THE EFFECTS OF CHRONIC IGF-I, IGF-II OR LONG R^3 IGF-I INFUSION ON THE POSTNATAL GROWTH OF RATS AND GUINEA PIGS.

By

Michael Allan Conlon, B.Sc.(Hons).

A thesis submitted on the 24th day of November 1995 to the University of Adelaide, South Australia, for the degree of Doctor of Philosophy (Department of Biochemistry).

To the encouragers.

TABLE OF CONTENTS.

Page no.

ADDENDUM

1) The reference to Figure 3.2 on page 56 (last paragraph) should be changed to Figure 3.3 and the reference to Figure 3.3 in the same paragraph changed to Figure 3.2.

2) The following statement should be included in each of sections 4.2.4 (page 63), 5.2.4 (page 98) and 6.2.3 (page 115).

Halothane was used with an oxygen flow rate of 2L/min.

3) The following statement should be included in section 4.2.5 (page 63).

The centrifuge speed for the preparation of plasma from blood was 2000 x g for 10 minutes.

4) On page 68, line 7, in the statement referring to RIA Buffer, 0.2 M sodium phosphate should be changed to read 0.03 M sodium phosphate.

5) On page 68, line 16, polyethylene glycol 5000 should be changed to read polyethylene glycol 6000.

6) The statement in section 4.3 (page 73 and continued on page 77) should read as follows.

The ligand blots also showed that 104 μg of IGF-I/day was more potent than 104 μg of IGF-II/day at increasing IGFBP-3 and the 32 kDa IGFBP-3 (compare lanes 4 and 6 of Figure 4.3a;4.3b) and 260 μg of IGF-I/day may have been more potent than the same dose of IGF-II in stimulating IGFBP-3 (compare lanes 5 and 7 of Figure 4.3a).

7) On page 77, line 23 the reference to Table 4.5 should be changed to Table 4.4.

8) The following statement should be inserted in section 5.4, page 109, between the third to last and second to last sentences.

A failure to increase plasma IGFBP concentrations, even though total IGF concentrations had increased, may indicate that the guinea pig requires a greater increase in total circulating IGF concentrations in order to effect an increase in IGFBPs, especially IGFBP-3. That is, there may be a degree of resistance to IGFBP concentration increases following IGF concentration increases in this animal.

9) On page 112 the second to last sentence should be changed to read as follows.

I have identified some differences between the two species which demonstrates our lack of understanding of that role.

10) The following statement should be included in section 6.2.5 (page 116).

Weight/slope regression analyses were carried out using the SYSTAT computer software program (see page 71).

11) The start of the second paragraph on page 126 of section 6.3 should read as described below.

None of the treatments significantly increased body weight gain compared to that which was achieved with the vehicle (Figure 6.5; Table 6.3). There was also no significant difference in weight/time slopes over 14 days between treatment groups. There was no relationship between pretreatment body weight gains and those during treatment. The high S.E.M. values, especially in the IGF-I treatment group, may be masking significant effects.

The mean body weight gain over 14 days, for the 3 animals in the LR^3IGF-I treatment group.....

12) On pages 126 and 127 all references to Table 6.3 should instead be designated as Table 6.4. Similarly, references to Tables 6.4, 6.5, 6.6 and 6.7 should be changed to Tables 6.5, 6.6, 6.7 and 6.8 respectively.

13) The following statement should be included within section 7.2.2 (page 144).

The specific activity of ^{125}I-IGFs used for the binding studies was approximately 40 μCi/μg and the concentration was calculated to be approximately 100,000 cpm/μl.

14) The following statement should be included in the discussion of section 7.2.4.

The percentage of radiolabelled IGF bound is not 100% at the apparent saturation of binding by membrane preparations in some instances in Figure 7.1. It is possible that a substantial proportion of the solution containing radiolabelled IGFs contains either free ^{125}I, radiolabelled IGFs which cannot bind to the binding sites of these tissues for some unknown reason or a combination of both factors. Hence it does not appear possible to bind 100% of the radioactivity added to each tube.

15) The discussion that follows should be included in the general discussion of section 8 on page 155 between paragraphs 2 and 3.

Although IGF-II concentration is at the lower limits of detection in the blood of adult rats, it is possible that such low concentrations could have some physiological actions. There is also increasing evidence that IGFs can act in a paracrine and autocrine manner, in addition to their endocrine function. That is, it is also possible that in the adult rat IGF-II may be being produced and acting locally within tissues without reaching the circulation. However, the concentration of IGF-II in the tissues of the adult rat is also low but detectable (Lee *et al.* 1991; Geenen *et al.* 1993), and so again it is possible that such concentrations could exert biological actions.

Recently, a number of IGF-II transgenic mouse lines have been established (see section 1.10.4). These models enable the study of the autocrine/paracrine actions of IGFs by their over-expression in tissues. Indeed the fact that different tissues have grown as a result of the use of different promoters to express the IGF-II gene supports the possibility that IGF-II can act locally. The use of exogenous IGF-II to examine these aspects of growth is limited instead to examining mainly endocrine effects.

16) On page 163, line 13, the word "is" should be inserted as follows.

...rats and humans, and this is thought to compensate for.....

17) On page 166, line 5 should be changed to read as follows.

.....blood IGF-II concentrations in the rat because it has a low concentration of IGF-II in circulation.

TABLE OF FIGURES.

CHAPTER 3.

CHAPTER 4.

CHAPTER 5.

CHAPTER 6.

CHAPTER 7.

APPENDIX B (Supporting chapter 5).

TABLE OF TABLES.

CHAPTER 4.

CHAPTER 5.

CHAPTER 6.

APPENDIX A (Supporting chapter 4).

APPENDIX B (Supporting chapter 5).

APPENDIX C (Supporting chapter 6).

STATEMENT OF ORIGINALITY.

I declare that this thesis does not include material which has been used for the award of any other degree or diploma in any other university or academic institution. This thesis also does not contain material which has been published by any other person except where appropriate reference has been made. Permission is granted by the author for this thesis to be made available for loan and photocopying.

Michael Allan Conlon.

ACKNOWLEDGEMENTS.

I would like to thank Drs. John Ballard and John Wallace for giving me the opportunity to do a PhD within a research group which was later to become the Cooperative Research Centre for Tissue Growth and Repair. I also wish to acknowledge the financial support of the Pig Research and Development Corporation who made the PhD possible by awarding me a scholarship. Thanks go to Dr. Phil Owens who encouraged me to do a PhD.

The work of this thesis has been made possible by the efforts and assistance of many people. Firstly, by the insight and direction often provided by my supervisors Dr. Frank Tomas, Dr. John Ballard and Dr. John Wallace. Also, Geoff Francis for his help in enabling me to produce IGF-II, and Dr. Phil Owens, Dr. Paul Walton, Dr. Chris Goddard and Gordon Howarth for their advice on various matters. Thanks also go to Kerrie McNeil and Steve Milner for their technical advice during the IGF-II purification, and Andrew Talbot of GroPep Pty. Ltd. for carrying out assays of the purified IGF-II. I also thank Dr. Frank Tomas, Judy Burgoyne, Paul Rogers, Kerry Wright (who assisted me in animal surgery and who also measured the fat content of the guinea pig carcasses), Vera Barney and the technical and animal care staff of CHRI, including Kerry Penning, Leanne Srpek and Anna Mercorella, all of whom assisted in some way during the collection and weighing of tissues from animals on kill days and/or provided technical advice on gut, 3-MH, creatinine and nitrogen measurements. Thanks also go to the laboratory of Dr. Julie Owens for carrying out plasma glucose and urea measurements.

Comic relief was usually provided by Rob Falconer, Phil Owens and Paul Walton. The team in Frank Tomas' lab and the people in the CRC as a whole have generally provided a fun and pleasant atmosphere in which to work in.

I wish to thank my family and friends for their support over the years of this PhD.

ABSTRACT.

The aim of the work described in this thesis was to gain a greater understanding of the actions of IGF-II in postnatal animals. To do this, the abilities of IGF-I and IGF-II to affect growth and alter metabolism of animals following chronic infusion were compared. This comparison was carried out at first in the rat, a traditional small animal model for IGF research which has a negligible circulating concentration of IGF-II during adulthood, and then in the guinea pig, an animal which has a substantial concentration of IGF-II in blood during postnatal life. The guinea pig has not previously been reported as having been treated with the IGFs. It was hypothesised that animals which lack circulating IGF-II may respond differently to animals which do have large amounts of IGF-II in blood. LR^3IGF-I, an analogue of IGF-I which has a greatly reduced affinity for the IGFBPs, has been included in the guinea pig studies to provide information on the role of the IGFBPs in controlling the growth of these animals.

To enable me to carry out this study, I first prepared sufficient amounts of recombinant human (rh)IGF-II for infusion into rats.

In the first experiment, the effects of continuous 14 day infusion of rhIGF-I (104 or 260 µg/day) or rhIGF-II (104, 260 or 650 µg/day) were compared in young female rats. In particular, this study examined whether IGF-II can promote the growth of a non-hypophysectomised rat, since only the body weights of hypophysectomised rats have been reported to increase significantly in response to this peptide. IGF-I and IGF-II, at equivalent doses, increased the concentrations of the respective peptides in plasma to a similar degree, but IGFBP concentrations were increased to a greater degree by IGF-I treatment. There was no obvious difference in the way that IGF-I or IGF-II affected the concentrations of individual types of IGFBPs in plasma when assessed by Western ligand blotting. Significant increases in body weight gain and feed conversion efficiency were achieved by 260 µg of IGF-I/day or 650 µg of IGF-II/day. Feed intakes were not significantly affected, although regression analysis

showed that it was significantly associated with body weight gain, as was feed conversion efficiency. These effects were associated with increased nitrogen retention and increases in the fractional weights of kidneys, spleen, total gut and individual gut regions. There was an increase in the size of villi and muscularis lining the jejunum, suggesting an increased absorptive capacity of the gut. However there were no significant changes in the amount of faecal nitrogen excretion when expressed as a percentage of nitrogen intake. Thus, changes in feed conversion efficiency could not be fully explained by increased absorption of nitrogen from food in the gut. IGF-II was at least as potent as IGF-I in increasing the depth of jejunal crypts. Carcass composition was not altered by any treatment. It is concluded that IGF-II can promote the growth of normal young rats, although generally less potently than IGF-I.

