiration, radioactivity in the pellet was measured in a gamma scintillation spectrometer. Affinity constants were estimated by Scatchard analysis (Scatchard 1949). The specificity of antibodies was characterized by the competition for LR$^3$IGF-I tracer binding to antibodies by related IGF peptides (IGF-I, IGF-II, long-IGF-I, R$^3$IGF-I and LR$^3$IGF-I).

### 3.2.3 Standard operating procedure LR$^3$IGF-I enzyme-linked immunosorbent assay

The following operating procedure for the LR$^3$IGF-I ELISA was developed after optimisation studies in which the effects of factors such as time, temperature, amount of antibodies, plate types and blocking conditions on specific and nonspecific binding were investigated (not shown).

Plates (Costar 96-well vinyl assay plates, Cat. No. 2595, Cambridge, MA, U.S.A.) were coated with mouse IgG 1A7-F5-E5 by adding 100 µl of ascites fluid diluted 1/200 (date 7/10/94) in coating buffer to each well and incubated standing for 3 h at 37 °C. After washing four times with PBS-Tween 20, each well was blocked by incubating with 100 µl of 20 mg/ml BSA in PBS-Tween 20 standing overnight at 4 °C. The plates were then washed four times with the same buffer. One hundred microlitres of 5 mg/ml BSA in PBS-Tween 20

containing either 1/30 diluted plasma from cows intravenously infused with $LR^3IGF$-I at 12 $\mu g.h^{-1}.kg^{-1}$ or $LR^3IGF$-I standard (0 to 6 ng/ml) with the same concentration of normal bovine plasma were then added to triplicate wells. The plates were incubated standing for 2 h at 37 °C and washed as above. Subsequently, 100 µl of Nelson rabbit anti-$LR^3IGF$-I immunoglobulins diluted 1:1000 in 5 mg/ml BSA in PBS-Tween 20 was added and incubated standing at 37 °C for 2 h. After washing four times as above, 100 µl of peroxidase-conjugated goat anti-rabbit immunoglobulins diluted 1 in 2000 with 5 mg/ml BSA in PBS-Tween 20 were added to the wells, followed by incubation for further 1.5 h standing at 37 °C. The plates were washed four times as above and 100 µl of peroxidase substrate solution (5.5 mmol/l o-phenylenediamine and 10 mmol/l hydrogen peroxide in 100 mmol/l citrate/phosphate buffer pH 5.0) was added. After 10 to 30 min incubation standing at room temperature, the reaction was stopped by addition of 50 µl of 1 mol/l sulphuric acid and the absorbance at 490 nm was measured using an automated microplate reader (BIO-TEK Instrument Inc., Highland Park, U.S.A.).

### 3.2.4 Characterisation of the $LR^3IGF$-I ELISA

#### 3.2.4.1 Specificity of $LR^3IGF$-I ELISA

The crossreactivities of IGF-I and IGF-II were determined by simultaneously testing solutions of IGF-I (0 to 1000 ng/ml) and IGF-II (0 to 5000 ng/ml), appropriately diluted in 5 mg/ml BSA in PBS-Tween 20, in addition to $LR^3IGF$-I.

#### 3.2.4.2 RECOVERY AND PRECISION OF LR$^3$IGF-I ELISA

Recovery was determined by measuring the increase in activity in the LR$^3$IGF-I ELISA produced by adding 100 µl of 10 ng/ml LR$^3$IGF-I assay standard to 30 µl of eleven different specimens of plasma from cows treated with this analog (Owens *et al.* 1994). These solutions were then diluted to a total volume 1 ml with 5 mg/ml BSA in PBS-Tween 20 and triplicates of 100 µl were assayed. Intra- and inter-assay variances were determined from the same analyses.

### 3.2.5 Effects of IGF-binding proteins on the LR$^3$IGF-I ELISA

The effects of IGF-binding proteins in plasma on the ELISA response to LR$^3$IGF-I were investigated by (i) adding LR$^3$IGF-I free plasma to LR$^3$IGF-I before assay; (ii) comparing ELISA measurements of the concentrations of LR$^3$IGF-I in plasma from LR$^3$IGF-I treated animals with and without prior chromatographic removal at pH 2.5 of IGF-binding proteins; and (iii) examing the effects of addition of pure IGF-binding protein-2 or -3 on the ELISA response to LR$^3$IGF-I.

The effect of blood plasma on this assay was assessed in two ways. Firstly, LR$^3$IGF-I assay standard at 5 ng/ml in different concentrations of normal bovine plasma was measured by ELISA. Secondly, LR$^3$IGF-I standard in the range of 0.1 to 5 ng/ml with or without 10% (vol/vol) normal bovine plasma was also measured. To determine whether IGF-binding proteins in plasma affect the estimates of LR$^3$IGF-I obtained by ELISA of diluted, unextracted plasma (*i.e.* assaying 100 ul of 3 % [vol/vol] plasma), the LR$^3$IGF-I content of plasma from cows infused with LR$^3$IGF-I was measured with and without size exclusion liquid chromatography at pH 2.5 prior to assay (Kind *et al.* 1995). For this experiment, 0.2

ml of 50% (vol/vol) plasma in mobile phase was chromatographed and the eluent containing free IGFs (*i.e.* unbound, dissociated from binding proteins) was collected in a volume of 2.25 ml. Triplicate aliquots were neutralised with Tris-base as above and 100 µl assayed for $LR^3IGF$-I by ELISA. Unextracted aliquots of the same plasma specimens were diluted to 3 % (vol/vol) in Tris-neutralised chromatography mobile phase. In this assay, the $LR^3IGF$-I standard was also prepared in neutralised mobile phase. Ovine IGF-binding protein-3 purified from plasma (Carr *et al.* 1994) and bovine IGF-binding protein-2 (a generous gift from Dr. Briony Forbes, Department of Biochemistry, University of Adelaide) were also added directly to the $LR^3IGF$-I standard and their effects on the ELISA response were determined.

### 3.2.6 RIA for $LR^3IGF$-I

$LR^3IGF$-I in blood plasma was also measured by RIA (Conlon *et al.* 1995*b*). Plasma was acidified to pH 2.5 to dissociate complexes between IGFs and binding proteins which were then separated by size exclusion high performance liquid chromatography at pH 2.5 (Gargosky *et al.* 1990*a*, Owens *et al.* 1990, Carr *et al.* 1995, Kind *et al.* 1995). The eluate containing IGFs was neutralized with Tris-base and its $LR^3IGF$-I content was measured by RIA. This step is necessary to completely remove IGF-binding proteins before $LR^3IGF$-I RIA because although unextracted plasma from rats treated with $LR^3IGF$-I (Tomas *et al* 1992, 1993*a*) produces significant activity in this RIA and the results correlate reasonably well with those obtained for the same specimens following size exclusion chromatography in acid ($r = 0.9$, $n = 34$, $p < 0.001$), the RIA of unextracted plasma produces estimates of the concentration of $LR^3IGF$-I that are ~40% lower than those obtained by this assay when IGF-binding proteins have been completely removed by the chromatographic procedure at pH 2.5

(Owens, Quinn & Knowles, unpublished observations). The LR$^3$IGF-I RIA was performed as described for IGF-I RIA (Carr *et al.* 1995, Kind *et al.* 1995) except that LR$^3$IGF-I was used as standard and for the preparation of radio-iodinated ligand and GroPep rabbit anti-LR$^3$IGF-I serum was used as primary antibody as reported previously (Conlon *et al.* 1995*b*).

## 3.3 RESULTS

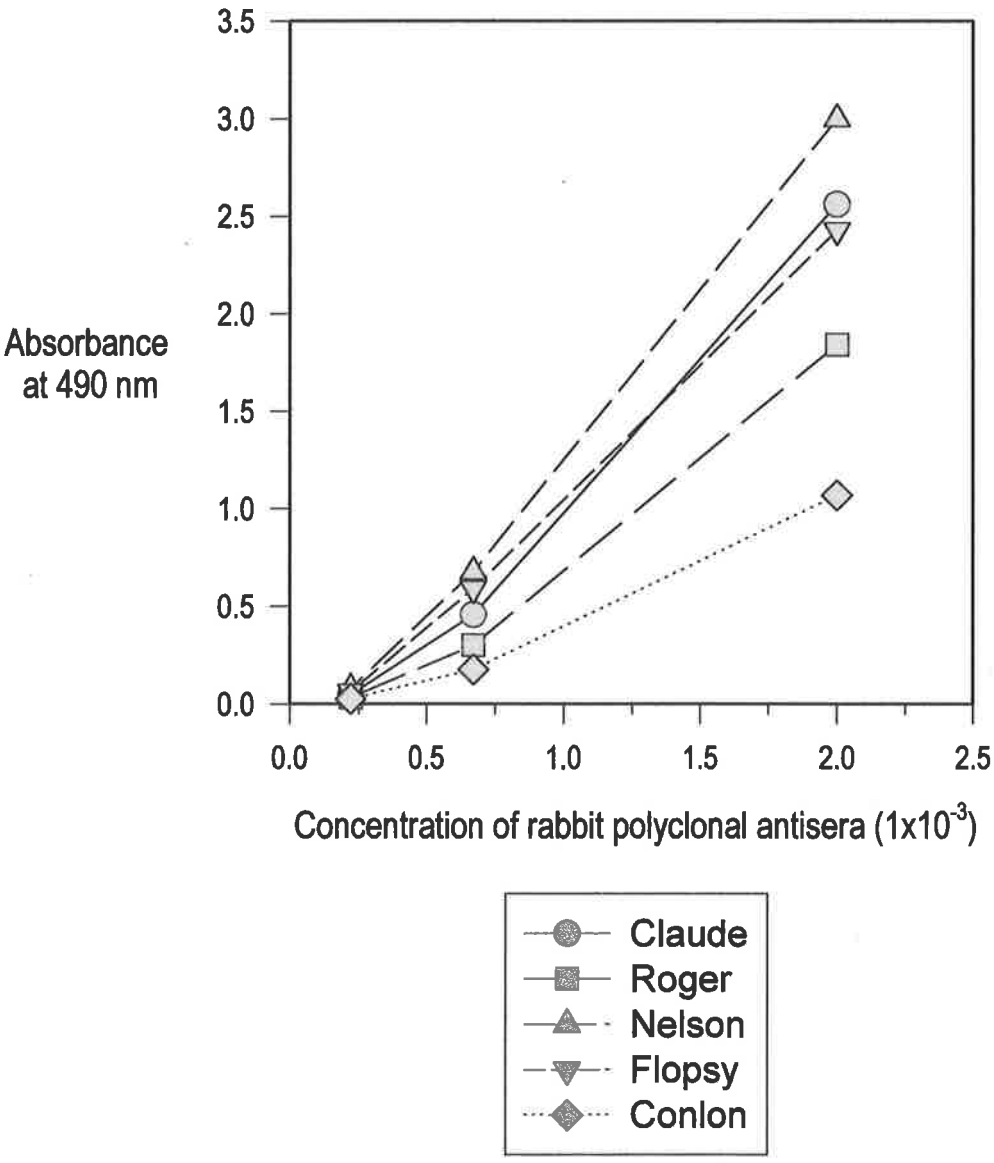
The abilities of different antibodies to bind simultaneously to LR$^3$IGF-I were determined by testing eight ascites fluids containing different mouse monoclonal antibodies as the solid phase antibody and six different rabbit polyclonal antibodies or antisera (all raised against either LR$^3$IGF-I or IGF-I) as the solution phase antibodies. In the presence of LR$^3$IGF-I, plates coated with monoclonal antibodies 1A7-F5-E5 and 1A7-F5-E8 were able to sequester five different rabbit polyclonal antibodies raised against either LR$^3$IGF-I or IGF-I. The combination between mouse IgG 1A7-F5-E5 (or 1A7-F5-E8) and Nelson rabbit anti-LR$^3$IGF-I immunoglobulins gave the highest sensitivity for LR$^3$IGF-I (Figure 3.1). None of the mouse antibodies could capture GroPep rabbit anti-LR$^3$IGF-I serum in the presence of LR$^3$IGF-I. The titre of 1A7-F5-E5 ascites fluid was higher than that of 1A7-F5-E8, and therefore 1A7-F5-E5 was selected for further evaluation in the development of an LR$^3$IGF-I ELISA.

The specificities of the complementary antibodies 1A7-F5-E5 and Nelson were compared with that of the non-complementary GroPep antiserum. This was done by solution phase procedures in which the abilities of unlabelled IGF peptides to inhibit antibody binding of [$^{125}$I]-LR$^3$IGF-I were measured (Figure 3.2). Scatchard analyses of the LR$^3$IGF-I binding

Sandwich ELISA for $LR^3IGF$-I using the combination between mouse IgG 1A7-F5-E5 as solid phase antibody and five different rabbit polyclonal antibodies raised against either $LR^3IGF$-I or IGF-I (namely, Claude, Roger, Nelson, Flopsy and Conlon) as solution phase antibody in the presence of $LR^3IGF$-I (100 µl of 10 ng/ml).

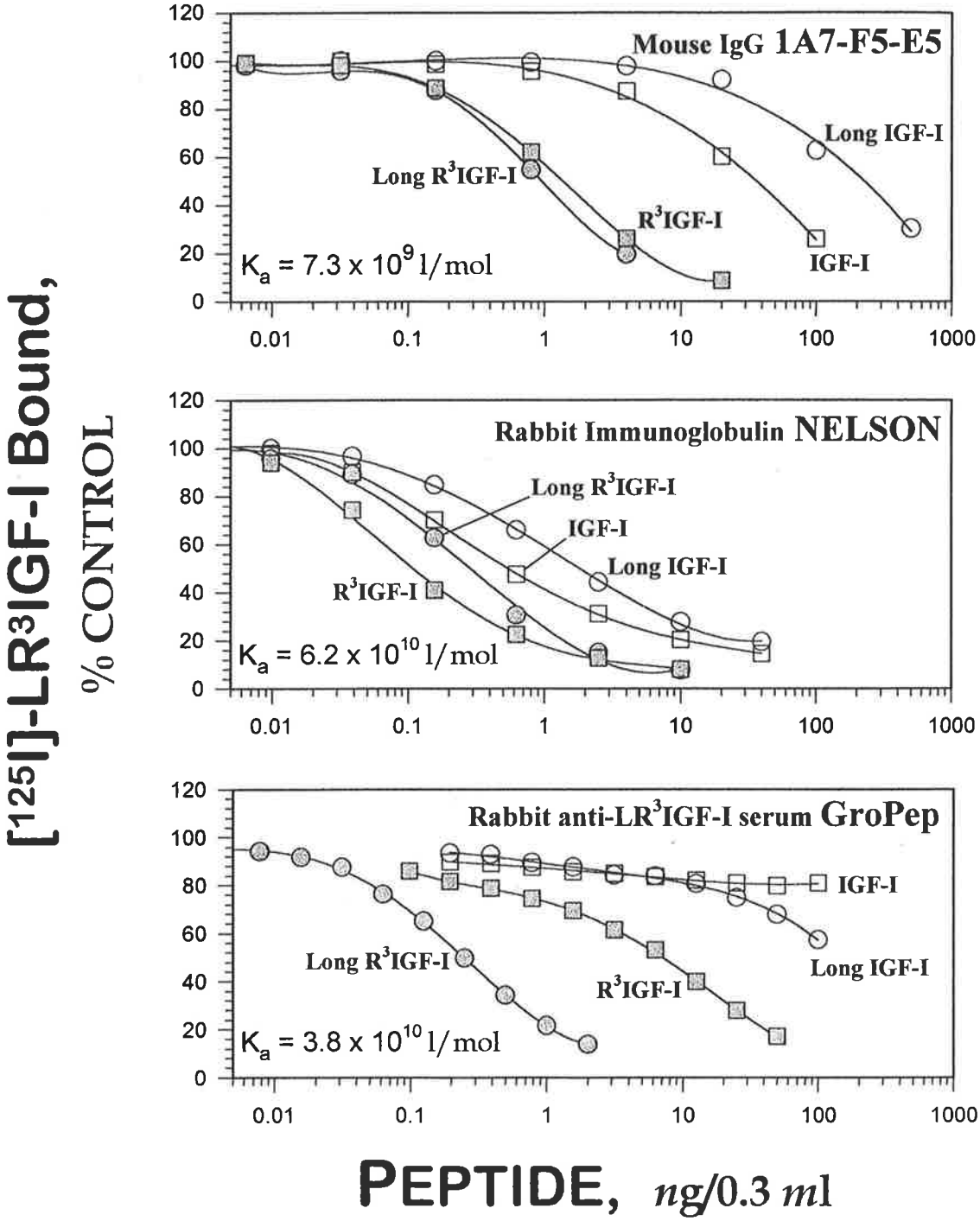
## Figure 3.1

**Detection of rabbit immunoglobulins captured by $LR^3IGF\text{-}I$ bound to immobilized mouse IgG 1A7-F5-E5**



Inhibition of [$^{125}$I]-LR$^3$IGF-I binding to mouse IgG 1A7-F5-E5 (upper panel), Nelson rabbit immunoglobulin (middle panel) and GroPep rabbit LR$^3$IGF-I antiserum (lower panel) in triplicate solutions of 300 µl by 20 pmol/l to 150 pmol/l of the unlabelled peptides (closed circle) LR$^3$IGF-I, (closed square) R$^3$IGF-I, (open circle) LongIGF-I, (open square) IGF-I and (not shown) IGF-II. The affinity constants (Ka) were determined by Scatchard analysis of the unlabelled LR$^3$IGF-I isotherm.

## Figure 3.2 ANTIBODY SPECIFICITY



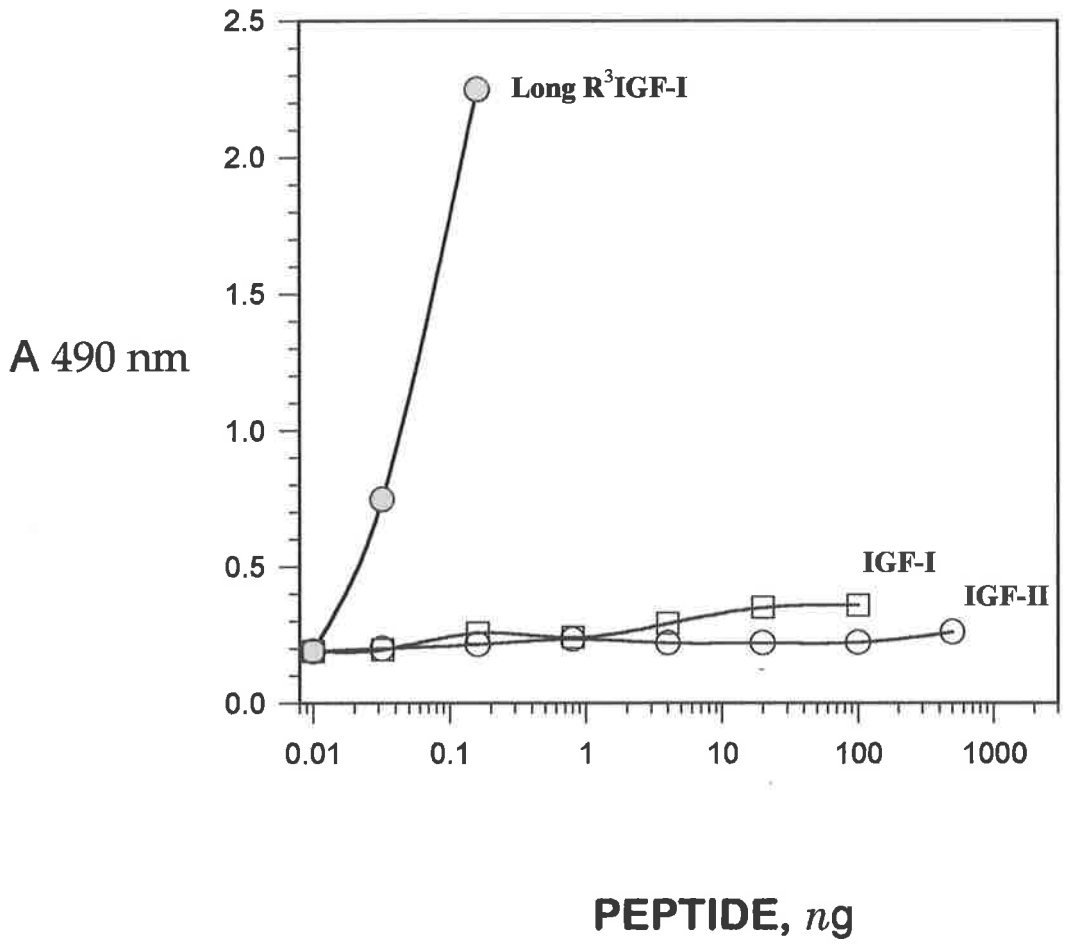
equilibrium data were also performed. For mouse IgG 1A7-F5-E5, which is the solid phase antibody used in the LR$^3$IGF-I ELISA, the relative potencies of LR$^3$IGF-I, R$^3$IGF-I, IGF-I and long-IGF-I respectively were 1.0 : 0.78 : 0.028 : 0.005 on a mass basis and the equilibrium constant for association with LR$^3$IGF-I was 7.3 x $10^9$ l/mol (Figure 3.2, upper panel). Significant competition from IGF-II (not shown) could not be detected at concentrations as high as 1 µg/ml. For Nelson rabbit immunoglobulin, which is the solution phase antibody used in the LR$^3$IGF-I ELISA, relative potencies of LR$^3$IGF-I, R$^3$IGF-I, IGF-I, long-IGF-I (Figure 3.2, middle panel) and IGF-II (not shown) were 1.0 : 2.5 : 0.4 : 0.15 : 0.0005 respectively and the $K_a$ was 62 x $10^9$ l/mol. For GroPep anti-LR$^3$IGF-I serum, which is the primary antibody used in the RIA for LR$^3$IGF-I (Conlon *et al.* 1995*b*) and which cannot bind to LR$^3$IGF-I while the latter is complexed to mouse IgG 1A7-F5-E5, the relative potencies of LR$^3$IGF-I, R$^3$IGF-I, IGF-I and long-IGF-I respectively were 1.0 : 0.03 : <0.001 : 0.002 (Figure 3.2, lower panel), while competition from IGF-II (0.01 to 1 µg/ml) could not be detected (not shown). The $K_a$ was 38 x $10^9$ l/mol for GroPep anti-LR$^3$IGF-I serum.

An ELISA for LR$^3$IGF-I was developed using the combination of mouse IgG 1A7-F5-E5 as solid phase antibody and Nelson rabbit anti-LR$^3$IGF-I immunoglobulins as solution phase antibody. The assay was optimized for maximal sensitivity and minimal non-specific activity by varying the concentration of mouse IgG 1A7-F5-E5 added to the plate in the coating step and by varying the concentration of Nelson rabbit immunoglobulin added after addition of LR$^3$IGF-I polypeptide. IGF-I and IGF-II have less than 0.01% of the activity of LR$^3$IGF-I in this system (Figure 3.3). A semi-linear LR$^3$IGF-I response (Figure 3.4, lower panel) was obtained over the range of 0.5 to 5 ng/ml (i.e. 50 pg to 500 pg LR$^3$IGF-I per 100 µl assayed) and plasma was found to slightly inhibit this response (Figure 3.4, upper and lower panels).

Spectrophotometric absorbance at 490 nm produced by triplicate 100 µl solutions of (closed circle) $LR^3IGF$-I, (open square) IGF-I and (open circle) IGF-II in the $LR^3IGF$-I ELISA.

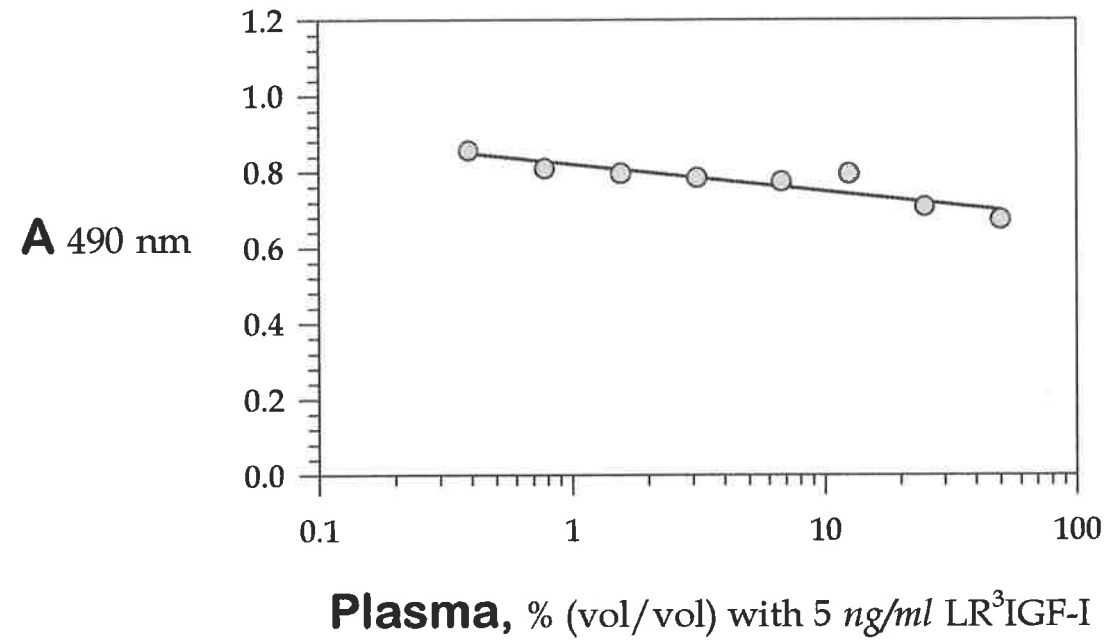
## Figure 3.3

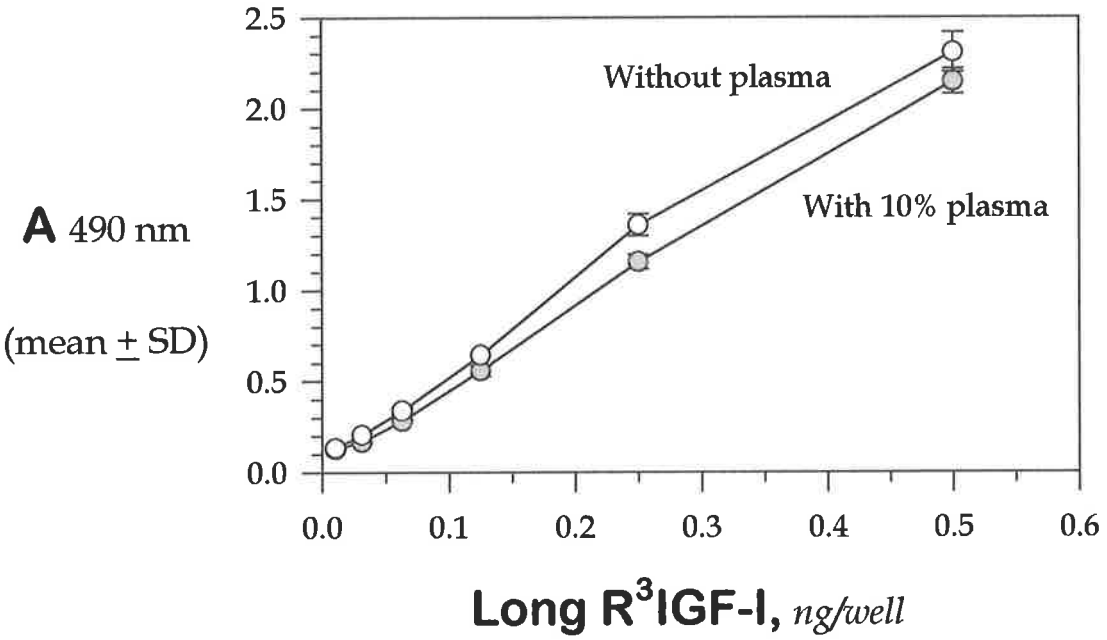
**ELISA SPECIFICITY**



## Figure 3.4

### EFFECT OF BLOOD PLASMA ON LR$^3$IGF-I ELISA




The upper panel shows the effect of 0.39% to 50% (v/v) normal bovine plasma on the response produced by triplicate 100 ul solutions also containing 5 ng/ml LR$^3$IGF-I. The lower panel compares the responses produced by triplicate 100 ul solutions of 0.1 to 5 ng/ml LR$^3$IGF-I (open circle) in the absence and (closed circle) in the presence of 10% (v/v) normal bovine plasma.

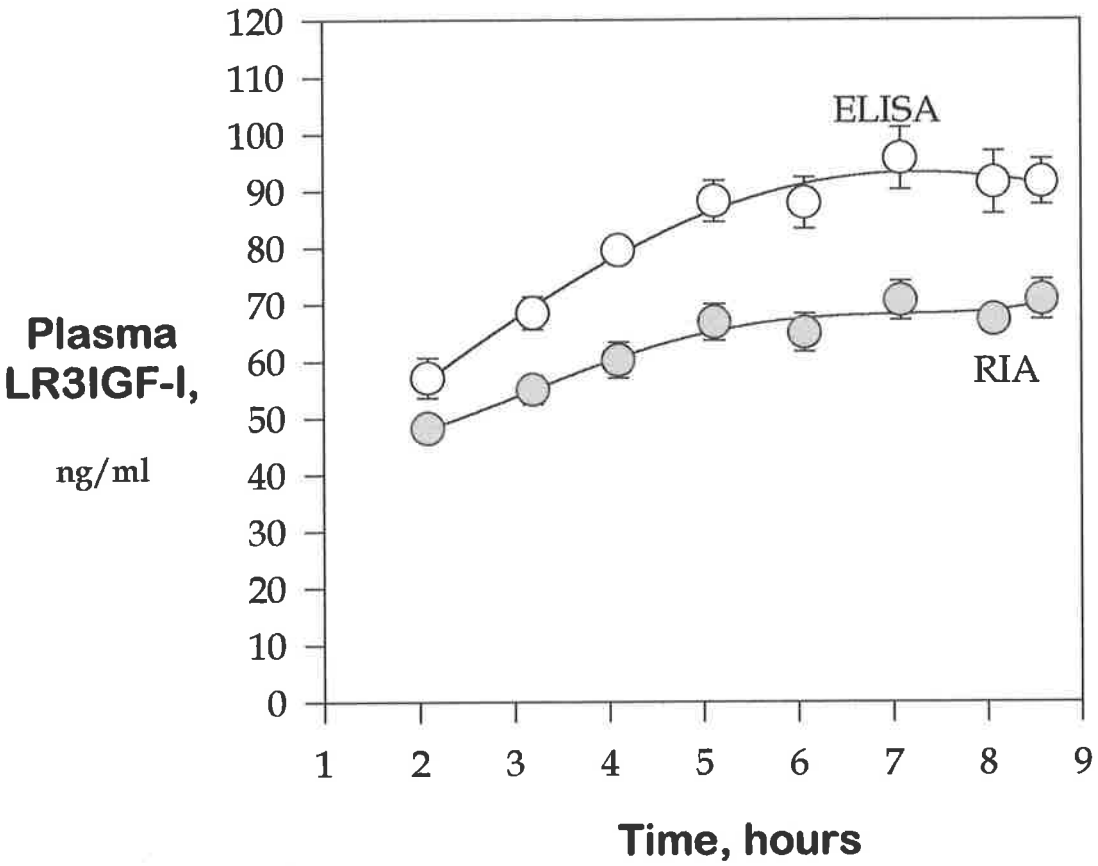
Based on the increase in activity detected in the ELISA due to *in vitro* addition of the assay standard to eleven different specimen of plasma from cows treated with LR$^3$IGF-I, the recovery was 91 ± 11% (mean ± SD, n = 11). The intra-assay CV (n = 12 replicates) was 2.8 % and the inter-assay CV (n = 6 assays) was 7.3 % for a plasma sample obtained from an LR$^3$IGF-I treated cow whose average concentration of LR$^3$IGF-I was 79 ng/ml of which 100 µl of a 3 % (vol/vol) solution was measured in triplicate.

The effect of endogenous plasma IGF-binding proteins on the measurement of LR$^3$IGF-I in 100 µl of 3 % (vol/vol) plasma was estimated by ELISA measurement of LR$^3$IGF-I in plasma from cows treated with this IGF analogue before ($x$) and after chromatography under acidic conditions that completely dissociate and separate IGFs from binding proteins ($y$). Regression analysis showed a slope that was not significantly different from unity and an intercept that was not significantly different from zero ($y = 0.99x + 6.3$, $r = 0.70$, $n = 12$, $p < 0.02$), considering the intra-assay CV is 2.8%. The results for unextracted plasma were therefore not significantly different from those obtained for IGF-binding protein-free column eluates. Inclusion of pure ovine IGFBP-3 at 9 ng/well (equivalent to 3 µg/ml plasma) with LR$^3$IGF-I at 300 pg/well (equivalent to 100 ng/ml plasma) as well as pure bovine IGFBP-2 at 3 ng/well with LR$^3$IGF-I at 300 pg/well also had no significant effect on the ELISA response (data not shown).

The concentrations of LR$^3$IGF-I in plasma from cows treated with LR$^3$IGF-I were measured using both ELISA of diluted unextracted plasma and LR$^3$IGF-I RIA (Figure 3.2, bottom panel) of binding protein-free extracts obtained by size exclusion chromatography of plasma at pH 2.5. The concentrations of LR$^3$IGF-I measured by extracted RIA were lower than those measured by unextracted ELISA (Figure 3.5) but there was a similar trend of

**Figure 3.5**

**COMPARISON OF RIA AND ELISA MEASUREMENTS**




Effect of intravenous infusion of $LR^3IGF\text{-}I$ at 12 $ug.kg^{-1}.h^{-1}$ in cows (n=6) on the concentrations of the infused polypeptide (open circle) as measured by ELISA of unextracted 3% (v/v) plasma and (closed circle) as measured by RIA after size exclusion high performance liquid chromatography of plasma in acid.

$LR^3IGF$-I concentration over time detected by both methods. Plasma levels of $LR^3IGF$-I were maximal after 7 h intravenous infusion at 12 $\mu g.kg^{-1}.h^{-1}$ at 95.7 ± 5.5 ng/ml (mean ± sem, n=6) by ELISA and at 71.7 ± 3.5 ng/ml by RIA. $LR^3IGF$-I was undetectable by both assays in the plasma from placebo-treated cows. There was a positive correlation between the results of the two $LR^3IGF$-I assays ($r = 0.54$, $n = 48$, $p < 0.001$).

## 3.4 DISCUSSION

The abilities of mouse IgG 1A7-F5-E5 and Nelson rabbit immunoglobulins to simultaneously bind to $LR^3IGF$-I indicate that these antibodies associate with different, and presumably remote, epitopes on the $LR^3IGF$-I protein. The inability of GroPep rabbit anti-$LR^3IGF$-I serum and mouse IgG 1A7-F5-E5 to simultaneously bind $LR^3IGF$-I indicate that these antibodies bind to similar or adjacent epitopes. These conclusions are consistent with the specificities of these antibodies. The arginine$^3$ substitution markedly increases the affinities of IGF-I and long-IGF-I for both mouse IgG 1A7-F5-E5 and GroPep rabbit anti-$LR^3IGF$-I serum. $LR^3IGF$-I is ~200-times as potent as long-IGF-I and $R^3IGF$-I is ~30-times as effective as IGF-I at inhibiting the binding of [$^{125}$I]-$LR^3IGF$-I to mouse IgG 1A7-F5-E5. With GroPep rabbit anti-$LR^3IGF$-I serum these ratios are similar, *i.e.* $LR^3IGF$-I:long-IGF-I ≈ 500:1 and $R^3IGF$-I:IGF-I ≈ 30:1. The specificity of Nelson rabbit immunoglobulin is very different. Firstly, $R^3IGF$-I, not $LR^3IGF$-I, is the peptide most effective at inhibiting [$^{125}$I]-$LR^3IGF$-I binding to Nelson immunoglobulin. Secondly, the arginine$^3$ substitution has a much smaller effect on the ability of either long-IGF-I or IGF-I to inhibit binding of [$^{125}$I]-$LR^3IGF$-I to this antibody, *i.e.* $LR^3IGF$-I:long-IGF-I ≈ 7:1 and $R^3IGF$-I:IGF-I ≈ 6:1, compared to its effect on the equivalent reactions with the other two antibodies. This suggests that the active antibodies in the Nelson rabbit immunoglobulin bind to different

epitopes in LR$^3$IGF-I than do those of mouse IgG 1A7-F5-E5 and GroPep rabbit anti-LR$^3$IGF-I. This is consistent with the simultaneous binding of LR$^3$IGF-I observed with 1A7-F5-E5 and Nelson that was not seen with the combination of 1A7-F5-E5 IgG and the GroPep antiserum.

Studies of the effects of *in vitro* incubations of LR$^3$IGF-I and normal bovine blood plasma showed that the ELISA detects LR$^3$IGF-I in the presence of plasma, although high concentrations of plasma (> 5% vol/vol) slightly reduce the response. However, the high assay sensitivity combined with the relatively high circulating concentrations achieved by intravenous infusion of LR$^3$IGF-I at a pharmacologically active dose rate permitted a 33-fold dilution of the test plasma before analysis by ELISA. At this concentration (3 % plasma, vol/vol) the effect of LR$^3$IGF-I-free plasma on the response to LR$^3$IGF-I was very small. This effect does not appear to be due to plasma IGF-binding proteins because elimination of IGF-binding proteins from specimens tested in the ELISA by prior acidic size exclusion chromatography had no significant effect on the ELISA measurements and addition of pure IGF-binding protein-2 and -3 had no effect on the ELISA response to LR$^3$IGF-I. None-the-less, standards were routinely prepared in 3 % normal bovine plasma for these measurements.