In the second study, female guinea pigs of 350 g body weight were continuously infused for 7 days with rhIGF-I or rhIGF-II (120 or 360 µg/day) or LR^3IGF-I (120 µg/day). Despite increases in plasma IGF-I or IGF-II concentrations following IGF-I or IGF-II treatment respectively, total IGF concentrations were significantly increased by only the highest dose of IGF-II. Plasma IGF-I concentration was lowered by IGF-II treatment as was IGF-II concentration by IGF-I infusion. LR^3IGF-I infusion lowered both IGF-I and IGF-II concentrations, and, unlike IGF-I or IGF-II treatment, lowered plasma IGFBP concentrations. Western ligand blots show that the concentration of IGFBP-3 in plasma was clearly reduced by LR^3IGF-I, and an IGFBP which is most likely to represent IGFBP-1 was increased in concentration. Body weight gain, feed intake, feed conversion efficiency and carcass composition were not significantly affected by IGF-I or IGF-II treatment. Amongst the tissues examined only the fractional weight of the adrenals was increased by IGF-I, and that only by 360 µg/day. IGF-II had no significant effects. However, the fractional weight of the adrenals, gut, kidneys and spleen were significantly increased by LR^3IGF-I, but again overall growth was not stimulated. This study demonstrated for the first time that IGFs can stimulate the growth

of guinea pig tissues *in vivo* and indicated that there may be some differences in the way that these animals and rats respond to the IGFs.

In a second guinea pig study, higher doses of IGFs than those of the first guinea pig study were used to determine if body weight gain can be increased by IGF treatment in these animals. This experiment was also designed to determine whether IGF-I and IGF-II act better in combination or alone. Consequently, the response of 370 g guinea pigs to 14 days of continuous infusion of either vehicle (0.1M acetic acid), 720 µg of rhIGF-I or rhIGF-II/day, 360 µg of rhIGF-I/day plus 360 µg of rhIGF-II/day (720 µg/day total) or 240 µg of LR^3IGF-I/day was examined. Of the 6 animals treated with LR^3IGF-I, 3 developed symptoms indicative of hypoglycaemia mid-way through the treatment period and were killed before 14 days of treatment had been completed. A plasma glucose assay later confirmed that these animals were hypoglycaemic, but other treatments had no effect on blood glucose concentration. Results from animals treated with LR^3IGF-I were not included in statistical analyses, but have nevertheless been included for comparison with results from other treatment groups. Plasma IGF-I concentrations were significantly increased by treatment with IGF-I or the IGF combination and decreased by IGF-II treatment. IGF-II concentrations in plasma were increased significantly by IGF-II treatment and significantly decreased by IGF-I. Only LR^3IGF-I seemed to affect the total IGF concentration of plasma. As in the previous study, the concentration was decreased (in animals which survived a full 14 days treatment and in those which did not), a result of a low IGF-I and a low IGF-II concentration. Total plasma IGFBP concentration was also significantly increased in animals infused with IGF-I or the IGF combination, when measured by interference in the IGF-I RIA, and appeared lower in the LR^3IGF-I treated animals. Western ligand blots demonstrated that IGFBP-3 had decreased and IGFBP-1 had increased in the guinea pigs treated with the analogue. Body weight gains, feed

intakes and feed conversion efficiencies were not significantly affected by any treatment. However, regression analysis revealed that body weight gain of animals in this experiment was significantly related to both feed intake and feed conversion efficiency, as it was in the rat study. IGF-II alone significantly increased fractional lung weight above that of the control. There were no other significant effects of treatment on the fractional weights of tissues nor on the weights of individual regions of the gut. However, the gut, kidneys and spleen seem to have grown in response to LR^3IGF-I administration, as they did in the earlier guinea pig study. Carcass composition was not altered by any treatment, nor was nitrogen retention or plasma urea concentration. The failure of almost 2 mg/kg/day of IGF-I to stimulate growth of the guinea pig has indicated that guinea pigs are generally more resistant to growth stimulation by IGF-I than rats. It is not clear whether IGF-II is generally more or less potent than IGF-I in the guinea pig, although the greater potency of IGF-II in stimulating lung growth indicates that IGF-II may have some functions separate to those of IGF-I in this species. The lowering of IGF-I, IGF-II and IGFBP concentrations in blood as well as the greater susceptibility of guinea pigs to hypoglycaemia following LR^3IGF-I treatment (compared to its reported effects in rats), suggests a fundamental difference in the actions of IGFs between rats and guinea pigs that requires further investigation. I have been unable to clearly demonstrate a synergism between IGF-I and IGF-II in promoting the growth of the guinea pig.

The IGFs can lower blood glucose concentration when injected into a range of animals. In normal (non-diabetic) rats and humans this is likely to be a result of enhanced glucose uptake by peripheral tissues of the body, but there appears to be little effect on glucose production by the liver, probably because there are few type-1 IGF receptors in this tissue to mediate its actions. I have examined whether the higher hypoglycaemic potency of LR^3IGF-I in the guinea pig compared with the rat is due to a greater number of

type-1 IGF receptors in the liver of the guinea pig. That is, to see if the analogue could substantially inhibit hepatic glucose production. Liver membranes were prepared from rats and guinea pigs of similar age to those reported in the current studies, and using ^{125}I-IGF-I it was shown that the number of IGF-I binding sites in guinea pig liver membranes were about double those in the membranes from rats. That is, specific binding to rat and guinea pig liver membranes was 1.4 % and 3.1 % respectively. Thus the heightened hypoglycaemic sensitivity of guinea pigs to LR^{3}IGF-I, compared to that for rats, could be due to greater insulin-like actions in the liver, such as the inhibition of glucose production, through a greater number of binding sites in this organ. The binding of ^{125}I-IGF-II to liver membranes was also compared. There were approximtely twice as many binding sites for IGF-II than IGF-I in guinea pig liver membranes but about 8 times as many sites in membranes derived from the rat. It is probable that LR^{3}IGF-I will bind very poorly to the type-2 IGF receptor and hence the concentration of these receptors is unlikely to mediate any insulin-like effects of the analogue in the liver.

The effects of IGF-II on body weight gain, feed conversion efficiency and organ growth in the rat, as well as the effects of IGF-II on lung growth in the guinea pig, demonstrates that it can play a part in the postnatal growth of animals, and in a sufficiently high dose can replace IGF-I in stimulating many aspects of the postnatal growth of the rat. It is not yet clear if IGF-II is generally less potent than IGF-I in stimulating the growth of the guinea pig, but the studies of this thesis indicate that this animal may be more resistant to the anabolic actions of the IGFs than the rat. The more potent effects of LR^{3}IGF-I in the guinea pig than IGF-I or IGF-II suggests that the IGFBPs may play a significant part in the apparent resistance of these animals to IGF treatment.

PUBLICATIONS ARISING FROM THESIS RESEARCH.

Conlon MA, Francis GL, Tomas FM, Wallace JC, Howarth GS & Ballard FJ 1995. Continuous 14 day infusion of IGF-II increases the growth of normal female rats, but exhibits a lower potency than IGF-I. *Journal of Endocrinology* **144** 91-98.

Conlon MA, Tomas FM, Owens PC, Wallace JC, Howarth GS & Ballard FJ 1995. Long R^3 insulin-like growth factor-I (IGF-I) infusion stimulates organ growth but reduces plasma IGF-I, IGF-II and IGF binding protein concentrations in the guinea pig. *Journal of Endocrinology* **146** 247-253.

ABSTRACTS OF THESIS RESEARCH PRESENTED AT SCIENTIFIC MEETINGS.

Conlon MA, Burgoyne JL, Wright K, Aplin SE, Owens PC, Tomas FM, Wallace JC & Ballard FJ 1993. Continuous 14 day infusion of IGF-I or IGF-II and growth and nitrogen balance of young rats. *Manipulating Pig Production IV. Proceedings of the Fourth Biennial Conference of the Australasian Pig Science Association* 184.

Conlon MA, Burgoyne JL, Wright K, Aplin SE, Tomas FM, Wallace JC & Ballard FJ 1994. IGF-II infusion increases growth and plasma IGF levels in normal rats but less potently than IGF-I. *Proceedings of the Third International Symposium on Insulin-like Growth Factors* In: *Growth Regulation 4* supplement 1 abstract II280.

Conlon MA, Tomas FM, Wallace JC & Ballard FJ 1994. Effects of continuous 7 day infusion of IGF-I, IGF-II or LR^3IGF-I on growth, plasma IGF and IGFBP levels in guinea pigs. *Proceedings of the Endocrine Society of Australia* abstract 100.

Conlon MA, Tomas FM, Owens PC, Wallace JC & Ballard FJ 1994. Long R^3 Insulin-like Growth Factor-I (LR^3IGF-I) infusion stimulates organ growth but reduces plasma IGF-I, IGF-II and IGF Binding Protein concentrations in the guinea pig. *2nd Annual International Scientific Meeting of DSL* abstract 10.

ABBREVIATIONS.

A_{215}: absorbance at a wavelength of 215 nm.

A_{280}: absorbance at a wavelength of 280 nm.

ACTH: adrenocorticotrophic hormone.