An advantage of the new ELISA over the established RIA is the elimination of the need for an extraction step to remove IGF-binding proteins from the specimens to be assayed. IGF-binding protein contamination is a major problem in all IGF-I and IGF-II RIAs (Hintz & Liu 1977, Powell *et al.* 1986, Daughaday *et al.* 1987, Owens *et al.* 1990, Breier *et al.* 1991, Crawford *et al.* 1992). In principle, IGF-binding proteins cause different problems in competitive tracer binding systems such as those used for RIA than those caused in noncompetitive ELISA systems. Because RIA procedures depend on competition between fixed amounts of labelled antigen (tracer) and variable amounts of unlabelled antigen for

binding to fixed amounts of antibody, additional reactions that affect binding to antibody of either tracer or unlabelled antigen can cause estimation errors in these analyses. IGF-binding proteins in RIA procedures can bind to either radiolabelled or unlabelled IGFs and this can inhibit binding of these ligands to the antibody. Inhibition of labelled antigen (tracer) binding to antibody due to formation of complexes between binding proteins originating in the test specimens and tracer added to the incubation with primary antibody will produce overestimation artefacts in RIA procedures. Conversely, inhibition of unlabelled antigen (*i.e.* the target test material) binding to antibody due to formation of complexes between binding proteins and unlabelled IGFs can produce underestimation errors. Artefacts of both types have been observed for IGF-I RIAs, although underestimation errors are most common. When the LR$^3$IGF-I RIA was used to measure LR$^3$IGF-I in plasma from rats treated for one week with this IGF analogue (Tomas *et al.* 1992, 1993*a*), the results obtained for diluted unextracted plasma were found to correlate reasonably well ($r = 0.9$, $p < 0.001$) with those obtained for the same specimens when IGF-binding proteins were completely removed from the assay system by prior size exclusion chromatography of plasma under conditions that dissociate IGFs from IGF-binding proteins. However, the values obtained by RIA of unextracted plasma were consistently about 40% lower than those obtained by the same LR$^3$IGF-I RIA of the same plasma samples after acidic size exclusion chromatography. This indicates that some of the unlabelled LR$^3$IGF-I has formed complexes with IGF-binding proteins in the primary RIA incubation, which has reduced the ability of LR$^3$IGF-I to react with the primary antibody and thus reduced its ability to inhibit tracer binding to this antibody. Overestimation errors were never observed in the LR$^3$IGF-I RIA, indicating that [$^{125}$I]-iodo-LR$^3$IGF-I, unlike unlabelled LR$^3$IGF-I, does not significantly associate with IGF-binding proteins under the same assay conditions. This would occur if [$^{125}$I]-iodo-LR$^3$IGF-I has a lower affinity than unlabelled LR$^3$IGF-I for IGF-binding proteins. This is possible

because iodination of IGFs does alter their binding characteristics. For example, iodination of IGF-I has been shown to reduce the affinity of IGF-I for binding to IGFBP-3 from pregnant human serum (Suikkari & Baxter 1991) and radio-iodination of IGF-II abolishes its ability to bind to type-I IGF receptors on cultured rat myoblasts (Francis *et al.* 1989*a*). In the LR$^3$IGF-I ELISA, the presence of IGF-binding proteins in specimens added to the assay did not reduce the ability of LR$^3$IGF-I to form ternary complexes with the assay antibodies. This has also been observed with IGF-I ELISA (Khosravi *et al.* 1996). This difference in performance of the RIA and the ELISA is not due the differences in affinities of different antibodies for LR$^3$IGF-I because the GroPep rabbit antiserum used in the RIA has a higher affinity ($3.8 \times 10^{10}$ l/mol) than the solid phase monoclonal IgG ($0.73 \times 10^{10}$ l/mol) used in the ELISA. It is most likely due to the differences in the concentrations of antibodies to which the antigen is exposed in the two systems. In the RIA, the antiserum is highly diluted to achieve maximum sensitivity, so that the primary antibody can be almost completely saturated (*i.e.* no radioligand bound) by the highest concentration of unlabelled standard and test ligand. Conversely in the ELISA, maximum sensitivity is achieved at the highest possible concentrations of antibodies, which are added in excess (Khosravi *et al.* 1996).

In conclusion, the results presented in this chapter show that a sensitive, specific non-isotopic assay for an analog of insulin-like growth factor-I that detects biologically active concentrations of this growth factor in animals has been developed. This new assay of LR$^3$IGF-I in blood plasma does not require extraction of plasma specimens. This assay permits discrimination between the exogenous synthetic IGF-I analog and the endogenous native IGF-I and IGF-II in animals treated with this growth factor.

# CHAPTER 4

## Molecular forms of $LR^3IGF$-I in blood plasma from animals treated with pharmacologically active doses of this growth factor analog

### 4.1 INTRODUCTION

$LR^3IGF$-I is more potent than IGF-I under many conditions *in vitro* and *in vivo*. The higher potency in rats of $LR^3IGF$-I compared to wild type IGF-I and IGF-II despite the slightly lower affinity of $LR^3IGF$-I for the type-1 IGF receptor appears to be due to its much lower affinity for IGF-binding proteins. The results of these and other studies (Lewitt *et al.* 1991, 1993*b*) support the view that IGF-binding proteins are generally inhibitory to the actions of IGFs in the blood of rats.

However, treatment with insulin-like growth factors does not generally promote growth of non-rodents. For example, continuous s.c. infusion of IGF-I at 6 $\mu g.kg^{-1}.h^{-1}$ into well fed lambs for 8 days did not affect growth rate although it significantly increased plasma IGF-I and reduced blood urea (Moritz *et al.* 1996). Also, similar treatment of well-fed guinea pigs for 7 days with IGF-I, IGF-II or $LR^3IGF$-I at 14 $\mu g.kg^{-1}.h^{-1}$ did not improve liveweight gain although the weights of several visceral organs were increased (Conlon *et al.* 1995*b*). Furthermore, 7 days s.c. infusion of IGF-I at 0.75, 2.25 and 7.5 $\mu g.kg^{-1}.h^{-1}$ into well fed adolescent pigs did not increase growth rate and identical treatment with $LR^3IGF$-I actually reduced the rate of gain of live weight (Walton *et al.* 1996).

It has been suggested that the higher potency in rats of $LR^3$IGF-I compared to IGF-I in terms of growth promotion could be ascribed to higher concentrations of IGFs in the free form during treatment with $LR^3$IGF-I than with IGF-I (Tomas *et al.* 1992, 1993*a*, 1993*b*, Lord *et al.* 1994). This may not necessarily be extended to other species because, although [$^{125}$I]-iodo-$LR^3$IGF-I exhibits extremely weak binding to proteins in rat plasma, it does form complexes with binding proteins when added to plasma from sheep, pig, human and chicken (Lord *et al.* 1994).

Several previous studies have examined the *in vivo* association of IGFs with binding proteins in blood by injection of trace amounts of [$^{125}$I]-iodinated IGFs and shown that free IGFs are cleared more rapidly from the circulation (*e.g.* Cohen & Nissley 1976, Drakenberg *et al.* 1990, Francis *et al.* 1990, Bastian *et al.* 1993). However, radio-iodination of IGFs changes their affinities for IGF-binding proteins (Suikkari & Baxter 1991, Moss *et al.* 1991) and IGF receptors (Ballard *et al.* 1987, Francis *et al.* 1989*a*, Gelato *et al.* 1989). Also, unlike injection of trace amounts of radio-iodinated IGFs, treatment with biologically active doses of IGFs alters the concentrations in blood of endogenous IGFs and IGF-binding proteins (Tomas *et al.* 1991*a*, 1992, Owens *et al.* 1994, Conlon *et al.* 1995*a*, 1995*b*). The relative concentrations of IGFs and IGF-binding proteins in blood are likely to affect the formation of complexes between IGF-binding proteins and administered IGFs. Incubation of IGF-I with binding proteins prior to injection reduces the pharmacological effect of IGF-I on blood glucose concentrations (Lewitt *et al.* 1991, 1993*b*). Therefore the *in vivo* formation of complexes between IGF-binding proteins and IGF-I is likely to affect both the endocrine actions of endogenous IGF-I and the pharmacological actions of administered IGF-I.

The molecular form(s) of $LR^3IGF$-I in blood during treatment of animals with pharmacologically active doses of this growth factor were therefore investigated in the studies described in this chapter. Blood plasma was obtained from fasted heifers acutely infused intravenously with $LR^3IGF$-I (Owens *et al.* 1994) and from guinea pigs chronically infused subcutaneously with this IGF analog (Conlon *et al.* 1995*b*).

Acute intravenous infusion of $LR^3IGF$-I into cows produced a number of metabolic responses (Owens *et al.* 1994). After 7 h of intravenous infusion of $LR^3IGF$-I at 12 $\mu g.kg^{-1}.h^{-1}$ into fasted heifers, plasma glucose concentrations (1.15 ± 0.13 μmol/l, mean ± SEM, n=6) were significantly lower ($p<0.001$) than those in similar animals infused with vehicle (3.81 ± 0.49 μmol/l, n=6). This treatment with $LR^3IGF$-I also reduced the concentration in blood of histidine, leucine, lysine, isoleucine, phenyl-alanine, tryptophan, methionine, valine, tyrosine, alanine, glycine, threonine, glutamine, serine and glutamate ($p<0.02$). The plasma concentrations of IGF-I and IGF-II in these animals were also reduced, being, respectively, 93 ± 8 ng/ml and 266 ± 15 ng/ml in vehicle-treated animals and 53 ± 7 and 136 ± 19 ng/ml in animals treated with $LR^3IGF$-I. Treatment of fasted heifers with $LR^3IGF$-I also increased plasma levels of an IGF-binding protein of ~31-32 kDa in this study (Owens *et al.* 1994).

Chronic treatment of guinea pigs with $LR^3IGF$-I affected the growth of several tissues (Conlon *et al.* 1995*b*). In well-fed guinea pigs, 7 days of s.c. infusion of $LR^3IGF$-I at 14 $\mu g.kg^{-1}.h^{-1}$ increased the fractional weights (*i.e.* organ weight as a proportion of total body weight) of adrenals, gut, kidneys and spleen (Conlon *et al.* 1995*b*). Treatment with $LR^3IGF$-I also reduced both IGF-I and IGF-II concentrations in plasma of these guinea pigs (mean ± SEM, n=8), being respectively 192 ± 27 ng/ml and 745 ± 60 ng/ml in vehicle treated animals

and 71 ± 9 ng/ml and 234 ± 30 ng/ml in $LR^3IGF$-I treated animals. $LR^3IGF$-I treatment also lowered concentrations of IGFBP-3 and increased those of ~28 and ~31 kDa binding proteins in guinea pig plasma as measured by western radioligand blotting (Conlon *et al.* 1995*b*).

The availability of a simple assay that measures $LR^3IGF$-I in blood plasma without significant crossreaction from either of the endogenous IGFs and without significant interference from IGF-binding proteins enabled the following investigations to be undertaken into the molecular size classes of circulating $LR^3IGF$-I formed *in vivo* during treatment of animals with biologically active doses of this IGF analog.

## 4.2 EXPERIMENTAL

### 4.2.1 Blood Plasma

Frozen plasma was obtained from fasted cows continuously infused intravenously with vehicle or with $LR^3IGF$-I at 12 $\mu g.kg^{-1}.h^{-1}$ for 7 hours (Owens *et al.* 1994) and from well fed guinea pigs continuously infused subcutaneously with vehicle or with $LR^3IGF$-I at 14 $\mu g.kg^{-1}.h^{-1}$ for 7 days (Conlon *et al.* 1995*b*). Limited quantities of guinea pig plasma were generously donated by Dr. Frank Tomas, CSIRO Division of Human Nutrition.

### 4.2.2 Fractionation of plasma by size exclusion liquid chromatography at pH 7.4

Plasma was chromatographed through an Ultraspherogel SEC 2000 column (7.5mm x 30 cm, Beckman, U.S.A.) under nondissociating conditions at physiological pH and salt concentrations. Plasma was delipidated before chromatography by extraction with an equal

volume of 1,1,2,-trichloro-1,2,2,-trifluoroethane. Fifty µl of defatted plasma was applied to the column and eluted at 1.0 ml/min in a mobile phase of phosphate-buffered saline pH 7.4 (38.8 mmol/l $NaH_2PO_4$, 61.2 mmol/l $Na_2HPO_4$, 150 mmol/l NaCl, containing 1 mmol/l EDTA, 0.05% [v/v] Tween-20 and 0.02% [w/v] $NaN_3$). Fractions of 250 µl were collected and replicates of each fraction were assayed for $LR^3IGF$-I content by ELISA (4.2.3) and by RIA (4.2.4). The column was calibrated with [$^{125}$I]-$LR^3IGF$-I, [$^{125}$I]-IGF-I complexed to pure recombinant bovine IGFBP-2 and with [$^{125}$I]-$LR^3IGF$-I complexed to mouse IgG 1A7-F5-E5. Bovine IGFBP-2 was kindly provided by Dr. Briony Forbes, Department of Biochemistry, University of Adelaide.

To determine whether guinea pig plasma could alter the apparent molecular size of $LR^3IGF$-I *in vitro*, normal guinea pig plasma was incubated with $LR^3IGF$-I before chromatography as follows. Plasma was pooled from the vehicle-treated guinea pigs in the study of Conlon *et al.* (1995*b*) and defatted as above. Two hundred and twenty-five µl of this guinea pig plasma pool was incubated with 25 µl of 600 ng/ml $LR^3IGF$-I assay standard in pH 7.4 chromatography mobile phase for 1 h at room temperature. Fifty µl of this solution of 90% [v/v] plasma containing 60 ng/ml $LR^3IGF$-I was fractionated by size exclusion chromatography at pH 7.4 and the eluate analysed by ELISA and RIA (4.2.3, 4.2.4).

### 4.2.3 $LR^3IGF$-I enzyme-linked immunosorbent assay of chromatography eluates

The ELISA for $LR^3IGF$-I was performed essentially as described in 3.2.3. Microtitre plates were coated with mouse IgG 1A7-F5-E5, washed and "blocked" with BSA. The plates were then again washed and 100 µl of undiluted fractions of column eluate or $LR^3IGF$-I standard (0 to 5 ng/ml) in pH 7.4 mobile phase were added to duplicate wells. For assay of

$LR^3IGF$-I in unfractionated plasma, 100 µl of 5 mg/ml BSA in washing buffer containing 1/30 diluted plasma from cows or 1/20 diluted plasma from guinea pigs was used and $LR^3IGF$-I standard (0 to 5 ng/ml) was prepared in the same solution containing normal plasma (either cow or guinea pig as appropriate) at the same concentration as the test plasma from $LR^3IGF$-I treated animals. The plates were incubated standing for 2 h at 37 °C, washed and incubated standing for 2 h at 37 °C with Nelson rabbit anti-$LR^3IGF$-I immunoglobulins diluted 1:1000 in washing buffer containing 5 mg/ml BSA. After washing, 100 µl of peroxidase-conjugated goat anti-rabbit immunoglobulins (1:2000) in washing buffer containing 5 mg/ml BSA was added to the wells, followed by incubation for a further 1.5 h standing at 37 °C. The plates were washed again and peroxidase activity measured (3.2.3).

### 4.2.4 $LR^3IGF$-I RIA of chromatography eluates

The above analyses were compared to those obtained by RIA. For analysis of $LR^3IGF$-I in Beckman SEC 2000 pH 7.4 column fractions by RIA, 50 µl of undiluted column fractions, 50 µl of rabbit anti-$LR^3IGF$-I serum (GroPep, diluted in RIA buffer as recommended by the distributor), 50 µl of sheep anti-rabbit immunoglobulin diluted 1/20 in RIA buffer and 10 µl of rabbit IgG diluted 1/200 in RIA buffer were used (3.2.6).

### 4.2.5 Characterization of high molecular weight form(s) of $LR^3IGF$-I

#### 4.2.5.1 Conversion of high molecular weight forms of $LR^3IGF$-I in guinea pig plasma to low molecular weight forms by *in vitro* addition of IGF-I and IGF-II

Replicates of plasma (50 µl) from a guinea pig infused with $LR^3IGF$-I were acidified to pH 2.5 by addition of 6.7 µl of 1 mol/l HCl and incubated standing for 80 min at 37 °C to dissociate any complexes between IGFs and binding proteins. Ten µl of mobile phase of phosphate-buffered saline pH 7.4 (4.2.2) containing either 25 µg/ml IGF-I, IGF-II, insulin or no peptides was then added. The pH of these mixtures was then adjusted to 7.4 by the addition of 12 µl of 0.427 mol/l Tris-base. The solutions were incubated standing at 37 °C for 60 min before chromatography at pH 7.4 (4.2.2). Duplicate one hundred µl aliquots of each fraction were assayed for $LR^3IGF$-I by the ELISA procedure (4.2.3).

#### 4.2.5.2 Determination by Western ligand blotting of the IGF-binding protein content of pH 7.4 chromatography eluates of plasma containing a high molecular weight form of $LR^3IGF$-I

Fractions obtained from size exclusion hplc at pH 7.4 of plasma from guinea pigs treated with $LR^3IGF$-I were analysed by Western-radioligand blotting (Hossenlopp *et al.* 1986). Fifty µl aliquots of column fractions were incubated with 17 µl of 4 x SDS loading buffer pH 6.9 (250 mmol/l Tris-base, 8% [w/v] SDS, 20% [v/v] glycerol and 0.06 mmol/l bromophenol blue) at 65 °C for 20 min. Twenty µl of these solutions were subjected to discontinuous SDS-polyacrylamide gel electrophoresis. $^{14}C$-labelled Rainbow molecular weight markers (Amersham, Bucks, UK) and unfractionated plasma from a guinea pig infused

with $LR^3IGF$-I were included on this gel. Plasma (5 µl) was mixed with 145 µl of water and 50 µl of 4 x SDS loading buffer and heated to 65 °C for 20 min. Twenty µl of this solution (equivalent to 0.5 µl of plasma) was also subjected to discontinuous SDS-polyacrylamide gel electrophoresis. This was carried out according to the method of Laemmli (1970) using a 4% stacking gel and a 10% separating gel (140 mm wide, 160 mm deep and 1.5 mm thick). All steps were performed at room temperature. Following electrophoresis overnight at 10 mA, proteins were electroeluted from the gel onto a 0.45 µm nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany) at 250 mA for 3.5 h in Tris/glycine buffer (0.2 mol/l Tris-base, 0.15 mol/l glycine, 20% [v/v] ethanol). The nitrocellulose filter was allowed to dry in air overnight. The following incubations were performed on a shaker at 150 rpm. The filter was washed for 0.5 h in 200 ml of Tris-saline (150 mmol/l NaCl and 10 mmol/l Tris-base) containing Triton X-100 (1% v/v), "blocked" with 250 ml of 1% (w/v) BSA in Tris-saline for 1.5 h and washed with 200 ml of Tris-saline containing 0.1% (v/v) Tween-20 for 10 min. The nitrocellulose filter was then incubated with [$^{125}$I]-iodo-*h*IGF-II (~4 x $10^6$ cpm, ~60 Ci/g) in 250 ml Tris-saline containing 1% (w/v) BSA for 2.5 h. The radiolabelled nitrocellulose filter was then washed with 200 ml of Tris-saline containing 0.1% (v/v) Tween-20 for 1.5 h. The filter was then dried in air overnight and exposed to X-ray film (Fuji RX, Tokyo) at -80 °C with intensifying screens (Cornex Hi-Plus, Du Pont) for 14 days.

## 4.3 RESULTS

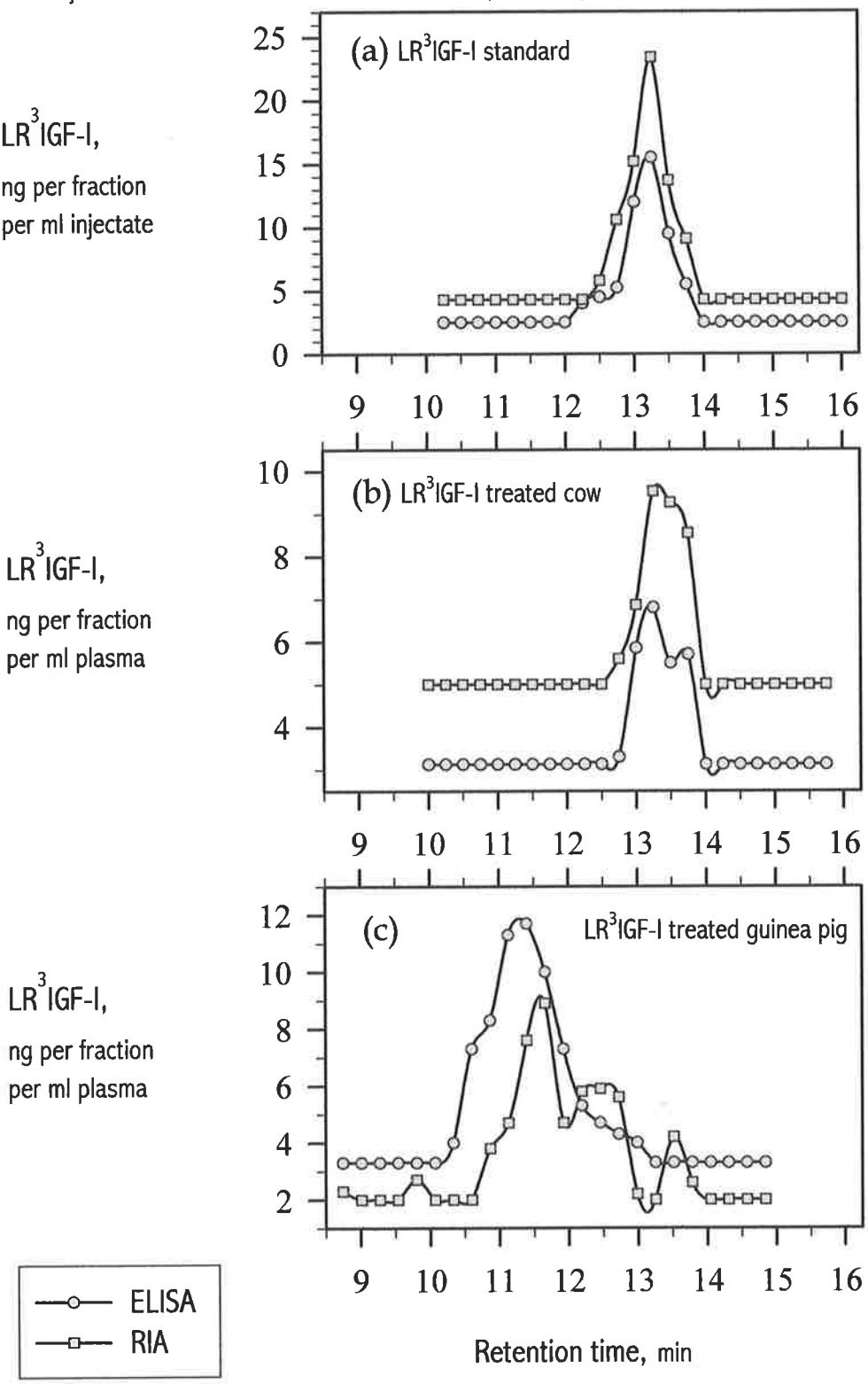
The concentrations of $LR^3IGF$-I measured in unfractionated plasma by ELISA were 96 ± 6 ng/ml (n=6) in the fasted heifers intravenously infused with $LR^3IGF$-I at 12 $\mu g.kg^{-1}.h^{-1}$ for 7 h and 42 ± 5 ng/ml (n=8) in well fed guinea pigs infused subcutaneously with $LR^3IGF$-I at

## Figure 4.1

**Size distribution of immunoreactive $LR^3IGF$-I in plasma from a cow and a guinea pig treated with this IGF analog.**

Size exclusion liquid chromatography through BECKMAN Ultraspherogel SEC 2000 at pH 7.4 in 0.15 mol/ NaCl of $LR^3IGF$-I assay standard and plasma from a cow and a guinea pig treated with $LR^3IGF$-I.

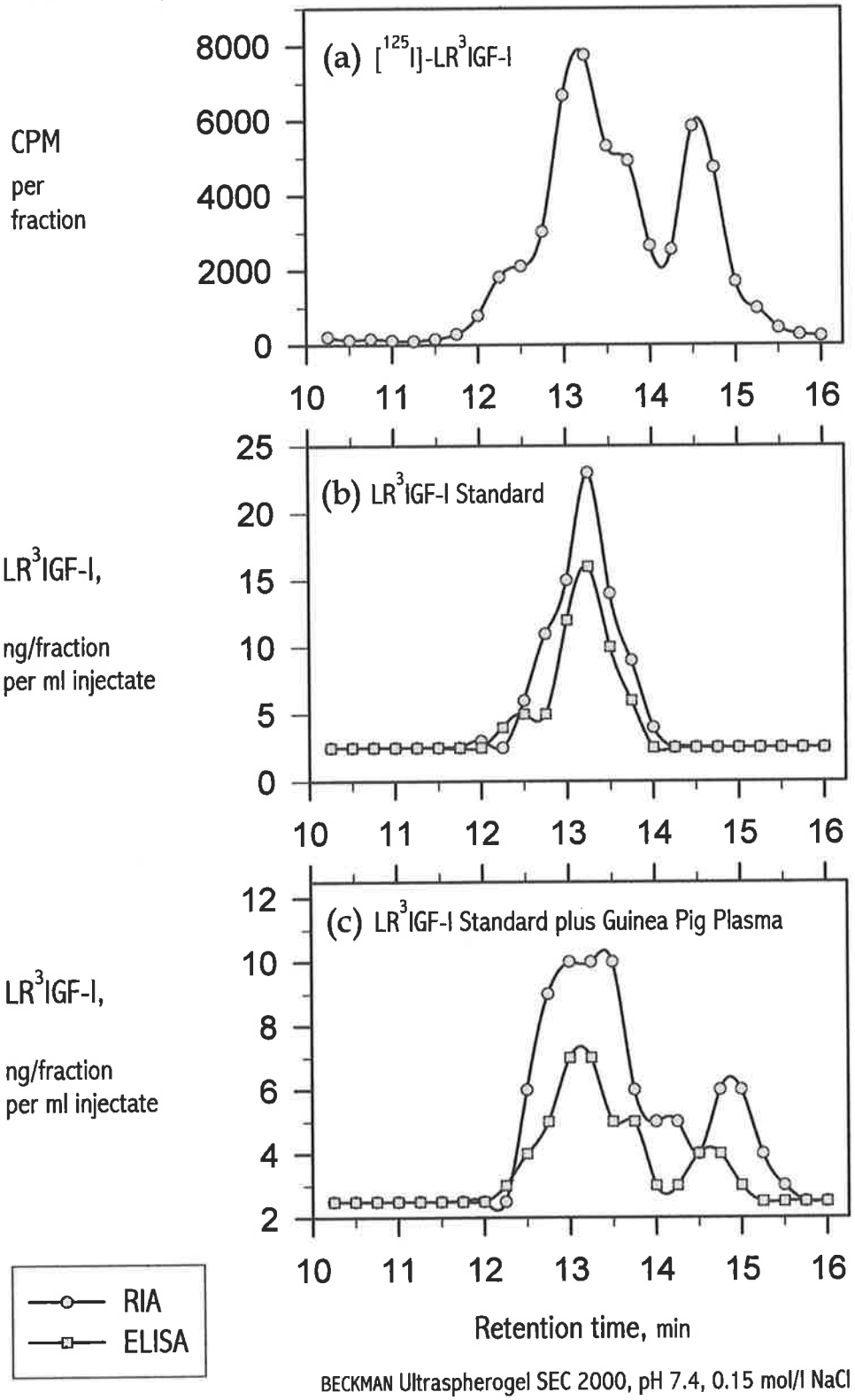
Results shown represent a single chromatographic fractionation in each case (a, b and c) and triplicate aliquots of the eluted fractions were subjected to ELISA and RIA for $LR^3IGF$-I. Means of these triplicates are plotted.



## Figure 4.2

### Size distribution of immunoreactive LR$^3$IGF-I after *in vitro* incubation with normal guinea pig plasma

Results shown represent a single chromatographic fractionation in both cases (b) and (c) and triplicate aliquots of the eluted fractions were subjected to ELISA and RIA for LR$^3$IGF-I. Means of these triplicates are plotted.




BECKMAN Ultraspherogel SEC 2000, pH 7.4, 0.15 mol/l NaCl

14 $\mu g.kg^{-1}.h^{-1}$ for 7 days. No activity was detected by $LR^3IGF$-I ELISA in vehicle-treated animals.

Molecular forms of $LR^3IGF$-I in plasma from $LR^3IGF$-I-treated cows and guinea pigs were characterised by chromatographing plasma from treated animals through a size exclusion column under non-dissociating conditions at physiological pH and salt concentration. Plasma from vehicle-treated animals was chromatographed as negative controls. The eluted fractions were assayed for $LR^3IGF$-I without further extraction by both ELISA and RIA (Fig. 4.1).

When $LR^3IGF$-I standard was chromatographed alone, both ELISA and RIA detected a peak of activity eluting from the Ultraspherogel SEC 2000 column with the same retention time, 13.25 min, at pH 7.4 (Fig. 4.1*a*). The retention time of the unlabelled $LR^3IGF$-I standard was the same as that of radioiodinated $LR^3IGF$-I (Fig. 4.2*a*,*b*). Neither the ELISA nor the RIA detected any activity in the chromatography eluates of plasma from vehicle-treated cows or guinea pigs (not shown).

Both RIA and ELISA showed that the $LR^3IGF$-I in plasma from fasted cows infused intravenously with this peptide at 12 $\mu g.kg^{-1}.h^{-1}$ for seven hours had a similar retention time at pH 7.4 as the $LR^3IGF$-I standard (Fig. 4.1*b*). However, in guinea pigs infused subcutaneously with the analog at 14 $\mu g.kg^{-1}.h^{-1}$ for one week, most of the $LR^3IGF$-I activity detected was present in a form that eluted from the column much earlier than the $LR^3IGF$-I standard, regardless of the $LR^3IGF$-I assay method used (Fig. 4.1*c*). The peak of the activity detected by $LR^3IGF$-I ELISA eluted with a retention time of ~11.25 min. A similar, but more variable, elution profile was seen by $LR^3IGF$-I RIA in the same eluate produced by size exclusion

chromatography at pH 7.4 of plasma from the LR$^3$IGF-I treated guinea pig. The RIA detected LR$^3$IGF-I activity eluting with peak retention times of 11.5 min, ~12.5 min and 13.5 min.

To determine whether guinea pig plasma could increase the apparent molecular size of LR$^3$IGF-I *in vitro*, plasma from a vehicle-treated guinea pig was incubated with LR$^3$IGF-I before size exclusion chromatography at pH 7.4 and LR$^3$IGF-I assay of the eluate. Radioiodinated and unlabelled LR$^3$IGF-I were chromatographed as calibration markers immediately before the mixture of LR$^3$IGF-I and normal guinea pig plasma. When [$^{125}$I]-iodo-LR$^3$IGF-I was chromatographed alone, two peaks of radio-activity eluted from the column (Fig. 4.2*a*). The first peak of radioactivity eluting after 13.25 min has the same retention time as that of the pure unlabelled LR$^3$IGF-I assay standard under the same conditions as determined by LR$^3$IGF-I assay of the eluate (Fig. 4.2*b*, 4.1*a*). The peak of radioactivity eluting 14.5 min after the injection of [$^{125}$I]-iodo-LR$^3$IGF-I is likely to be due to breakdown products because this batch of tracer had been radio-iodinated ~2.5 months previously.

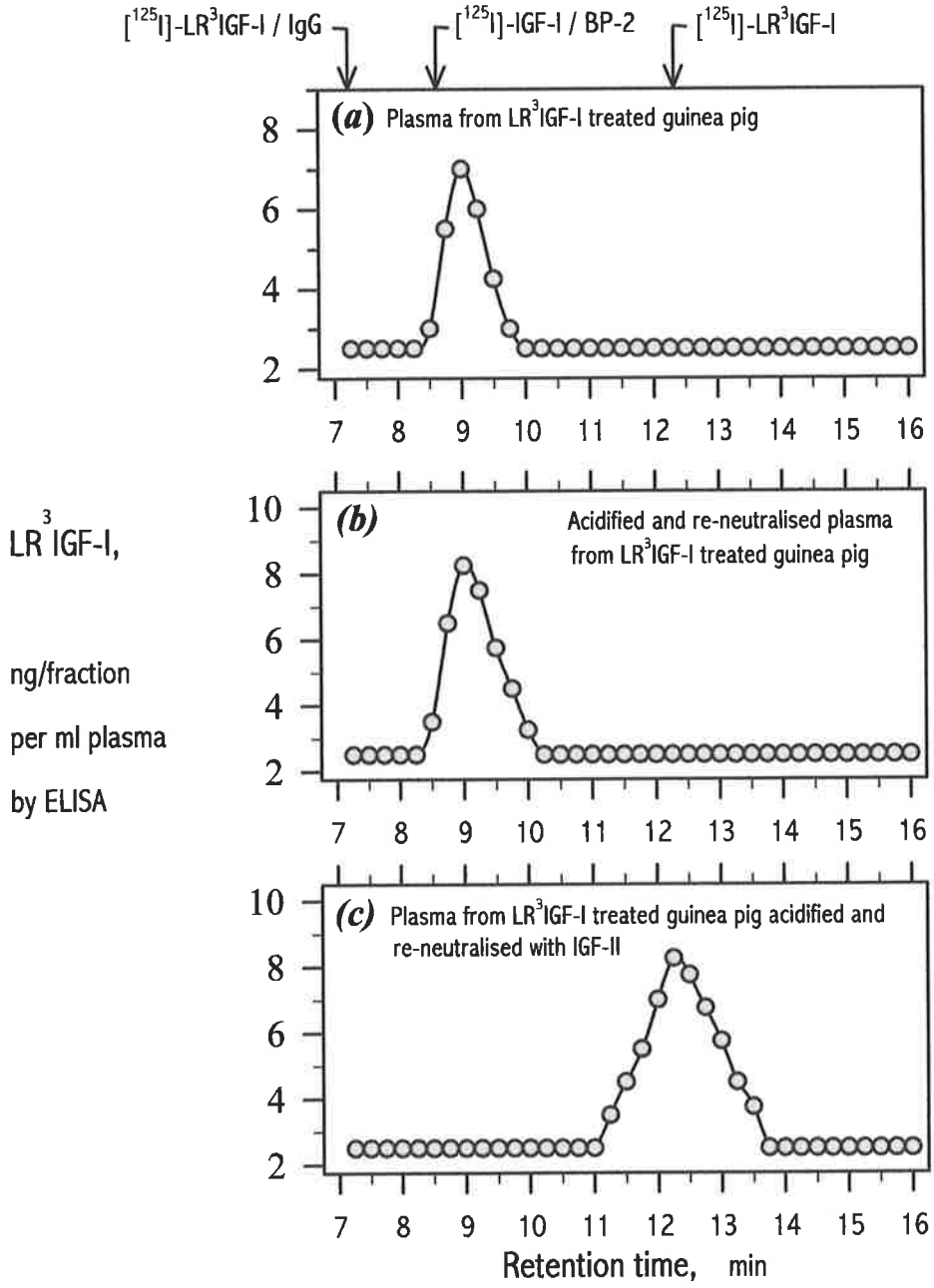
*In vitro* incubation of LR$^3$IGF-I at 60 ng/ml in 90% plasma from vehicle-treated guinea pigs produced two peaks of eluted activity in both LR$^3$IGF-I ELISA and RIA when fractionated by size exclusion chromatography at pH 7.4 (Fig. 4.2*c*). The larger peak of activity eluted with a similar retention time (~13.0 to 13.5 min) to both [$^{125}$I]-LR$^3$IGF-I and the pure unlabelled LR$^3$IGF-I assay standard, indicating that this material is unmodified free LR$^3$IGF-I.

Both the ELISA and the RIA detected smaller peaks of immunoreactive LR$^3$IGF-I eluting ~14.5 to 15 min after injection of LR$^3$IGF-I pre-incubated with (Fig. 4.2*c*) but not

## Figure 4.3

**Effect of *in vitro* incubation with excess IGF-II on the size distribution at pH 7.4 of immunoreactive $LR^3IGF$-I in plasma from a guinea pig treated with this IGF-I analog *in vivo*.**

Fractionation of guinea pig plasma by chromatography at pH 7.4 through BECKMAN Ultraspherogel SEC 2000




Replicates of plasma from a guinea pig treated with $LR^3IGF$-I were either ***(a)*** diluted by 36% with chromatography mobile phase, ***(b)*** diluted by a total of 36% with 1 mol/l HCl, followed by pH 7.4 buffer, and finally neutralised with Tris-base, or ***(c)*** diluted by a total of 36% with 1 mol/l HCl, then 3,200 ng/ml IGF-II in pH 7.4 buffer and finally Tris-base neutralising solution, and incubated for 60 min at 37 °C prior to chromatography.

The arrows above the upper panel indicate the retention times of the following calibration markers:
[$^{125}$I]-$LR^3IGF$-I pre-incubated with mouse IgG 1A7-F5-E5 (160 kDa, 7.25 min),
[$^{125}$I]-IGF-I pre-incubated with recombinant bovine IGF-binding protein-2 (37.5 kDa, 8.5 min) and
[$^{125}$I]-$LR^3IGF$-I alone (9.1 kDa, 12.25 min).
Each chromatogram represents one experiment.

without (Fig. 4.2*b*) normal guinea pig plasma *in vitro*. This is similar to the retention time observed for the breakdown products of [$^{125}$I]-iodo-LR$^3$IGF-I (Fig. 4.2*a*), suggesting that limited proteolysis or other modification of LR$^3$IGF-I may have occurred during *in vitro* incubation of the peptide with normal guinea pig plasma. The "big" forms of immunoreactive LR$^3$IGF-I seen during similar analysis of plasma from a guinea pig treated with the growth factor analog *in vivo* (Fig. 4.1*c*) were not detected by either ELISA or RIA after *in vitro* incubation of LR$^3$IGF-I with normal guinea pig plasma.

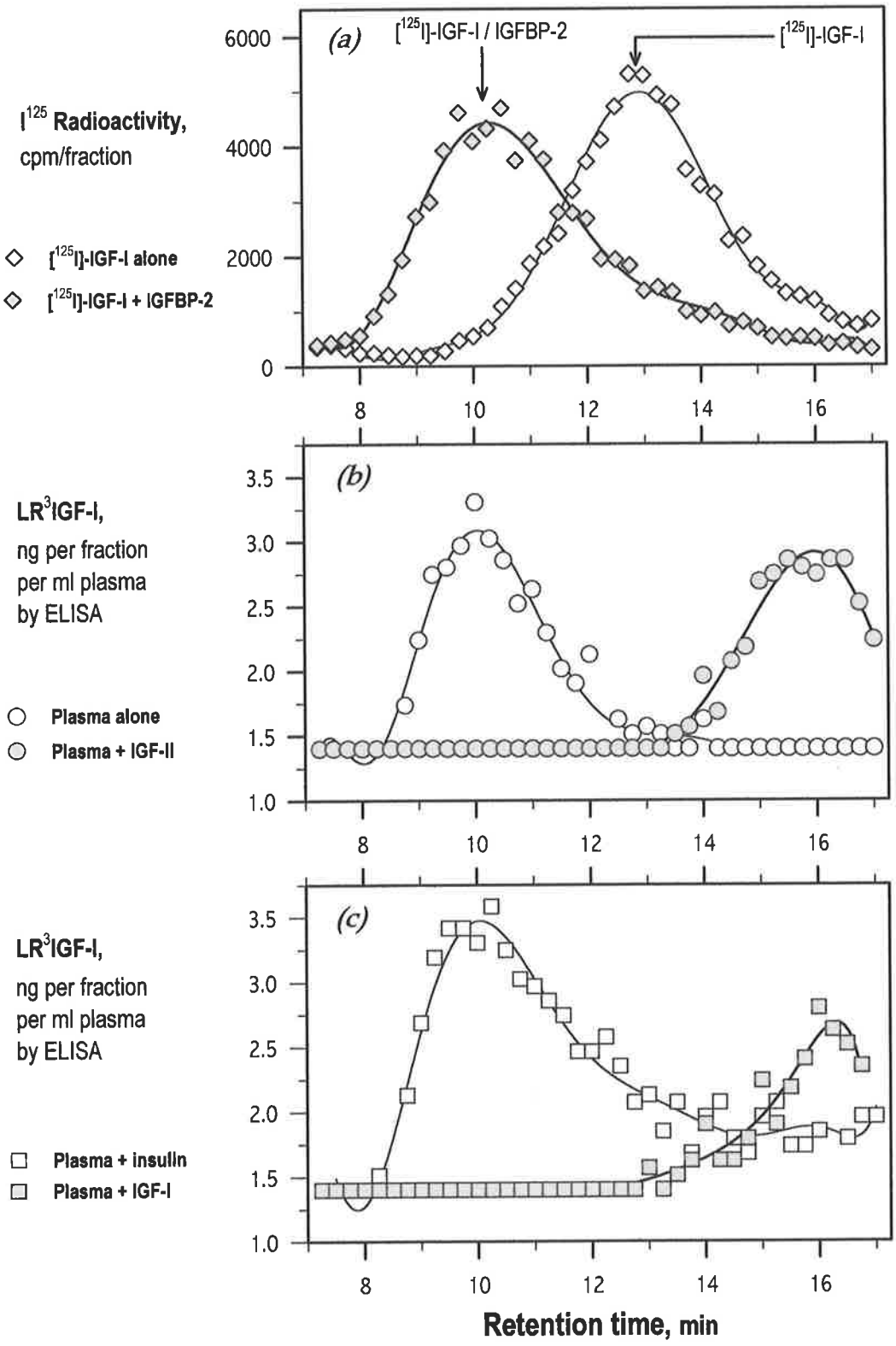
In order to determine whether the high molecular weight forms of LR$^3$IGF-I in plasma from guinea pigs treated with this analog were due to LR$^3$IGF-I having formed complexes with IGF-binding protein(s) *in vivo*, the abilities of IGF-I, IGF-II and insulin to reduce the apparent molecular weight of "big" LR$^3$IGF-I in plasma from analog-treated guinea pigs were compared in two experiments. In the first experiment, replicates of plasma from an LR$^3$IGF-I treated guinea pig containing 44 ng LR$^3$IGF-I per ml were firstly acidified to dissociate potential complexes between LR$^3$IGF-I and any IGF-binding protein(s) and then reneutralized in the presence of IGF-II at a final concentration of either 0 or 3,200 ng per ml. The mixtures of 64% (v/v) plasma with and without excess IGF-II were fractionated at pH 7.4 through Ultraspherogel SEC 2000 and the eluates analysed for LR$^3$IGF-I by ELISA (Fig. 4.3) to determine whether acidification and reneutralisation in the presence of excess IGF-II could convert "big" LR$^3$IGF-I into "free" LR$^3$IGF-I. [$^{125}$I]-LR$^3$IGF-I, [$^{125}$I]-IGF-I preincubated with bovine IGFBP-2 and [$^{125}$I]-LR$^3$IGF-I preincubated with mouse IgG 1A7-F5-E5 were run as calibration markers immediately before the test samples.

All of the immunoreactive LR$^3$IGF-I present in plasma from the LR$^3$IGF-I treated guinea pig that was similarly diluted by 36% without acidification and reneutralisation eluted

## Figure 4.4

***In vitro* effects of IGFs and insulin on "big" LR$^3$IGF-I in plasma from a guinea pig treated with LR$^3$IGF-I *in vivo*.**

Size exclusion hplc at pH 7.4 through BECKMAN Ultraspherogel SEC 2000




**Chromatography of *(a)* radiolabelled markers or *(b,c)* plasma from guinea pigs treated with LR$^3$IGF-I. Each chromatographic profile represents a single experiment.**

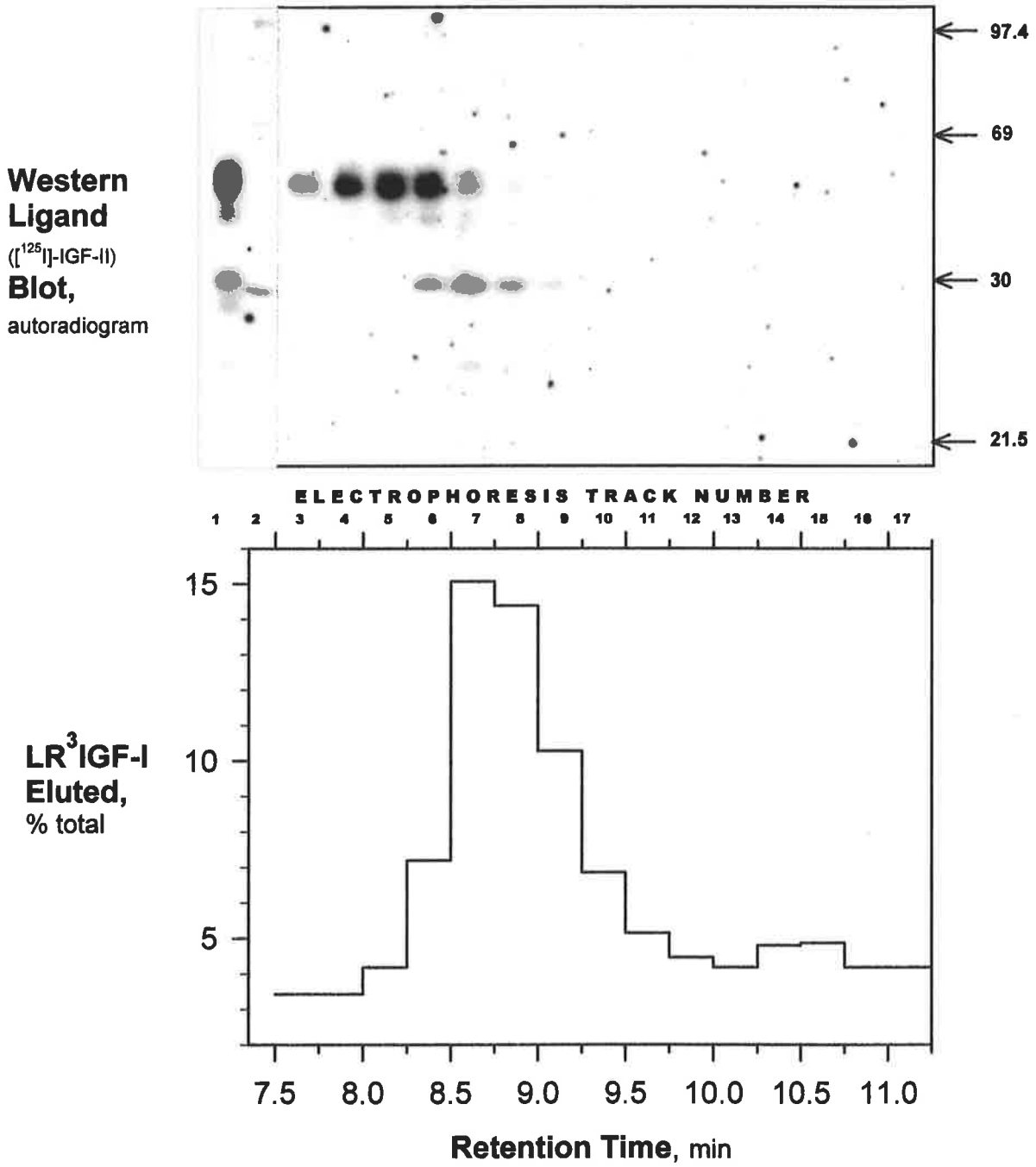
at pH 7.4 as a single peak 9 min after injection onto the Ultraspherogel column (Fig. 4.3*a*). This is 2.25 min earlier than observed when an aliquot of the same plasma sample had been previously chromatographed under the same conditions (Fig. 4.1*c*). Radio-iodinated $LR^3IGF$-I eluted much later (12.25 min) than the immunoreactive $LR^3IGF$-I in plasma from the treated guinea pigs. The tracer also eluted earlier than observed in the previous chromatography session (Fig. 4.2*a*). This suggests that column performance had changed since the previous chromatography session. The apparent molecular weight of the immunoreactive $LR^3IGF$-I in plasma from this treated guinea pig was slightly less than that of the complex formed by association of IGF-I with IGFBP-2 (37.5 kDa) as determined by the retention time (8.5 min) of the radioactivity eluted after injection of [$^{125}$I]-IGF-I preincubated with pure IGFBP-2. The "big" immunoreactive $LR^3IGF$-I was also considerably smaller in size than that of the complex formed between [$^{125}$I]-$LR^3IGF$-I and anti-$LR^3IGF$-I IgG (7.25 min) but considerably greater than the size of "free" [$^{125}$I]-$LR^3IGF$-I (12.25 min). Acidification and reneutralisation of this guinea pig plasma in the absence of excess unlabelled IGF-II did not alter the retention time of its immunoreactive $LR^3IGF$-I content during size exclusion chromatography at pH 7.4 (Fig. 4.3 *b*).

The high molecular weight form of $LR^3IGF$-I (Fig. 4.2*a,b*) could be converted to a form of apparent lower molecular weight of identical retention time to the [$^{125}$I]-$LR^3IGF$-I size calibration marker by *in vitro* addition of IGF-II during neutralisation after acidification of plasma from this $LR^3IGF$-I treated guinea pig (Fig. 4.2*c*).

In a second experiment of the same design, replicates of plasma from another guinea pig similarly treated with $LR^3IGF$-I that contained 56 ng of $LR^3IGF$-I per ml were acidified and reneutralised in the presence of 3,200 ng/ml of IGF-I, IGF-II, insulin or no peptide,

## Figure 4-5

**Size distribution of IGF-binding proteins and $LR^3IGF-I$ in plasma from a well-fed guinea pig treated with $LR^3IGF-I$**




Western ligand blot (*upper panel*) and $LR^3IGF-I$ ELISA (*lower panel*) of fractions obtained by chromatography at pH 7.4 through Ultraspherogel SEC 2000 of 0.05 ml plasma from a well fed guinea pig treated with $LR^3IGF-I$.

In the western ligand blot (UPPER PANEL): unchromatographed plasma was run in the lane on the extreme left (track #1) and the arrows on the extreme right indicate the mass markers (KDa) run in track #2.

Aliquots of the column eluate equivalent to 6% of each fraction were electrophoresed commencing in track #3. .

chromatographed as above and the LR$^3$IGF-I content of their eluents measured by ELISA (Fig. 4.4). [$^{125}$I]-IGF-I (n=3) and [$^{125}$I]-IGF-I preincubated with bovine IGFBP-2 (n=3) were chromatographed as calibration markers immediately before, between, and immediately after injection of the test samples. Radioactivity (mean of three injections each) eluted as a single peak ~13 min after injection of [$^{125}$I]-IGF-I and ~10 min after injection of complexes formed by preincubation of [$^{125}$I]-IGF-I with IGFBP-2 (Fig. 4.4*a*).

The retention time of the [$^{125}$I]-IGF-I/IGFBP-2 complexes observed during this chromatography session (Fig. 4.4*a*) were ~1 min later than that observed in the preceding session (Fig. 4.3).

When plasma from this LR$^3$IGF-I treated guinea pig was acidified and reneutralised in the absence of added peptides, immunoreactive LR$^3$IGF-I eluted as a single peak (Fig. 4.4*b*) with a similar retention time at pH 7.4 (~10 min) to that of [$^{125}$I]-IGF-I/IGFBP-2 complexes (Fig. 4.4*a*). This is similar to the result observed in the previous experiment that used plasma from a different but identically treated animal (Fig. 4.3*b*). However, as observed with [$^{125}$I]-IGF-I/IGFBP-2 complexes, the "big" immunoreactive LR$^3$IGF-I also eluted from the column with a retention time (~10 min) different from that observed in the first (11.25 min, Fig. 4.1*c*) and second (~9 min, Fig. 4.3*a*,*b*) chromatography sessions. As observed in the previous experiment, inclusion of IGF-II during neutralisation of acidified plasma from an LR$^3$IGF-I treated guinea pig abolished the peak of "big" LR$^3$IGF-I and produced a similar sized peak of immunoreactive LR$^3$IGF-I eluting much later than [$^{125}$I]-IGF-I from the column (Fig. 4.4*b*). Addition of IGF-I, but not insulin, produced a similar result (Fig. 4.4*c*).

The high molecular weight form of $LR^3IGF$-I in well fed guinea pigs treated with this analog was further characterized by subjecting the neutral column chromatography fractions of plasma from a $LR^3IGF$-I treated guinea pig to Western-radioligand blotting analysis (Fig. 4.5, upper panel). Unchromatographed plasma from the same guinea pig contained IGF-binding proteins of 48, 43, 31, 28 and 25 kDa (Fig.4.5, upper panel, track 1). Western-radioligand blotting analysis of neutral column chromatography fractions from this $LR^3IGF$-I treated guinea pig (Fig. 4.5, upper panel) showed that an IGF-binding protein of ~48 kDa eluted between 7.5 and 9 min after injection, with the highest peak being at ~8.25-8.5 min. An IGF-binding protein of ~43 kDa eluted between 7.75 and 8.75 min after injection, with the highest peak at 8.5 min. An IGF-binding protein of ~31 kDa eluted between 8.25 and 9.25 min after injection, with the highest peak at 8.75 min. Another IGF-binding protein of ~25 kDa eluted between 8.5 and 9 min after injection. The IGF-binding protein of ~28 kDa seen in the original plasma was not detected in any chromatography fractions. $LR^3IGF$-I was measured by ELISA in these same column fractions (Fig. 4.5, lower panel). The $LR^3IGF$-I activity eluted between 8.0 and 9.75 min after injection with the peak at 8.75 min (Fig. 4.5, lower panel). Therefore, the column fractions that contained the high molecular weight form of $LR^3IGF$-I also contained IGF-binding proteins of approximately 48, 43, 31 and 25 kDa.

## 4.4 DISCUSSION

When plasma from animals treated with bioactive doses of $LR^3IGF$-I was chromatographed through a size exclusion column under non-dissociating conditions, $LR^3IGF$-I was detected in both free form and bound to IGF-binding protein(s).

ELISA and RIA produced similar but not identical results for chromatography of plasma from guinea pigs treated with $LR^3IGF$-I. In $LR^3IGF$-I treated guinea pig plasma, although a similar peak of "big" $LR^3IGF$-I activity was detected by both ELISA (retention time 11.25 min) and by RIA (retention time 11.5 min), the RIA also detected two other $LR^3IGF$-I activity peaks eluting later at 12.5 min and 13.5 min whereas ELISA did not. These extra peaks of $LR^3IGF$-I activity found in the RIA might be due to different forms of free $LR^3IGF$-I produced by *in vivo* modification of $LR^3IGF$-I. ELISA and RIA for $LR^3IGF$-I might have different abilities to detect different modified forms of $LR^3IGF$-I due to the different specificities of these assays (Figure 3.2 and Figure 3.3).

*In vitro* addition of excess IGF-I and IGF-II, but not insulin during neutralisation of acidified plasma from $LR^3IGF$-I treated guinea pigs converted the high molecular weight $LR^3IGF$-I activity into a low molecular weight form of identical size to pure free $LR^3IGF$-I. Since IGFBPs have high affinity and specificity for IGFs but do not bind to insulin, the high molecular weight $LR^3IGF$-I in plasma from $LR^3IGF$-I treated guinea pig is therefore associated with IGF-binding protein(s).

In guinea pig plasma which had been acidified and re-neutralised without *in vitro* addition of excess IGFs, $LR^3IGF$-I remained in the high molecular form. This suggests that either this IGF-binding protein may bind $LR^3IGF$-I better than endogenous IGF-I or IGF-II or there is an excess of this binding protein in an unsaturated form that is available for formation of $LR^3IGF$-I/IGFBP complexes. The relative concentrations of IGF-I, IGF-II, $LR^3IGF$-I and IGF-binding proteins achieved during these *in vivo* experiments are therefore worthy of consideration. For these well fed guinea pigs, s.c. infusion of $LR^3IGF$-I for 7 days reduced plasma IGF-I by 65% and lowered IGF-II by 70% (Conlon *et al.* 1995*b*). Total plasma IGF-

binding proteins were also reduced by ~40% in LR$^3$IGF-I treated guinea pigs mainly due to a substantial reduction in the IGF-binding protein doublet bands of approximately 40 to 50 kDa, presumed to be isoforms of guinea pig IGFBP-3 (Conlon *et al.* 1995*b*). However, LR$^3$IGF-I treament also increased the minor ~28 and ~31 kDa IGF-binding proteins in plasma from these animals. Thus LR$^3$IGF-I treatment reduced the concentrations of its competitors for complex formation, IGF-I and IGF-II, by ~65-70%, reduced IGFBP-3 and increased the amounts of IGF-binding proteins of ~28 and ~31 kDa with which it could potentially associate in guinea pig. In contrast, LR$^3$IGF-I was detected in the free form after *in vitro* incubation of LR$^3$IGF-I with normal guinea pig plasma which contained higher amounts of IGF-I, IGF-II and IGFBP-3, and lower amounts of ~28 and ~31 kDa IGF-binding proteins than that from LR$^3$IGF-I treated guinea pigs. The concentrations of IGF-I, IGF-II and IGF-binding proteins may therefore influence the formation of complexes between LR$^3$IGF-I and IGF-binding proteins in guinea pigs.

Western ligand blotting analysis of fractions obtained by chromatography at pH 7.4 of plasma from a guinea pig treated with LR$^3$IGF-I detected IGFBPs of approximately 48, 43, 31 and 25 kDa in the column fraction that contained the greatest concentration of the high molecular weight form of LR$^3$IGF-I (retention time 8.75). Therefore any or all of these binding proteins could be associated with LR$^3$IGF-I. The ~48 and ~43 kDa IGFBPs are likely to be isoforms of IGFBP-3 as observed in rat (Baxter & Martin 1987) and these peaked at 8.25-8.5 min after injection. Therefore the peaks of IGFBP-3 and "big" LR$^3$IGF-I did not coelute from the column. Moreover, IGFBP-3 was decreased by treatment with LR$^3$IGF-I in these guinea pigs (Conlon *et al.* 1995*b*). On the other hand, LR$^3$IGF-I treatment increased ~28 and ~31 kDa binding proteins (Conlon *et al.* 1995*b*). The IGF-binding protein of 28 kDa was not detected in any column fractions but due to its molecular weight, this IGFBP should elute

at a retention time between 8.5-9.25 min as did IGFBPs of ~31 and ~25 kDa. This suggests that the 28 kDa IGFBP might stick to the column. If LR$^3$IGF-I is associated with IGFBP of 28 kDa, it should therefore not elute from the column. The recovery of LR$^3$IGF-I was found to be 77%. Therefore 23% of the LR$^3$IGF-I which was lost in the column may have been present in complexes with the 28 kDa IGFBP. LR$^3$IGF-I which eluted from the column (77%) should therefore be associated with other binding proteins. The highest amount of ~31 kDa and ~25 kDa IGF-binding protein(s) eluted in the same fraction that contained the highest amount of LR$^3$IGF-I suggesting that LR$^3$IGF-I might also associate with these IGFBPs *in vivo*.

It was not possible to estimate the molecular weight of LR$^3$IGF-I/IGFBP complex from size exclusion chromatography at pH 7.4 because the elution time was not consistent with the molecular weight in this system and varied between sessions. For example, LR$^3$IGF-I which has a $M_r$ of 9.1 kDa eluted later than [$^{125}$I]-IGF-I (7.5 kDa). Further studies used a different column system.

LR$^3$IGF-I was present in the free form in plasma from fasted cows infused i.v. for 7 hours. Treatment with LR$^3$IGF-I in these cows reduced IGF-I by 45% and IGF-II by 50%, increased a 31-32 kDa IGF-binding protein and had no effect on IGFBP-3 (Owens *et al.* 1994). Considering that LR$^3$IGF-I treatment reduced the concentrations of its competitors for complex formation and increased the amount of low molecular weight IGF-binding protein, it is therefore surprising that LR$^3$IGF-I detected in plasma from the cows was in the free form.

The mechanism responsible for the formation of IGFBP/LR$^3$IGF-I complexes are not clear. Factors which regulate the production of endogenous IGFs and IGF-binding proteins appear likely to have a significant effect on the formation of complexes between the

administered IGF and circulating IGF-binding proteins. Animal species differences in affinity of binding proteins for LR$^3$IGF-I or differences in phosphorylation status of binding proteins might also affect complex formation. Factors responsible for *in vivo* complex formation between LR$^3$IGF-I and IGF-binding proteins were further investigated in the next chapter.

# CHAPTER 5.

## Age and nutrition affect the apparent molecular size of plasma $LR^3IGF\text{-}I$ *in vivo*

### 5.1 INTRODUCTION

In the previous chapter, it was established that when $LR^3IGF\text{-}I$ was administered to animals in pharmacologically active doses, it can be present in either the free form or bound to IGF-binding proteins in the circulation. The mechanisms responsible for controlling the formation of IGF-binding protein/IGF complexes are not clear. However, factors which regulate the production of endogenous IGFs and IGF-binding proteins are likely to have a significant effect on the formation of complexes between administered IGFs and circulating IGF-binding proteins.

Nutrition is one of the major regulators of synthesis, secretion and blood levels of insulin-like growth factor-I (IGF-I) and IGF-binding proteins (Clemmons & Underwood 1991, Thissen *et al.* 1994). Plasma IGF-I peptide concentrations and hepatic IGF-I mRNA abundance are both decreased when growth is reduced by restriction of dietary energy or protein in rats (Takahashi *et al.* 1990, Thissen *et al.* 1991, VandeHaar *et al.* 1991, Miura *et al.* 1992, Jones & Clemmons 1995), indicating that hepatic biosynthesis of IGF-I is a key factor in nutritional regulation of growth in this species. The effect of dietary protein on serum IGF-I is age-related, being more marked in younger rats (Fliesen *et al.* 1989). IGF-binding proteins are also affected by dietary manipulation in rats. In general, dietary restriction decreases IGFBP-3 and increases IGFBP-1 and IGFBP-2 concentrations in blood. Moreover,

the abundance of mRNAs for IGFBP-1 (Umezawa *et al.* 1991, Takenaka *et al.* 1993, Lemozy *et al.* 1994, Takenaka *et al.*1996) and IGFBP-2 (Straus & Takemoto 1990*b*, Lemozy *et al.* 1994, Takenaka *et al.*1996) are increased in livers from nutritionally restricted or deprived rats.

Treatment of animals with IGFs also alters hepatic mRNA abundances and blood concentrations of IGF-I, IGF-II and IGF-binding proteins. Administration of IGF-I to GH-deficient dwarf rats (Butler *et al.* 1994), malnourished rats (Schalch *et al.* 1989), hypophys-ectomized rats (Gosteli-Peter *et al.* 1994) and fetal sheep (Kind *et al.* 1996) reduces hepatic IGF-I mRNA. IGF-I administration increases IGFBP-3 plasma levels in rats that are hypo-physectomized (Clemmons *et al.* 1989, Zapf *et al.* 1989), protein deprived (Clemmons *et al.* 1989), diabetic (Zapf *et al.* 1989, Tomas *et al.* 1991*a*, 1993*b*), nitrogen restricted (Tomas *et al.* 1991*b*) or dexamethasone-treated (Tomas *et al.* 1992). Gargosky *et al.* (1994) also observed that administration of IGF-I increases IGFBP-3 in ~60 kDa forms but not in the ~150 kDa IGFBP-3 forms in GH-deficient rats. Administration of IGF-I to patients with GH receptor-deficiency also fails to increase IGFBP-3 or the acid-labile subunit of the ~150 kDa complex in the circulation (Gargosky *et al.* 1993). Wilson *et al.* (1995) also found that IGF-I administration did not increase serum levels of IGFBP-3 in patients with growth hormone receptor deficiency. Walton & Etherton (1989) found that a daily intra-arterial injection of IGF-I into pigs for 3 days had no effect on IGFBP-3 as measured by specific RIA.

Nutrition and duration of IGF-I treatment appear to affect the plasma IGFBP responses to IGF-I administration. A single subcutaneous injection of human IGF-I into fasted healthy adults causes a rapid rise in plasma IGFBP-1 levels with little effect on IGFBP-3 (Baxter *et al.* 1993). However, when the same subjects were fed and were injected with IGF-I daily for 7

days, the increase in IGFBP-1 levels on day 1 was blunted by over 90%. Also, after the seventh day of IGF-I injections, a significant decrease in immunoreactive IGFBP-3 levels and acid-labile subunit and an increase in IGFBP-2 levels was observed in the same study (Baxter *et al.* 1993). An increase of IGFBP-2 after IGF-I infusion was observed in healthy adults and patients with extra-pancreatic tumour hypoglycaemia by Zapf et al. (1990). Kind *et al.* (1996) observed an increase in plasma IGFBP-1 (measured by Western ligand blotting) during IGF-I infusion in fetal sheep.

In addition to its effects on the synthesis and secretion of IGF-I and IGF-binding proteins, dietary protein restriction in rats alters the size distribution of IGF-I in the circulation. Thissen *et al.* (1992) found that the circulating size classes of [$^{125}$I]-IGF-I after its injection into well-fed or protein-restricted rats are not the same. In well-fed rats, IGF-I tracer was almost equally distributed between ~150 kDa and ~30 kDa binding protein complexes whereas in protein-restricted rats, IGF-I tracer was preferentially bound to IGFBPs in the smaller (~30 kDa) complexes. Takahashi *et al.* (1990) also found that [$^{125}$I]-IGF-I added *in vitro* to plasma of rats fed on a protein-depleted diet was found mainly in ~40 kDa fractions after gel-filtration whereas it was recovered in the free IGF-I fraction (7.5 kDa) in the plasma of rats fed on high-protein diets (gluten or casein). They suggested that IGF-binding protein(s) of molecular weight about 40 kDa were not saturated with endogenous IGF-I in the rats fed on the protein-free diet.

In contrast to the studies cited above which used tracer quantities of [$^{125}$I]-IGF-I to examine formation of IGF-I complexes with binding proteins, this thesis is an examination of the circulating forms of an IGF-I analog (LR$^3$IGF-I) formed *in vivo* during treatment with biologically active doses of this IGF analog. The aim of the study described in this chapter

was to determine whether dietary restriction and age affect the formation of complexes between the administered IGF-I analog and circulating IGF-binding protein(s) in rats.

## 5.2 EXPERIMENTAL

### 5.2.1 Animals

The animal experiment and specimen collections described in this chapter were performed by staff of CSIRO Division of Human Nutrition under the direction of Dr. F.M.Tomas. Male Hooded Wistar rats were housed individually in metabolism cages with unrestricted access to water and a casein/starch based ration containing 18% protein. Animals were introduced to the ration the day before transfer to metabolism cages. After 6 days of acclimatisation in the metabolism cage, animals, whose average body weights were 128 g (~5 week old rats) and 316 g (~12 week old rats), were allocated to one of nine treatment groups and one pre-treatment group (n=6 per group). Pre-treatment controls were then immediately sacrificed (see below). The remaining experimental animals were anaesthetised and an osmotic mini-pump (Alza Corp., Palo Alto, CA, USA, Model 2001) containing IGF peptide or vehicle was inserted s.c. as described previously (Tomas *et al.* 1991*a*). Feed was offered to these animals at either *ad libitum* rates or at 22% (moderately undernourished), 44% (severely undernourished) or 67% (extremely undernourished) less than the *ad libitum* rate for the 7 days of treatment with placebo or 0.91 $mg.kg^{-1}.d^{-1}$ of $LR^3IGF$-I. An additional group of 6 rats were fed at 67% less than the *ad libitum* rate and infused with human IGF-I at 0.91 $mg.kg^{-1}.d^{-1}$. The animals were then stunned, decapitated and trunk blood was collected into an iced heparinised tube. Plasma was recovered by centrifugation at 4 °C and stored at -20 °C. Organs were dissected and weighed fresh.

### 5.2.2 Measurement of endogenous rat IGF-I

Rat IGF-I was measured in plasma from all animals using a rat IGF-I RIA kit according to the manufacturer's instructions (Diagnostic Systems Laboratories Inc., Webster, TX, USA, Cat. No. DSL-2900). Briefly, 50 µl of plasma was mixed with 200 µl of assay kit extraction solution (ethanolic HCl) and incubated for 30 min at room temperature. The mixture was then centrifuged at 10,000 g for 3 min. One hundred microlitres of the extracted supernatant was neutralized by addition of 500 µl of assay kit neutralizing solution. Fifty microlitres of the standards, controls or unknowns, 100 µl of rat [$^{125}$I]-IGF-I assay kit reagent and 100 µl of assay kit goat antiserum to rat IGF-I were incubated at room temperature overnight. Assay kit precipitating reagent (donkey anti-goat gamma globulin) was then added and incubated at room temperature for 20 min. After centrifugation at 4000 g for 20 min, the tubes were decanted and counted in a gamma spectrometer and results analyzed using RIACALC (LKB).

To determine the validity of measurements obtained with the DSL rat IGF-I kit, plasma was pooled on the basis of age, nutrition and IGF peptide treatment (20 group pools of 6 animals per group pool) and IGF-I was measured by RIA (Carr *et al.* 1995) using anti-*h*IGF-I serum MAC89 after removal of IGF-binding proteins by high performance size exclusion liquid chromatography of plasma at pH 2.5 before assay. Chromatography of plasma at pH 2.5 was performed using established methods (Owens *et al.* 1990, Carr *et al.* 1995). To determine which fractions contain IGF-I and IGF-binding proteins for routine analytical collection, rat plasma pooled from different treatment groups was chromatographed on a Waters Protein-Pak 125 column (7.8 mm x 30 cm, Waters, Milford, MA). Two groups

of rats ($LR^3$IGF-I-treated *ad libitum* fed old rats and IGF-I treated 67% underfed young rats) were analysed in this way because they contained vastly different amounts of lower molecular mass IGF-binding proteins. IGF-I treated 67% underfed young rats (Figure 5.7) had much higher concentrations of ~29 kDa IGF-binding protein than $LR^3$IGF-I treated *ad libitum* fed old rats (Figure 5.6). IGF-binding proteins of similar molecular mass are not efficiently precipitated from plasma by acidic ethanol and are known to interfere with IGF-I radioimmunoassays (Daughaday *et al.* 1987, Crawford *et al.* 1992).

To determine the fraction parameters for routine chromatographic fractionation and analysis, 200 µl of plasma and 100 µl of water were mixed with 100 µl of 4-times concentrated mobile phase (800 mmol/l acetic acid and 200 mmol/l trimethylamine, pH 2.5). The diluted, acidified plasma solution was delipidated before chromatography by mixing with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (freon). The solution was centrifuged at 10,000 g for 10 min at 4 °C. The defatted acidic plasma solution was filtered through a fresh cellulose acetate membrane (0.22 µm, Alltech Associates Australia Pty Ltd, Homebush, NSW, Australia) by centrifugation at 10,000 g for 3 min. Two hundred µl of ultrafiltered 50% plasma was chromatographed on a Waters Protein-Pak 125 column at a flow rate of 1 ml/min in a mobile phase of 200 mmol/l acetic acid and 50 mmol/l trimethylamine, pH 2.5, containing 0.5% (v/v) Tween-20. Fractions were collected every 0.25 min from 6 to 15 min after injection. Fifty µl of each fraction was neutralised with Tris-base and subjected to IGF-I RIA using human IGF-I as standard and radio-iodinated ligand (~60 Ci/g) and rabbit antiserum MAC 89 at a final dilution of 1/60,000 (Carr *et al.* 1995). Analysis of pooled plasma from $LR^3$IGF-I treated *ad libitum* showed two well resolved peaks of activity (Figure 5.1*b*). The first eluted between 7.25 and 8 ml and the second peak eluted between 9 and 10.5 min. The second peak of immunoreactive IGF-I eluted with a retention time similar to that of

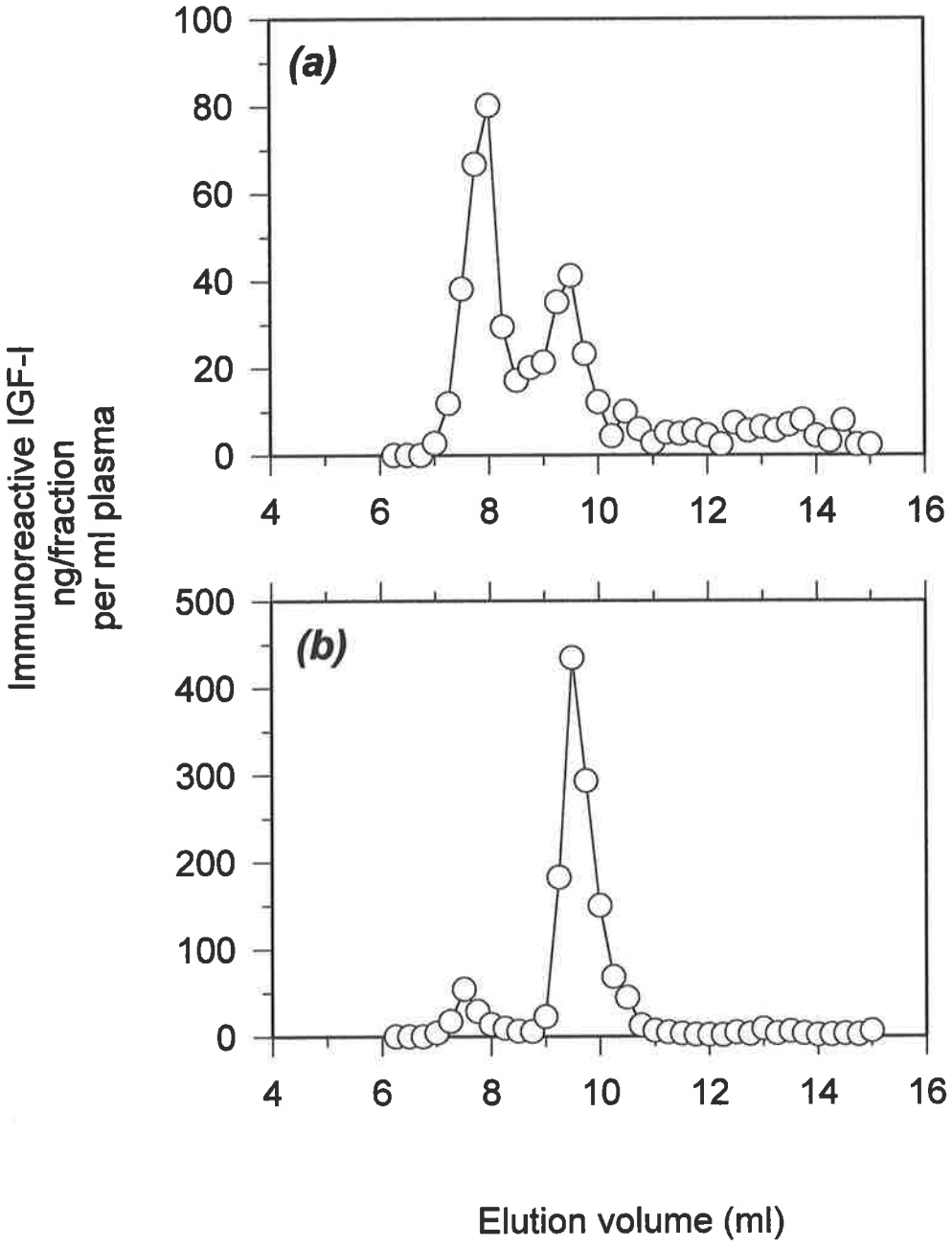
[$^{125}$I]-IGF-I (not shown). It therefore represents rat IGF-I that has been dissociated from plasma binding proteins. The first eluting peak of immunoreactive IGF-I, which appears to contain IGF-I of higher molecular mass, represents IGF-binding proteins which associate with [$^{125}$I]-IGF-I in the RIA incubation and therefore reduce the amount of radioactivity that can bind to the antibodies in the IGF-I RIA (Gargosky *et al.* 1990*a*, Owens *et al.* 1990). Similar analysis of pooled plasma from IGF-I treated 67% underfed young rats also showed two peaks of activity but these were not completely resolved (Figure 5.1*a*). The first peak eluted at 8 ml (Figure 5.1*a*) which was 0.5 ml later than the first peak of LR$^3$IGF-I treated *ad libitum* fed old rats (Figure 5.1*b*), consistent with the lower relative molecular mass of the major IGF-binding protein(s) in the former plasma pool (Figs. 5.6, 5.7). The second peak eluted with a similar retention time to [$^{125}$I]-IGF-I and the same elution volume as the second peak of immunoreactive IGF-I observed with plasma from LR$^3$IGF-I treated *ad libitum* fed old rats (Figure 5.1*a,b*).