ANOVA: analysis of variance.

Ala: Alanine.

Arg: Arginine.

Asp: Aspartic acid.

cm: centimetre.

c.p.m.: counts per minute.

CHRI: Child Health Research Institute.

CSIRO: Commonwealth Scientific and Industrial Research Organisation.

DMEM: Dulbecco's Modified Eagles Medium.

DNA: deoxyribonucleic acid.

E. coli: Escherichia coli.

EGF: Epidermal Growth Factor.

GH: growth hormone (bGH: bovine growth hormone).

g: gram.

x *g*: times the force of gravity.

Glu: Glutamic acid.

Gly: Glycine.

H.P.L.C.: high performance liquid chromatography.

hr: hour.

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.

kb: kilobase.

kDa: kilodalton.

kg: kilogram.

l: litre.

$LR^3IGF\text{-}I$: long R^3 insulin-like growth factor-I.

M: molar.

mA: milliampere.

[Met^1]: methionine at position 1.

mg: milligram.

3-MH: 3-methylhistidine.

min.: minute.

ml: millilitre.

mM: millimolar.

mm: millimetre

mRNA: messenger ribonucleic acid.

nm: nanometre.

P : probability.

Pro: Proline.

R^3: Arginine at position 3.

RGD: Arginine-Glycine-Aspartic acid.

rh: recombinant human.

RIA: radioimmunoassay.

S.E.M.: standard error of the mean.

Ser: Serine.

Tyr: Tyrosine.

µg: microgram.

µl: microlitre.

µm: micrometre.

Val-Asn-Phe-Ala-His-Tyr: Valine-Asparagine-Phenylalanine-Alanine-Histidine-Tyrosine.

v/v : volume per volume.

w/v : weight per volume.

CHAPTER 1.

LITERATURE REVIEW.

1.1 Introduction.

The main goal of the work described in this thesis has been to gain a greater understanding of the actions of IGF-II in postnatal growth. This has involved studying the effects of chronic infusion of IGF-II, IGF-I or LR^3IGF-I (an analogue of IGF-I) into the rat and the guinea pig. Hence, the information contained within the following literature review is intended to give the reader an understanding of the current knowledge pertaining to the actions of the IGFs, particularly their *in vivo* actions. The review also details information relating to the IGF receptors, IGF binding proteins and analogues of the IGFs, as these are studied in this thesis.

The review covers information published prior to May 1995.

1.2 Insulin-like growth factors (IGFs) can mediate the actions of Growth Hormone (GH).

The actions of GH in stimulating postnatal growth are often not direct but mediated by other factors. This was discovered in rats when it became apparent that an intermediate was required for the stimulation of cartilage growth by GH. Due to its role in mediating the growth of GH (somatotropin), the intermediate became known as a somatomedin (reviewed by Daughaday 1989).

It was also discovered that antibodies to insulin could only block a small percentage of the insulin-like activity of serum. The two agents responsible for this non-suppressible insulin-like activity were shown to be much more potent than insulin in stimulating DNA synthesis and cell division of fibroblasts (Morell & Froesch 1973), and following purification and protein sequencing were named insulin-like growth factor (IGF)-I and IGF-II (Rinderknecht & Humbel 1976; 1978a,b).

IGF-I was later shown to be a somatomedin, and responsible for mediating the effects of GH on numerous tissues (Daughaday 1989).

1.3 The somatomedin hypothesis.

In the somatomedin hypothesis proposed by Salmon & Daughaday (1957), a somatomedin, later to be recognised as IGF-I, is released from the liver in response to pituitary-derived GH present in blood. Once in circulation, IGF-I could then stimulate growth and metabolism of peripheral tissues, particularly that of the growth plate in bone.

This hypothesis proved to be an over-simplification of the roles of both GH and the IGFs. Both IGF-I and IGF-II are produced by, and have pleiotropic actions on, an extensive range of tissues in many animals throughout their life-cycle (reviewed by Cohick & Clemmons 1993; Jones & Clemmons 1995) yet IGF-II release by the tissues does not seem to be controlled by GH (Adams *et al.* 1983; LeRoith *et al.* 1991). Also, GH can act directly on tissues other than liver and stimulate IGF release (Werther *et al.* 1993). The IGFs can then act on the cells from which they are produced (an autocrine function), neighbouring cells (a paracrine or juxtacrine function), or, by transport via the circulatory system, act on cells at distant sites (an endocrine function) (Daughaday 1989). Though produced by other tissues, the majority of IGF-I in adult blood is thought to be derived from the liver (Schwander *et al.* 1983), but this may not be the case for chickens (McMurtry *et al.* 1994). The actions of IGFs are also mediated/controlled by a group of proteins which bind to the IGFs and which are known as IGF-binding proteins (IGFBPs) (reviewed by Rechler 1993). Additionally, IGFs are produced by cells in response to a number of hormones and growth factors (reviewed by Daughaday & Rotwein 1989).

Behringer *et al.* (1990) have shown that IGF-I is capable of stimulating nearly all aspects of growth postnatally in mice that are GH-deficient, all that is except for growth of the liver. This is strong support for the wide applicability of the somatomedin hypothesis to postnatal growth.

1.4 The IGFs and the insulin super-family of peptides.

The IGFs belong to a super-family of peptides that share strong structural and sequence homology with insulin. This family includes the relaxins and also several peptides found in invertebrates, namely bombyxins, locusta insulin-related peptide and molluscan insulin-like peptides (Murray-Rust *et al.* 1992; Smit *et al.* 1993). During evolution the structure of the IGFs has been strongly conserved, since 57 of 70 of the amino acid residues of IGF-I are the same in species as diverse as the human, rat, chicken and frog (Kajimoto & Rotwein 1990).

The IGFs differ from other members of the superfamily in that they are single-chain, not double-chain, peptides. The two chains (known as A and B) of the insulin dimer are connected by disulphide bridges. Proinsulin is produced as a single chain with a connecting peptide (called the C-peptide) that links the A and B chain sequences. The C-peptide is later excised to produce the mature insulin molecule (Murray-Rust *et al.* 1992). The structure of the IGFs closely resembles proinsulin. Regions of the IGFs with sequence homology to the A and B chains of insulin are known as A and B domains, and the connecting region, which is not excised, is known as the C domain. An extra region not found in insulin is at the C-terminal end and is known as the D domain. Human IGF-I and -II are 70 and 67 amino acid residues in length respectively (Rinderknecht & Humbel 1978a,b), and similar sizes are present in other species (Daughaday & Rotwein 1989).

The prohormone form of both IGFs contains a carboxy-terminal extension known as the E domain which is cleaved at the time of secretion (Jansen *et al.*1983; Rotwein 1986). Expression of alternative mRNA forms of IGF-I can give rise to E domains of varying length (eg. 35 or 77 residues in humans) (Rotwein 1986; Adamo *et al.* 1994).

The E domain of the IGF-II prohormone is 89 residues long in rats and humans (Jansen *et al.* 1983; Whitfield *et al.* 1984). Several alternative processing sites exist within this domain, enabling cells to produce "big" forms of IGF-II (Gowan *et al.* 1987). A 10 kDa form of IGF-II was found in the blood and spinal fluid of humans, and in other studies 15 kDa or 11.5 kDa forms of IGF-II have also been described (Haselbacher & Humbel 1982; Liu *et al.* 1993a; Zumstein *et al.* 1985; Gowan *et al.* 1987). The concentration of large forms of IGF-II is increased in the blood of patients with non-islet cell tumour hypoglycaemia and also in some other disease states (Daughaday *et al.* 1988; 1990).

A number of other naturally occurring variants of the IGFs have been isolated but these will not be discussed here.

1.5 The IGF Receptors.

1.5.1 General description.

There are 3 types of cell surface receptors that IGFs can bind to: the insulin receptor, to which both IGF-I and IGF-II bind weakly; the type-1 IGF receptor, to which both IGFs bind strongly and through which most of the biological responses of both peptides occur; and the type-2 IGF receptor to which only IGF-II binds with strong affinity (Kasuga *et al.* 1981; Czech 1989). The insulin receptor binds IGF-I with between 100 to 1000 times lower affinity than insulin, and the type-1 IGF receptor binds insulin

with a similar affinity (Gunn *et al.* 1977; Zapf *et al.* 1978; Rechler *et al.* 1980; Massague & Czech 1982; Czech *et al.* 1989).

1.5.2 The type-1 IGF receptor.

The type-1 IGF and insulin receptors share strong structural homology and are members of the tyrosine kinase receptor family (Nissley *et al.* 1985; Christoffersen *et al.* 1994). In both cases the receptor is a heterotetrameric glycoprotein that consists of two extracellular alpha sub-units (135 kDa) linked by disulphide bonds and two beta sub-units (90 kDa) which are also linked to each other and to the alpha sub-units by disulphide bonds. The alpha sub-units bind the corresponding ligand and activate a tyrosine kinase which is intrinsic to the beta sub-units that span the plasma membrane (Heyner *et al.* 1989; Le Roith *et al.* 1991). The autophosphorylation of the beta sub-unit is necessary to exert biological actions (McLain *et al.* 1987). The complex intracellular signalling pathways that follow are still being elucidated, but of particular note is the early activation of a 170 kDa protein known as insulin receptor substrate-1 (IRS-1) (Sun *et al.* 1991). IRS-1 subsequently activates numerous biochemical pathways that are critical to maintaining normal growth and glucose homeostasis (Lienhard 1994). Differences in the intracellular signalling pathways of the insulin and IGF receptors are likely to explain some or all of the differences in the functions of these peptides.