To overcome the poor resolution achieved with plasma that contained very high concentrations of ~29 kDa IGF-binding protein(s), less plasma pooled from IGF-I treated 67% underfed young rats was chromatographed (100 µl of 25% plasma instead of 200 µl of 50% plasma) and the flow rate was reduced (0.5 ml/min instead of 1 ml/min). Fractions were collected at 0.5 min intervals from 10 to 30 min after injection. Under these conditions, two well resolved peaks of activity were detected in pooled plasma from IGF-I treated 67% underfed young rats (Figure 5.2*b*). The first peak eluted between 7.25 and 8.25 ml and the second peak of IGF-I RIA activity eluted between 9 and 10 min (Figure 5.2*b*). The second peak of activity was eluted in a similar volume to that of [$^{125}$I]-IGF-I (Figure 5.2*a*).

## Figure 5.1

**Acid size-exclusion chromatography of pooled plasma from (*a*) IGF-I-treated 67% underfed young rats and (*b*) LR$^3$IGF-I treated *ad libitum* fed old rats followed by IGF-I RIA**

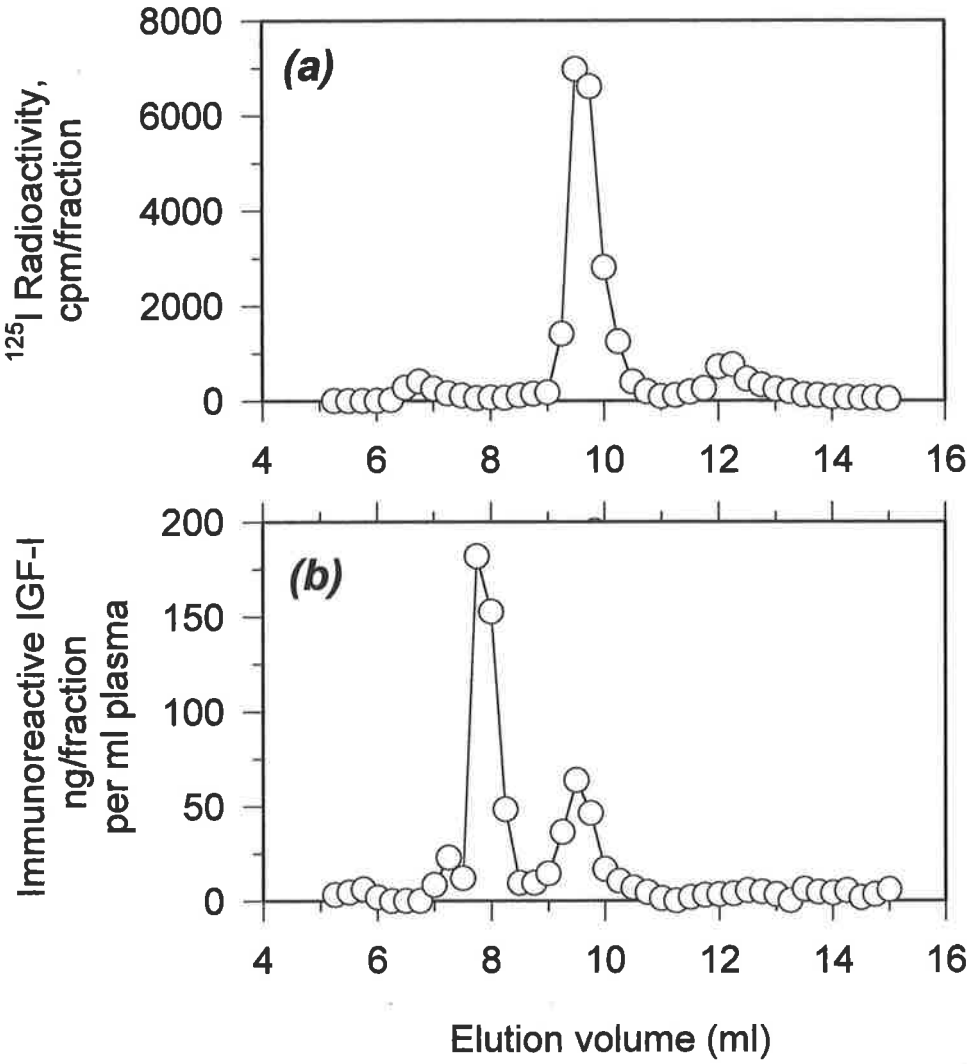
Each chromatographic profile represents a single experiment.
Triplicate aliquots of each eluted fraction were subjected to IGF-I RIA.
The means of these triplcate determinations are plotted.




One hundred ul of defatted plasma was chromatographed on a Waters Protein-Pak 125 at flow rate 1 ml/min in mobile phase of 200 mmol/l acetic acid and 50 mmol/l trimethylamine, pH2.5, containing 0.5% (v/v) Tween-20.

## Figure 5.2

**Acid size-exclusion chromatography of (*a*) [$^{125}$I]-IGF-I and (*b*) pooled plasma from IGF-I-treated 67% underfed young rats followed by IGF-I RIA**




Twenty five ul of defatted plasma was chromatographed on a Waters Protein-Pak 125 column at flow rate 0.5 ml/min in a mobile phase of 200 mmol/l acetic acid and 50 mmol/l trimethylamine, pH2.5, containing 0.5% (v/v) Tween 20.

The specimen chromatographed and analysed in the LOWER PANEL (*b*) is the same as that similarly treated and analysed in Figure 5.1(a)

Reducing the amounts of plasma injected and slowing the flow rate improved separation of IGF-binding protein and IGF-I peaks (Figs. 5.1*a*, 5.2*b*).

Therefore, 100 µl of a defatted and ultrafiltered 25% solution of acidified pooled plasma was injected onto the column and eluted at 0.5 ml/min at pH 2.5 as described above for routine analysis of mean rat IGF-I levels in each of the treatment groups. Five fractions were routinely collected and assayed for IGF-I by RIA. The first eluted between 6.5 and 7.5 ml and contained little or no activity. The second contained IGF-binding proteins (7.5-8.25 ml). The third was an intermediate fraction containing very little activity (8.25-8.75 ml). The fourth fraction eluted between 8.75 and 10.75 ml and contained IGF-I. The fifth was a trailing fraction (10.75-11.25 ml) and contained no activity.

Recombinant rat IGF-I is 70% as effective as human IGF-I at inhibiting the binding of [$^{125}$I]-human IGF-I to rabbit antiserum MAC 89. Results were determined using RIACALC and the human IGF-I standard and then converted from units of human IGF-I to rat IGF-I by multiplication of the IGF-I concentrations by 1.428. The cross reactivity of LR$^3$IGF-I in this assay system was determined to be 7%. The primary antibody bound ~30% of the added [$^{125}$I]-IGF-I in the absence of competing unlabelled IGF-I.

### 5.2.3 Analysis of IGF-binding proteins by Western ligand blotting

IGF-binding proteins in plasma pooled from each experimental group were analysed by Western-radioligand blotting (4.2.5.2). Briefly, 10 µl aliquots of plasma were incubated with 50 µl of 4-times concentrated SDS loading buffer pH 6.9 and 140 µl water at 65 °C for 20 min. Twenty µl of these solutions were subjected to discontinuous SDS-polyacrylamide

gel electrophoresis (Laemmli 1970) using a 4% stacking gel and a 10% separating gel. $^{14}C$-labelled "Rainbow" molecular weight markers (Amersham, Bucks, UK) were included on each gel. Proteins were transferred by electro-elution to nitrocellulose which was then probed with [$^{125}I$]-IGF-II (~ 4 x $10^6$ cpm) and exposed to X-ray film for 14 days.

Fractions obtained from size exclusion hplc at pH 7.4 of plasma from LR$^3$IGF-I treated young or old rats, which were fed at 44% less than the *ad libitum* rate, were also analysed for IGF-binding proteins by Western radio-ligand blotting (4.2.5.2).

### 5.2.4 Measurement of LR$^3$IGF-I by ELISA

The ELISA for LR$^3$IGF-I is described in section 3.2.3. For assay of LR$^3$IGF-I in fractions obtained by chromatography at pH 7.4, 100 µl of undiluted fractions of column eluate or LR$^3$IGF-I standard (0 to 5 ng/ml) in pH 7.4 mobile phase were used. For assay of LR$^3$IGF-I in unfractionated plasma, 100 µl of 5 mg/ml BSA in PBS-Tween 20 containing 1/100 diluted test rat plasma was used and LR$^3$IGF-I standard (0 to 5 ng/ml) was prepared in the same solution containing normal rat plasma at the same concentration as the test plasma.

### 5.2.5 Fractionation of plasma by size exclusion liquid chromatography at pH 7.4

Plasma pooled by treatment group was chromatographed through a Waters Protein-Pak 125 column (30 x 0.78 cm) under nondissociating conditions at physiological pH and salt concentration. Plasma (diluted 1/5 or 1/10 in mobile phase (see below) for younger or older rats, respectively) was delipidated before chromatography by extraction with an equal volume of 1,1,2,-trichloro-1,2,2,-trifluoroethane. Two hundred and fifty µl of defatted 1/5 or 1/10

diluted plasma was injected onto the column and eluted at 1.0 ml/min in a mobile phase of phosphate-buffered saline pH 7.4 (38.8 mmol/l $NaH_2PO_4$, 61.2 mmol/l $Na_2HPO_4$, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.05% [v/v] Tween-20 and 0.02% [w/v] $NaN_3$). Fractions were collected at 0.5 min intervals from 6 to 18 min after injection and duplicate aliquots (100 µl) of each fraction were assayed for $LR^3$IGF-I content by ELISA.

### 5.2.6 Statistics

Results are expressed as mean ± SEM with the number of animals in parentheses. Treatment effects were analysed by one way analysis of variance (ANOVA) using BMDP New System for Windows version 1.1 (BMDP Statistical Software, Inc, Los Angeles, CA, USA). Relationships between variables were tested using simple regression analysis and differences between groups were assessed by t-test using Sigma Stat for Windows version 1.0 (Jandel Scientific Software, Jandel Corporation, San Jose, CA, USA).

## 5.3 RESULTS

Body weights are shown in Table 5.1. Treatment with vehicle increased the body weights of *ad libitum* fed rats by ~6% in the older and by ~35% in the younger rats over 7 days compared to their weights at the start of treatment. The most extreme nutrient restriction (67% underfed) caused weight loss in both old (13% loss) and young rats (27% loss) treated with vehicle. The 44% underfed old rats lost 7% of body weight whereas similarly underfed young rats lost 4% of body weight. Older rats fed 22% less than *ad libitum* maintained their body weight whereas similarly fed younger rats gained 10% body weight during 7 days treatment with vehicle. In older rats infused with vehicle, body weight at the end of treatment

was progressively reduced by increased nutrient restriction, being respectively 6% ($P$=0.014), 12% ($P$=0.0001) and 18% less ($P$<0.0001) in moderately (22% underfed), severely (44% underfed) and extremely undernourished rats (67% underfed) than those of *ad libitum* fed rats. In younger rats infused with vehicle, body weight at the end of treatment was also progressively reduced by increased severity of undernutrition, being respectively 18% ($P$=0.0001), 29% ($P$<0.0001) and 46% less ($P$<0.0001) in moderately (22% underfed), severely (44% underfed) and extremely undernourished (67% underfed) rats compared to the body weights of *ad libitum* fed rats.

Compared to vehicle treatment, infusion of $LR^3IGF$-I for 7 days significantly increased body weight in *ad libitum* fed young rats (by 8%, $P$=0.029) and in moderately undernourished old rats (by 5%, $P$=0.030) but not in the other groups. The fractional weights (*i.e.* organ weight as % body weight) of carcass were reduced by treatment with $LR^3IGF$-I in all groups except for the most severely underfed young animals (not shown). The fractional weights of a number of organs were affected in different ways by treatment with $LR^3IGF$-I and these responses were affected by both age and nutrition. $LR^3IGF$-I treatment increased the fractional weights of the spleen, the intestine and the combined weights of head, tail and paws regardless of age or nutrition (not shown). $LR^3IGF$-I also increased fractional weights of kidneys and thymus in an age-dependent manner and nutrition had opposite effects on their growth responses. These issues are dealt with in considerable detail below (Table 5.2, Figs. 5.13 and 5.14). $LR^3IGF$-I reduced the fractional weights of liver in both old and young rats (not shown). Individual muscles were carefully excised and weighed in the younger rats only. In the 22% and 44% underfed young rats, treatment with $LR^3IGF$-I reduced the fractional weights of the soleus, the extensor digitorum longus and the gastrocnemius muscles (not shown).

Table 5.1 Effects of $LR^3IGF$-I treatment of rats at 0.91 mg $kg^{-1}$ $d^{-1}$ for 7 days on body weight.

The values represent mean ± S.E.M. for 6 animals in each treatment group.

| | Body weight (g) | | | |
|---|---|---|---|---|
| | Older rats | | Younger rats | |
| Nutrition | Vehicle | $LR^3IGF$-I-treated | Vehicle | $LR^3IGF$-I-treated |
| ad-lib fed rats | 335.32 ± 5.32 | 352.59 ± 7.53 | 172.83 ± 4.53 | 187.06 ± 3.24$^{\star}$ |
| 22% underfed | 313.74 ± 4.94$^{\star\star}$ | 329.94 ± 4.06$^{\star}$ | 141.18 ± 2.76$^{\star\star\star}$ | 144.65 ± 1.91 |
| 44% underfed | 294.39 ± 4.40$^{\star\star\star}$ | 307.01 ± 3.93 | 122.76 ± 1.93$^{\star\star\star}$ | 126.23 ± 1.92 |
| 67% underfed | 274.81 ± 5.12$^{\star\star\star}$ | 283.36 ± 4.52 | 92.82 ± 1.25$^{\star\star\star}$ | 97.38 ± 3.08 |

$^{\star}p<0.05$, $^{\star\star}p<0.02$, $^{\star\star\star}p<0.001$ significantly different from vehicle group (ANOVA test)

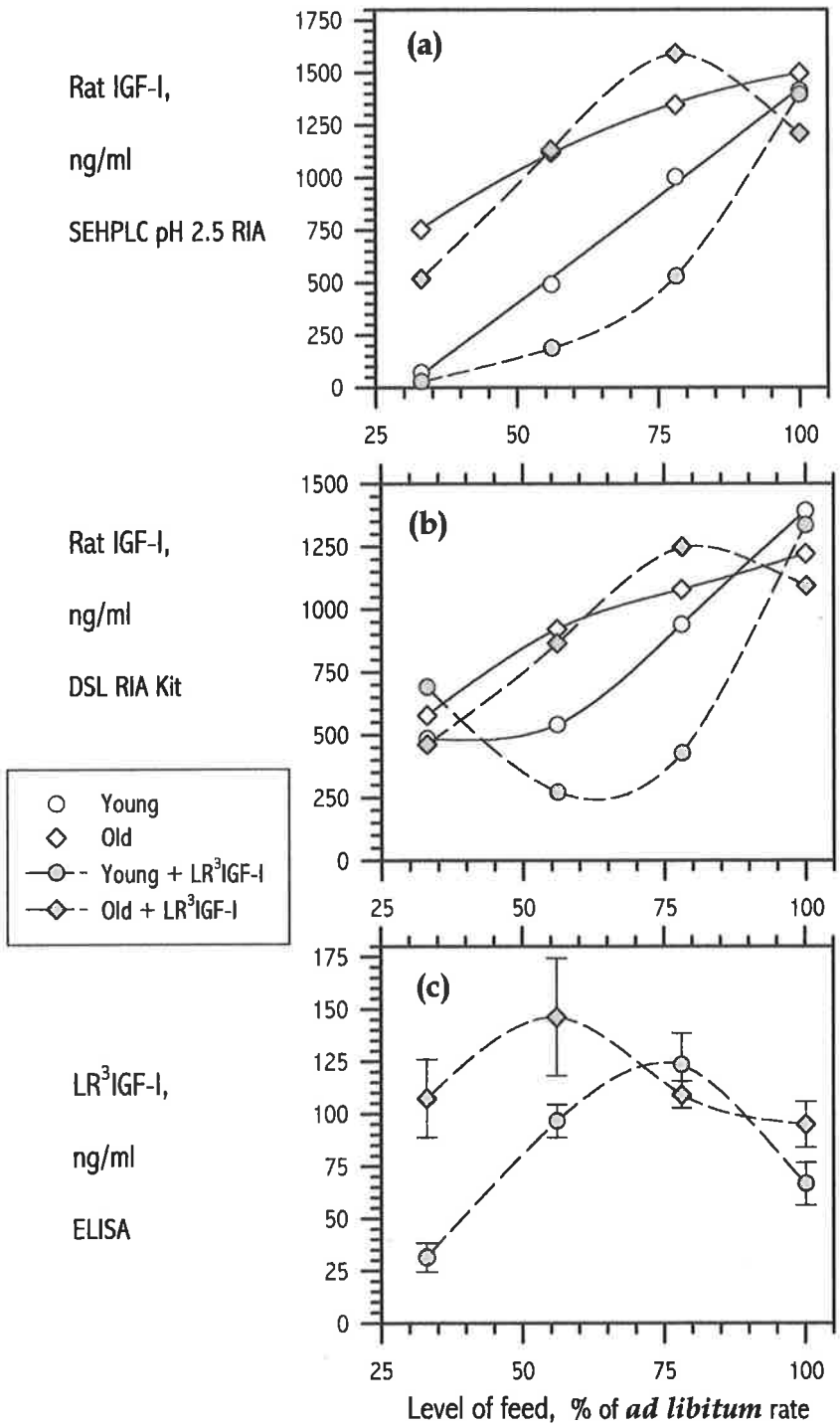
$^{\star}p<0.05$, $^{\star\star}p<0.02$, $^{\star\star\star}p<0.001$ significantly different from ad-lib group (ANOVA test)

The mean weights of older rats and younger rats were 316 and 128 g, respectively, at the start of treatment

Data kindly supplied by Dr. Frank M. Tomas, CSIRO Division of Human Nutrition.

## Figure 5.3

**Effects of age, nutrition and treatment with $LR^3IGF\text{-}I$ on plasma IGF-I in male rats**




Rat IGF-I was measured by RIA calibrated with recombinant rat IGF-I in eluates from size exclusion chromatography at pH 2.5 of plasma pooled from 6 rats in each treatment group (UPPER PANEL, a)
Rat IGF-I was also measured by DSL Rat RIA Kit which uses recombinant rat IGF-I as standard in acidic ethanol extracts of plasma (according to the manufacturer's instructions) from all animals in the study (MIDDLE PANEL, b, means only depicted)
$LR^3IGF\text{-}I$ was measured in diluted plasma by ELISA (LOWER PANEL, c, mean $\pm$ SEM depicted)

IGF-I was measured in individual specimens of rat plasma in accordance with the manufacturer's instructions using a radioimmunoassay kit designed specifically for this purpose by Diagnostic Systems Laboratories Inc. The DSL rat IGF-I RIA kit employs recombinant rat IGF-I as assay standard and requires extraction of plasma with acidic ethanol before assay. These measurements showed that in comparison to vehicle treated older rats fed *ad libitum*, feed restriction had no significant effect on plasma rat IGF-I for older animals fed 22% ($P$>0.4) and 44% ($P$>0.1) less than *ad libitum* but rat IGF-I levels were reduced by ~50% in 67% underfed older rats ($P$<0.01). In vehicle-treated younger rats, feed restriction decreased plasma IGF-I by 33% ($P$<0.01), 61% ($P$<0.001) and 65% ($P$<0.001) in 22%, 44% and 67% underfed rats respectively in comparison to *ad libitum* fed younger rats as determined by the DSL rat IGF-I RIA kit. These results were compared to those obtained by measurement of rat IGF-I by a different RIA of plasma pools extracted by acidic column chromatography (Figure 5.3*a*). Both assays detected generally similar responses of plasma rat IGF-I to both feed restriction and treatment with $LR^3$IGF-I except in the younger rats on the lowest levels of feed.

Treatment with $LR^3$IGF-I had no significant effect on endogenous IGF-I concentrations in plasma at any feed level in older rats as determined by the DSL rat IGF-I kit assay of plasma specimens from individual rats in the study ($P$>0.2, Figure 5.3*b*). Similar results were obtained with the alternate IGF-I RIA after size exclusion chromatography of plasma from older rats pooled by treatment group (Figure 5.3*a*). However in younger rats, treatment with $LR^3$IGF-I reduced endogenous IGF-I in moderately ($P$<0.001) and severely ($P$<0.01) undernourished rats by 55% and 50%, respectively, compared to vehicle-treated rats but had no significant effect on endogenous IGF-I in *ad libitum* fed animals ($P$=0.57) according to the DSL rat IGF-I RIA kit (Figure 5.3*b*). In the extremely undernourished young

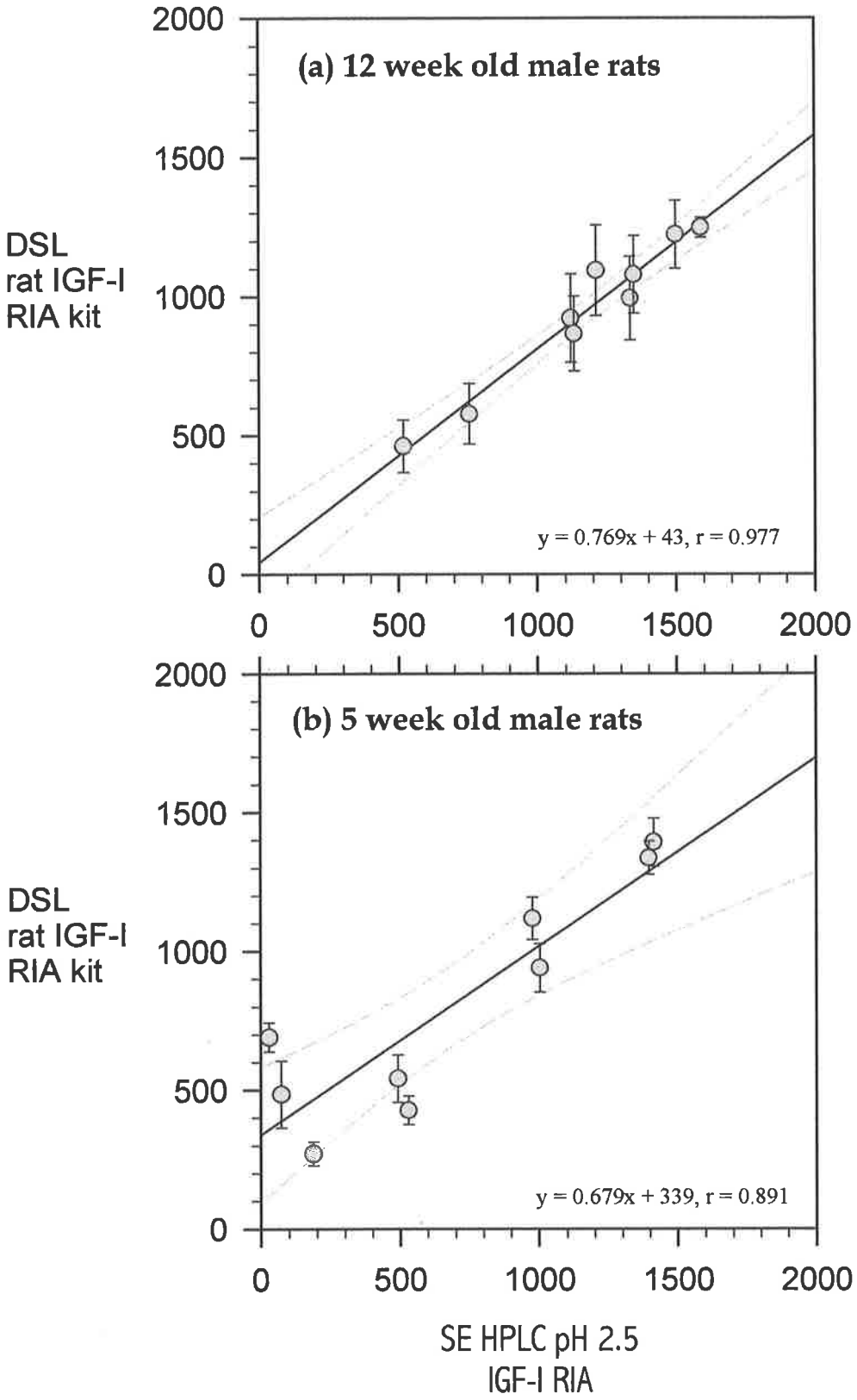
rats (67% underfed), treatment with $LR^3IGF$-I appeared to increase endogenous IGF-I by 42% compared to vehicle treatment as determined by the DSL kit assay (Fig. 5.3*b*) but this difference is not statistically significant ($P$=0.12). When rat IGF-I was measured by the alternate RIA after size exclusion chromatography of plasma from young rats pooled by treatment group, infusion of $LR^3IGF$-I was found to decrease the mean concentration of rat IGF-I in 22% underfed, 44% underfed and 67% underfed rats by 47%, 62% and 58%, respectively, compared to vehicle-treated rats (Figure 5.3*a*).

Plasma concentrations of rat IGF-I in extremely undernourished rats (67% underfed) tended to be lowered by infusion with human IGF-I in older (578 ± 100 in vehicle-treated rats and 352 ± 66 in IGF-I treated rats, $P$=0.08) but not in younger rats (485 ± 110 in vehicle-treated rats and 515 ± 134 in IGF-I treated rats, $P$=0.86) according to results from the commercial kit. The concentrations of rat IGF-I in these 67% underfed rats treated with human IGF-I can not be determined by alternate IGF-I RIA after size exclusion chromatography because this assay measures both rat and human IGF-I.

There was a positive correlation between results obtained by the two methods of measuring rat IGF-I in plasma of both older (r=0.977, n=9, $P$=0.0001, Fig. 5.4*a*) and younger rats (r=0.891, n=9, $P$=0.001, Fig. 5.4*b*). However, there was considerably more deviation in results from the two assays for plasma from the younger animals (Figure 5.4*b*). In the 67% underfed younger rats, mean rat IGF-I concentrations were 485 ng/ml (vehicle treated) and 690 ng/ml ($LR^3IGF$-I treated) according to the DSL assay kit for rat IGF-I whereas they were 73 ng/ml and 30 ng/ml, respectively, as determined by IGF-I RIA after size exclusion chromatography of plasma pooled from individual specimens in these groups. Because rat IGF-I concentrations in $LR^3IGF$-I and vehicle treated 67% underfed young rats measured by

Correlation between the concentrations of rat IGF-I measured by DSL rat IGF-I RIA kit (y) and by the alternate "in house" RIA after size exclusion chromatography at pH 2.5 (x) in plasma from (a) 12 week old male rats ($r = 0.977$, $P = 0.0001$, $n = 9$) or (b) 5 week old male rats ($r = 0.891$, $P = 0.001$, $n = 9$).

**Figure 5.4**
**Correlation between results obtained by two different methods of measurement of rat IGF-I in plasma**



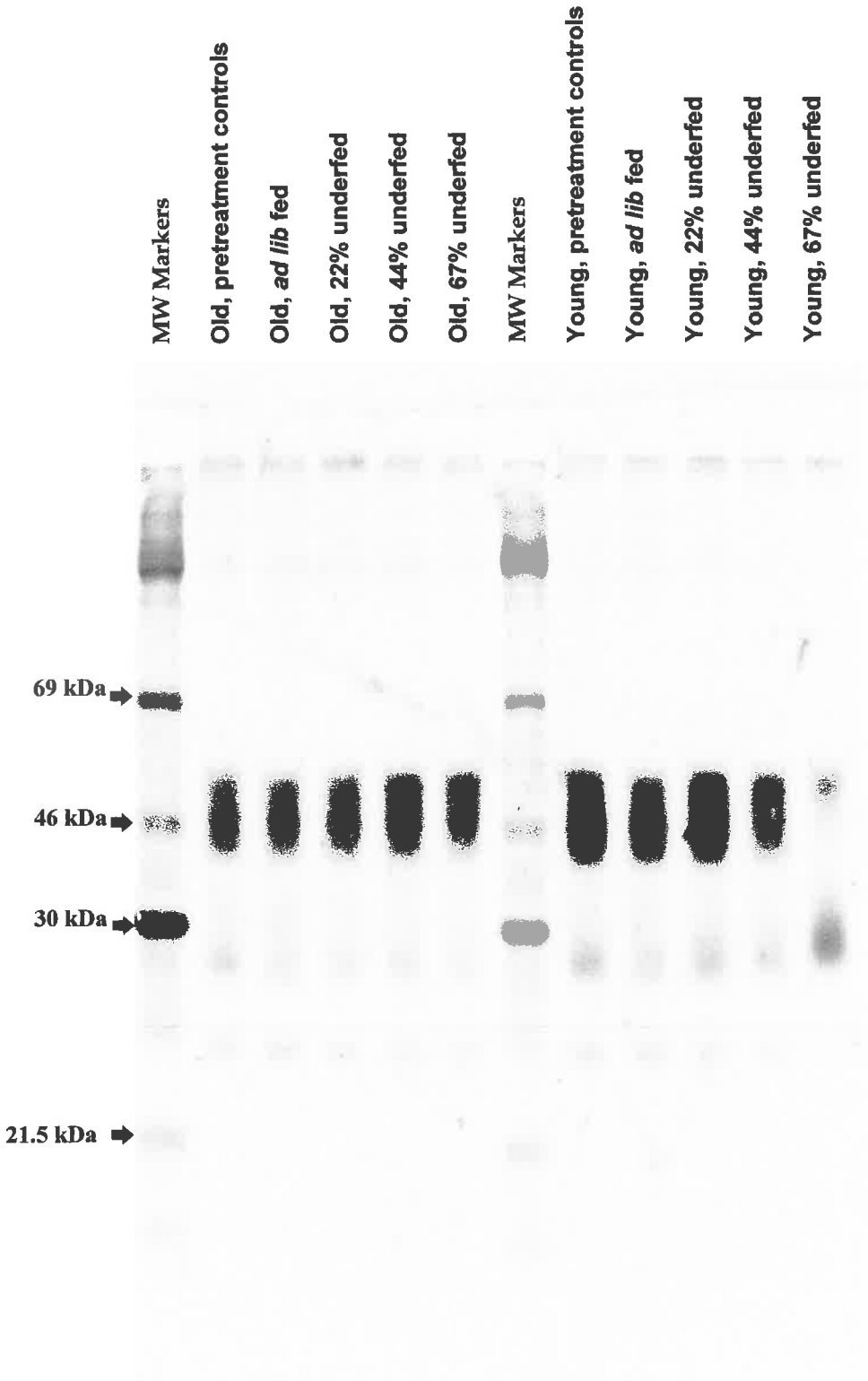

Dotted lines indicate the 95% confidence limits

DSL assay kit ($y$) are much higher than those obtained by IGF-I RIA after size exclusion chromatography ($x$), regression analysis between results obtained by these two different methods in young rats showed an intercept significantly greater than zero ($y = 0.679x + 339$, r = 0.891, Figure 5.4*b*). Omitting the data from 67% underfed younger rats treated with vehicle and with $LR^3IGF$-I from this comparison lowered the intercept of the linear regression ($y = 0.942x + 53$, r=0.977) to one similar to that obtained from the analysis of the older animals (y = 0.769x + 43, r = 0.977, Fig. 5.4*a*).

Plasma $LR^3IGF$-I was affected by the level of feed in younger rats (one way analysis of variance, $P< 0.0001$) but not older rats ($P$=0.18) (Fig. 5.3*c*). Plasma $LR^3IGF$-I was higher in older rats than younger rats overall (t-test, $P$=0.006). Comparisons between the plasma levels of $LR^3IGF$-I achieved in older and younger rats were also made within each feed level. For *ad libitum* fed rats, there was a tendency for $LR^3IGF$-I to be higher in the older animals than the younger animals ($P$=0.066). For moderately undernourished rats (22% underfed), the concentration of $LR^3IGF$-I was not significantly different between the older and the younger animals ($P$>0.3). For severely undernourished rats (44% underfed), there was a tendency for $LR^3IGF$-I to be higher in the older than the younger rats ($P$=0.092). For extremely undernourished rats (67% underfed), the concentration of $LR^3IGF$-I was significantly lower in younger rats ($P$<0.002).

Nutrition had little or no effect on plasma concentrations of IGF-binding proteins in older animals (Fig. 5.5). In contrast, reduction of feed intake to 67% less than *ad libitum* in younger rats decreased plasma IGF-binding proteins of ~40-50 kDa and ~24 kDa and increased a ~29 kDa IGF-binding protein (Figs. 5.5 and 5.7). Reduction of feed intake to 44% less than *ad libitum* in younger rats slightly decreased plasma IGF-binding proteins of ~40-50

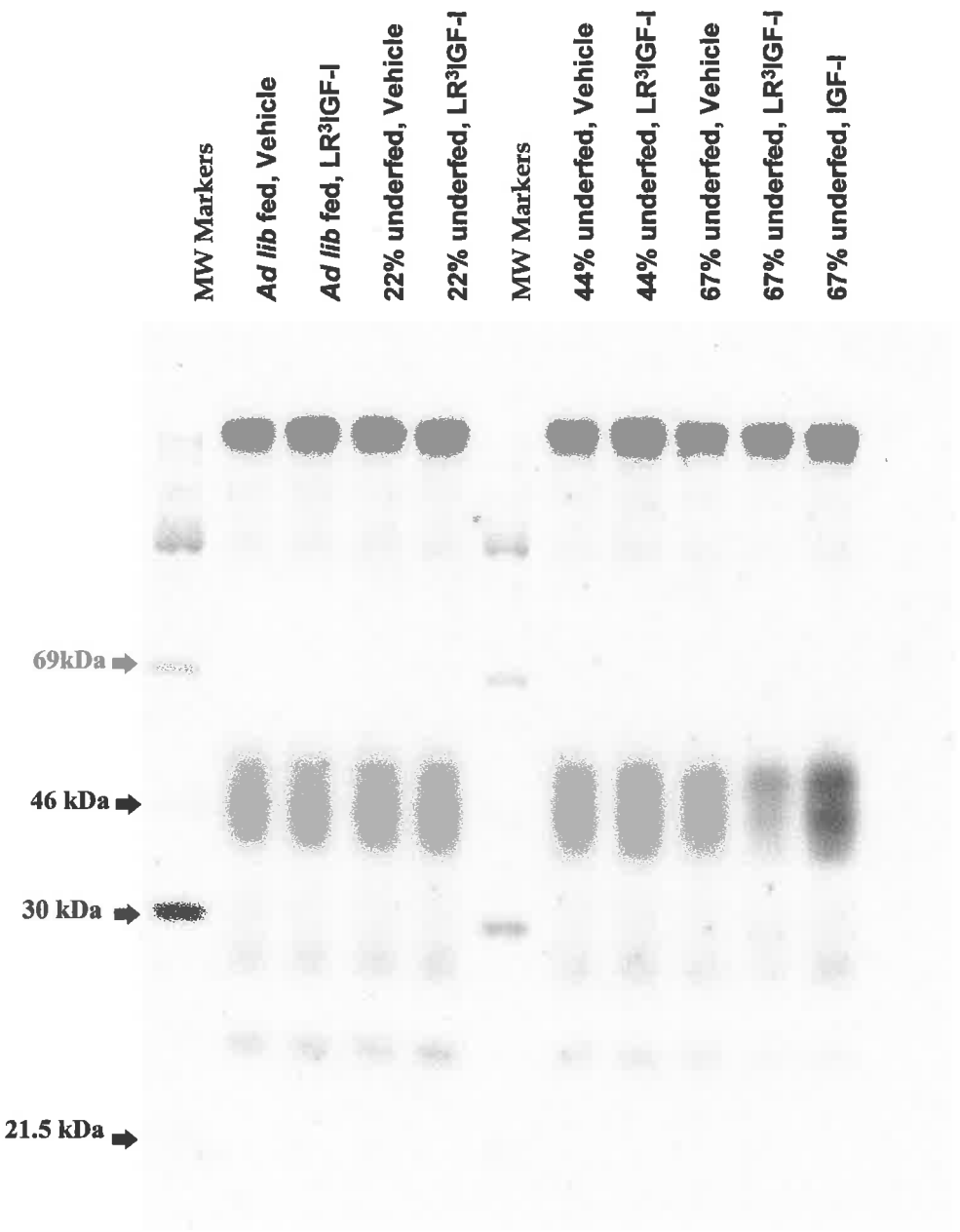
# Figure 5.5
**Effects of age and nutrition on IGF-binding proteins in male rats**




Autoradiogram after 14 days exposure of western ligand blot filter probed with [$^{125}$I]-hIGF-II

## Figure 5.6

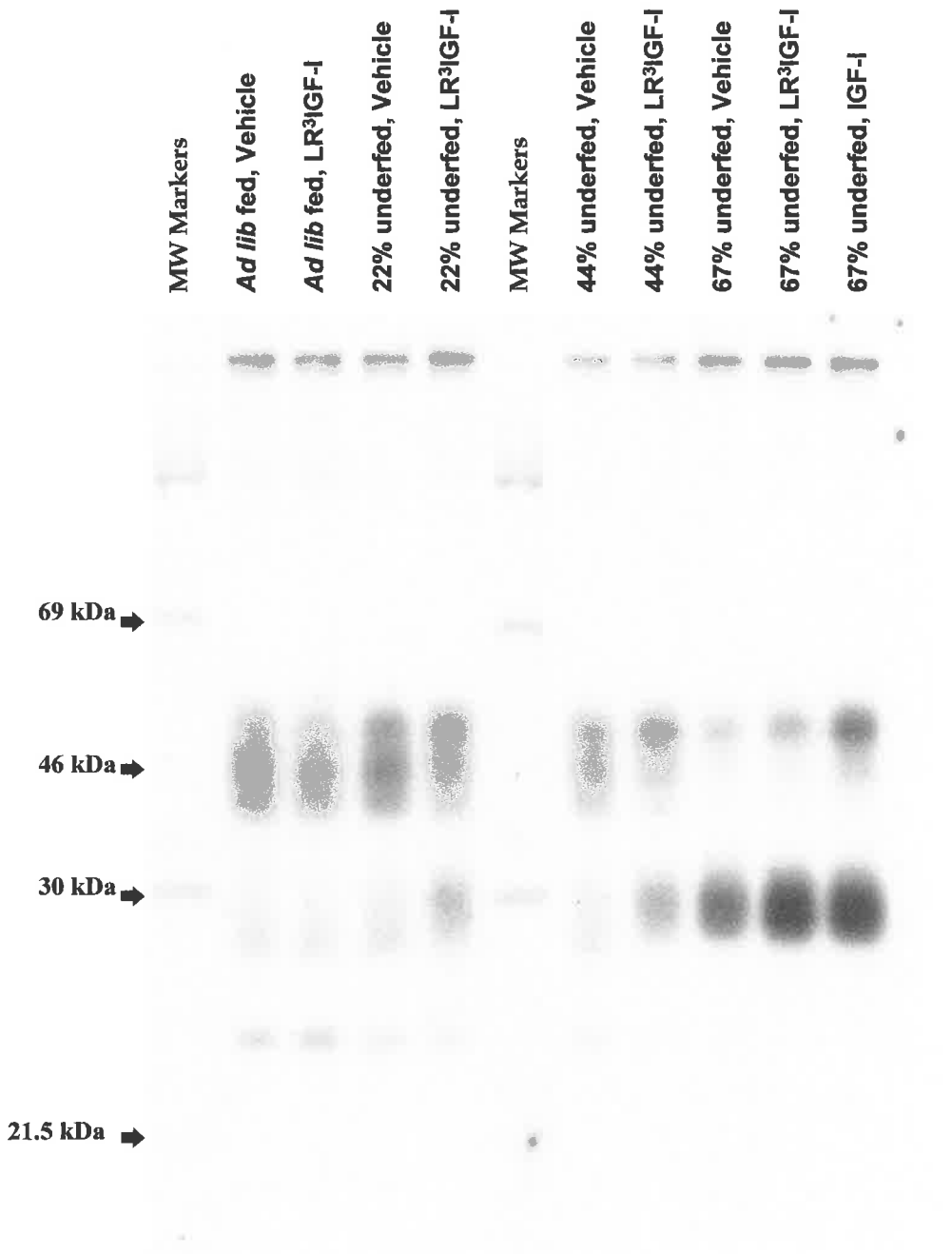
**Effects of nutrition and treatment with LR³IGF-I on IGF-binding proteins in 12 week old male rats**




Autoradiogram after 14 days exposure of a western ligand blot filter probed with [$^{125}$I]-hIGF-II

## Figure 5.7

**Effects of nutrition and treatment with LR³IGF-I on IGF-binding proteins in 5 week old male rats**




Autoradiogram after 14 days exposure of western ligand blot fiter probed with [$^{125}$I]-hIGF-II

kDa but had no effect on other binding proteins (Figs. 5.5 and 5.7). In older rats, treatment with $LR^3IGF$-I had no obvious effect on plasma concentrations of IGF-binding proteins regardless of nutrition whereas similar treatment of extremely undernourished older rats with human IGF-I increased plasma IGF-binding proteins of ~40-50 kDa (Fig. 5.6). In contrast, treatment with $LR^3IGF$-I in younger rats increased the concentrations of a ~29 kDa IGF-binding protein in all feed-restricted groups but not in *ad libitum* fed rats (Fig. 5.7). Treatment with $LR^3IGF$-I also slightly decreased the concentrations of ~40-50 kDa and ~24 kDa IGF-binding proteins in 44% underfed younger rats but not in the other groups. Treatment with IGF-I increased plasma concentrations of IGF-binding proteins of ~40-50 kDa and a ~29 kDa IGF-binding protein in the extremely undernourished younger rats.

The apparent molecular weight of the IGF-binding protein migrating slightly faster than the 30 kDa marker by Western ligand blotting of SDS-PAGE gels, referred to above as the ~29 kDa IGF-binding protein, was estimated from four experiments. The electrophoretic mobility (Rf) was expressed as a fraction of the distance migrated by the $^{14}C$-labelled "Rainbow" molecular weight marker of 21.5 kDa. The mean ± SEM of electrophoretic mobilities of $^{14}C$-labelled Rainbow molecular weight markers (n = 4) are shown in Figure 5.8.

There was neither an effect of nutrition nor of treatment with $LR^3IGF$-I on apparent molecular weight of the IGF-binding protein migrating slightly faster than the 30 kDa marker by Western ligand blotting of SDS-PAGE gels in older rats. In plasma pooled from these older animals, this IGF-binding protein had an apparent $M_r$ of 28.45 kDa under these conditions. In contrast, there were effects of nutrition and treatment with $LR^3IGF$-I on the apparent molecular weight of this IGF-binding protein by Western ligand blotting of SDS-

## Figure 5.8

**Calibration plot for estimation of molecular weight of IGF-binding proteins from Western ligand blots**

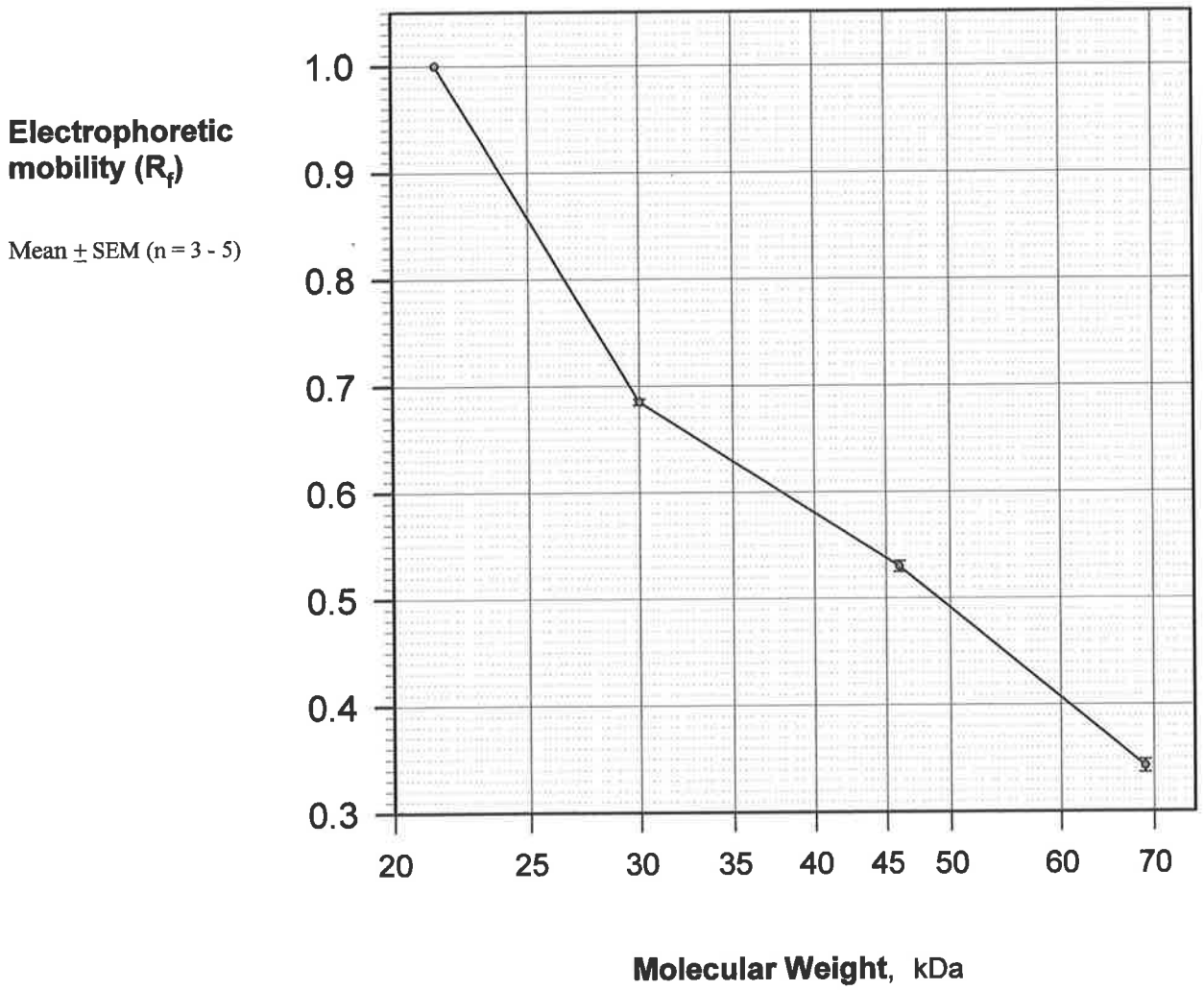
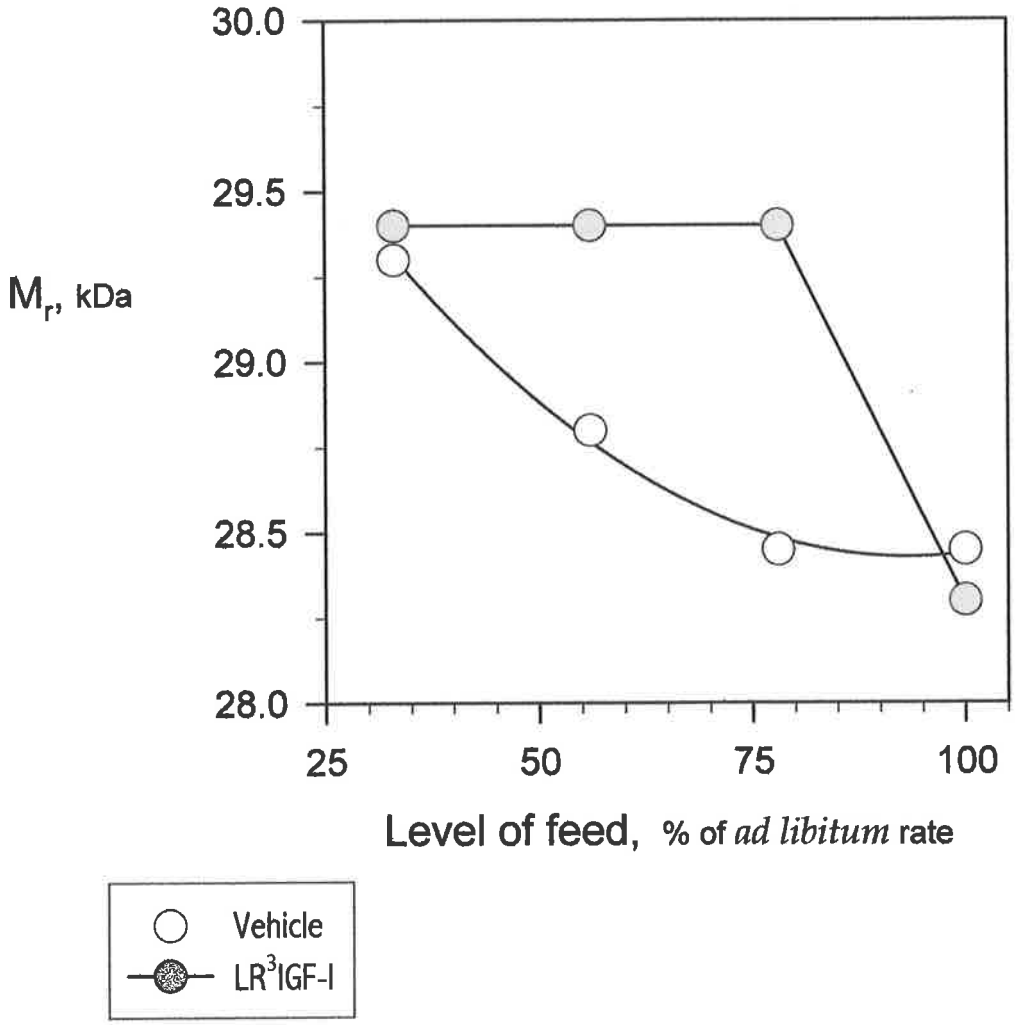


Figure 5.9
Effects of nutrition and treatment with $LR^3IGF\text{-}I$ in young rats on apparent $M_r$ of the IGF-binding protein migrating slightly faster than the 30 kDa marker by Western ligand ($[^{125}I]$-IGF-II) blotting of SDS-PAGE gels

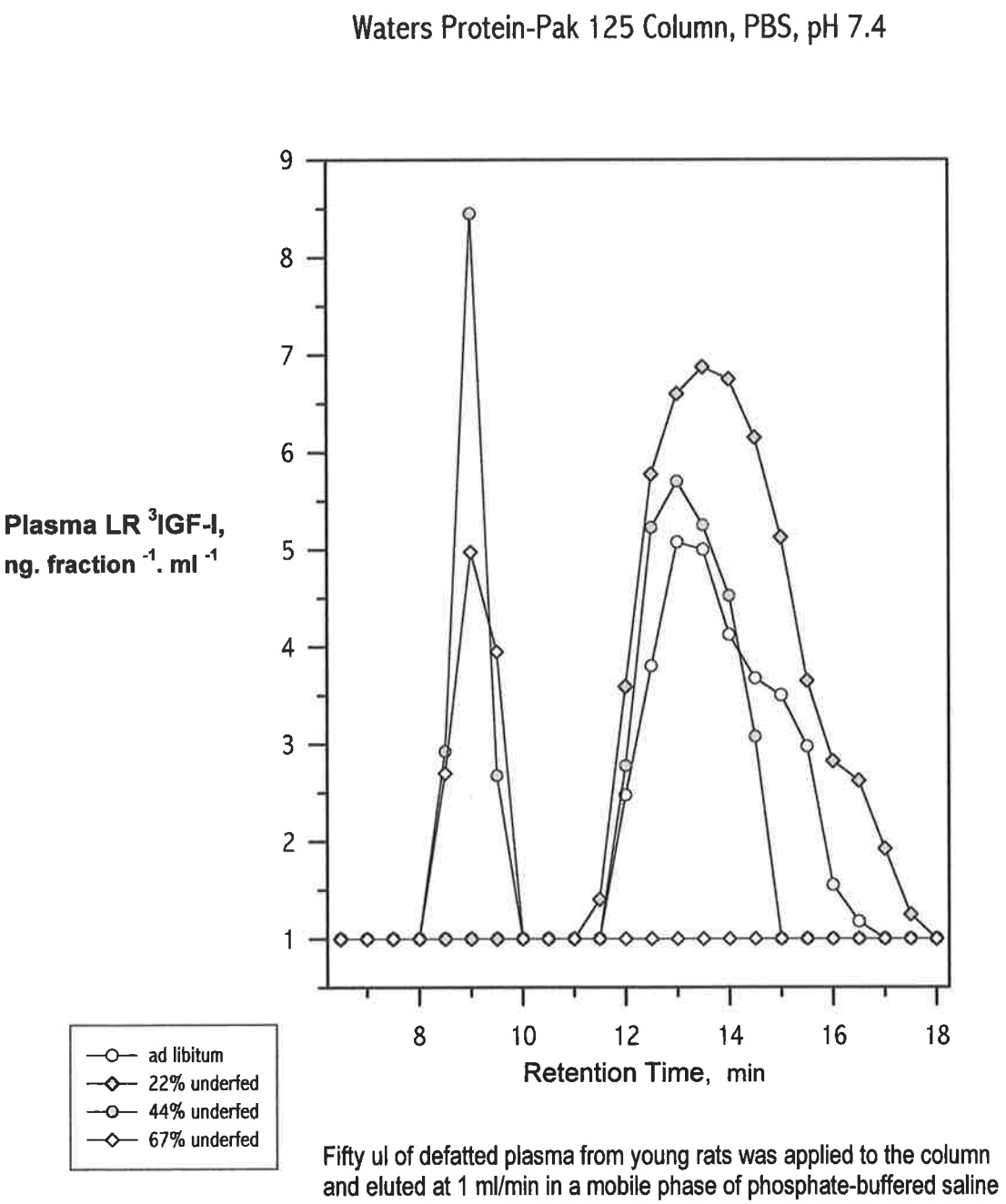


PAGE gels in young rats (Fig. 5.9). Vehicle-treated *ad libitum* fed and 22% underfed young rats contained an IGF-binding protein of the same molecular weight (28.45 kDa) which is slightly lower than that of vehicle treated 44% underfed (28.8 kDa). Vehicle treated 67% underfed young rats contained an IGF-binding protein of slightly higher molecular weight (29.3 kDa) than those of the less undernourished and well-fed young rats. $LR^3IGF$-I treated undernourished young rats (fed 22%, 44% and 67% less than *ad libitum*) had an IGF-binding protein of the same molecular weight (29.4 kDa) whereas $LR^3IGF$-I treated well-fed young rats had IGF-binding of lower molecular weight (28.3 kDa).

The molecular forms of $LR^3IGF$-I in plasma from $LR^3IGF$-I treated young and old rats in each nutrition group were characterised by chromatographing plasma pooled by age and treatment through a size exclusion column under non-dissociating conditions at physiological pH and salt concentration. The eluted fractions were assayed for $LR^3IGF$-I by ELISA. In $LR^3IGF$-I treated twelve week old rats at all feed levels, the $LR^3IGF$-I activity in blood plasma was present in a single peak of retention time ~13-14 min (data not shown). In $LR^3IGF$-I treated five week old rats which were fed either *ad libitum* or at 22% less than the *ad libitum* rate, all of the detected $LR^3IGF$-I activity in plasma was also eluted at a retention time of ~13-14 min (Fig. 5.10) after size exclusion high performance liquid chromatography at pH 7.4. However, two peaks of $LR^3IGF$-I activity were eluted with plasma pooled from the 44% underfed younger rats treated with $LR^3IGF$-I. For plasma pooled from these animals, ~30% of the $LR^3IGF$-I activity was eluted at ~9 min and 70% of $LR^3IGF$-I activity was eluted at ~13 min (Fig. 5.10). In $LR^3IGF$-I treated extremely undernourished young rats (67% underfed), a single peak of $LR^3IGF$-I activity was detected with a retention time of ~9 min.

## Figure 5.10

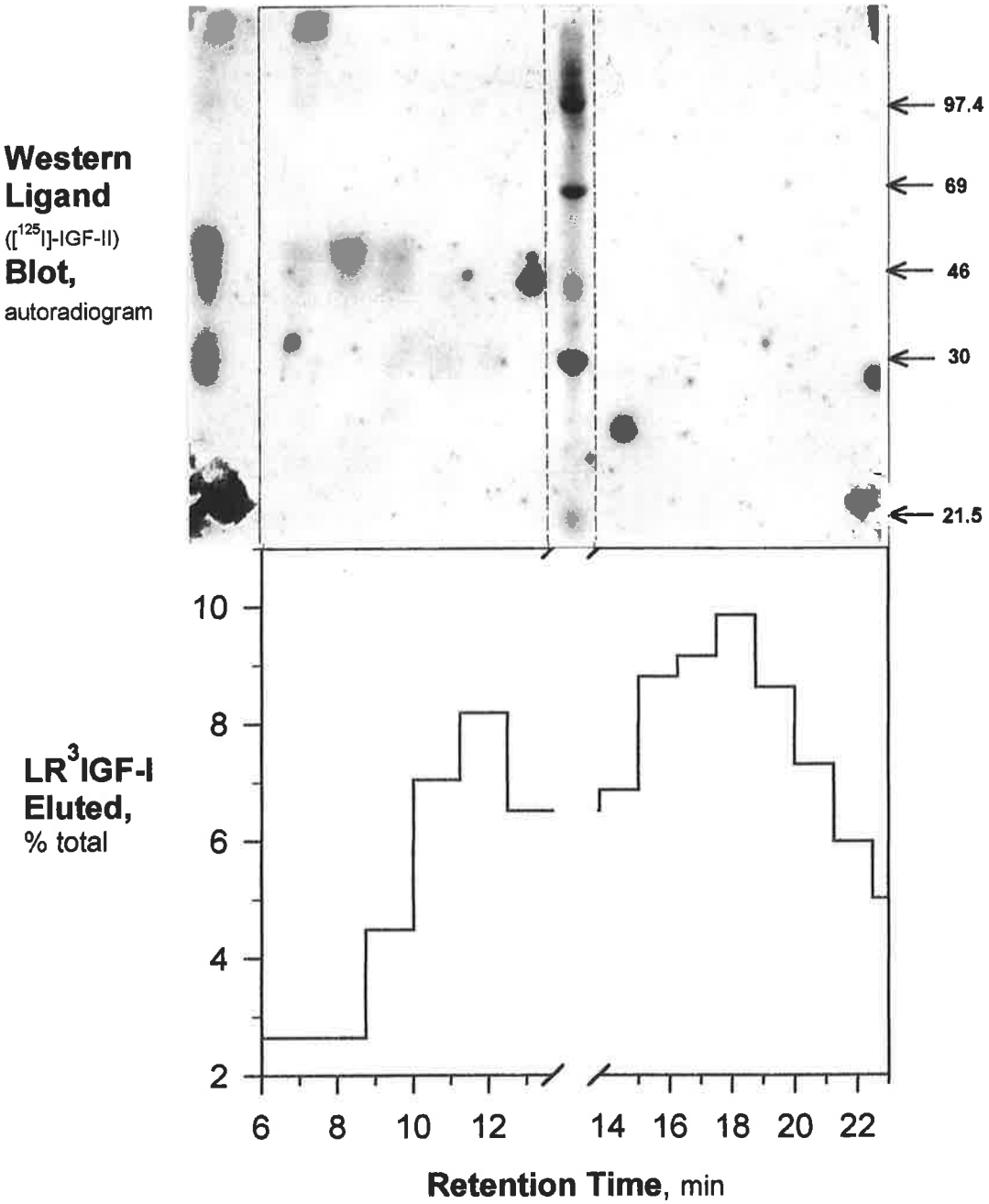
**Effect of nutrition on apparent MW of immunoreactive LR$^3$IGF-I in plasma from young rats treated with LR$^3$IGF-I**




Fifty ul of defatted plasma from young rats was applied to the column and eluted at 1 ml/min in a mobile phase of phosphate-buffered saline , pH 7.4. One hundred ul of undiluted fractions of column eluate was assayed for LR$^3$IGF-I by ELISA in duplicate.

## Figure 5-11

Size distribution of IGF-binding proteins and $LR^3IGF-I$ in plasma from a young rat underfed by 44% and treated with $LR^3IGF-I$



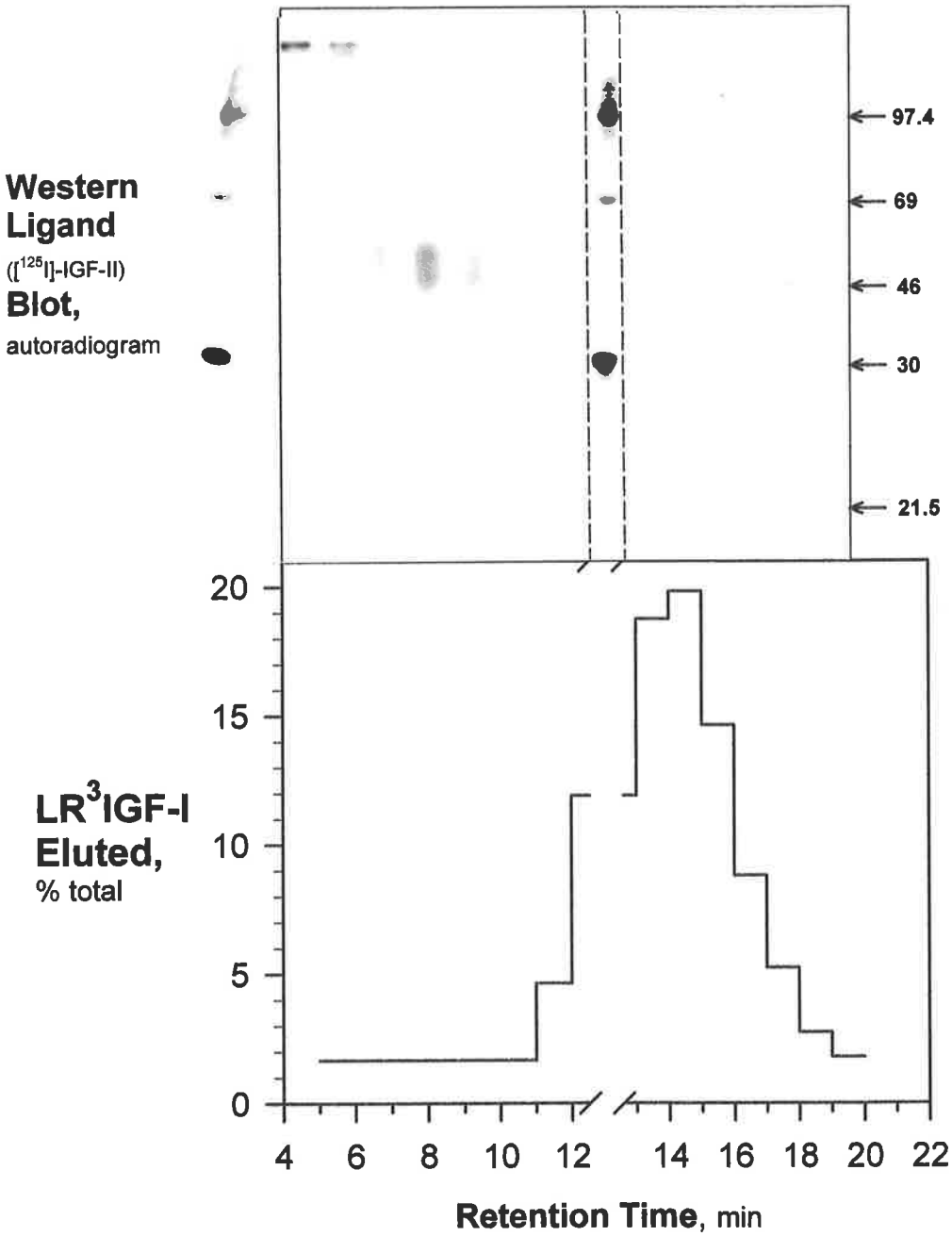

Western ligand blot (*upper panel*) and $LR^3IGF-I$ ELISA (*lower panel*) of fractions obtained by chromatography at pH 7.4 through ProteinPak 125 of 0.05 ml plasma from a 44% underfed young rat treated with $LR^3IGF-I$.

In the western ligand blot (UPPER PANEL): unchromatographed plasma was run in the lane on the extreme left and the arrows on the extreme right indicate the mass markers (KDa) run between fractions eluting at 13.75 and 15.0 ml.

The IGF-binding protein content of the different molecular size classes of immunoreactive $LR^3IGF$-I in plasma from younger rats treated with this peptide were analysed by western radio-ligand blotting (Fig. 5.11). Unchromatographed plasma from a $LR^3IGF$-I treated 44% underfed young rat contained major IGF-binding proteins of ~40-50 kDa and a minor binding protein of ~29 kDa (Fig. 5.11, upper panel, track 1). $LR^3IGF$-I in pH 7.4 column chromatography fractions from this rat was measured by ELISA (Fig. 5.11, lower panel). Two peaks of $LR^3IGF$-I activity were eluted with plasma from this $LR^3IGF$-I treated 44% underfed younger rat. These two peaks of $LR^3IGF$-I activity were not well separated. Approximately 27% of the $LR^3IGF$-I activity eluted in a peak at 11.25 min and 73% of $LR^3IGF$-I activity eluted in a peak at 17.5 min (Fig. 5.11, lower panel). The retention times of these two peaks from plasma from this individual rat were different from those previously obtained from similar chromatography of pooled plasma from $LR^3IGF$-I treated 44% underfed young rats which were eluted earlier at ~9 and ~13 min, respectively (Fig. 5.10). Western radio-ligand blotting analysis of pH 7.4 column chromatography fractions from this $LR^3IGF$-I treated 44% underfed young rat showed that IGF-binding proteins of ~40-50 kDa eluted between 7.5 and 10 min after injection (Fig. 5.11, upper panel). A weak IGF-binding protein signal of ~29 kDa eluted between 10 and 12.5 min. Therefore, the column fraction that contained the highest concentration of the high molecular weight form of $LR^3IGF$-I (retention time 12.5 min) also contained the highest concentration of ~29 kDa IGF-binding protein (Fig. 5.11, lower and upper panel). The fractions that contained the low molecular weight form of $LR^3IGF$-I (peak retention time 17.5 min) did not contain any detectable IGF-binding proteins (Figure 5.11, lower and upper panel).

## Figure 5.12

Size distribution of IGF-binding proteins and $LR^3IGF$-I in plasma from an old rat underfed by 44% and treated with $LR^3IGF$-I




Western ligand blot (*upper panel*) and $LR^3IGF$-I ELISA (*lower panel*) of fractions obtained by chromatography at pH 7.4 through ProteinPak 125 of 0.05 ml plasma from a 44% underfed old rat treated with $LR^3IGF$-I.

In the western ligand blot (UPPER PANEL) the arrows on the extreme right indicate the mass markers (KDa) run on the extreme left (track #1) and between the fractions eluting at 13 and 14 min (track #9).

Similar analysis of the size distribution of IGF-binding proteins and immunoreactive $LR^3IGF$-I by, respectively, western radio-ligand blotting and $LR^3IGF$-I ELISA of fractions obtained by pH 7.4 chromatography through a Waters Protein Pak 125 column of plasma from a twelve week old $LR^3IGF$-I treated 44% underfed rat was also performed (Fig. 5.12) because previous analysis of the size distribution at pH 7.4 of immunoreactive $LR^3IGF$-I showed that this activity was present exclusively in the lower molecular weight form in plasma from older rats. One peak of $LR^3IGF$-I activity was eluted 15 min after injection with plasma from a $LR^3IGF$-I treated 44% underfed old rat (Fig. 5.12). Western radio-ligand blotting of these column chromatography fractions showed that IGF-binding proteins of ~40-50 kDa eluted between 7 and 9 min after injection (Figure 5.12, upper panel). There were no other IGF-binding proteins detected in any fractions. Therefore, the fractions that contained low molecular weight forms of $LR^3IGF$-I (retention time 15 min) did not contain any detectable IGF-binding proteins (Figure 5.12, lower and upper panel).

To determine whether the plasma concentrations of free $LR^3IGF$-I might be related to the effects of $LR^3IGF$-I treatment, associations between plasma concentrations of free as well as total $LR^3IGF$-I and weight responses were investigated for the weights of whole body, carcass, pelt, kidneys, liver, spleen, thymus, combined weights of the head, tail and paws, and the empty gut in older and younger rats, as well as the gastrocnemius muscle, soleus muscle and extensor digitorum longus muscle in the younger rats only. Plasma concentrations of free $LR^3IGF$-I were calculated from the proportion of the total $LR^3IGF$-I in individual specimens measured by ELISA of unfractionated plasma that was determined to be free, characterized by

Table 5.2

Linear regressions between organ size (*dependent variable*) and plasma concentrations of $LR^3IGF$-I (*independent variable*) in young rats (n=24) treated with $LR^3IGF$-I and fed at variable rates.

**Probability, sign *and* r**

| ORGAN OR TISSUE | Total $LR^3IGF$-I | Free $LR^3IGF$-I |
|---|---|---|
| Body weight | ns (p>0.09) | **p =0.009, + r =0.521** |
| Carcass weight | **p =0.045, + r =0.413** | **p =0.004, + r =0.570** |
| % Carcass weight | ns (p>0.8) | ns (p>0.5) |
| % Pelt weight | ns (p>0.4) | ns (p>0.1) |
| % Kidney weight | **p =0.0005, - r =0.656** | **p <0.0001, - r =0.761** |
| % Liver weight | ns (p>0.5) | ns (p>0.1) |
| % Spleen weight | ns (p>0.1) | **p =0.032, + r =0.440** |
| % Thymus weight | **p =0.0001, + r =0.699** | **p <0.0001, + r =0.737** |
| % Head, tail and paws weight | **p =0.013, - r =0.499** | **p =0.0011, - r=0.626** |
| % Empty gut weight | ns (p>0.1) | ns (p>0.2) |
| % Gastrocnemius muscle weight | ns (p>0.1) | **p =0.015, - r =0.490** |
| % Soleus muscle weight | **p =0.023, - r =0.463** | **p =0.014, - r =0.494** |
| % Extensor digitorum longus muscle weight | ns (p>0.2) | **p =0.053, - r =0.399** |

In young rats free $LR^3IGF$-I concentrations in plasma were strongly positively dependent ($r=0.954$, $y = 1.12x - 22$) on the total plasma concentration of $LR^3IGF$-I ($p<0.0001$).

%(organ) weight indicates the fractional organ weight as a percentage of total body weight.

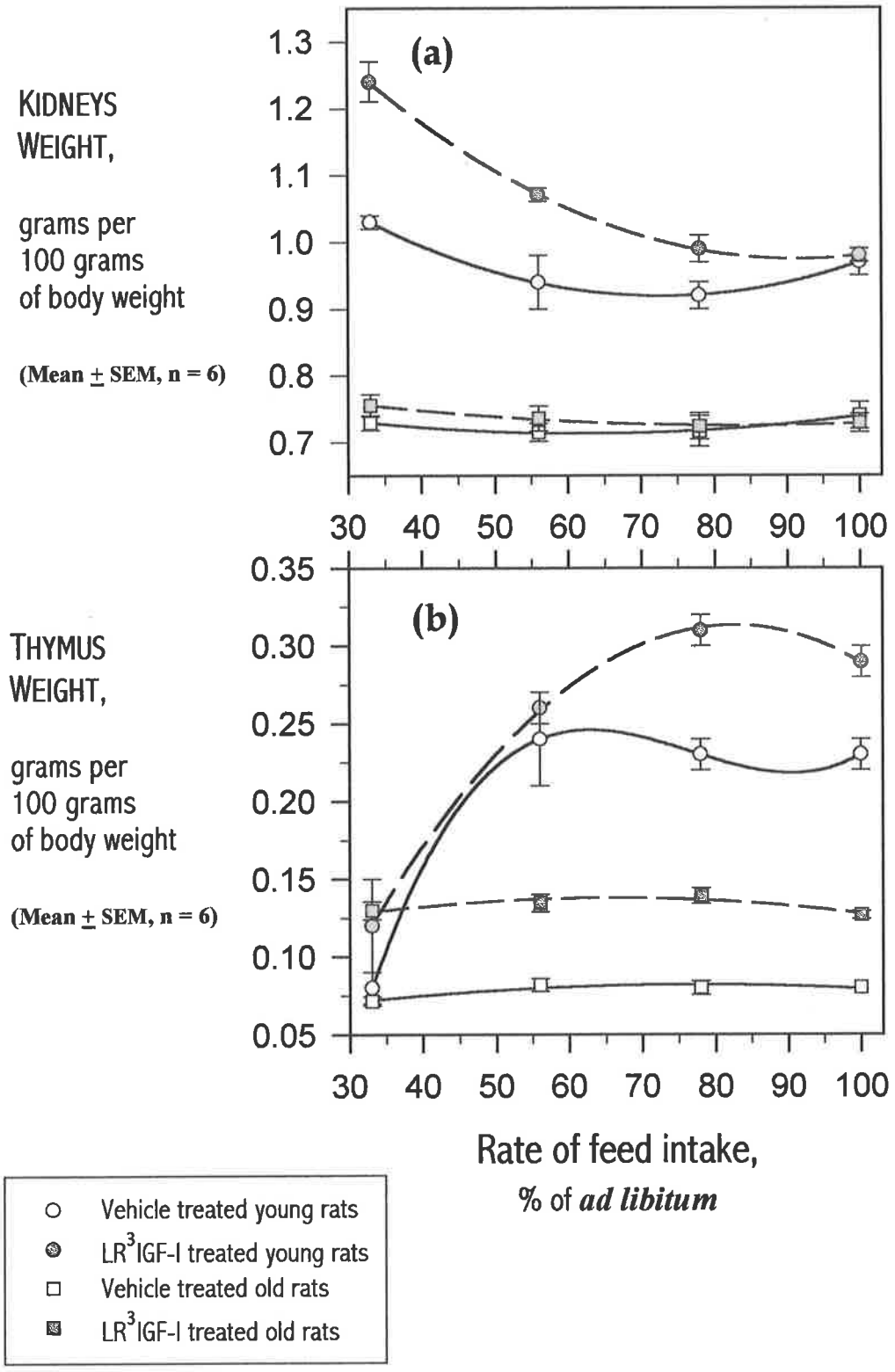
Organ weights provided by Dr. Frank M. Tomas, CSIRO Division of Human Nutrition.

chromatographing plasma pooled from treated animals through a size exclusion column under non-dissociating conditions at pH 7.4 and measuring the eluted $LR^3IGF$-I by ELISA (Fig. 5.10). For example, 100% of the $LR^3IGF$-I in plasma was free in $LR^3IGF$-I treated older rats at all feed levels and in the younger rats treated with $LR^3IGF$-I and fed either *ad libitum* or 22% less than *ad libitum*. Therefore free and total $LR^3IGF$-I concentrations are the same for plasma from these animals. $LR^3IGF$-I treated 44% underfed young rats contained 70% free $LR^3IGF$-I on average whereas $LR^3IGF$-I treated 67% underfed young rats contained no free $LR^3IGF$-I (Fig. 5.10).