Type-1 IGF receptors mediate the biological actions of both IGF-I and IGF-II (Ewton *et al.* 1987; Kiess *et al.* 1987; Ballard *et al.* 1988; Adashi *et al.* 1990) and are distributed widely throughout the tissues of animals in order to facilitate the wide-ranging actions of the IGFs. Since the IGFs mediate growth, it is of no surprise that type-1 receptor levels are high in the rapidly growing tissues of the fetus and neonate and decline thereafter (Werner *et al.* 1989; Bondy *et al.* 1990; Lee *et al.* 1993). In mice in which the

type-1 IGF receptor is absent due to knock-out of the gene, mice are smaller than normal at birth but die not long after (Liu *et al.* 1993b). Thus, although the receptor is required to maintain a normal growth rate during development of mice, it is absolutely essential for postnatal life. In adult rats and humans the levels of this receptor in the liver are very low compared to those in many other tissues such as muscle (Poggi *et al.* 1979; Rechler *et al.* 1980; Caro *et al.* 1988). Adipocytes also have few type-1 IGF receptors (Zapf *et al.* 1981). Despite low concentrations of the type-1 IGF receptor in liver and adipose tissue, insulin receptor concentrations remain substantial in these tissues. However, it is not likely that physiological concentrations of IGFs are sufficient to exert biological actions via the insulin receptor in these tissues (see Ballard *et al.* 1994).

1.5.3 The type-2 IGF receptor.

This receptor is structurally unrelated to the type-1 IGF and insulin receptors and has been shown to be identical to the cation-independent mannose 6-phosphate receptor in mammals (Morgan *et al.* 1987; Kiess *et al.* 1988; MacDonald *et al.* 1988). It is a single 250 kDa polypeptide chain comprising an N-terminal extracellular domain of over 2000 amino acid residues, a short 22-23 residue transmembrane domain and a 163-167 residue carboxy terminal cytoplasmic domain (MacDonald *et al.* 1989). The cytoplasmic domain can be cleaved to produce a truncated form of the receptor that circulates in blood (MacDonald *et al.* 1989). The type-2 receptor has been found in the blood of a range of species including rats (Kiess *et al.* 1987b), monkeys (Gelato *et al.* 1988), pigs (Lee *et al.* 1993) and sheep (Gelato *et al.* 1989). The extracellular domain binds IGF-II or ligands containing mannose 6-phosphate groups at separate sites. Binding of ligands containing mannose 6-phosphate, such as beta-galactosidase, can sterically hinder the binding of

IGF-II (Kiess *et al.* 1988). In chickens and frogs the cation-independent mannose 6-phosphate receptor does not have a binding site for IGF-II (Clairmont & Czech 1989).

The main function ascribed to the type-2 receptor is to transport lysosomal enzymes, which have a mannose 6-phosphate attachment, from their site of synthesis in the Golgi/trans-Golgi network of cells to an acidic prelysosomal compartment (Braulke *et al.* 1990). The lysosomal enzymes can be secreted if they are unable to bind to the receptor. At least 10% of the type-2 receptors of a cell are at the surface where they bind and endocytose ligands (Braulke *et al.* 1990). Treatment of cells with ligands for this receptor (or for example with IGF-I or insulin) can rapidly increase the number of cell surface receptors by redirecting intracellular receptors (Braulke *et al.* 1989), but IGF-II can also decrease the levels of receptor on the surface of endothelial cells (Hu *et al.* 1990). While IGF-II can induce an increase in cell surface receptors the resulting decrease in intracellular receptors does not affect the synthesis or targeting of lysosomal enzymes in the cell (Braulke *et al.* 1990; Braulke *et al.* 1991).

The receptor can bind several other growth factors with mannose 6-phosphate moieties and plays a part in the activation of latent transforming growth factor beta (TGF beta), probably by internalising the latent complex and activating it by acidification in prelysosomal compartments (Jirtle *et al.* 1991).

A number of studies suggest that IGF-II can induce biological actions via this receptor. Actions noted include increased growth of human erythroleukemia cells (Tally *et al.* 1987), increased motility of rhabdomyosarcoma cells (Minniti *et al.* 1992), stimulation of influxes of Ca^{++} ions into cells (Nishimoto *et al.* 1987), activation of G-proteins (Murayama *et al.* 1990) and the triggering of inositol triphosphate and diacylglycerol production (Rogers *et al.* 1990). By using an analogue of IGF-II which binds poorly to insulin and type-1 IGF receptors, but preferentially to the type-2 receptor,

Rosenthal *et al.* (1994) have demonstrated that the differentiation of muscle cells by autocrine secretion of IGF-II may involve both type-1 and type-2 IGF receptor mediated actions. Nevertheless, most of the biological actions of both IGF-I and IGF-II appear to be mediated by the type-1 IGF receptor and not the type-2 IGF receptor in a number of cell types (Ewton *et al.* 1987; Kiess *et al.* 1987a; Ballard *et al.* 1988; Adashi *et al.* 1990; Raile *et al.* 1994).

In rats, the developmental expression of the type-2 receptor roughly follows that of IGF-II in that it is expressed in high levels in most tissues of the fetus, and then declines substantially between postnatal days 20 and 40 (Sklar *et al.* 1989). The concentration of the circulating form of the receptor also follows a similar developmental pattern (Kiess *et al.* 1987b). A postnatal decline in the tissue levels of the receptor also occurs in other animals (Kar *et al.* 1993; Lee *et al.* 1993), although the concentration is still substantial in most tissues during postnatal life. Unlike the type-1 IGF receptor, the type-2 IGF receptor is abundant in the liver of adult rats (Massague & Czech 1982; Scott & Baxter 1987). The type-2 IGF receptor and IGF-II are expressed in a co-ordinate fashion during muscle cell differentiation in the rat (Tollefsen *et al.* 1989).

One of the more popular theories about the role of the type-2 IGF receptor is that it acts as a sink for IGF-II. This is supported by *in vitro* studies that demonstrate that antibodies that block the receptor also inhibit IGF-II degradation (Kiess *et al.* 1987a). Also, the lower biological potency of IGF-II compared to IGF-I can be accounted for, at least in part, by its binding to the type-2 IGF receptor (Francis *et al.* 1993).

1.6 Imprinting of the IGF-II and type-2 IGF receptor genes.

DeChiara *et al.* (1991) demonstrated that the gene for IGF-II is only expressed by the paternally derived allele in mice and that ablation of this allele results in pups which

are stunted in growth at birth. In contrast, the mouse gene for the type-2 IGF receptor is expressed only by the maternal allele, and deletion of this allele is lethal to the developing fetus (Barlow *et al.* 1991). Thus, expression is dependent on which parent the gene is derived from, a phenomenon which is known as gene imprinting.

Imprinting studies support the notion that the type-2 IGF receptor acts as a sink for IGF-II. The most compelling evidence for this is the study by Filson *et al.* (1993). They demonstrated that mice that would normally die during development, because they lack the maternally derived type-2 IGF receptor gene, can live if they also lack the IGF-II gene derived from the father. This suggested that an over-abundance of IGF-II results when it cannot be cleared via the type-2 receptor, resulting in death.

Opposite imprinting of the IGF-II and type-2 receptor genes does not occur in all mammals. In humans, the IGF-II gene is paternally imprinted (Ohlsson *et al.* 1993) but the type-2 receptor is not imprinted (Ogawa *et al.* 1993b). The genes for IGF-I and the type-1 receptor are not imprinted.

1.7 The IGF Binding Proteins.

1.7.1 General Description.

Most of the IGFs in circulation (up to 98%) (Hintz 1984) are normally non-covalently bound to binding proteins (IGFBPs) (Froesch *et al.* 1963; Burgi *et al.* 1966; Jakob *et al.* 1968; Zapf *et al.* 1975; Hintz & Liu 1977; Daughaday *et al.* 1982). These IGF-IGFBP complexes prolong the half-life of IGFs considerably and act as a reservoir of IGFs for use by the tissues (Hodgkinson *et al.* 1987; Ballard *et al.* 1991). Regulation of the IGFBPs is also a means of controlling the activities of the IGFs. It has been estimated that the total circulating serum IGF concentration in humans, if not restrained by IGFBPs, would have a hypoglycaemic potency approximately 80 times greater than that of the

circulating insulin (Lewitt & Baxter 1991). While IGFBPs usually inhibit the actions of IGFs by preventing binding to cell receptors, they may in some cases potentiate the actions of IGFs (Elgin *et al.* 1987; Blum *et al.* 1989; Conover *et al.* 1994).

To date, 6 classes of IGFBP have been identified. These are known as IGFBP-1, -2, -3, -4, -5 and -6 (Ballard *et al.* 1990a, Rechler 1993). These IGFBPs, while sharing strong structural homology, have unique characteristics and have distinct developmental and tissue sites of expression in a wide range of animals (Funk *et al.* 1992; Rechler 1993; Gosteli-Peter *et al.* 1994). Although only 6 classes of IGFBP have been clearly identified to date, numerous modifications, including glycosylation, phosphorylation and proteolysis, increase the heterogeneity and therefore probably the functions of these proteins. This was highlighted by the study of Chan & Nicoll (1994) who discovered 25 forms of IGFBP in rat serum, one of which, a 50 kDa form, appears unrelated to IGFBPs 1 to 6.

1.7.2 The 150 kDa IGFBP complex.

Studies in which serum from humans, pigs, rats, sheep and other species have been subjected to size exclusion chromatography under neutral pH conditions, demonstrate that the majority of IGF-I and IGF-II in blood is bound to IGFBP-3 and that 70 to 80 % of this is carried in an approximately 150 kDa complex that contains no other class of IGFBP (Froesch *et al.* 1963; Burgi *et al.* 1966; Jakob *et al.* 1968; Hintz & Liu 1977; Zapf *et al.* 1990; Walton *et al.* 1991; Gargosky *et al.* 1993; Hodgkinson *et al.* 1994). The remainder of the IGFs are carried by the other IGFBPs that elute in an approximately 30 - 50 kDa pool, which also contains IGFBP-3.