Table 5.2 shows linear regressions between organ weights and plasma concentrations of $LR^3IGF$-I in young rats treated with $LR^3IGF$-I and fed at variable rates. In the old rats, there were no significant associations between plasma concentrations of total (= free) $LR^3IGF$-I with body weight, carcass weight or any organ weights ($P>0.1$) were observed. In contrast, there were several significant associations between plasma concentrations of total and/or free $LR^3IGF$-I with several organ weights in young rats.

In young rats, free $LR^3IGF$-I concentrations in plasma were naturally strongly positively dependent on the total plasma concentration of $LR^3IGF$-I. Three types of associations were found in young rats: no association, positive associations and negative associations. In every instance that there were significant associations between plasma concentrations of total or free $LR^3IGF$-I with organ weights, the associations between plasma concentrations of free $LR^3IGF$-I and organ weights were stronger than the association between plasma concentrations of total $LR^3IGF$-I and organ weights.

**Figure 5.13**
**Effect of nutrition, age and treatment with LR$^3$IGF-I on thymus and kidney growth**



There was no association between plasma concentration of either total or free $LR^3IGF$-I and carcass fractional weight, pelt fractional weight, liver fractional weight and empty gut fractional weight.

Positive associations were found between plasma concentration of total as well as free $LR^3IGF$-I and carcass weight and thymus fractional weight. Positive associations were also found between plasma concentration of free $LR^3IGF$-I and body weight and also spleen fractional weight.

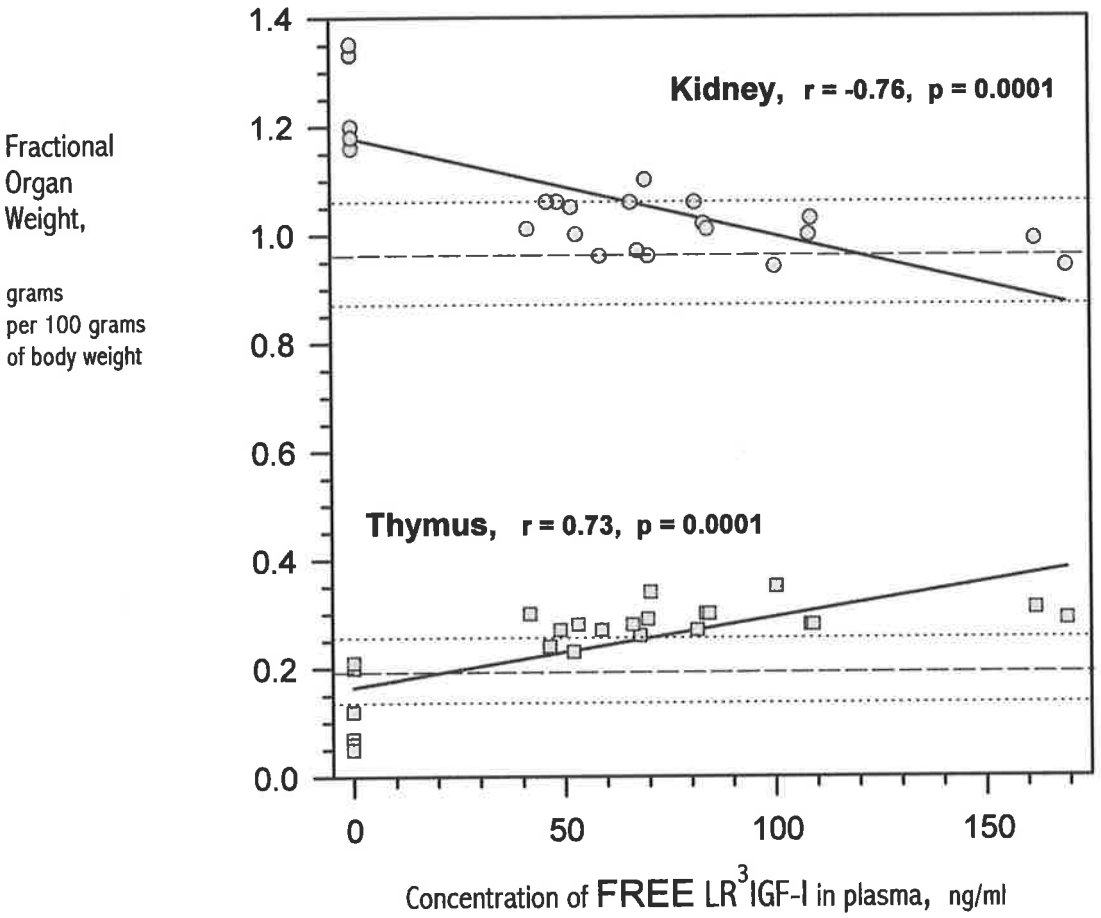
Negative associations were found between plasma concentration of total as well as free $LR^3IGF$-I and kidney fractional weight, combined fractional weights of head, tail plus paws and soleus muscle fractional weight. Negative associations were also found between plasma concentrations of free $LR^3IGF$-I and fractional weights of gastrocnemius muscle and extensor digitorum longus muscle.

The fractional weight of kidneys was significantly increased by $LR^3IGF$-I treatment in 22% underfed young rats ($P$=0.015), 44% underfed young rats ($P$=0.0079) and 67% underfed young rats ($P$=0.0002) but not in *ad libitum* fed young rats and old rats at all feed levels (Figure 5.13*a*).

The fractional weight of thymus was significantly increased by $LR^3IGF$-I in the older rats at all feed levels ($P<1x10^{-6}$), in *ad libitum* fed young rats ($P$=0.0016) and in 22% underfed young rats ($P$=0.0001) but not in 44% and 67% underfed younger rats ($P>0.6$ and $P>0.25$, respectively) (Figure 5.13*b*).

## Figure 5.14

**Relationship between circulating free $LR^3IGF\text{-}I$ and growth of kidneys and thymus in 24 variably fed young rats treated with $LR^3IGF\text{-}I$**




The mean and 95% confidence limits of the fractional weights of kidneys and thymus from vehicle-treated rats are indicated by the horizontal lines.

As reported above (Table 5.2), there was a positive correlation (illustrated in Figure 5.14) between the concentrations of free $LR^3IGF$-I and fractional weights of thymus in younger rats treated with $LR^3IGF$-I. Similar relationships were observed for carcass and spleen (Table 5.2). In contrast, there was a negative correlation (illustrated in Figure 5.14) between the concentrations of free $LR^3IGF$-I and weights of kidneys in young rats. Similar relationships were observed for combined weight of head, tail and paws, and for individual muscles (Table 5.2).

## 5.4 DISCUSSION

In both younger and older rats infused with vehicle, body weights were lower in feed restricted animals compared to those of similarly aged animals fed *ad libitum*. Treatment with $LR^3IGF$-I increased body weight gain in *ad libitum* fed young rats and in moderately undernourished old rats but not in the other groups. Thissen *et al.* (1991) suggested that dietary restriction might impair the growth promoting actions of IGF-I. They reported that infusion with recombinant human IGF-I by osmotic mini-pump at 3 $mg.kg^{-1}.d^{-1}$ for a week into 4 week-old protein-restricted rats did not stimulate carcass growth, despite the normalisation of serum IGF-I concentrations although growth of the spleen and kidneys was enhanced. This result was different to that obtained with human IGF-I infusion at 1.5 $mg.kg^{-1}.d^{-1}$ into well nourished hypophysectomized rats, where a significant growth response of both carcass and internal organs was observed (Thissen *et al.*1991). They suggested that dietary protein restriction caused organ-specific resistance to the growth promoting properties of exogenous IGF-I. In contrast, Tomas *et al.* (1991*b*) reported that exogenous IGF-I can partly protect body protein reserves during nitrogen restriction. They reported that treatment with 1.2 $mg.kg^{-1}.d^{-1}$ human IGF-I reduced body weight loss due to dietary protein deficiency,

after differences in food intake had been taken into account by analysis of variance. The fractional rate of muscle protein synthesis was increased significantly but the rate of muscle protein breakdown was not significantly altered by the treatment (Tomas *et al.* 1991*b*). In the same report they showed that treatment with a higher dose of human IGF-I (2.9 $mg.kg^{-1}.d^{-1}$) increased kidney weight and body water content and decreased body fat content (Tomas *et al.* 1991). Recently, Oddy and Owens (1996) reported that acute direct infusion of IGF-I at 0.3 $mg.kg^{-1}.d^{-1}$ into muscle of lambs improved nitrogen balance of muscle regardless of the level of feed provided. More recently, treatment of adolescent pigs with $LR^3$IGF-I at 0.2 $mg.kg^{-1}.d^{-1}$ for 15 days was found to reduce live weight gain and suppress plasma levels of IGF-I, IGF-II and IGF-binding protein-3 measured by specific RIA (Walton *et al.* 1997). Also, analysis of endogenous rat IGF-I and exogenous IGFs by specific RIAs in normal, in glucocorticoid treated and in hypoinsulinemic rats showed that the effects of treatment with human IGF-I, human des(1-3)IGF-I and $LR^3$IGF-I for one week on live weight and on blood levels of exogenous IGFs, endogenous rat IGF-I and IGF-binding proteins varies with pathophysiology (Owens *et al.* 1997).

In the present study, $LR^3$IGF-I treatment promoted liveweight gain in only two of the eight age-nutrition groups: the 22% underfed older rats and the *ad libitum* fed younger rats. Since nutrition affected both endogenous rat IGF-I levels and exogenous $LR^3$IGF-I levels, the following analysis was undertaken to see whether the overall growth responses could be explained by comparison of the plasma concentrations of rat IGF-I in vehicle treated animals with the total IGF concentration (*i.e.* rat IGF-I plus $LR^3$IGF-I) in the animals that were treated with the IGF-I analog. Before this exercise could be performed, the validity of the rat IGF-I measurements (Fig. 5.4) must be considered.

In the present study, age affected the responses to undernutrition and treatment with $LR^3IGF$-I of blood plasma concentrations of rat IGF-I, IGF-binding proteins and $LR^3IGF$-I. Rat IGF-I was measured in plasma using two different methods (*i.e.* by DSL rat IGF-I RIA kit which uses an acid-ethanol extraction and by an alternate "in house" IGF-I RIA which uses acid size exclusion chromatography [Figs. 5.1, 5.2]). In all groups except the younger rats on the lowest level of feed, there was reasonable agreement between the results obtained by the two methods of rat IGF-I measurement. The results for vehicle and $LR^3IGF$-I treated 67% underfed young rats were vastly different (Fig. 5.3), being very much higher when measured by the DSL rat IGF-I RIA kit (> 500 ng/ml) than by "in house" method (< 100 ng/ml). Plasma from these two groups also contained very high concentrations of ~29 kDa IGF-binding protein (Figs. 5.5, 5.6, 5.7), higher than in any other group of rats studied.

Crawford *et al.* (1992) showed that more than 98% of IGFBP-3 was removed from rat plasma by acid-ethanol extraction methods but only 24-40% of the lower molecular weight IGF-binding proteins (~24 to 32 kDa) were removed by this method. This suggests that in the present study the acid-ethanol extraction does not effectively eliminate IGF-binding proteins from the DSL rat IGF-I RIA system. This might cause problems in the assay especially when high concentrations of ~29 kDa IGF-binding protein are present in the plasma specimens. This IGF-binding protein could bind radiolabelled IGF-I in the RIA which would inhibit its binding to the primary antibody in the assay and therefore produce overestimation artefacts.

This does not appear to cause significant problems for plasma from the older rats in the present study because they have very low concentrations of low molecular mass IGF-binding proteins (Fig. 5.5, 5.6). Rivero *et al.* (1994) also found a significant correlation between serum IGF-I levels measured by RIA after acid-ethanol cryo-extraction and after gel

filtration in fed and undernourished adult rats (70-day-old) but not in well-fed or undernourished neonatal rats (10- and 20-day old). They also suggested that the small molecular mass IGF-binding proteins, whose plasma concentrations were higher in the neonates than the adults, were not eliminated from the IGF-I RIA by acid-ethanol cryo-precipitation and interfered in the IGF-I RIA. The most effective and reliable method for separation of IGF-binding proteins from IGFs prior to assay is size exclusion chromatographic extraction under acidic, dissociating conditions (Hintz & Liu 1977, Powell *et al.* 1986, Daughaday *et al.* 1987, Owens *et al.* 1990, Breier *et al.* 1991, Crawford *et al.* 1992). Therefore, the rat IGF-I measurements obtained by RIA after acid size exclusion chromatography of plasma pooled from treatment groups were used for further analysis. Since there is only one such measurement for each treatment group, because of limitations on the volume of plasma available, statistical treatment of the data cannot be included in this discussion.

In younger *ad libitum* fed rats, the sum of the group mean concentrations of rat IGF-I (determined by "in house" RIA after acid column chromatography of plasma pooled by treatment group) plus that of LR$^3$IGF-I (the group mean of individual ELISA measurements) in LR$^3$IGF-I treated animals (1463 ng/ml) was only 3.5% different from the mean concentration of rat IGF-I in similar vehicle treated rats (1414 ng/ml). Therefore treatment with LR$^3$IGF-I promoted live weight gain in younger rats fed *ad libitum* without markedly increasing the total concentration of IGFs in blood. In all other nutrition treatments of younger rats, LR$^3$IGF-I appeared to reduce the total plasma concentration of IGFs (LR$^3$IGF-I plus rat IGF-I). In 22% underfed young rats, the combined concentrations of rat IGF-I plus LR$^3$IGF-I in the treated animals (653 ng/ml) were ~35% lower than the concentrations of rat IGF-I in vehicle treated rats (1004 ng/ml). Similarly in 44% underfed young rats these were 284 ng/ml (rat IGF-I plus LR$^3$IGF-I) in the treated and 492 ng/ml (rat IGF-I) in the control animals. In the 67% underfed young rats they were 61 ng/ml (rat IGF-I plus LR$^3$IGF-I) in the

rats treated with LR$^3$IGF-I and 73 ng/ml (rat IGF-I) in the vehicle treated rats. The failure of LR$^3$IGF-I treatment to promote live weight gain in the 5 week old rats may therefore be explained by the failure of the treatment to increase the total IGF concentration in circulation. However, despite apparently the lower total levels of IGFs in blood in all underfed groups of the younger rats due to treatment with LR$^3$IGF-I, the treated young rats did not gain live weight more slowly than the vehicle treated animals of the same age and nutritional status.

In 22% underfed but in no other group of 12 week old rats, treatment with LR$^3$IGF-I promoted live weight gain and the combined concentration of rat IGF-I plus LR$^3$IGF-I in the LR$^3$IGF-I treated rats (1700 ng/ml) appeared ~25% higher than the concentration of rat IGF-I in similarly fed vehicle treated rats (1348 ng/ml). However, the combined concentrations of rat IGF-I plus LR$^3$IGF-I were ~13% lower than the concentration of rat IGF-I in vehicle treated *ad libitum* fed old rats (1307 and 1499 ng/ml, respectively) and in vehicle treated 67% underfed old rats (625 and 755 ng/ml, respectively, an apparent decrease of ~17% due to treatment with LR$^3$IGF-I), consistent with their failure to gain weight in response to LR$^3$IGF-I treatment. However, the effect of LR$^3$IGF-I treatment on total plasma IGFs cannot readily explain the lack of a growth response to LR$^3$IGF-I treatment in 44% underfed older rats. In these animals, the combined concentration of rat IGF-I plus LR$^3$IGF-I in the LR$^3$IGF-I treated rats (1279 ng/ml) appeared to be somewhat higher (~14%) than the concentration of rat IGF-I in similarly fed vehicle treated rats (1121 ng/ml).

Thus, as observed with the younger rats, the overall growth responses to treatment with LR$^3$IGF-I cannot be readily explained by the circulating concentrations of IGFs. Therefore, either circulating IGFs do not directly promote growth of rats or the growth promoting bioactivity of circulating IGFs varies with nutrition and age.

In younger animals, undernutrition reduced plasma concentrations of IGF-I, ~40-50 kDa IGF-binding proteins and a ~24 kDa IGF-binding protein but increased a ~29 kDa IGF-binding protein. The ~40-50 kDa IGF-binding proteins have previously been identified as isoforms of IGFBP-3 (Baxter & Martin 1987). It has been suggested that the band with molecular mass of 24 kDa is IGFBP-4 (Shimasaki *et al.* 1990). Treatment with $LR^3IGF$-I reduced the circulating concentrations of rat IGF-I and increased that of the ~29 kDa IGF-binding protein in these younger rats. In the older animals, neither undernutrition nor treatment with $LR^3IGF$-I had more than a minor effect on the concentrations of IGF-binding proteins. However, feed restriction to 67% less than *ad libitum* significantly reduced the concentrations of rat IGF-I in the vehicle treated older rats.

Thissen *et al.* (1994) reviewed the mechanisms by which undernutrition reduces endogenous IGF-I. Firstly, nutrient deprivation decreases hepatic IGF-I production by diminishing IGF-I gene expression. Secondly, dietary restriction also increases the clearance and degradation of serum IGF through changes in the levels of circulating IGF-binding proteins. Thirdly, fasting results in a marked decrease in the number of somatogenic receptors, indicating that undernutrition blocks the ability of growth hormone to stimulate IGF-I gene expression via the growth hormone receptor. In addition, dietary restriction also causes serum insulin concentrations and availability of amino acids to decline and this might be involved in the decline of serum IGF-I.

Fliesen *et al.* (1989) showed that malnutrition impaired growth and reduced serum IGF-I levels but this effect was attenuated with increasing age in rats. Oster *et al.* (1996) also found that 4 week-old male rats which were fed 40% of the caloric intake of controls had reduced serum IGF-I, IGF-binding protein-3, acid-labile subunit and growth hormone-binding protein levels and this reduction was significantly greater than observed with similarly diet

restricted 8 week-old rats. They concluded that the suppression of the IGF-I system in response to chronic undernutrition is greater in younger rats.

Age appeared to effect the response of plasma IGF-I levels to treatment with $LR^3IGF$-I in the present study. $LR^3IGF$-I had little or no effect on the concentrations of endogenous IGF-I in older rats. Previous studies have shown no effect of treatment with $LR^3IGF$-I on plasma IGF-I levels in rats of similar age to the younger animals in the present study (*i.e.* 90 to 140 g body weight) including dexamethasone-treated rats, normal rats and streptozotocin hypoinsulinemic rats (Tomas *et al.* 1992, 1993*a*, 1993*b*). However, in younger rats in the present study, treatment with $LR^3IGF$-I reduced endogenous IGF-I in undernourished rats but not in *ad libitum* fed rats. Reduction of endogenous IGF-I and IGF-II by infusion of $LR^3IGF$-I has recently been observed in cows (Owens *et al.* 1994), guinea pigs (Conlon *et al.* 1995*b*), pigs (Walton *et al.* 1997) and rats (Owens *et al.* 1997). A decrease of endogenous IGF-I levels by $LR^3IGF$-I administration was also found in neonatal calves (Hammon & Blum 1997). The mechanisms responsible for the reduction of endogenous IGFs by treatment with $LR^3IGF$-I may include inhibition of GH secretion, inhibition of IGF-I secretion and enhanced IGF clearance and delivery of IGFs to tissues by altered circulating IGF-binding proteins.

The effects of nutrition and $LR^3IGF$-I treatment on IGF-binding protein concentrations in blood were also affected by age. The plasma concentrations of binding proteins were not markedly altered by food intake in the older rats, but IGFBP-3 and 24 kDa IGF-binding protein concentrations were decreased and concentrations of a ~29 kDa IGF-binding protein were increased in the undernourished younger rats compared to those of the *ad libitum* fed rats

of the same age. Previous reports also claimed that protein deprivation decreased plasma concentrations of IGFBP-3 and 24 kDa IGF-binding protein whereas those of a 30 kDa IGF-binding protein were elevated (Umezawa *et al.* 1991, Takenaka *et al.* 1996). In the present study, nutritional deprivation increased plasma levels of a ~29 kDa IGF-binding protein in the younger rats and this was further increased by $LR^3IGF$-I treatment. In guinea pigs, treatment with $LR^3IGF$-I also led to an increase in the minor ~28 and ~31 kDa IGF-binding proteins but it reduced IGF-binding proteins of ~40-50 kDa in plasma (Conlon *et al.* 1995*b*). Treatment with $LR^3IGF$-I in cows also increased a 31-32 kDa IGF-binding protein but had no effect on IGFBP-3 (Owens *et al.* 1994). Subcutaneous injection of $LR^3IGF$-I into neonatal calves increased the 35 kDa IGF-binding protein band which was identified as IGFBP-2 whereas it had no effect on IGFBP-3 (Hammon & Blum 1997).

The effects of nutrition and treatment with IGF-I or $LR^3IGF$-I on circulating concentrations of IGF-binding proteins therefore are different for individual binding proteins and vary with age and animal species. The results presented in this chapter show that age and nutrition affect the molecular form(s) of immunoreactive $LR^3IGF$-I found in blood plasma from rats treated with biologically active doses of this IGF-I analog.

. $LR^3IGF$-I ELISA of eluates obtained by size exclusion hplc at pH 7.4 of plasma pooled by treatment group showed that all of the circulating $LR^3IGF$-I was in the free form in all groups of older rats and in the better fed groups of young rats. However, in the younger animals whose feed was restricted by 44% and 67%, $LR^3IGF$-I was present in plasma in high molecular weight forms. For plasma pooled from $LR^3IGF$-I treated 44% underfed young rats, ~30% of the $LR^3IGF$-I activity was in a high molecular weight form and 70% was in the free form. In $LR^3IGF$-I treated extremely undernourished young rats (67% underfed), a single

peak of high molecular weight $LR^3IGF$-I activity was detected. Western radioligand blotting analysis of IGF-binding proteins in neutral column fractions obtained from chromatographic separation of plasma from $LR^3IGF$-I treated young rats which were fed at 44% less than the *ad libitum* rate proved that the column fractions that contained most of the high molecular weight form of $LR^3IGF$-I also contained the highest concentrations of an IGF-binding protein whose molecular weight was approximately 29 kDa. Unlike the studies of guinea pig plasma in the previous chapter, the conversion of high molecular weight forms of $LR^3IGF$-I in rat plasma to low molecular weight forms by *in vitro* addition of IGF-I and IGF-II could not be performed due to the limited quantities of rat plasma. However, it is likely that the high molecular weight form of $LR^3IGF$-I was due to $LR^3IGF$-I having formed complexes with an IGF-binding protein of ~29 kDa in rat blood.

The age dependent effects of nutrition on formation of complexes between $LR^3IGF$-I and IGF-binding protein(s) in young and old animals can be explained by the different IGF-I and IGF-binding protein responses to undernutrition and $LR^3IGF$-I treatment at these two ages. Complex formation between $LR^3IGF$-I and an ~29 kDa IGF-binding proteins in undernourished younger rats (44% and 67% underfed) may be promoted by the very low plasma concentrations of rat IGF-I and the high concentrations of ~29 kDa IGF-binding protein. This may result in high availability of unsaturated ~29 kDa binding proteins *in vivo*. Both undernutrition and $LR^3IGF$-I treatment also produced small physicochemical changes in the ~29 kDa binding protein, increasing its apparent molecular mass on SDS-PAGE gels from <29 kDa to >29 kDa. It is possible that these changes in structure of the protein also increase its affinity for $LR^3IGF$-I.

In older animals, treatment with $LR^3IGF$-I and undernutrition had little or no effect on the concentrations of endogenous IGF-I and IGF-binding proteins in plasma and the concentrations of ~29 kDa binding protein were very low. Therefore, there would only be very small amounts, if any, of unsaturated ~29 kDa IGF-binding protein available for binding to $LR^3IGF$-I in the 12 week old rats.

It was not possible to conclusively define which, if any, rat IGF-binding protein bound $LR^3IGF$-I in plasma from poorly fed young rats from the investigation in this chapter. Rat IGF-binding protein-1 and binding protein-2 have very similar apparent molecular masses on SDS-PAGE gels within the 28 to 32 kDa range Also, levels of IGFBP-1 and -2 are nutritionally and developmentally regulated. Hepatic mRNA levels of IGFBP-1 and IGFBP-2 are increased by fasting or protein deprivation in rats (Straus & Takemoto 1990*b*, Umezawa *et al.* 1991, Takenaka *et al.* 1993, Lemozy *et al.* 1994, Takenaka *et al.* 1996). Also, serum levels and hepatic mRNAs of IGFBP-1 and IGFBP-2 decrease with increasing developmental age in rats (Cohick & Clemmons 1993, Lewitt *et al.* 1994). Therefore, it is most likely that IGF-binding protein-1 or -2 complexed to $LR^3IGF$-I but it was not possible to identify it in the present experiment.

Previous studies have shown stimulation of renal growth by IGF-I administration. Kidney size is increased by IGF-I infusion in lit/lit mice (Gillespie *et al.* 1990), hypophysectomised rats (Guler *et al.* 1988), dexamethasone treated rats (Tomas *et al.* 1992), gut resected rats (Lemmey *et al.* 1991) and rats with mild renal insufficiency (Martin *et al.* 1991). $LR^3IGF$-I treatment also increased the fractional weight of kidney in well-fed normal female rats (Tomas *et al.* 1993*a*) and guinea pigs (Conlon *et al.*1995*b*). In contrast, $LR^3IGF$-I

treatment in the early phase of acute renal failure had no effect on kidney weight in rats (Martin *et al.* 1994).

IGF-I is claimed to increase the weight of thymus in young normal rats (Tomas *et al.* 1993*a*), hypophysectomised rats (Guler *et al.* 1988) and dwarf mice (Pell & Bates 1992). IGF-I can also restore the growth of the atrophied thymus in rats which are diabetic (Binz *et al.* 1990). The effect of $LR^3$IGF-I on increasing thymus growth was substantial in normal female rats (Tomas *et al.* 1993*a*) but not in dexamethasone-treated rats (Tomas *et al.* 1992).

The association between complex formation of $LR^3$IGF-I and IGF-binding protein and the biological responses to $LR^3$IGF-I-treatment were investigated. In all groups of animals in which circulating $LR^3$IGF-I was exclusively in the free form, treatment with this IGF-I analog increased thymus weight. Specifically, treatment with $LR^3$IGF-I did not increase thymus weight in the 5 week old rats fed 67% less and 44% less than *ad libitum*, and these were the only groups in which $LR^3$IGF-I appeared to be complexed to a ~29 kDa IGF-binding protein. Conversely, free $LR^3$IGF-I appeared to be associated with reduced growth response of kidney weight.

In most instances, IGF-binding proteins are observed to inhibit the action of IGF-I via its receptor, but enhancement of IGF-I action by IGF-binding proteins has also been reported. For kidney, both inhibitory and enhancing effects of IGFBP-1 on the action of IGF-I have been suggested. Chin and Bondy (1994) found that rats which were fed high-protein diets had high IGF-I mRNA and decreased IGFBP-1 mRNA levels in the medullary thick ascending limb of the kidney. Conversely, they also found that feeding low protein diets to rats decreased IGF-I and increased IGFBP-1 mRNA levels in this area of the kidney. The increase

in renal IGF-I mRNA was closely paralleled by significant increases in fractional renal weight, DNA synthesis and length of the medullary thick ascending limb. This implies inhibition of IGF-I action in the kidney by IGFBP-1, which is the reverse of the observations made in this chapter. However, a number of IGF-binding proteins are synthesized in the kidney (Ooi *et al.* 1990, Chin *et al.* 1992) and there is evidence that these binding proteins can enhance, rather than inhibit, the cell proliferative actions of IGF-I. IGFBP-1 in particular is co-localized with IGF-I in adult rat kidney as determined by immunohistochemistry using a rabbit anti-human IGFBP-1 antibody and a rabbit anti human IGF-I antibody (Kobayashi *et al.* 1991). Both peptides are located in the same nephron segments in the same cell types. In rats with an increased tubular work load induced by furosemide, increased immunocytochemical staining of IGF-I and IGFBP-1 was observed in the distal convoluted tubules and collecting ducts, the areas which were especially hypertrophied by the treatment (Kobayashi *et al.* 1995). Because IGFBP-1 increased three fold without significant changes in IGF-I mRNA, they suggested that hypertrophic stimuli might induce the synthesis of IGF binding proteins which function to trap extracellular IGF-I. Moreover, Flyvbjerg *et al.* (1992) showed that hypertrophy of diabetic kidneys is associated with co-increases of kidney IGF-I, 30 kDa IGF-binding protein and 38-47 kDa IGF-binding proteins. IGFBP-1 therefore might be involved in the renotropic action of IGF-I in diabetic kidney hypertrophy. Bach *et al.* (1992) found an association between increasing IGF-binding protein mRNA expression and a marked local increase in proximal tubular binding of [$^{125}$I]-IGF-I. They suggested that IGFBP-1 is cell associated and that the increased levels of IGFBP-1 present in early diabetes contribute towards an accumulation of IGF-I in the kidney.

In conclusion, the studies in this chapter show that age and nutrition, which are regulators of endogenous plasma IGF-I and IGF-binding proteins, are determinants of the molecular size of $LR^3$IGF-I in blood of animals treated with this IGF-I analog. They strongly suggest that age and nutrition affect the *in vivo* formation of complexes between $LR^3$IGF-I and IGFBP(s). The results also suggest that complex formation may affect the pharmacological activity of $LR^3$IGF-I on different tissues in different ways.

# CHAPTER 6.

# Studies aimed at identifying binding proteins associated with $LR^3IGF$-I in plasma from rats treated with this IGF-I analog

## 6.1 INTRODUCTION

The previous chapter established that $LR^3IGF$-I was present in the free form in blood plasma from all $LR^3IGF$-I treated groups of twelve week old rats and in the better nourished groups of similarly treated five week old rats. However, some of the $LR^3IGF$-I in blood plasma from the 44% underfed younger rats and all in that from the extremely underfed younger animals (fed 67% less than *ad libitum*) was present in a high molecular weight form. Studies in Chapter 4 in which plasma was incubated with excess IGF-I, IGF-II or insulin showed that "big" immunoreactive $LR^3IGF$-I in plasma from guinea pigs treated with this IGF-I analog consisted of a complex or complexes formed between $LR^3IGF$-I and an IGF-binding protein. Indirect evidence suggests that $LR^3IGF$-I also formed complexes with a low molecular mass IGF-binding protein in blood of rats as in guinea pigs. Firstly, high molecular mass forms of $LR^3IGF$-I were observed only in rats in which the plasma concentrations of ~29 kDa IGF-binding protein were high and in which the concentrations of endogenous (rat) IGF-I were low, as measured by RIA after size exclusion hplc at pH 2.5. Secondly, plasma from the 67% underfed younger rats contained a greater proportion of high molecular mass $LR^3IGF$-I than that from similarly treated young rats underfed by 44%. The more undernourished of these two groups also had higher plasma levels of ~29 kDa IGF-binding protein and lower levels of rat IGF-I.

Thus the presence of high molecular mass immunoreactive $LR^3IGF$-I in rat plasma is associated with high levels of ~29 kDa IGF-binding protein and low levels of rat IGF-I in plasma. This is consistent with increased amounts of complex formed between $LR^3IGF$-I and ~29 kDa IGF-binding protein *in vivo* as the amount of unsaturated ~29 kDa IGF-binding protein increases. Thirdly, chromatographic size fractionation of plasma components at physiological pH and salt concentration showed that ~29 kDa IGF-binding protein and "big" $LR^3IGF$-I co-eluted.

Since this ~29 kDa rat IGF-binding protein has a molecular mass similar to that reported for both rat IGFBP-1 and rat IGFBP-2 and because the concentrations of both these binding proteins in blood are increased by undernutrition in an age dependent manner (Donovan *et al.* 1991), similar to that observed for the ~29 kDa IGF-binding protein, it seems most likely that if $LR^3IGF$-I has formed complexes with a rat binding protein in blood then IGFBP-1 and/or IGFBP-2 are the complex partners.

The aim of the studies described in this chapter is to identify which, if any, binding protein is complexed to $LR^3IGF$-I in blood from young underfed rats. Four different strategies were investigated. Firstly, the ability of rat plasma to promote capture of antisera raised against IGFBP-1 and IGFBP-2 to solid phase mouse IgG 1A7-F5-E5, the monoclonal antibody used as solid phase antibody in the ELISA for $LR^3IGF$-I, was examined. Secondly, the ability of this antibody to precipitate IGF-binding proteins from rat plasma was investigated. Thirdly, the ability of antiserum raised against rat IGFBP-1 to precipitate $LR^3IGF$-I from rat plasma was investigated. Finally, the effect of low pH, which dissociates IGFs from IGF-binding proteins, on the molecular size of immunoreactive $LR^3IGF$-I in plasma from rats treated with the analog was investigated.

## 6.2 EXPERIMENTAL

### 6.2.1 ELISAs for detecting LR$^3$IGF-I/IGFBP-1 and LR$^3$IGF-I/IGFBP-2 complexes

The system was identical to that described for LR$^3$IGF-I ELISA (3.2.3) except that either rabbit antiserum raised against rat IGFBP-1 (a generous gift from Prof. R.C. Baxter, Sydney) or rabbit antiserum raised against bovine IGFBP-2 (Upton *et al.* 1990) was used as solution-phase antibody instead of Nelson rabbit anti-LR$^3$IGF-I immunoglobulin. Wells of a microtitre plate were "coated" by incubating 100 µl of 2 µg/ml of pure IgG 1A7-F5-E5 in coating buffer (3.2.3) for 3 h at 37°C. After washing with PBS-Tween 20 (3.2.3), each well was "blocked" by incubating overnight at 4°C with 100 µl of 20 g/l BSA in PBS-Tween 20. The plates were then washed with the same buffer. One hundred µl of 5 g/l BSA in PBS-Tween 20 containing 1/25, 1/50 or 1/100 diluted plasma from rats in the studies described in Chapter 5 were then added (see below). Plates were incubated with diluted pooled rat plasma for 2 h at 37°C and washed as above. Subsequently, 100 µl of rabbit anti-rat IGFBP-1 or rabbit anti-bovine IGFBP-2 diluted 1/1000, 1/2000 or 1/4000 in 5 g/l BSA in PBS-Tween 20 were added. Nelson rabbit anti-LR$^3$IGF-I immunoglobulin diluted 1/4000 in the same buffer was also included in this experiment to measure LR$^3$IGF-I concentrations in these plasma samples and to provide a positive control for the testing procedure. Plates were incubated with solution phase rabbit antibodies at 37°C for 2 h. After washing, 100 µl of peroxidase-conjugated goat anti-rabbit immunoglobulins (diluted 1/2000 in 5 g/l BSA in PBS-Tween 20) was added followed by incubation for 1.5 h at 37°C. Substrate for peroxidase was *o*-phenylene diamine as described before in section 3.2.3. The reaction was stopped after 30 min incubation at 37°C by addition of 50 µl of 1 mol/l sulphuric acid and absorbance at 490 nm was measured using an automated microplate reader.

The plasma tested by this method was pooled from four groups: (i) $LR^3IGF$-I treated 44% underfed young rats, which contains "big" $LR^3IGF$-I, (ii) vehicle treated 44% underfed young rats, which contains no $LR^3IGF$-I, (iii) $LR^3IGF$-I treated *ad libitum* fed young rats, which contains only the free form of $LR^3IGF$-I and (iv) IGF-I treated 67% underfed rats, which contains no $LR^3IGF$-I but a very high concentration of ~29 kDa IGF-binding protein.

### 6.2.2 Immunoprecipitation of $LR^3IGF$-I and IGF-binding proteins from rat plasma with IgG 1A7-F5-E5

The objective of this experiment was to determine whether anti-$LR^3IGF$-I IgG could precipitate IGF-binding proteins from rat plasma that contains "big" $LR^3IGF$-I.

Before examining the ability of mouse IgG 1A7-F5-E5 to precipitate IGF-binding proteins in plasma from $LR^3IGF$-I treated rats that contains high molecular mass forms of $LR^3IGF$-I, its ability to precipitate $LR^3IGF$-I from rat plasma was examined. The following experiment was therefore conducted to determine the amounts of plasma, the concentration of $LR^3IGF$-I antibody and the amounts of cellulose coated with goat anti-mouse serum needed to obtain satisfactory recovery of $LR^3IGF$-I from rat plasma by immuno-precipitation with IgG 1A7-F5-E5 and resuspension in SDS.