The 150 kDa complex is composed of 3 sub-units, IGF-I or IGF-II, IGFBP-3 and an acid-labile sub-unit (Baxter & Martin 1989a,b). Assembly of the complex can occur in blood but requires IGF-I or IGF-II to bind IGFBP-3 first, and this binary complex then

binds with the acid-labile sub-unit. Thus all of the 150 kDa complex is saturated with IGF (Baxter & Martin 1989a). The acid-labile sub-unit circulates in molar excess of the 150 kDa complex in blood, as evidenced by the fact that infusion of IGF/IGFBP-3 complexes into normal rats enables 150 kDa complex formation within 2 minutes (Lewitt *et al.* 1994).

Zapf *et al.* (1995) have shown that injection of IGF-I/IGFBP-3 complexes into hypophysectomised rats results in their elution in a 40 kDa pool following neutral chromatography, but in a pool corresponding to 150 kDa in normal rats. Blood glucose was lowered and synthesis of glycogen in the diaphragm was stimulated only in the hypophysectomised animals. The complexes not associated with the acid-labile sub-unit were probably able to leave circulation far more readily and thereby exert greater insulin-like actions. These findings corroborate results of Francis *et al.* (1988), Guler *et al.* (1989b) and Lewitt *et al.* (1994) who demonstrated that IGF-I bound to IGFBP-3 in an approximately 50 kDa pool is cleared more rapidly than when in the 150 kDa complex in lambs, humans and rats respectively.

1.7.3 IGFBP-3.

IGFBP-3 is a glycoprotein with a molecular mass of 40 to 42 kDa in rats and humans (Martin & Baxter 1986) and 46 kDa in sheep (Carr *et al.* 1994), although it appears as bands corresponding to somewhere between 40 to 53 kDa on Western ligand blots. Bands of similar size to that expected of IGFBP-3 are present in Western ligand blots of plasma from a wide range of animals. In rats, humans and cattle it has a consensus glycosaminoglycan binding site which may enable it to bind to extracellular matrix (Hodgkinson *et al.* 1994). It lacks the Arg-Gly-Asp (RGD) cell adhesion sequence of some other IGFBPs (Rechler 1993). The structure of IGFBP-3 appears highly

conserved between species, particularly in regard to the position of the 18 disulphide bonded cysteine residues, which are also present in most of the other IGFBP classes (Rechler 1993).

IGFBP-3 mRNA is found in a greater number of rat tissues than that of the acid-labile sub-unit mRNA at birth, indicating that IGFBP-3 has functions separate to that of the 150 kDa complex (Chin *et al.* 1994). In addition to the liver, the IGFBP-3 gene is expressed strongly in the central nervous system (Lamson *et al.* 1989), spleen, kidney and white adipose tissue (Gosteli-Peter *et al.* 1994). Gene expression can be controlled differently in the various organs since protein restriction reduces liver but not kidney mRNA levels in rats (Lemozy *et al.* 1994).

The age-dependent expression pattern of IGFBP-3 varies between species. IGFBP-3 is the predominant serum IGFBP in the adult of most normal animals. IGFBP-3 only appears in the blood of the postnatal rat, whereas in other species such as pigs (Lee *et al.* 1993) and humans this protein is detectable in blood during fetal development and in the latter it predominates by 30 weeks of gestation (D'Ercole *et al.* 1980). A study by Hahnel & Schultz (1994) has shown that genes for IGFBP-3 and most other IGFBPs are transcribed in pre-implantation mouse embryos. This suggests that IGFBP-3 is playing a role in most, if not all, stages in the life-cycle of animals. Hogg *et al.* (1994) demonstrated that mRNA for IGFBP-3 was also present in high amounts in liver and pancreas in the fetus of rats. Thus even though serum IGFBP-3 may not be detectable, this IGFBP is probably still playing autocrine/paracrine roles in development.

There appears to be a strong association between the concentrations of IGFBP-3, IGF-I and GH in blood. Administration of GH to rats (Clemmons *et al.* 1989), pigs (Owens *et al.* 1990) and humans (Laron 1993) results in increases in blood concentrations of IGF-I and IGFBP-3 (or IGFBPs likely to represent IGFBP-3). Conversely,

immunoneutralisation of GH in neonatal rats (Gardner & Flint 1990) leads to large falls in serum IGF-I concentrations. Hypophysectomy significantly reduces IGFBP-3 and IGF-I concentrations (Glasscock *et al.* 1990) and IGFBP-3 concentration is low in humans that do not respond to GH due to a defective GH receptor (Laron syndrome) and high in those with acromegaly (Laron 1993). The association between blood GH and IGFBP-3 is such that the latter can be used as a clinical marker for the rate of GH secretion from the pituitary (Blum *et al.* 1993). An advantage of this is that even though GH is released episodically into circulation, IGFBP-3 concentrations do not appear to fluctuate significantly (Cheetham *et al.* 1994). Serum IGF-I and IGFBP-3 increase concomitantly in children, reach their maximum during puberty and then fall through adulthood (Rosenfield *et al.* 1983; Luna *et al.* 1987; Wilson *et al.* 1992; Argente *et al.* 1993). Substantial concentrations of IGFBP-3 appear in the blood of rats about 20 days after birth (Glasscock *et al.* 1990), a time when IGF-I concentrations are also increasing (Donovan *et al.* 1989). Similar observations have been made in other animals including pigs (Lee *et al.* 1993).

Although IGFBP-3 and IGF-I concentrations are closely related, there is probably an even greater relationship between IGFBP-3 (or total IGFBP) concentration and the sum of IGF-I and IGF-II (total IGF) in blood, as has been demonstrated in humans (Baxter 1988; Argente *et al.* 1993) and pigs (Owens *et al.* 1991). This is because IGFBP-3 is the primary carrier of IGFs and also because it appears to carry both IGFs with roughly equal affinity (Martin & Baxter 1986). Even though IGF-II expression appears GH-independent (Adams *et al.* 1983), GH probably indirectly influences IGF-II concentration in blood by its ability to regulate IGFBP-3 concentration.

Notwithstanding the strong positive correlation between IGFBP-3 and IGF-I, administration of IGF-I to animals does not always result in an increase in IGFBP-3.

Treatment has increased IGFBP-3 (or again, based on the size of bands on Western ligand blots, what is likely to represent IGFBP-3) in normal (Tomas *et al.* 1993a), diabetic (Tomas *et al.* 1991a) and nitrogen-restricted (Tomas *et al.* 1991b) rats, diabetic humans (Cheetham *et al.* 1994), fasted lambs (Douglas *et al.* 1991a), goats (Prosser *et al.* 1995) and mini-poodles (Guler *et al.* 1989a). However, daily injection of IGF-I into humans over 7 days (Baxter *et al.* 1993) or infusion of the potent IGF-I analogue $LongR^3IGF\text{-}I$ into growing pigs (Walton *et al.* 1994) has decreased blood IGFBP-3 concentrations. Although Guler *et al.* (1989a) demonstrated that constant infusion of IGF-I into mini-poodles was associated with a blunting of GH secretion, there was still an increase in IGFBP-3 levels in blood. Also, the reduction in IGFBP-3 by IGF treatment of humans was not associated with changes in urinary excretion of GH (Baxter *et al.* 1993). Therefore some other agents may be contributing to the fall in IGFBP-3 levels. IGF-II has also been shown to affect GH release from the pituitary *in vitro* (Simes *et al.* 1991) and may be an important factor contributing to the concentrations of GH and IGFBP-3 in blood, but this has not been studied in any detail *in vivo*.

IGFBP-3 concentrations are lowered by a wide range of treatments or disorders including nitrogen restriction (Tomas *et al.*1991b), diabetes (Tomas *et al.* 1991a; Lewitt *et al.* 1993; Cheetham *et al.* 1994) and hypothyroidism (Nanto-Salonen *et al.* 1993). In the latter case, serum IGFBP-3 was restored in rats by GH treatment, indicating that the effects of thyroid hormones on IGFBP-3 may be GH mediated. Thyroid hormones are also known to stimulate IGFBP-3 production by rat calvariae *in vitro* (Schmid *et al.* 1992). Glucocorticoids, and hence the adrenals, are able to control IGF concentration in rats, but this control is not mediated by IGFBP-3 since adrenalectomy of normal or diabetic rats has no effect on serum IGFBP-3 concentration (Unterman *et al.* 1993a). Expression of the gene for this IGFBP, along with that for IGFBP-1, varies throughout

the oestrous cycle in the uterus and other tissues in rats (these changes are not detectable in serum) and appears inversely regulated by oestradiol in human breast cancer (Shao *et al.* 1992; Molnar & Murphy 1994).

1.7.4 IGFBP-1.

IGFBP-1, also known as placental protein 12 and human pregnancy associated endometrial alpha 1 globulin (Chard 1989), is a non-glycosylated protein with a core molecular mass of about 25 kDa, but which appears as an approximately 28 kDa band on Western ligand blots (Ballard *et al.* 1990a; Rechler 1993). It has a carboxy-terminal RGD sequence which enables it to bind to integrin receptors (Ruoslahti & Pierschbacher 1987; Rechler *et al.* 1993) and it is able to move out of circulation and into tissues (Bar *et al.* 1990). IGFs bound to IGFBP-1 are likely to be part of a pool in blood with a half-life of only about 25 minutes (Guler *et al.* 1989b). IGFBP-1 binds IGF-I and IGF-II with roughly equal affinity (Baxter *et al.* 1987).

The concentration of IGFBP-1 can fluctuate rapidly in the blood of adults and increase overnight, possibly due to lack of food intake during that time (Cotterill *et al.* 1988; Lewitt & Baxter 1991; Jorgensen *et al.* 1993). Fasting has been shown to decrease plasma IGF-I and insulin concentrations in blood while increasing IGFBP-1 and -2 (Ooi *et al.* 1990). In Diabetes Mellitus, serum IGFBP-1 concentrations are often high (Holly *et al.* 1990), and in rats, insulin deficiency stimulates liver IGFBP-1 mRNA expression (Ooi *et al.* 1990; Seneviratne *et al.* 1990). These observations are explained by the fact that IGFBP-1 is strongly controlled by blood insulin concentration (Suikkari *et al.* 1988; Thissen *et al.* 1994). As a consequence, IGFBP-1 is also controlled by a wide range of other factors involved in metabolic regulation. It is negatively regulated by glucose, insulin, amino acid availability, IGF-I and GH (Seneviratne *et al.* 1990; Lewitt & Baxter

1991; Powell *et al.* 1992; Thissen *et al.* 1994) and positively regulated by cAMP, glucagon and glucocorticoids (Lewitt & Baxter 1989; Luo *et al.* 1990; Conover *et al.* 1993).

Injection of human IGFBP-1 into humans causes the glucose concentration of blood to increase (Lewitt *et al.* 1991) and suggests inhibition of the insulin-like activity of the IGFs (Lewitt *et al.* 1994). Zierath *et al.* (1992) demonstrated that IGFBP-1 suppresses the effects of IGF-II on glucose transport in human skeletal muscle. IGFBP-1 also inhibits the effects of IGF-I when injected into rats (Lewitt *et al.* 1993). In the glucocounter-regulatory role of IGFBP-1, postulated by Lewitt & Baxter (1991), the normally IGF-unsaturated IGFBP-1 acts to rapidly bind the IGFs from the tissues, under conditions when insulin and glucose are low, in order to prevent undesirable insulin-like actions, and then delivers the IGFs to the large pool of IGFBP-3 in circulation. The transfer of IGFs from tissues to blood is aided by the higher affinity of IGFBP-3 for IGFs than those of the other IGFBPs such as IGFBP-1.

Treatment of normal rats or lactating goats with IGF-I results in an increase in a plasma IGFBP that has a size expected of IGFBP-1 (Tomas *et al.* 1993; Prosser *et al.* 1995). IGF-I infusion into diabetic or nitrogen restricted rats (Tomas *et al.* 1991a,b) or diabetic humans (Bach *et al.* 1994; Cheetham *et al.* 1994) also appears to increase IGFBP-1 concentrations. GH treatment increases IGFBPs, likely to include IGFBP-1, in the plasma of pigs (Owens *et al.* 1990), but injection of GH into humans can lower blood IGFBP-1 concentration (Tapanainen *et al.* 1991). Although IGFBP-1 is thought to be inversely regulated by GH and IGF-I *in vivo*, exogenous treatment of animals with these peptides often seems to have caused increases in the blood concentration an IGFBP likely to represent IGFBP-1.

The gene is expressed in a range of tissues but predominantly by the liver in rats and humans (Lewitt & Baxter 1991; Rechler 1993) and to a greater extent in the fetus than in the adult (Murphy *et al.* 1990; Straus *et al.* 1991; Wang *et al.* 1991). It is also strongly expressed in decidualised uterus (Ritvos 1988). In the human fetus, IGFBP-1 is the main serum IGFBP until 26 weeks of age (D'Ercole *et al.* 1980) and during the second trimester is expressed in an identical range of tissues to IGF-I (Hill *et al.* 1989a), implying a crucial role for this IGFBP in mediating effects of IGF-I on development (Wang *et al.* 1991). Indeed, the concentration of IGFBP-1 in maternal and umbilical cord serum of humans is inversely related to birth weight (Chard 1989), indicating that it is a crucial factor in determining the growth rate of the fetus. In support of this, the fetal growth retardation that results from glucocorticoid excess appears to be a result of increased serum IGFBP-1 (Luo *et al.* 1990).

1.7.5 IGFBP-2.

This protein has as an approximate size of 30 to 34 kDa when analysed by western ligand blotting (Romanus *et al.* 1986; Mottola *et al.* 1986; Coleman & Etherton 1991; Funk *et al.* 1992; Gosiewska *et al.* 1994), although its molecular mass deduced from its nucleotide sequence is closer to 30 or 31 kDa (Rechler 1993). Like IGFBP-1, it contains an RGD sequence for binding to integrin receptors and is capable of moving out of the circulation (Bar *et al.* 1990; Ooi *et al.* 1991). While genes for both IGFBP-1 and -2 are expressed strongly during development, their tissue-specific patterns of expression are usually not overlapping (Funk et al. 1992; Zhou & Bondy 1993; Chard *et al.* 1994; Gosteli-Peter *et al.* 1994; Green *et al.* 1994).

IGFBP-2 has greater affinity for IGF-II than for IGF-I, but it is not certain by how much (Ross *et al.* 1989; Clemmons *et al.* 1991; Rechler *et al.* 1993). It is therefore not

surprising that IGF-II and IGFBP-2 show similar developmental patterns of expression in tissues from a range of animals (Straus *et al.* 1991; Delhanty & Han 1993). Since IGF-II is believed to play a greater role in the fetus than in adult life, then IGFBP-2 may contribute to that role. In rats, serum IGFBP-2 (Romanus *et al.* 1986; Donovan *et al.* 1989) and liver IGFBP-2 mRNA levels are highest in late fetal development and during the neonatal period (Brown *et al.* 1989), as are concentrations of the protein in the cerebrospinal fluid where it is the predominant IGFBP (Ocrant *et al.* 1990). High expression of IGFBP-2 in the fetal and neonatal periods followed by declines in adulthood have also been demonstrated in sheep, rhesus monkeys (Sullivan & Feldman 1994), humans (Chard *et al.* 1994) and pigs (Lee *et al.* 1993). As for IGFBP-1, IGFBP-2 expression is greater in the liver than in other tissues during the peri-natal period of the rat (Ooi *et al.* 1990).

During rat embryogenesis, IGFBP-2 is produced by tissues which are highly mitotic or which appear to direct the development of adjacent tissues (Wood *et al.* 1992; Streck *et al.* 1992), and is expressed largely in tissues derived from the ectoderm or endoderm, while IGF-II is largely expressed by mesoderm derived tissues, highlighting the complementarity of these 2 peptides (Wood *et al.* 1992).

In adult rats, IGFBP-2 continues to be strongly expressed along with IGF-II in the brain at a time which correlates strongly with growth of this tissue (Sullivan & Feldman 1994). IGFBP-2 is also predominant in the brain of humans (Funk et al. 1992). This IGFBP is also likely to play a part in the control of kidney development and/or function, since kidney-derived cell lines from a range of species preferentially secrete IGFBP-2 (Ooi *et al.* 1990).

Diabetes in rats increases serum IGFBP-2, since hepatic IGFBP-2 mRNA is inversely regulated by insulin (Boni-Schnetzler *et al.* 1990). IGFBP-2 is relatively GH

independent (Laron *et al.* 1993). Hypophysectomy of rats does not affect hepatic IGFBP-2 mRNA levels (Gosteli-Peter *et al.* 1994) and GH treatment of these rats does not change its expression (Boni-Schnetzler *et al.* 1990). During fasting, when blood IGF-I concentration decreases, IGFBP-2 concentration and hepatic gene expression increase (LeRoith *et al.* 1991; Lemozy *et al.* 1994). In humans, blood IGFBP-2 seems independent of normal changes in insulin and glucose concentrations and also short term GH deprivation (Jorgensen *et al.* 1993).

Treatment of pigs with GH is able to dose-dependently decrease a 34 kDa IGFBP that is likely to be IGFBP-2 and increases IGF-I and IGFBP-3 (Coleman & Etherton 1991). Thus circulating GH can affect IGFBP-2 expression even though hypophysectomy studies of rats suggests relative GH independence. Treatment of normal rats with IGF-I results in an increase in a band approximating IGFBP-2 by western ligand blotting (Tomas *et al.* 1993). Western ligand blots of plasma from nitrogen-restricted or diabetic rats indicates a lowering of the IGFBP in these animals and IGF-I treatment partly restores the concentration to that in normal rats (Tomas *et al.* 1991a,b). Treatment of normal humans with IGF-I for 7 days causes IGFBP-2 to increase and IGFBP-3 to decrease in blood (Baxter *et al.* 1993). IGF-I administered to GH receptor deficient patients also increases IGFBP-2 (Vaccarello *et al.* 1993).

The regulation of IGFBP-2 mRNA expression is controlled differently by nutrition in the various tissues. Expression is increased in the liver, decreased slightly in the kidney (Lemozy *et al.* 1994) but unaffected in the brain (Straus & Takemoto 1990) of fasted rats.

There is often an inverse relationship between IGF-I and IGFBP-2 in blood. Development associated increases in IGF-I and IGFBP-3 in neonatal rats is accompanied by decreases in IGFBP-2 and -1 (Donovan *et al.* 1989). Therefore, plasma IGFBP-3 concentrations, linked largely to IGF-I concentration, are also usually inversely related to

IGFBP-2. In GH-deficient and normal humans there is a significant inverse correlation between nocturnal IGFBP-2 and -3 concentrations (Jorgensen *et al.* 1993).

1.7.6 IGFBP-4, IGFBP -5 and IGFBP-6.

IGFBP-4 is N-glycosylated, has 20 cysteines and a predicted molecular mass close to 26 kDa (Rechler *et al.* 1993). It appears as a 24 (non-glycosylated) or 29 (glycosylated) kDa band by western ligand blotting of plasma from sheep, pigs and humans (Binoux *et al.* 1991; Walton *et al.* 1991; Carr *et al.* 1994; Hodgkinson *et al.* 1994). Unlike most other IGFBPs, no glycosaminoglycan sequence is present in the human and rat forms of this IGFBP (Hodgkinson *et al.* 1994) yet it binds well to the matrix of bone (Mohan & Baylink 1991a). In humans, IGFBP-4 has been postulated to have a crucial part in bone remodelling by controlling IGF-II actions (Mohan & Baylink 1991a).