The specimen used to optimize immunoprecipitation of $LR^3IGF$-I from rat plasma with mouse IgG 1A7-F5-E5 was normal rat plasma (100 or 200 µl) spiked with 28 ng/ml $LR^3IGF$-I standard. These were incubated with 50 or 100 µl of pure mouse IgG 1A7-F5-E5 (GroPep) at 0, 2, 4, 5, 8, 20 and 80 µg per ml in 10 g/l BSA in PBS. After incubation at 4°C overnight, 50 or 100 µl of a suspension of cellulose coated with goat anti-mouse serum (Sac-Cel AA-SAC4)

was added, mixed and incubated for 30 min at room temperature. One ml of water was added and the precipitate recovered by centrifugation at 10,000 rpm for 10 min at room temperature. After washing the pellet by resuspending in 1 ml of PBS containing 2.5 g/l BSA followed by centrifugation as above, the pellet was resuspended in SDS loading buffer (see below) to dissociate the expected ternary complexes between solid phase goat antibodies, solution phase mouse IgG and $LR^3IGF$-I, and then heated at 65°C for 20 min as would be required for analysis of IGF-binding proteins by western radio-ligand blotting. The suspension was again centrifuged as above and the supernatant diluted 1/30 in PBS/Tween 20 containing 5 g/l BSA and then assayed for $LR^3IGF$-I by ELISA (3.2.3). For the $LR^3IGF$-I ELISA of the diluted supernatant obtained from immunoprecipitation, $LR^3IGF$-I assay standard was prepared in 5 g/l BSA in PBS/Tween 20 containing SDS loading buffer at the same concentration as in the test samples. For assay of $LR^3IGF$-I in the original spiked plasma specimen, plasma was diluted 1/10 in 5 g/l BSA in PBS/Tween 20 and $LR^3IGF$-I assay standard was prepared in 5 mg/ml BSA in PBS/Tween 20 containing 10% normal unspiked rat plasma.

Following optimisation of the immunoprecipitation of $LR^3IGF$-I from plasma, the following experiment was conducted to determine whether IGF-binding proteins could be detected in mouse IgG 1A7-F5-E5 immunoprecipitates prepared as above from rat plasma that contained "big" $LR^3IGF$-I (*i.e.* putative $LR^3IGF$-I/binding proteins complexes). Plasma (200 µl) pooled from (a) $LR^3IGF$-I treated 44% underfed young rats, in which ~30% of the immunoreactive $LR^3IGF$-I is in the high molecular weight form, (b) vehicle treated 44% underfed young rats, which contains no $LR^3IGF$-I and (c) $LR^3IGF$-I treated *ad libitum* fed young rats, which contains only free $LR^3IGF$-I was incubated at 4°C overnight with 100 µl of 10 g/l BSA in PBS containing 5 µg/ml pure mouse IgG 1A7-F5-E5. One hundred µl of a suspension of cellulose coated with goat anti-mouse serum (Sac-Cel AA-SAC4) was then

added and incubated for 30 min at room temperature. One ml of water was added and the cellulose was pelleted by centrifugation at 10,000 rpm for 10 min at room temperature. Pellets were washed by addition of 1 ml of PBS containing 2.5 g/l BSA and centrifuged as above. Pellets were than resuspended in 60 µl of SDS loading buffer and heated at 65°C for 20 min, vortexed and centrifuged as above. Twenty µl of supernatant was subjected to discontinuous SDS-polyacrylamide gel electrophoresis through a 4% stacking gel and 10% separating gel at 12 mA for 20 h. After transfer onto nitrocellulose membranes, the proteins were incubated with $9x10^6$ cpm [$^{125}$I]-IGF-II for 2.5 h at room temperature, washed and exposed to X-ray film. An additional negative control was employed in this experiment by using 5 µg/ml mouse IgG S1-F2, raised against rat IGF-II, instead of 1A7-F5-E5 antibody. IgG S1-F2 was a generous gift from Dr. K Nishikawa, Kanaza Medical Centre, Ishikawa, Japan. Postnatal rat plasma contains very little, if any, IGF-II.

### 6.2.3 Immunoprecipitation of LR$^3$IGF-I with rabbit anti-rat IGFBP-1 serum

The objective of this experiment was to determine whether LR$^3$IGF-I is bound to IGFBP-1 in plasma from 44% and 67% underfed young rats. If the high molecular weight form of LR$^3$IGF-I occurs because it is associated with IGFBP-1, then LR$^3$IGF-I should be co-precipitated with IGFBP-1 by antiserum raised against rat IGFBP-1.

Plasma (50 µl) pooled from (1) LR$^3$IGF-I treated 44% underfed young rats, which contains "big" LR$^3$IGF-I, (2) vehicle-treated 67% underfed young rats, which contains no LR$^3$IGF-I but has high concentrations of ~29 kDa IGF-binding protein and (3) LR$^3$IGF-I treated 22% underfed young rats, which contains only the free form of LR$^3$IGF-I, were incubated at 4°C overnight with 100 µl of rabbit antiserum raised against rat IGFBP-1 diluted

1/80, 1/160, 1/320, 1/640 or 1/1,280 in 10 g/l BSA in PBS. Fifty µl of a suspension of cellulose coated with donkey anti-rabbit serum (Sac-Cel AA-SAC1) was then added and incubated for 30 min at room temperature. One ml of water was added and the cellulose beads were pelleted by centifugation at 10,000 rpm for 10 min at room temperature. The pellet was washed by addition of 1 ml of PBS containing 10 g/l BSA and centrifuged as above. The pellet was resuspended in 150 µl of 200 mmol/l acetic acid and 50 mmol/l trimethylamine pH 2.5, containing 0.5% (v/v) Tween-20 to dissociate $LR^3IGF$-I from IGF-binding proteins and stood at room temperature for 30 min. The pH of these mixtures was then adjusted to 7.4 by the addition of 90 µl of 0.4 M Tris-base containing 10 µg/ml IGF-II. The IGF-II was added in order to prevent any reassociation between $LR^3IGF$-I and IGF-binding proteins in case rat IGFBP-1 produces artefacts in the $LR^3IGF$-I ELISA. IGF-II has very poor crossreactivity in the $LR^3IGF$-I ELISA (Figure 3.3). The sample was vortexed and then centrifuged as above. One hundred µl of supernatant was assayed for $LR^3IGF$-I in an ELISA in duplicate (3.2.3). $LR^3IGF$-I standards were prepared in neutralized acidic buffer (5 volumes of pH 2.5 buffer to 3 volumes of 0.4 M Tris).

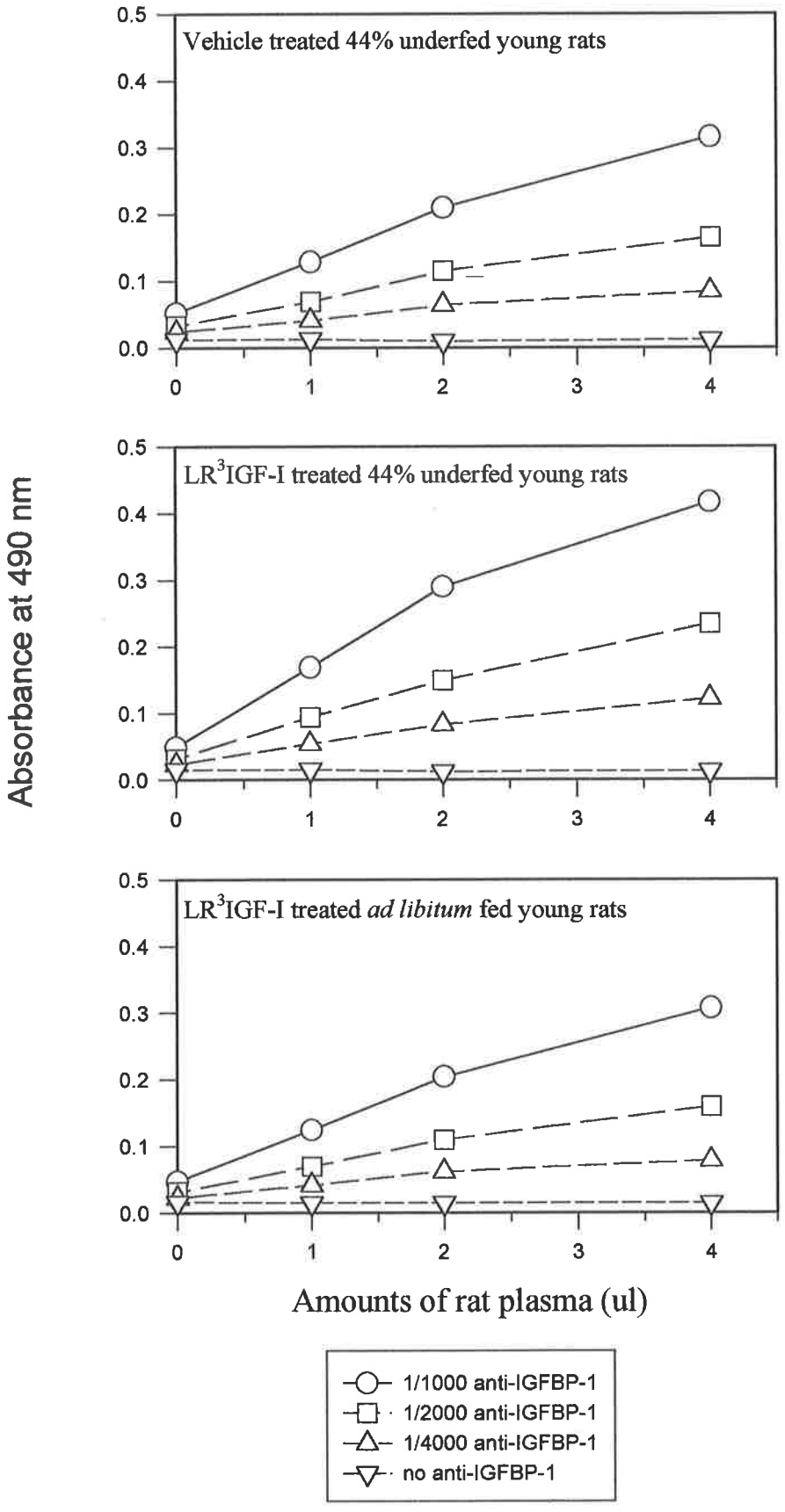
To determine the molecular mass of any IGF-binding proteins precipitated by this rabbit polyclonal antibody against rat IGFBP-1 under the above conditions, the immuno-precipitates were also analysed for IGF-binding proteins by Western radio-ligand blotting. Thirty µl of resuspended immunoprecipitate (or 20 µl plus 10 µl of water, depending on how much was left over from the above experiment) was incubated with 10 µl of 4-times concentrated SDS loading buffer (250 mmol/l Tris-base pH 6.9, 8% [w/v] SDS, 20% [v/v] glycerol and 0.06 mmol/l bromophenol blue) at 65 °C for 20 min. Thirty two µl of these solutions (equivalent to ~5 µl or 3.33 µl plasma) were subjected to discontinuous SDS-polyacrylamide gel electrophoresis through a 4% stacking gel and 10% separating gel

(4.2.5.2) at 20 mA for 1.5 h and then 40 mA for another 2.5 h. The native pooled plasma specimens from which the immunoprecipitates were derived were also included on this gel to compare the amounts of IGFBP-1 in plasma with the amounts in the precipitates by antiserum to rat IGFBP-1. Ten µl of plasma was mixed with 140 µl of water and 50 µl of 4-times concentrated SDS loading buffer, and 20 µl of this solution was subjected to gel electrophoresis. After transfer onto nitrocellulose membranes, the proteins were probed for IGF binding activity by incubation of the membrane with ~$4x10^6$ cpm [$^{125}$I]-IGF-II for 2.5 h at room temperature, washed and visualized by autoradiography.

### 6.2.4 Dissociation of LR$^3$IGF-I from IGF-binding proteins by acidification

Plasma (210 µl) from rats treated with LR$^3$IGF-I (below) was acidified to pH 2.5 by the addition of 70 µl of 4-times concentrated acidic mobile phase (below), defatted and ultrafiltered as described above, and 200 µl of the solution was subjected to size exclusion hplc by injection onto a ProteinPak 125 column (Waters) and fractionation at 0.5 ml/min with a mobile phase of 200 mmol/l acetic acid, 50 mmol/l trimethylamine pH 2.5 containing 0.5% (v/v) Tween-20. Fractions were collected at 1 min intervals from 10 to 30 min after injection, and duplicate aliquots of 200 µl were neutralised with 120 µl of 0.4 mol/l Tris-base and 150 µl was assayed for LR$^3$IGF-I by ELISA. The specimens analysed in this manner were plasma pools from 5 week old rats treated with LR$^3$IGF-I and fed either ad libitum or under fed by 67%. The size distribution of immunoreactive LR$^3$IGF-I in these specimens was previously characterised by size exclusion hplc at pH 7.4 (Fig. 5.10). LR$^3$IGF-I standard (30 ng/ml) was similarly analysed. The column system was calibrated with [$^{125}$I]-IGF-I.

**Figure 6.1**
**Sandwich ELISA for LR$^3$IGF-I/IGFBP-1 complexes**



## 6.3 RESULTS

### 6.3.1 Sandwich ELISAs for complexes between LR$^3$IGF-I and IGFBP-1 or IGFBP-2

Attempts to detect LR$^3$IGF-I/IGFBP-1 complexes or LR$^3$IGF-I/IGFBP-2 complexes by sandwich ELISA were carried out using the combination of mouse IgG 1A7-F5-E5 as solid phase antibody and either rabbit polyclonal anti-rat IGFBP-1 or anti-bovine IGFBP-2 as solution phase antibody.

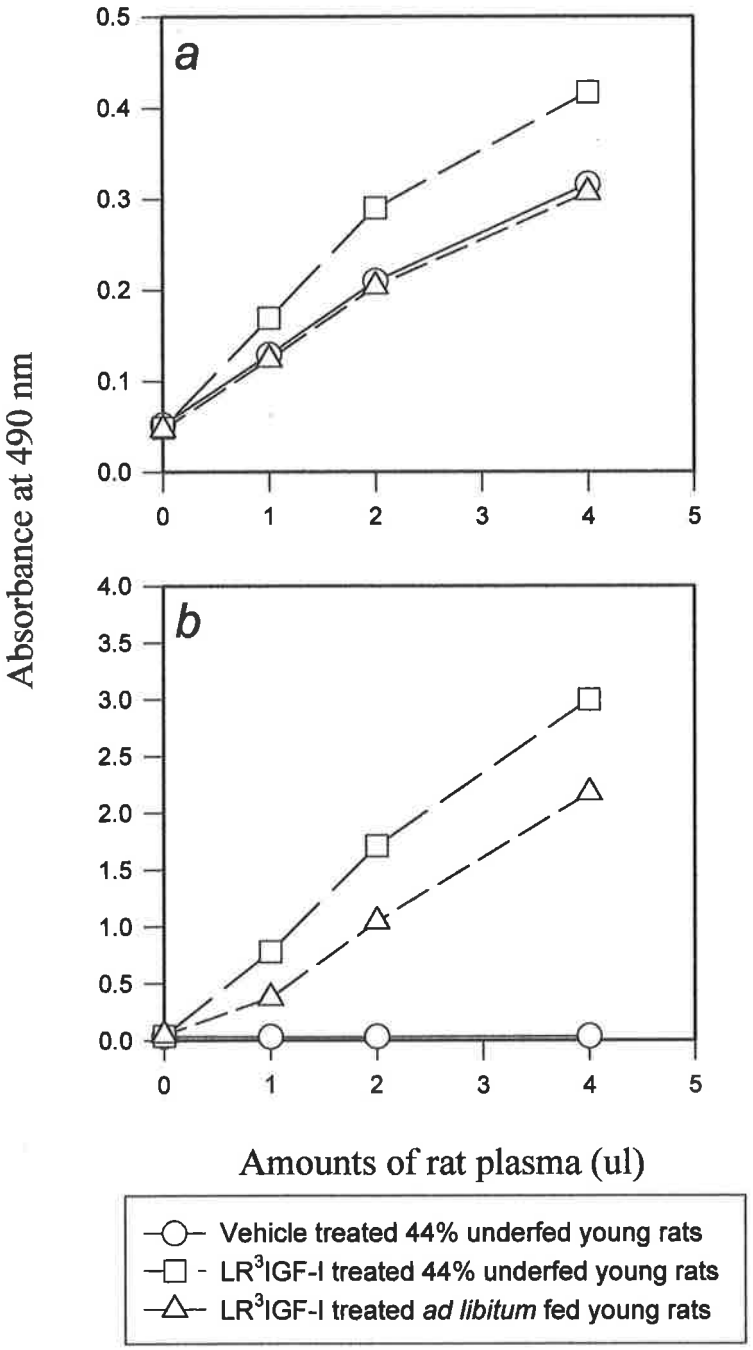
With mouse IgG anti-LR$^3$IGF-I (1A7-F5-E5) as solid phase antibody and rabbit anti-rat IGFBP-1 serum as solution phase antibody, no significant activity was detected when the assay contained neither plasma nor rabbit polyclonal anti-rat IGFBP-1 (Fig. 6.1), and the amount of rabbit immunoglobulin captured increased as the dilution of rabbit anti-IGFBP-1 serum was decreased and as the amount of rat plasma was increased.

However, all plasma specimens tested gave positive results in this system, including plasma from vehicle treated 44% underfed young rats (which contains no LR$^3$IGF-I, Fig. 6.2b) and plasma from LR$^3$IGF-I treated *ad libitum* fed young rats (which contains only the free, low molecular weight form of LR$^3$IGF-I). The reaction response increased when the concentrations of plasma or rabbit anti-rat IGFBP-1 serum were increased. Plasma from LR$^3$IGF-I treated 44% underfed young rats (which contains "big" LR$^3$IGF-I) gave a significantly stronger reaction than those from vehicle treated 44% underfed young rats and LR$^3$IGF-I treated *ad libitum* fed young rats (Fig. 6.2*a*).

# Figure 6.2
## Sandwich ELISA for LR$^3$IGF-I/IGFBP-1 complexes and sandwich ELISA for LR$^3$IGF-I

a) Sandwich ELISA for LR$^3$IGF-I/IGFBP-1 complex using the combination between mouse IgG anti-LR$^3$IGF-I (1A7-F5-E5) as a solid phase antibody and rabbit polyclonal anti-rat IGFBP-1 (1:1000) as a solution phase antibody

b) Sandwich ELISA for LR$^3$IGF-I using the combination between mouse IgG anti LR$^3$IGF-I (1A7-F5-E5) as a solid phase antibody and Nelson rabbit anti-LR$^3$IGF-I immunoglobulin (1:4000) as a solution phase antibody



Plasma pooled from $LR^3IGF$-I treated 44% underfed young rats had higher concentrations of $LR^3IGF$-I than that from $LR^3IGF$-I treated *ad libitum* fed young rats whereas vehicle-treated 44% underfed young rats contained no $LR^3IGF$-I (Fig. 6.2*b*) as observed previously (Fig. 5.3*c*).

To determine whether the stronger reaction found with plasma from $LR^3IGF$-I treated 44% underfed young rats is due to $LR^3IGF$-I/IGFBP-1 complexes and not due to $LR^3IGF$-I independent capture of IGFBP-1, plasma pooled from human IGF-I treated 67% underfed young rats (which contains no $LR^3IGF$-I but very high amounts of ~29 kDa IGF-binding protein) was also subjected to this assay (Fig. 6.3). The relative potencies of plasma pools in this ELISA system were: IGF-I treated 67% underfed young rats > $LR^3IGF$-I treated 44% underfed young rats > $LR^3IGF$-I treated *ad libitum* fed young rats.

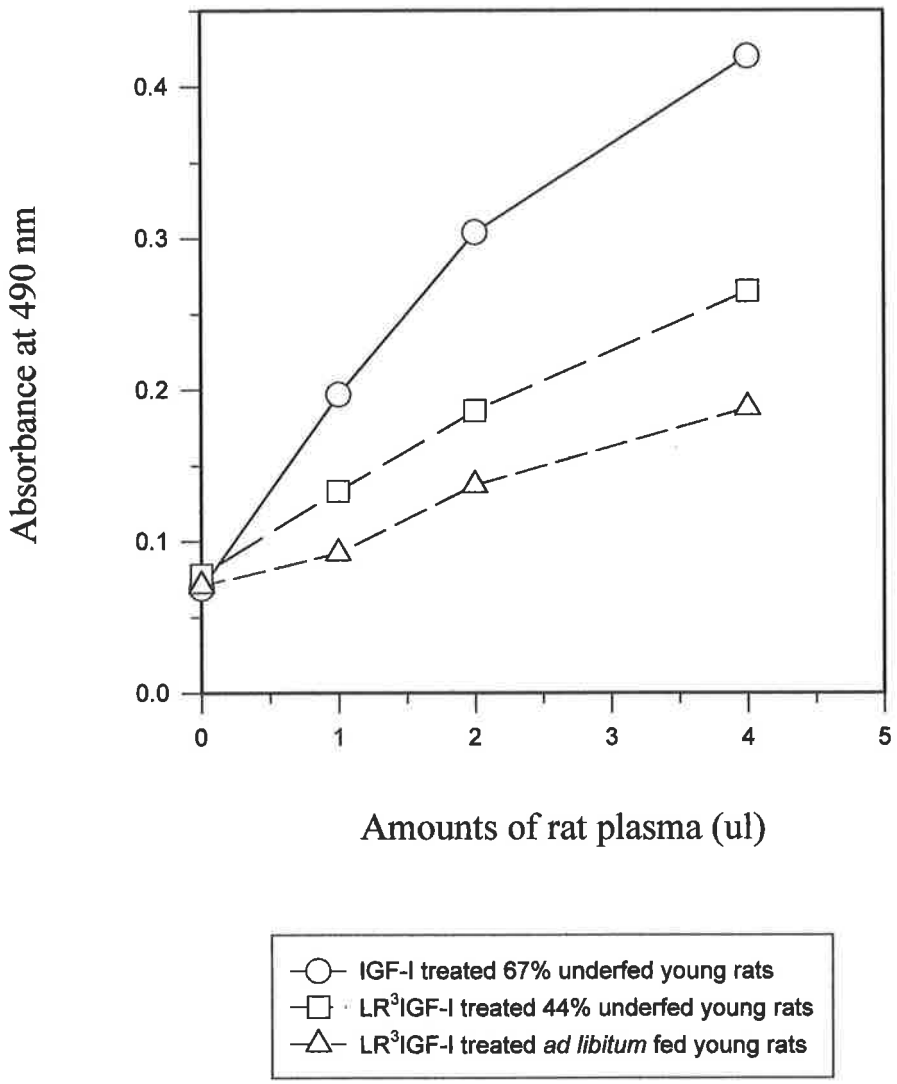
Using rabbit anti-bovine IGFBP-2 serum as solution phase antiserum instead of rabbit anti-rat IGFBP-1 in a similar ELISA system, there was no significant reaction with any plasma pool tested (data not shown).

### 6.3.2 Immunoprecipitation of binding proteins in rat plasma with IGF antibodies

Using the mouse monoclonal IgG 1A7-F5-E5 at 5 μg/ml gave approximately 100% recovery of $LR^3IGF$-I after immunoprecipitation. The $LR^3IGF$-I concentration in the original spiked plasma sample was found to be 27.7 ng/ml while the mean $LR^3IGF$-I concentration obtained after immunoprecipitation was found to be 28.3 ng/ml (n =3). Neither the amounts of plasma or Sac-Cel had significant effects on the recovery of $LR^3IGF$-I. Therefore 200 μl of plasma, 100 μl of 5 μg/ml mouse IgG 1A7-F5-E5 and 100 μl of cellulose coated with goat

# Figure 6.3
## Sandwich ELISA for LR$^3$IGF-I/IGFBP-1 complexes

Sandwich ELISA for LR$^3$IGF-I/IGFBP-1 complexes using the combination between mouse IgG anti-LR$^3$IGF-I (1A7-F5-E5) as a solid phase antibody and rabbit polyclonal anti-rat IGFBP-1 (1:2000) as a solution phase antibody



anti-mouse serum were then selected for use in an experiment conducted to determine whether IGF-binding proteins could be co-precipitated with antibody 1A7-F5-E5 from rat plasma that contained high molecular weight forms of $LR^3IGF$-I (Fig. 6.4). Pooled native plasma from $LR^3IGF$-I treated *ad libitum* fed young rats (track 2), $LR^3IGF$-I treated 44% underfed young rats (tracks 3 and 10) and vehicle treated 44% underfed young rats (tracks 4 and 11) were also included in this gel.

Plasma from all treatments examined contained IGF-binding proteins of ~40-50 kDa, ~29 kDa and ~24 kDa. Plasma pooled from $LR^3IGF$-I treated 44% underfed young rats (tracks 3 and 10) had lower concentrations of IGF-binding proteins of ~40-50 kDa than that from $LR^3IGF$-I treated *ad libitum* fed young rats (track 2) as previously observed (Fig. 5.7). $LR^3IGF$-I treated *ad libitum* fed young rats (track 2) had similar plasma concentrations of IGF-binding proteins of ~40-50 kDa to vehicle treated 44% underfed young rats (tracks 4 and 11). The concentration of IGF-binding protein of ~29 kDa in plasma was higher in $LR^3IGF$-I treated 44% underfed young rats (tracks 3 and 10) than in $LR^3IGF$-I treated *ad libitum* fed young rats (track 2). Treatment of 44% underfed young rats with $LR^3IGF$-I (tracks 3 and 10) increased the plasma concentration of ~29 kDa IGF-binding protein compared to vehicle treated animals (tracks 4 and 11). The concentrations of ~24 kDa IGF-binding protein were highest in $LR^3IGF$-I treated *ad libitum* fed young rats (track 2), lower in vehicle treated 44% underfed young rats (tracks 4 and 11) and lowest in $LR^3IGF$-I treated 44% underfed young rats (tracks 3 and 10).

IgG 1A7-F5-E5 precipitated IGF-binding proteins of ~40-50 kDa from all tested plasma (Fig. 6.4). These include plasma pooled from $LR^3IGF$-I treated *ad libitum* fed young rats (contains only free $LR^3IGF$-I, track 6), plasma from $LR^3IGF$-I treated 44% underfed

young rats (contains "big" and free $LR^3IGF$-I, track 7) and that from vehicle treated 44% underfed young rats (contains no $LR^3IGF$-I, track 9).

IgG 1A7-F5-E5 also precipitated IGF-binding proteins of ~29 kDa from plasma from $LR^3IGF$-I treated 44% underfed young rats (track 7, which contain "big" $LR^3IGF$-I)) and vehicle treated 44% underfed young rats (track 9, which contain no $LR^3IGF$-I).

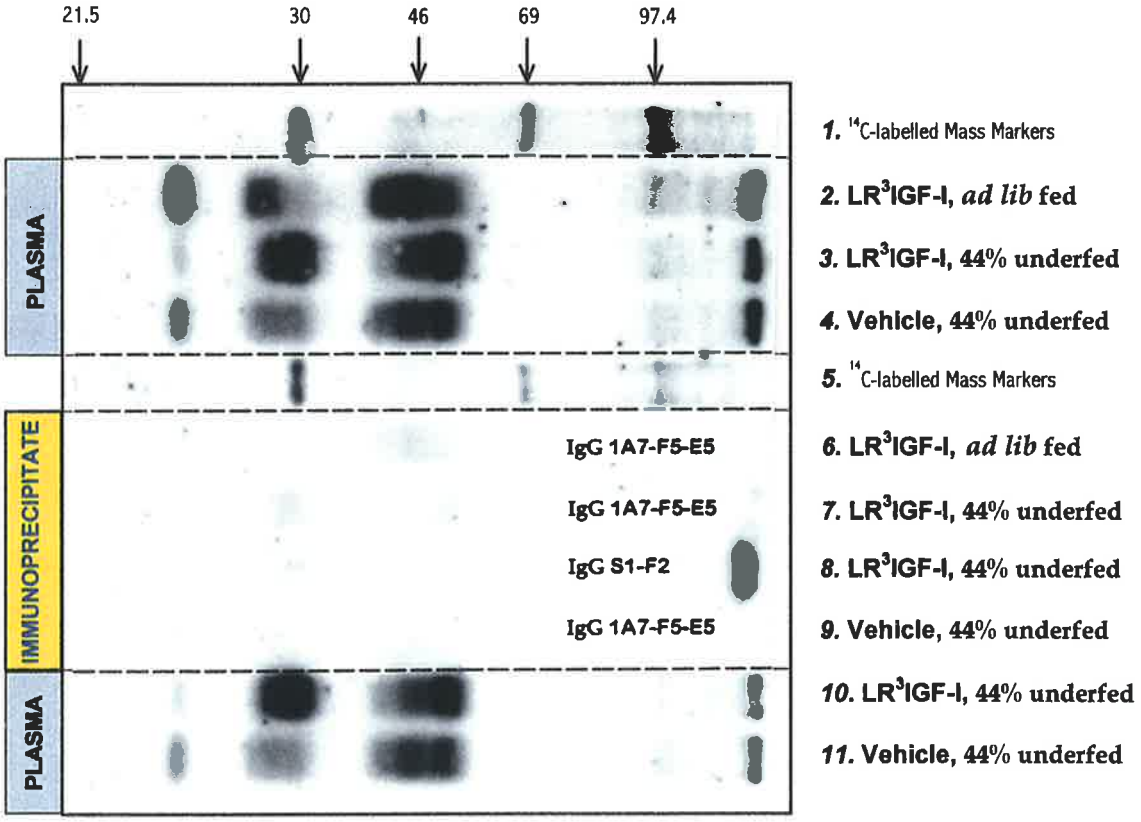
Intensities of IGF-binding protein bands in plasma precipitated with IgG 1A7-F5-E5 appear to be determined by the concentration of the IGF-binding proteins in plasma, rather than the presence of "big" $LR^3IGF$-I. The highest amounts of IGF-binding proteins of ~40-50 kDa were immunoprecipitated from plasma of $LR^3IGF$-I treated *ad libitum* fed young rats (track 6) compared to those from $LR^3IGF$-I treated 44% underfed (track 7) and vehicle treated 44% underfed young rats (track 9). This result corresponds to the concentrations of ~40-50 kDa IGF-binding proteins in the original plasma specimens.

Plasma from $LR^3IGF$-I treated 44% underfed young rats, which had the highest concentrations of ~29 kDa IGFBP in the groups tested, also showed the highest concentration of this binding protein after precipitation with IgG 1A7-F5-E5 (Fig. 6.4, track 7).

An IGF-binding protein of ~29 kDa was also precipitated from plasma of $LR^3IGF$-I treated 44% underfed young rats by anti-IGF-II IgG (track 8). In addition, a high intensity band of MW ~150-200 kDa was found after precipitation of plasma from $LR^3IGF$-I treated 44% underfed young rats with monoclonal mouse anti-rat IGF-II IgG S1-F12. The latter is

**Figure 6.4**
Immunoprecipitation of IGF-binding proteins from plasma of young rats with anti-LR$^3$IGF-I and anti-IGF-II antibodies

**Autoradiogram of a western ligand blot ([$^{125}$I]-IGF-II)**




Plasma was pooled by treatment group from rats infused with vehicle or with LR$^3$IGF-I and fed either ***ad libitum*** or 44% less than ***ad libitum***.

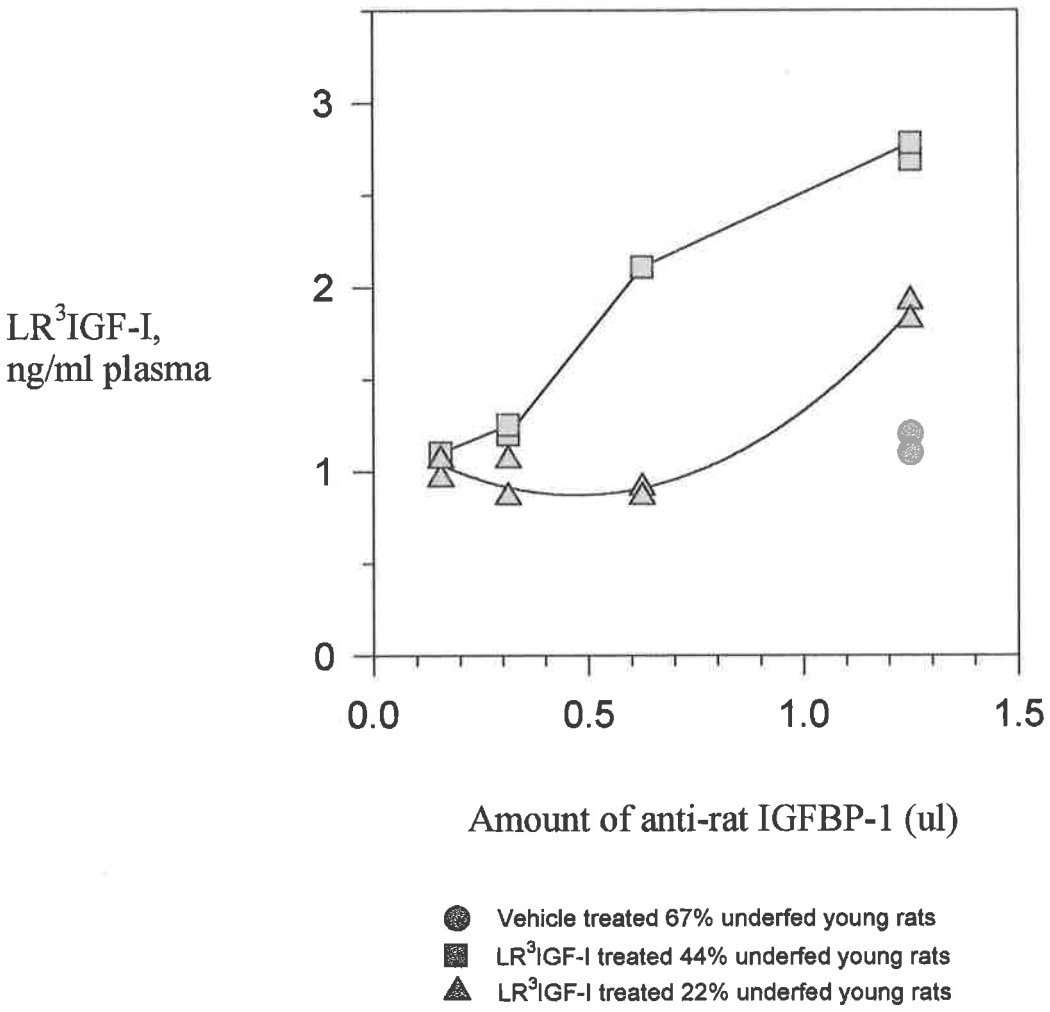
Plasma (20 ul of a 5% [v/v] solution in SDS) was run in tracks 2-4 and tracks 10-11.

Tracks 6-9 contain resuspended pellets prepared from the same plasma pools by immunoprecipitation with either mouse IgG 1A7-F5-E5 or mouse IgG S1-F2.

The arrows indicate the migration positions (kDa) of molecular mass markers run in tracks 1 and 5.

## Figure 6.5

**Immunoprecipitation of $LR^3IGF$-I with rabbit antiserum raised against rat IGFBP-1**



probably due to [$^{125}$I]-IGF-II binding to IgG S1-F12. Because there are negligible amounts of IGF-II in their blood, it is unlikely that anti-rat IGF-II precipitated soluble type-II IGF receptors complexed with IGF-II.

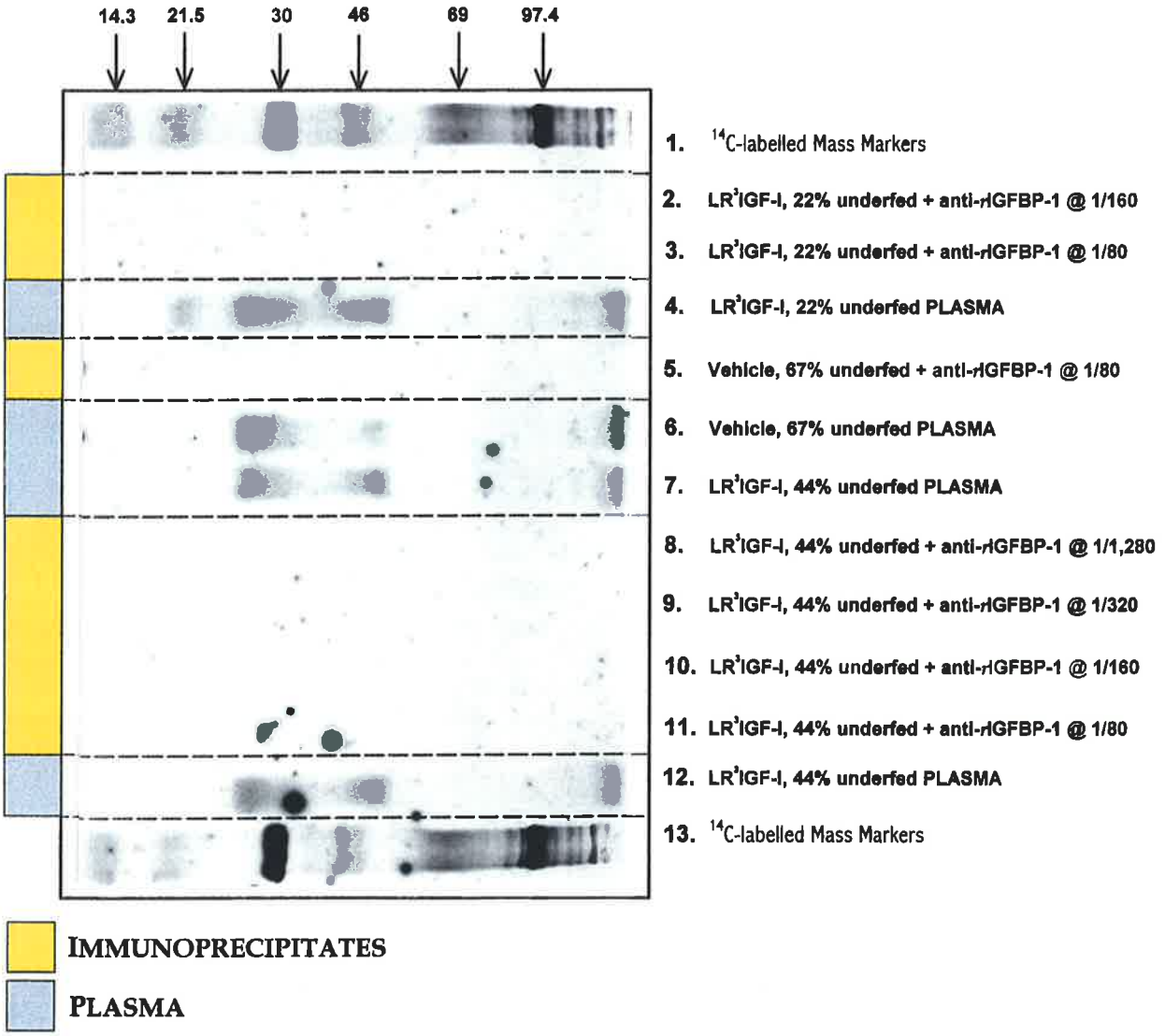
### 6.3.3 Immunoprecipitation of LR$^3$IGF-I from rat plasma with rabbit antiserum raised against IGFBP-1

In this experiment, LR$^3$IGF-I was measured after immunoprecipitation with antiserum raised against IGFBP-1 of plasma pooled from (i) LR$^3$IGF-I-treated 44% underfed young rats (containing high molecular weight LR$^3$IGF-I); (ii) vehicle-treated 67% underfed young rats (containing no LR$^3$IGF-I but a high level of ~29 kDa IGF-binding protein) and (iii) LR$^3$IGF-I treated 22% underfed young rats (containing only the free form of LR$^3$IGF-I).