The developmental expression and role of IGFBP-4 is not well characterised, but it is produced by a number of tissues, including bone, liver, muscle and kidney (Mohan & Baylink 1991a; Gosteli-Peter *et al.* 1994; McCusker & Clemmons 1994; Molnar & Murphy 1994) In rats, hepatic IGFBP-4 mRNA is developmentally regulated, increasing substantially after postnatal day 21, although in the pancreas expression decreases after that time (Hogg *et al.* 1994). Also, Carr *et al.* (1994) have shown IGFBP-4 mRNA to be developmentally regulated in the tissues of sheep.

The molecular masses of IGFBP-5 and -6 are approximately 28 and 21/22 kDa respectively (Rechler 1993), and of the 6 classes of IGFBPs these are the least studied.

IGFBP-5 has a high number of glycosaminoglycan sequences (Hodgkinson *et al.* 1994) suggesting strong extracellular matrix interactions. Indeed it is the most abundant IGFBP stored in human bone, where it binds strongly to hydroxyapatite (Bautista *et al.*

1991; Kanzaki *et al.* 1994). It is expressed in many rat tissues but is most plentiful in the kidney, white adipose and skeletal muscle (Gosteli-Peter *et al.* 1994). It plays an important role in the differentiation of muscle (Green *et al.* 1994) and is expressed in the notochord/floor plate of the fetus (Wood *et al.* 1992).

IGFBP-6 has a low number of cysteine residues compared with other IGFBPs, it has only 14 in rats and 16 in humans, and has a much greater affinity for IGF-II than for IGF-I (60 to 70 times greater) (Rechler 1993). It is expressed in many tissues but most notably in the lung (Rechler *et al.* 1993). Moats-Staats *et al.* (1995) have shown that it is expressed in the adult rat lung at a level up to 50 times that found in the fetus, pointing to a role in adult lung function.

1.7.7 Proteolysis of IGFBPs.

During pregnancy proteases can act on IGFBP-3 and other IGFBPs in the maternal circulation (Gargosky *et al.* 1990; Suikkari & Baxter 1991). Such proteolysis is also induced in animals in some catabolic states (Cwyfan-Hughes *et al.* 1992; Davenport *et al.* 1992). This modification decreases the affinity of IGFs for IGFBPs (Lalou *et al.* 1994), thereby, presumably, making the IGFs more available to the tissues receptors. Serum from pregnant but not non-pregnant women can also proteolyse IGFBP-4 and -5, but not IGFBP-1 or -6 (Claussen *et al.* 1994). The serine proteases or matrix metalloproteinases implicated in these actions (Fowlkes *et al.* 1994) have been shown to be secreted by a number of cell types *in vitro* and could be the same as those involved in the proteolysis of IGFBPs that occurs during bone formation and resorption (Lalou *et al.* 1994).

1.7.8 Phosphorylation of IGFBPs.

Phosphorylation of IGFBP-1 alters its affinity for the IGFs. Jones *et al.* (1991) demonstrated that four to five phosphorylated isoforms of IGFBP-1 could be isolated from human serum or amniotic fluid. These isoforms, the main forms secreted by decidual cells, had 6 times greater affinity for IGF-I than unphosphorylated forms. Levels of phosphorylated IGFBP-1 are increased in patients with Insulin-Dependent Diabetes Mellitus or severe ketoacidosis, and it is speculated that the GH and IGF-I resistance reported in some catabolic states may be a result of increased concentrations of highly phosphorylated IGFBP-1 (Frost *et al.* 1994). IGFBP-3 and IGFBP-5 may also undergo phosphorylation (see Jones & Clemmons 1995).

1.8 Natural variants and analogues of the IGFs and their interaction with IGFBPs and receptors.

The use of naturally occurring variants or analogues of the IGFs has contributed to our understanding of the roles of IGFBPs and IGF receptors in IGF action.

Analogues of IGF-I which bind poorly to IGFBPs have shown that the IGFBPs act to inhibit the actions of the IGFs. The discovery of a naturally occurring variant of IGF-I, des(1-3)IGF-I (Carlsson-Skwirut *et al.* 1986; Francis *et al.* 1986; Sara *et al.* 1986), led to an understanding of the importance of the N-terminal tripeptide of IGF-I for interaction with the IGFBPs. That is, des(1-3)IGF-I was shown to have a greater biological potency than IGF-I *in vitro* because it binds poorly to IGFBP-3 and low molecular weight IGFBPs which are secreted by cells in culture (Ballard *et al.* 1987; Forbes *et al.* 1988; Francis *et al.* 1988b; Bagley *et al.* 1989; Carlsson-Skwirut *et al.* 1989; Ross *et al.* 1989; Adashi *et al.* 1992). The reduced binding to IGFBPs enables the variant to have greater access to the type-1 IGF receptor. The low affinity of des(1-3)IGF-I is largely due to the absence

of the glutamate at position 3 of IGF-I, which appears critical for IGFBP binding (Bagley *et al.* 1989). The analogue [Gln^3 Ala^4 Tyr^{15} Leu^{16}]IGF-I, in which the Glu^3 of IGF-I has been replaced with Gln, also has a low affinity for IGFBPs (Bayne *et al.* 1988; Oh *et al.* 1993). King *et al.* (1992) have demonstrated that by replacing the negatively charged Glu at position 3 of IGF-I with a glycine (which has a neutral charge), the affinity for IGFBPs was decreased. However, the affinity for IGFBPs was lowered even further by substituting the Glu with a positively charged Arg (ie. R^3IGF-I). The addition of 13 amino acids (11 of which are the first 11 amino acids of porcine GH) to the N-terminus of human IGF-I (the extended peptide is known as LongIGF-I) reduces the affinity of IGF-I for the IGFBPs (Francis *et al.* 1992). By combining R^3IGF-I with the Long extension, Francis *et al.* (1992) produced an analogue (LongR^3IGF-I or simply LR^3IGF-I) which is at least, if not more, potent than des(1-3)IGF-I in its capacity to stimulate growth of cells or metabolism *in vitro*. The increased potency is again due to reduced IGFBP affinity.

As in the *in vitro* studies, the IGF-I analogues are more potent than IGF-I *in vivo*. Cascieri *et al.* (1988) showed that the [Gln^3 Ala^4 Tyr^{15} Leu^{16}]IGF-I analogue, which has reduced IGFBP binding affinity, was more potent than IGF-I in its ability to promote glucose incorporation into the glycogen of the rat diaphragm. Also, des(1-3)IGF-I or LR^3IGF-I are more potent than IGF-I in stimulating growth or metabolism of *lit/lit* mice (Gillespie *et al.* 1990), gut-resected rats (Lemmey *et al.* 1991; Vanderhoof *et al.* 1992), diabetic rats (Tomas *et al.* 1991a), nitrogen-restricted rats (Tomas *et al.* 1991b), dexamethasone-treated rats (Tomas *et al.* 1992), nephrectomised rats (Martin *et al.* 1991) and normal rats (Tomas *et al.* 1993a). Des(1-3)IGF-I is also more potent than IGF-I in its ability to stimulate blood flow in the mammary gland of the lactating goat (Prosser *et al.*1995). The increased potency of these analogues is likely to be due to their more rapid clearance from the blood to the tissues, a result of their decreased affinity for IGFBPs. In

rats, des(1-3)IGF-I is cleared more rapidly from the blood than IGF-I or IGF-II (Ballard *et al.* 1991), as is [Gln^3 Ala^4 Tyr^{15} Leu^{16}]IGF-I (Cascieri *et al.* 1988) and LR^{3}IGF-I (Bastian *et al.* 1993). Des(1-3)IGF-I has also been demonstrated to be cleared from blood at a faster rate than IGF-I when injected into lambs (Francis *et al.* 1988a) but the clearance rate of the variant was not significantly greater than IGF-I when administered to lactating goats (Prosser et al. 1995). While the affinity of des(1-3)IGF-I and other analogues for IGFBPs is substantially reduced, they can nevertheless still bind. Radiolabelled LR^{3}IGF-I can bind to the IGFBPs in plasma from a range of species (Lord *et al.* 1994), and radiolabelled des(1-3)IGF-I can bind to IGFBP-3, but not to other IGFBPs, in the plasma of goats (Prosser *et al.* 1995).

No naturally occurring variant of IGF-II that is equivalent to des(1-3)IGF-I has been discovered. However, two variants of IGF-II that have been purified from human Cohn fraction IV, and which have insertions of three or four extra amino acids at the Serines of positions 29 and 33, have increased (171%) and decreased (34 %) affinity to IGFBP-3 respectively compared with full-length IGF-II (Luthi *et al.* 1992). Francis *et al.* (1993) have replaced the Glu at position 6 of IGF-II with an Arg (ie. R^{6}IGF-II) or, like Luthi *et al.* (1992), have removed the six N-terminal amino acids (ie. des(1-6)IGF-II) and have been able to substantially reduce the affinity for IGFBPs and increase the biological potency relative to IGF-II *in vitro*. This indicates that the N-terminal region of IGF-II plays a role in IGFBP interaction like it does in IGF-I.

Despite the evidence that circulating IGFBPs act to inhibit the actions of IGFs, there is also some evidence which suggests that the IGFBPs can potentiate IGF actions (eg. Elgin *et al.* 1987; Blum *et al.* 1989; Koistinen *et al.* 1990; Andress *et al.* 1992; reviewed by Jones & Clemmons 1995). It is evident that a lot more work is required before the role(s) of IGFBPs is clearly understood.