For LR$^3$IGF-I treated 44% underfed young rats, there was a dose response between the concentrations of precipitated LR$^3$IGF-I and the amounts of anti-IGFBP-1 used (Figure 6.5). LR$^3$IGF-I was precipitated from plasma of LR$^3$IGF-I treated 22% underfed young rats when the highest concentrations of antiserum raised against rat IGFBP-1 (1.25 µl) were used, but this pooled specimen was previously observed to contain only free LR$^3$IGF-I (Fig. 5.10). The presence of "big" LR$^3$IGF-I was, however, associated with increased precipitation of LR$^3$IGF-I by antiserum to rat IGFBP-1. More LR$^3$IGF-I was precipitated from plasma pooled from LR$^3$IGF-I treated 44% underfed young rats than those obtained from LR$^3$IGF-I treated 22% underfed young rats. Vehicle treated 67% underfed young rats produced a reaction similar to the blank (Figure 6.5).

**Figure 6.6**
IGF-binding protein analysis of immunoprecipitates of plasma from young rats prepared with rabbit antiserum to *rat* IGF-binding protein-1

**Autogradiogram of a western ligand blot** ([$^{125}$I]-IGF-II)




Plasma was pooled by treatment group from rats infused with vehicle or with LR$^3$IGF-I and fed either 22%, 44% or 67% less than ***ad libitum***.

Plasma was run in tracks 4, 6, 7 and 12 as described in Figure 6.6.

Tracks 2, 3, 5, 8, 9, 10,and 11 contain resuspended pellets prepared from the same plasma pools by immunoprecipitation with rabbit antiserum raised against rat IGFBP-1.

IGF-binding proteins were also analysed in the immunoprecipitates to determine the molecular mass of any IGF-binding proteins immunoprecipitated by this antiserum to rat IGFBP-1 (Figure 6.6). Plasma samples pooled from $LR^3IGF$-I treated 22% underfed young rats (track 4), from vehicle treated 67% underfed young rats (track 6) and from $LR^3IGF$-I treated 44% underfed young rats (tracks 7 and 12) were also analysed for IGF-binding proteins, without addition of anti-IGFBP-1, in this gel.

As previously shown (Figure 5.7), $LR^3IGF$-I treated 22% underfed young rats (track 4) had higher concentrations of IGFBP of ~40-50 kDa than $LR^3IGF$-I treated 44% underfed young rats (tracks 7 and 12) whereas vehicle treated 67% underfed young rats (track 6) had the least concentrations of these binding proteins. Vehicle treated 67% underfed young rats had the highest of concentrations of ~29 kDa IGFBP compared to $LR^3IGF$-I treated 22% underfed young rats and $LR^3IGF$-I treated 44% underfed young rats. There was no IGF-binding protein detected in any immunoprecipitates of plasma from young rats including $LR^3IGF$-I treated 22% underfed young rats (tracks 2 and 3), vehicle treated 67% underfed young rats (track 5) or $LR^3IGF$-I treated 44% underfed young rats (tracks 8 to 11) using rabbit antiserum to rat IGFBP-1 at the concentration of 1/1280-1/80.

### 6.3.4 Dissociation of $LR^3IGF$-I from IGF-binding proteins by acidification

Size exclusion liquid chromatography of plasma pooled from $LR^3IGF$-I treated 67% underfed young rats at pH 2.5 followed by $LR^3IGF$-I ELISA of the neutralised effluent revealed a single peak of activity eluting after ~22 min (Fig. 6.7b). However, unlike the observation made when this sample was similarly fractionated at pH 7.4 (Fig. 5.10), the immunoreactive $LR^3IGF$-I in this specimen now eluted with a similar retention time to that of

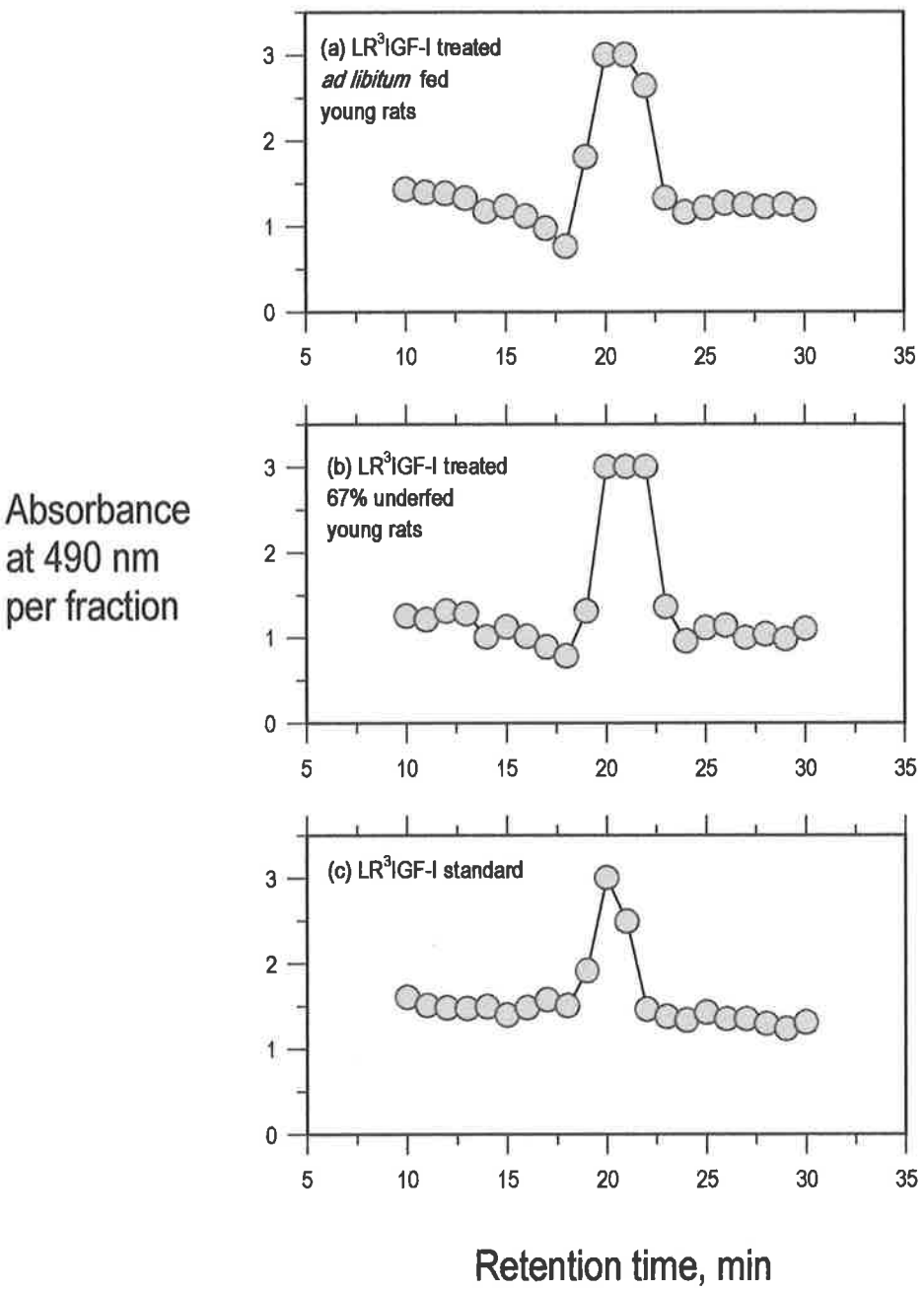
the $LR^3IGF$-I standard (Fig. 6.7c) and that of the free $LR^3IGF$-I in plasma pooled from *ad libitum* fed young rats treated with the analog (Fig. 6.7a, 5.10). [$^{125}$I]-IGF-I eluted 21 min after injection under these conditions (not shown).

## 6.4 DISCUSSION

The high molecular mass form of immunoreactive $LR^3IGF$-I present at physiological pH in plasma from the most severely undernourished young rats could be converted to free $LR^3IGF$-I by acidification to pH 2.5. This indicates that the "big" $LR^3IGF$-I in rat plasma is indeed associated with an IGF-binding protein, since this treatment also dissociates IGF-I and IGF-II from similar complexes. It remains possible that "big" $LR^3IGF$-I is caused by self association and that this is inhibited under acidic conditions. However, this appears unlikely considering that it is not observed in other groups of rats treated with the analog.

The sandwich ELISA using mouse anti-$LR^3IGF$-I (IgG 1A7-F5-E5) as solid phase and rabbit anti-rat IGFBP-1 serum as solution phase antibody failed to confirm that "big" $LR^3IGF$-I formed *in vivo* in blood plasma from poorly nourished young rats is a binary complex between $LR^3IGF$-I and rat IGFBP-1. Positive activity in this ELISA was obtained for all tested samples regardless of whether they contained no $LR^3IGF$-I, free $LR^3IGF$-I or large molecular forms of $LR^3IGF$-I. The reaction in this ELISA was higher for plasma from IGF-I treated 67% underfed young rats (which contain no $LR^3IGF$-I) than for plasma from $LR^3IGF$-I treated 44% underfed young animals (which contain 97 ng/ml $LR^3IGF$-I, ~30% of which is "big") whose response is similar to that of $LR^3IGF$-I treated *ad libitum* fed young rats (which contain 67 ng/ml $LR^3IGF$-I, all of which is free).

## Figure 6.7
**Effect of acidification on "big" immunoreactive $LR^3IGF$-I in rat plasma**




Acid size-exclusion chromatography of pooled plasma from (*a*) $LR^3IGF$-I treated *ad libitum* fed young rats, (*b*) $LR^3IGF$-I treated 67% underfed young rats and (*c*) $LR^3IGF$-I standard followed by $LR^3IGF$-I ELISA

Reactivity in the "anti-LR$^3$IGF-I/anti-IGFBP-1" ELISA did not follow the concentrations of total LR$^3$IGF-I or those of "big" LR$^3$IGF-I but rather followed the concentrations of ~29 kDa IGF-binding protein in these samples (Figure 5.7). The higher capture of rabbit antiserum to rat IGFBP-1 by plasma that have higher levels of ~29 kDa IGF-binding protein suggests that the ~29 kDa IGF-binding protein is indeed IGFBP-1 but that it is being captured by the plate in a manner that is not dependent on LR$^3$IGF-I.

The mechanism responsible for the response to rat plasma in the IgG 1A7-F5-E5/anti-rat IGFBP-1 serum ELISA system is not clear. Because the response is related to the western radioligand blotting intensity of ~29 kDa IGF-binding protein, it is likely that this IGF-binding protein is bound non-specifically to the plate despite the blocking with 20 g/l BSA. It is also possible that mouse IgG 1A7-F5-E5 captured complexes between IGF-I and IGFBP-1 despite the weak affinity of this antibody for IGF-I (Figure 3.2*a*).

There was no significant reaction obtained from any sample tested using a sandwich ELISA with mouse IgG anti-LR$^3$IGF-I (1A7-F5-E5) as solid phase and rabbit anti-bovine IGFBP-2 serum as solution phase antibody. It is not possible, however, to conclude that LR$^3$IGF-I did not form complexes with IGFBP-2 in LR$^3$IGF-I treated underfed young rats because there is also no evidence that the combination of these two antibodies in this assay can capture LR$^3$IGF-I/IGFBP-2 complexes. Clearly, there were no significant reactions from plasma specimens that contained high levels of ~29 kDa IGFBP in this ELISA. This implies that rabbit anti-rat IGFBP-1 serum recognized ~29 kDa IGFBP whereas rabbit anti-bovine IGFBP-2 serum did not. This antiserum raised against bovine IGFBP-2 detected a major immunoreactive band (29 kDa) and a minor band (14 kDa) in fetal rat serum but not in adult

rat serum (Upton *et al.* 1990). It also detected rat IGFBP-2 in conditioned media from rat liver-derived BRL3A cells (Gargosky *et al.* 1990*c*).

In the second strategy, the ability of mouse IgG 1A7-F5-E5 to precipitate IGF-binding proteins from rat plasma was investigated. In both RIA format and ELISA format, this antibody has high specificity for $LR^3$IGF-I (Figs. 3.2, 3.3). This IgG precipitated IGF-binding proteins of ~40-50 kDa (IGFBP-3) and ~29 kDa (IGFBP-1) from tested plasma regardless of whether they contained no $LR^3$IGF-I, free $LR^3$IGF-I or high molecular forms of $LR^3$IGF-I. The IGF-binding protein content of immunoprecipitates prepared with IgG 1A7-F5-E5 was related to the total IGF-binding protein content of the plasma added to the system. This indicates that IgG 1A7-F5-E5 reacts with rat IGF-I in plasma and co-immunoprecipitates IGF-binding proteins associated with IGF-I. The IGF-binding protein of ~29 kDa in plasma of $LR^3$IGF-I treated 44% underfed young rat was also precipitated by IgG S1-F2, an antibody rasied against rat IGF-II that is highly specific for IGF-II in RIA format (Carr *et al.* 1995). This is not due to the immunoprecipitation of IGF-II/IGF-binding protein complexes by anti-IGF-II because mature postnatal rats contain negligible concentrations of IGF-II in their blood (Donovan *et al.* 1989). It seems that both the mouse monoclonal antibodies 1A7-F5-E5 (anti-$LR^3$IGF-I) and S1-F2 (anti-IGF-II) can react to some extent with IGF-I, therefore IGF-I/IGF-binding protein complexes were precipitated in all samples.

Thirdly, the ability of anti-IGFBP-1 antiserum to precipitate $LR^3$IGF-I from rat plasma was investigated. More $LR^3$IGF-I was immunoprecipitated with antiserum to IGFBP-1 from plasma that contained $LR^3$IGF-I in both high and low molecular mass forms (*i.e.* $LR^3$IGF-I treated 44% underfed young rats) than from that which contained only free $LR^3$IGF-I (*i.e.* $LR^3$IGF-I treated 22% underfed young rats) and the response increased with increasing

amounts of plasma. The plasma that contained no $LR^3IGF$-I (*i.e.* vehicle treated 67% underfed young rats) produced the same response as the blank. A baseline or blank level of $LR^3IGF$-I activity of ~1 ng/ml was detected from all tested samples after immunoprecipitation with anti-IGFBP-1. Excess amounts of IGF-II which were added to prevent the reassociation between $LR^3IGF$-I and IGF-binding proteins might cross react slightly in this assay and account for the blank.

However, the stronger reaction in the $LR^3IGF$-I ELISA produced by anti-IGFBP-1 immunoprecipitates from plasma of $LR^3IGF$-I treated 44% underfed young rats is certainly not due to the concentrations of $LR^3IGF$-I or IGFBP-1 alone because plasma from $LR^3IGF$-I treated 44% underfed young rats, which gave the strongest response contained neither the highest concentrations of $LR^3IGF$-I nor the highest concentrations of ~29 kDa IGFBP of the plasma specimens tested. $LR^3IGF$-I levels were higher in plasma pooled from $LR^3IGF$-I treated 22% underfed young rats (123 ng/ml) than in that from $LR^3IGF$-I treated 44% underfed young rats (97 ng/ml). Plasma pooled from the vehicle treated 67% underfed young rats, whose response in this test was not different from the baseline, had higher concentrations of ~29 kDa IGFBP than $LR^3IGF$-I treated 44% underfed young rats. Plasma from $LR^3IGF$-I treated 22% underfed young rats had the lowest concentrations of this IGF-binding protein. Therefore the stronger reaction, which was found in $LR^3IGF$-I treated 44% underfed young rats, is likely to be due to $LR^3IGF$-I/IGFBP-1 complexes.

However, no IGF-binding proteins could be detected by Western ligand blotting in these immunoprecipitates of plasma from young rats using rabbit antiserum to rat IGFBP-1. Several factors might cause failure to detect IGF-binding proteins in these rat plasma immunoprecipitates with antiserum to rat IGFBP-1. The conditions used for precipitating

IGFBP-1 from young rat plasma using rabbit antiserum to rat IGFBP-1 in this experiment were the same as those used for immunoprecipitating IGF-binding proteins from plasma of young rats with mouse monoclonal antibodies except a suspension of cellulose coated with donkey anti-rabbit serum was used instead of a suspension of cellulose coated with goat anti-mouse serum. A suspension of cellulose coated with goat anti-mouse serum precipitated mouse anti-rat IGF-II which was observed as a high intensity band of MW ~150-200 kDa on the Western ligand blot using [$^{125}$I]-IGF-II as a probe (Figure 6.4, track 8). Therefore a suspension of cellulose coated with donkey anti-rabbit serum should precipitate rabbit antiserum to rat IGFBP-1. The next question is whether IGFBP-1 was denatured during the process of immunoprecipitation by rabbit antiserum to rat IGFBP-1. The immunoprecipitated pellet was resuspended in a pH 2.5 buffer to dissociate LR$^3$IGF-I from IGF-binding proteins in case rat IGFBP-1 produces artefacts in the LR$^3$IGF-I ELISA. The acidic buffer should not inactivate IGFBP-1 because an acidic solution was also used during the process of purification of rat IGFBP-1 (Lewitt *et al.* 1992). Similarly, the electrophoresis and western ligand blotting itself should not be a problem in this regard because Lewitt *et al.* (1994) previously reported SDS-PAGE of rat IGFBP-1 in serum from fasted rats during nonreducing conditions.

In this experiment, 32 µl of immunoprecipitate solution containing 10.4% or 15.5% plasma equivalent was subjected to Western radioligand blotting. If 100% of ~29 kDa IGF-binding protein was precipitated by antiserum to IGFBP-1, then the intensities in tracks 2, 3, 5, 8, 9, 10 and 11 of Figure 6.6 should be 3-5 times higher than those of the plasma (1 µl loaded) on tracks 4, 6, 7, 12. This is not the case. It is possible that only a small proportion of ~29 kDa IGF-binding protein was precipitated by this antibody and that it is below the minimum detectable limit of the western ligand blotting procedure. However, it is also

possible, but less likely, that the ~29 kDa IGF-binding protein is not IGFBP-1, therefore it cannot be precipitated by anti-rat IGFBP-1.

Because of the limitations of available antibodies and the procedures employed, it is difficult to identify the binding protein present in $LR^3$IGF-I/IGF-binding protein complexes in rats with certainty. However, the immunoprecipitation of $LR^3$IGF-I with rabbit antiserum raised against IGFBP-1 suggests that $LR^3$IGF-I might bind to IGFBP-1 in plasma of $LR^3$IGF-I treated 44% underfed young rats.

# CHAPTER 7

## General Discussion

Insulin-like growth factors are almost exclusively found *in vivo* associated with soluble binding proteins. The *in vivo* formation of complexes between the different IGF-binding proteins and IGFs and the physiological functions of different types of complexes are not well understood. In this thesis, an ELISA was developed for a synthethic IGF-I analog, LR$^3$IGF-I, in which the native endogenous IGF-I and IGF-II have no significant activity. This assay was used in combination with size exclusion chromatography of plasma at pH 7.4 to determine the molecular size distribution of LR$^3$IGF-I in blood during its administration to animals.

It was found that LR$^3$IGF-I could be present in either free form or in high molecular weight form in blood during its administration to animals at pharmacologically active doses. The high molecular weight form of LR$^3$IGF-I which was detected in plasma from guinea pigs treated with bioactive doses of this analog was proved to be LR$^3$IGF-I/IGFBP complexes. This was not anticipated because LR$^3$IGF-I binds with very poor affinity to IGF-binding proteins including ovine IGFBP-3, rat IGFBP-3, total rat plasma IGFBPs, ovine IGFBP-4 and the binding proteins released from rat L6 myoblast cells (Francis *et al.* 1992, Ballard *et al.* 1993). However, Lord *et al.* (1994) showed that although [$^{125}$I]-LR$^3$IGF-I exhibits extremely weak binding to binding proteins in rat plasma, it does bind significantly to binding proteins in plasma from sheep, pig, human and chicken.

Age, nutrition and LR$^3$IGF-I treatment, which are factors that affect synthesis of endogenous IGF-I and IGF-binding proteins, were shown to affect the molecular weight forms of circulating LR$^3$IGF-I in rats. LR$^3$IGF-I was observed in free form in twelve week old rats regardless of nutrition. However, nutrition affected the molecular size distribution of LR$^3$IGF-I in blood plasma of five week old rats. About 30% of the LR$^3$IGF-I in blood plasma from the 44% underfed younger rats and ~100% in 67% underfed younger animals, was present in plasma in a high molecular weight form or forms. The high molecular weight form of LR$^3$IGF-I in LR$^3$IGF-I treated 67% underfed young rats was converted to the free form of identical retention time to that of LR$^3$IGF-I standard and LR$^3$IGF-I in LR$^3$IGF-I treated *ad libitum* fed young rats upon acidification. This evidence is consistent with one property of IGFBP/IGF complexes, for which acidification dissociates IGFBP from IGFs. It is also possible that the high molecular weight form of LR$^3$IGF-I was due to self aggregation of LR$^3$IGF-I and that acidification dissociated the aggregate. However, if LR$^3$IGF-I was aggregated in plasma from LR$^3$IGF-I treated 44% and 67% underfed young rats, it should also be found in plasma from other groups of rats with similar levels of LR$^3$IGF-I. This is not the case. Therefore, it is most likely that the high molecular weight form of LR$^3$IGF-I in plasma of LR$^3$IGF-I treated 44% and 67% underfed young rats was due to the formation of complexes between LR$^3$IGF-I and IGF-binding proteins. Moreover, in LR$^3$IGF-I treated 44% and 67% underfed young rats, the concentrations of endogenous IGF-I are lower and the concentrations of the ~29 kDa IGF-binding protein are higher than those of other groups.

Reducing feed intake progressively decreased plasma concentrations of rat IGF-I in vehicle treated younger rats. Treatment with LR$^3$IGF-I further reduced the concentrations of rat IGF-I in plasma of undernourished younger rats by ~40-50% compared to vehicle treated animals.

Reduction of feed intake to 67% less than *ad libitum* in younger rats decreased plasma concentrations of IGF-binding proteins of ~40-50 kDa (IGFBP-3) and ~24 kDa but increased levels of ~29 kDa IGF-binding protein. Reduction of feed intake to 44% less than *ad libitum* in younger rats decreased plasma concentrations of IGF-binding proteins of ~40-50 kDa but had no effect on other binding proteins. Treatment with $LR^3IGF$-I increased the concentrations of ~29 kDa IGF-binding protein in all feed-restricted groups of young rats but not in *ad libitum* fed rats. Since undernourished younger rats (44% and 67% underfed) had very low concentrations of rat IGF-I and IGFBP-3 and higher concentrations of ~29 kDa IGF-binding protein, this IGF-binding protein would be present in an unsaturated form so that it could form complexes with the infused $LR^3IGF$-I *in vivo*. This was also supported by the fact that fractions containing the high molecular weight form of $LR^3IGF$-I also contained this ~29 kDa IGF-binding protein.

In contrast, in vehicle treated older rats, only the extremely undernourished (i.e. reducing feed intake to 67% less than *ad libitum*) but not 44% or 22% less than *ad libitum* had significantly decreased plasma levels of rat IGF-I.

Reducing feed intake had no significant effect on plasma levels of IGF-binding proteins in older animals. Treatment with $LR^3IGF$-I did not affect plasma concentrations of rat IGF-I and IGF-binding proteins at any levels of feed intake in older rats. Therefore, older rats did not have unsaturated IGF-binding proteins available for binding to infused $LR^3IGF$-I.

The effect of nutrition as well as age on the complex formation between [$^{125}$I]-IGF-I and IGF-binding proteins has been reported previously. In protein-restricted rats, IGF-I bound preferentially with IGF-binding protein(s) in small (40 kDa) complexes (Takahashi *et al.* 1990, Thissen *et al.* 1992). These authors suggested that the small molecular weight IGF-

binding protein(s) were not saturated with IGF-I in rats fed on a protein-free diet, resulting in an increase in availability of IGF-binding sites on low molecular weight IGF-binding proteins and hence formation of a ~40 kDa complex in blood. Drakenberg *et al.* (1990) studied the binding of [$^{125}$I]-IGF-I to the IGFBPs in serum from untreated adult and 10 day old rats. In neonatal rats whose blood predominantly contained low molecular weight binding proteins, [$^{125}$I]-IGF-I bound mainly to the low molecular weight binding proteins whereas in adult rats, it bound mainly to high molecular weight binding proteins (Drakenberg *et al.* 1990).

The ~29 kDa IGF-binding protein is likely to be the IGF-binding protein that formed complexes with LR$^3$IGF-I in plasma of LR$^3$IGF-I treated 44% and 67% underfed young rats because this binding protein was increased by undernutrition and was also found in the fractions containing high molecular weight LR$^3$IGF-I. The bands at about 28-30 kDa on western ligand blots may contain several IGF-binding proteins such as IGFBP-1, -2, -5, -6 and glycosylated IGFBP-4. However, only IGFBP-1 and IGFBP-2 have been reported to be increased in nutritionally restricted or deprived rats (Straus & Takemoto 1990*b*, Umezawa *et al.* 1991, Takenaka *et al.* 1993, 1996, Lemozy *et al.* 1994). Molecular weights of IGFBP-1 or -2 reported in the literature are slightly different in different laboratories. Takenaka *et al.* (1996) observed that the amount of 30 kDa IGF-binding protein increased in protein-deprived rats using western ligand blot analysis. By using specific antisera against IGFBP-1 and IGFBP-2, they found that plasma concentrations of IGFBP-1 increased greatly and IGFBP-2 increased slightly in these protein-deprived rats. Lewitt *et al.* (1994) observed that the rat IGFBP-1 standard and rat IGFBP-1 in serum appeared as a doublet with molecular masses at 31 and 33 kDa by SDS-PAGE. Yang *et al.* (1990) showed that the 30 kDa IGF-binding protein in media from BRL-3A, Clone 9 (a normal rat liver cell line) and TRL12-15 cells (derived from the livers of 10-day-old Fischer 344 rats) was rat IGFBP-2 whereas the 30 kDa binding protein in H35 hepatoma cells was rat IGFBP-1. Differences in the molecular weight

of IGFBP-1 or -2 reported from each laboratory might be due to the differences in SDS-PAGE protocol or differences in molecular weight markers used. For example, 10% SDS-polyacrylamide gels and $^{14}$C-labelled Rainbow molecular weight markers were used in the present studies whereas 12% SDS-polyacrylamide gels were used in the study of Lewitt *et al.* (1994). Lewitt *et al.* (1994) did not report the molecular weight markers which were used in their experiment whereas Takenaka *et al.* (1996) used Biorad markers in their study.

Examination of the apparent molecular masses of the IGF-binding protein showed that there were effects of nutrition and treatment with LR$^3$IGF-I on size of the ~29 kDa IGF-binding protein in young rats. Reducing feed intake increased the apparent molecular weight of this binding protein. Treatment with LR$^3$IGF-I further increased the apparent molecular weight of this binding protein in undernourished young rats but not in *ad libitum* fed young rats. Two obvious possibilities are discussed. Firstly, it is possible that the bands which run slower and faster in this ~29 kDa region on SDS-PAGE gels might be different IGF-binding proteins, e.g. IGFBP-2 and IGFBP-1. Yang *et al.* (1990) showed that rat IGFBP-2 from conditioned media of H35 hepatoma cells ran slightly faster than rat IGFBP-1 from conditioned media of clone 9 and from TRL12-15 cells in ~30 kDa region using western ligand blotting of SDS-PAGE gels. If the band that ran faster in the present study is IGFBP-2 and the band that ran slower was IGFBP-1, it means that reducing feed intake as well as treatment with LR$^3$IGF-I in younger rats decreased IGFBP-2 but increased IGFBP-1. This is very unlikely because both IGFBP-1 and IGFBP-2 are reported to be increased in undernourished rats (Straus & Takemoto 1990*b*, Umezawa *et al.* 1991, Takenaka *et al.* 1993, 1996, Lemozy *et al.* 1994). Secondly, the slight difference in the apparent molecular weight of this binding protein might be due to the phosphorylation of IGFBP-1. IGFBP-1 is reported to exist in a highly phosphorylated form in human serum after overnight fasting and refeeding

resulted in a reduction in the phosphorylation of IGFBP-1 in blood (Bereket *et al.* 1996). Phosphorylated IGFBP-1 has higher affinity for IGF-I than it does after dephosphorylation (Jones *et al.* 1991, Koistinen *et al.* 1993). If the ~29 kDa IGFBP which was most abundant in blood plasma from LR$^3$IGF-I treated 44% and 67% underfed young rats in the present study is phosphorylated IGFBP-1, it would also have increased affinity for IGF-I and, presumably, LR$^3$IGF-I. This supports the formation of complexes between ~29 kDa IGF-binding protein and LR$^3$IGF-I in blood of younger undernourished animals.

In the study in chapter 6, I have tried to identify which binding protein is bound to LR$^3$IGF-I in blood from LR$^3$IGF-I treated 44% underfed young rats. Even though the reactivity in ELISA for detecting LR$^3$IGF-I/IGFBP-1 complexes did not follow the concentrations of high molecular weight form of LR$^3$IGF-I (LR$^3$IGF-I/IGFBP complexes), it directly followed the concentrations of ~29 kDa IGF-binding protein in those samples (Figure 5.7). This suggests that the ~29 kDa IGF-binding protein is indeed IGFBP-1. Moreover, LR$^3$IGF-I was immunoprecipitated by rabbit antiserum raised against IGFBP-1 suggesting that LR$^3$IGF-I might bind to IGFBP-1 in plasma of LR$^3$IGF-I treated 44% and 67% underfed young rats.

IGFBP-1 and IGFBP-2 associate with IGF-I in smaller complexes (30-40 kDa) that can cross the capillary endothelium more effectively than IGFs in large (~150 kDa) complexes (Bar *et al.* 1990). IGFBP-1 and -2 also contain RGD (Arg-Gly-Asp) sequences (Shimasaki & Ling 1991) that may allow attachment to cell surface integrin receptors. Therefore, these small IGFBP complexes may facilitate transport of IGF-I from serum to tissues and increase its sequestration near receptor-containing cells. IGFBP-1 exerts both stimulatory and inhibitory effects on IGF-I actions. IGFBP-1 can inhibit the action of IGF-I on cells by forming a complex, which prevents IGF-I from binding to its receptor on the cell

membrane (Liu *et al.* 1991, McGuire *et al.* 1992, Figueroa *et al.* 1993). However, IGFBP-1 can also potentiate the action of IGF-I *in vitro* (Elgin *et al.* 1987, Koistinen *et al.* 1990, Kratz *et al.* 1992). The precise mechanism of the interaction of IGF-I and IGFBP-1 that results in stimulation or inhibition of IGF-I action is not well understood but resistance to proteolysis seems reasonable. The primary sites of IGFBP-1 metabolism and clearance is also not known. Human IGFBP-1 is cleared with a half life of ~12 min after infusion into rats while radioiodinated human IGFBP-1 is cleared with a half life of ~ 7.5 min in rats (reviewed by Lee *et al.* 1993).

There are limited investigations regarding IGFBP-1 action *in vivo*. Lee *et al.* (1993) reviewed that IGFBP-1 was elevated in intrauterine growth retardation, chronic renal failure and GH deficiency, whereas its blood levels are decreased in obesity. These pathophysiological correlations are consistent with an IGF-inhibitory role for IGFBP-1. Lewitt *et al.* (1991) showed that infusion of hIGF-I into male Wistar rats induced a fall in plasma glucose. Co-infusion of IGFBP-1 blocked the hypoglycemic response to intravenous IGF-I and IGFBP-1 increased blood glucose levels when administered alone. They suggested IGFBP-1 levels might modulate the hypoglycemic activity of unbound IGFs in the circulation. The effect of human IGFBP-1 on the action of administered IGF-I was also determined in rats (Lewitt *et al.* 1993*b*). They showed that, compared with a control group, hIGF-I significantly stimulated hexose uptake into heart, soleus and red quadriceps muscles and IGFBP-1 reversed this effect. There was no effect of administration of IGF-I or IGFBP-1 on hexose uptake into brain tissue.

However, the co-administration of IGF-I and IGFBP-1 stimulates wound healing in diabetic (*db/db*) mice and normal rabbits (Tsuboi *et al.* 1995). The authors suggested five possible explanations: 1) IGFBP-1 could extend the half life of IGF-I at the wound site; 2)

IGFBP-1 can cross the capillary boundaries and may act to transport IGF-I from the vasculature to IGF target cells; 3) IGFBP-1 binds to $\alpha_5\beta_1$ integrin on the cell membrane by means of its Arg-Gly-Asp sequence and IGF-I may associate with cell bound IGFBP-1 thereby being more accessible to IGF receptors; 4) the IGF-I independent migration stimulatory activity of IGFBP-1 may allow cells to respond more vigorously to IGF-I stimulation; 5) IGFBP-1 may protect IGF-I from proteolytic degradation at the wound site.

In this study, there were associations between plasma concentrations of free LR$^3$IGF-I with several organ weights in young rats that were consistently stronger than those seen with total LR$^3$IGF-I level. The association between levels of free LR$^3$IGF-I and kidney weight is an example of a negative association whereas the association between levels of free LR$^3$IGF-I and thymus weight is an example of a positive association.

For thymus, when LR$^3$IGF-I was present in the free form, it was then available to the receptor and promoted growth. When LR$^3$IGF-I was present in the high molecular weight form (presumably bound to IGFBP-1), it was not available to receptors in the thymus and did not promote growth of this tissue.

In contrast for kidney, when LR$^3$IGF-I was in the bound form (LR$^3$IGF-I/IGFBP-1), it was more available to the receptors in this organ and promoted kidney growth. On the other hand, free LR$^3$IGF-I was negatively associated with kidney growth. Free LR$^3$IGF-I might be degraded by proteases in kidney because kidney is also known to be a site for degradation of IGFs (Bhaumick & Bala 1987). Type-I IGF receptors were found in both kidney (Werner *et al.* 1989) and thymus (Verland & Gammeltoft 1989) in rats.

IGFBP-1 seems to have an important role in the kidney. IGFBP-1 mRNA is expressed primarily in liver, uterine endometrium and kidney (reviewed by Lee *et al.* 1993). IGF-I and IGFBP-1 are co-increased in hypertrophied distal convoluted tubules and collecting ducts when rats were treated with furosemide (Kobayashi *et al.* 1995). IGF-I and a 30 kDa IGF-binding protein were found to co-increase in hypertrophying diabetic kidneys (Flyvbjerg *et al.* 1992). Bach *et al.* (1992) found a positive association between IGF-binding protein mRNA expression and proximal tubular binding of [$^{125}$I]-IGF-I, suggesting that the accumulation of IGF-I in kidney is increased by binding to cell associated IGFBP-1. These previous studies and those in this thesis are consistent with a growth promoting role for IGFBP-1 in kidney.

The studies in this thesis suggest that the formation of complexes between LR$^3$IGF-I and IGF-binding protein is affected by factors that regulate synthesis of endogenous IGF-I and IGF-binding proteins, such as age and nutrition. It also suggests that IGFBP-1 inhibits the pharmacological activity of circulating LR$^3$IGF-I on thymus whereas it appears to stimulate the pharmacological activity of LR$^3$IGF-I in kidneys. It would be expected that the same results will be obtained if IGF-I was used instead of LR$^3$IGF-I.

The future directions of this study are suggested to be as following:

1) Even though it is most likely that IGFBP-1 is complexed to LR$^3$IGF-I, further investigation needs to be done. Purification of the 29 kDa IGF-binding protein from the plasma of LR$^3$IGF-I treated undernourished rats is one possible way to definitely prove the identity of this binding protein. Affinity column chromatography using an antibody against LR$^3$IGF-I to capture LR$^3$IGF-I/IGFBP could be used as a first step in the purification of this binding protein. Subsequent IGF-affinity chromatography and reverse-phase hplc would be expected

to yield purified 29 kDa IGF-binding protein suitable for identification by N-terminal sequencing and mass spectrometry.

2) The effects of the free form of LR$^3$IGF-I and the complex between LR$^3$IGF-I and IGF-binding protein on organ growth should be further investigated. This can be determined by administration of LR$^3$IGF-I, LR$^3$IGF-I/IGFBP or IGFBP to animals. Tissue distribution and the effect of these peptides on growth and specific organ weights can then be examined.

3) The ELISA for LR$^3$IGF-I in this study can detect both the free form and complexed form of LR$^3$IGF-I. If we can develop such an assay with additional monoclonal antibodies specific for IGF-I or IGF-II complexed with particular IGFBPs, it will be useful to examine the regulation and physiological functions of different types of complexes.

# CHAPTER 8

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