IGF analogues have also improved our knowledge of the interaction of the IGFs with receptors. Substitution of Tyr^{25} or Tyr^{24} with other amino acids in insulin or IGF-I respectively leads to a much reduced affinity of these peptides for the type-1 IGF and insulin receptors (Cara *et al.* 1988; Cascieri *et al.* 1988; Cascieri *et al.* 1989). A number of other studies have shown that disruption of the equivalent Tyr at position 27 of IGF-II also reduces type-1 and insulin receptor binding (Beukers *et al.*1991; Burgisser *et al.* 1991; Rosenfeld *et al.* 1991; Roth *et al.* 1991; Sakano *et al.* 1991). In these studies, analogues of IGF-II such as [Leu^{27}]IGF-II do not have a substantially altered affinity for the type-2 IGF receptor. The type-2 IGF receptor binding affinity of IGF-II has been lowered while retaining type-1 receptor binding affinity by changing only the Phe at position 48 or by changing to [Thr^{48} Ser^{49} Ile^{50}] IGF-II or [Arg^{54} Arg^{55}] IGF-II (Burgisser *et al.* 1991; Sakano *et al.* 1991). Thus these analogues have enabled us to discover which regions of the IGFs are important for receptor binding and function. The use of [Leu^{27}]IGF-II is likely to yield important information regarding the function of the type-2 IGF receptor. Indeed by using the analogue, Rosenthal *et al.* (1994) have demonstrated that this receptor is likely to be involved in the stimulation of muscle differentiation by IGF-II.

1.9 Actions of IGF-I.

1.9.1. Correlations between IGF-I and growth.

There is a substantial body of evidence which links IGF-I with growth rate. During fetal development there is a strong association between body weight and the blood concentration of IGF-I. That is, IGF-I concentration is low in the small for gestational age fetal rat (Vileisis *et al.* 1986; Unterman *et al.* 1993b) and in growth-retarded humans (Lassare *et al.* 1991) and sheep (Owens *et al.* 1994). Concentrations are high in macrosomic fetuses (Hill *et al.* 1989b). Breeds of mice selected for high blood IGF-I

concentrations show a correspondingly high birth weight (Kroonsberg *et al.* 1989). Transgenic mice which over-express IGF-I have a greater body weight than non-transgenic littermates (Mathews *et al.* 1988) and production of transgenic mice that do not express IGF-I has demonstrated that IGF-I is needed for fetal growth to proceed normally (Powell-Braxton *et al.* 1993).

There is often a correlation between IGF-I and postnatal growth as well. An increase in circulating IGF-I concentrations after birth appears related to increased levels of IGF-I mRNA in the liver of rodents (Lund *et al.* 1986; Singh *et al.* 1991) and other animals, although the postnatal increase in IGF-I concentration in pigs is not a result of increased liver IGF-I expression (Lee *et al.* 1993). Transgenic mice which overexpress GH and which grow larger than their non-transgenic counterparts also have an increased expression of IGF-I (Palmiter *et al.* 1983). Blood concentrations are highest during the pubertal growth spurts of rats (Maes *et al.* 1983) and humans (Rosenfield *et al.* 1983; Luna *et al.* 1987; Argente *et al.* 1993), and are often low during the poor growth associated with dwarfism (Merimee *et al.* 1981), hypophysectomy (Guler *et al.* 1988) and hypothyroidism (Thomas *et al.* 1993). It has been speculated that the natural decline in circulating IGF-I in the elderly may be associated with a decline in physical fitness and hence the fitness of the musculo-skeletal system (Canalis 1994). A fall in the serum concentration of IGF-I is linked to the slowing of growth following epidermal growth factor administration to neonatal rats (Chernausek *et al.* 1991). In deer, the rate at which velvet antler growth occurs is strongly related to the concentration of IGF-I in blood (Suttie *et al.* 1989). The linear growth of animals is also correlated with IGF-I expression (Hayden *et al.* 1994) and the difference in body size between dogs of different breeds is also correlated with blood IGF-I concentration (Eigenmann *et al.* 1984). However, differences in the sizes of the mini-pig and domestic pig (Zenobi *et al.* 1988) and strains of

pigs with different growth rates (Owens *et al.* 1990) are apparently not related to IGF-I concentration. Nevertheless, the concentration of IGF-I in blood is often related to aspects of animal growth.

1.9.2 Nutritional regulation of IGF-I.

Fasting or a low protein diet reduces plasma IGF-I concentration and conversely a high protein diet can increase its concentration. Such changes occur in humans and in animals such as rats, mice, guinea pigs, sheep, pigs, cattle, chickens and dogs (Prewitt *et al.* 1982; Isley *et al.* 1983; Breier *et al.* 1986; O'Sullivan *et al.* 1989; Palka *et al.* 1989; Bass *et al.* 1991; Nap *et al.* 1993; Hayden *et al.* 1994). Consequently, there is a strong relationship between plasma IGF-I concentration and nitrogen balance (Clemmons *et al.* 1985; Elsasser *et al.* 1989; Takahashi *et al.* 1990). In rodents, release of IGF-I by the liver, the principal site of production, is impaired during fasting, which may be contributed to by a lowering of GH receptor levels in that tissue (Maes *et al.* 1983b). The low plasma IGF-I concentration which results from a reduction in food, nitrogen or energy intake in a number of species is not associated with a low plasma GH concentration but may also be a result of regulation by insulin, glucose or amino acids (Kriel *et al.* 1992). Indeed, insulin and glucagon, both of which are produced by the pancreas, can control IGF-I production by liver cells, and are acutely sensitive to nutritional status (Denver & Nicoll 1994).

1.9.3 Effects of IGF-I on tissue growth and differentiation.

A vast body of evidence from cell culture studies and IGF-I mRNA measurements implicates IGF-I in mitogenesis and differentiation of cells in many tissues (Daughaday & Rotwein 1989; Cohick & Clemmons 1993; Jones & Clemmons 1995). Also, IGF-I can inhibit apoptosis (Rodriguez *et al.* 1992; Geier *et al.* 1993; Muta & Krantz 1993). IGF-I

contributes to the growth of adrenals (Feige & Baird 1991), bone and cartilage (Daughaday & Rotwein 1989; Canalis *et al.* 1991), gut (Steeb *et al.* 1994), heart and vasculature (Thomas *et al.* 1993; Haskell & Tucker 1994), kidneys (Rogers *et al.* 1991), the regenerating liver (Caro *et al.* 1988), muscle (DeVol *et al.* 1990), the nervous system, including the brain (Pahlman *et al.* 1991; Carson *et al.* 1993), ovaries (Yoshimura *et al.* 1994), skin (Gilhar *et al.*1994) and uterus (Murphy *et al.* 1988). This list of tissues is not exhaustive.

The growth of the tissues is under control of numerous hormones and growth factors besides IGF-I, and given the integral part of IGF-I in such growth, it is understandable that IGF-I is influenced by, or influences, those agents. In addition to GH these include cortisol, parathyroid hormone (McCarthy *et al.* 1990), epidermal growth factor, transforming growth factor alpha (Mondschein & Hammond 1988), estrogen (Murphy *et al.* 1988), glucagon (Kachra *et al.* 1994), gonadotrophins (Hsu & Hammond 1987), insulin (Boni-Schnetzler *et al.* 1989), testosterone (Parker *et al.* 1984), thyroid hormones (Wolf *et al.* 1989), transforming growth factor beta and IGF-II (Tremollieres *et al.* 1991). Again, this list is not exhaustive.

Clearly IGF-I has a central place in the interrelationships that exist between tissue growth and the endocrine system.

1.9.4 In vivo studies: general effects of IGF-I on growth.

Studies in which IGF-I has been administered to animals confirms that it can be both an effector and a mediator of the growth of tissues. This was first clearly demonstrated by Schoenle *et al.* (1982) who restored the growth of young hypophysectomised rats, in a dose-dependent manner, by IGF-I treatment. IGF-I also increases the growth of congenitally hypopituitary Snell dwarf mice (van Buul-Offers *et*

al. 1986). Injections of IGF-I into humans with dwarfism, resulting from a defective GH receptor (Laron syndrome), is able to improve growth when given over a one to two year period (Clemmons & Underwood 1994). A lack of a growth response to IGF-I has been noted for mini-poodles (Guler *et al.* 1989a) and also chickens (Tixier-Boichard *et al.* 1992).

The effects of IGF-I on tissue growth is dependent on the age and condition of the animal. In neonatal rats, body weight, brain, heart, liver, testes and tail length are increased following IGF-I treatment, as is erythropoiesis (Philipps *et al.* 1988). Also, precocious eye opening is stimulated, providing evidence for mediation of EGF effects *in vivo*. Steeb *et al.* (1994) have shown that the gut is also responsive to IGF-I in the neonatal rat and Glasscock *et al.* (1992) have shown that adrenals can also respond to treatment in these neonates. Infusion into slightly older male rats, starting at 23 days of age, has resulted in increased weights of kidneys, liver, testes and pituitary in addition to significant body weight gain (Hizuka *et al.* 1986). In young, rapidly-growing, normal rats of over 100g in weight, or hypophysectomised rats of similar weight, IGF-I increases body weight gain and selectively increases the growth of several organs, including gut, kidneys, spleen and thymus (Guler *et al.* 1988; Tomas *et al.* 1993a). IGF-I treatment also selectively increases the growth of kidneys, spleen or thymus in dwarf mice (Pell & Bates 1992) and dwarf rats (Skottner *et al.* 1989). The kidneys and spleen are also responsive in protein restricted rats (Thissen *et al.* 1991) and IGF-I can restore the growth of the atrophied thymus in rats which are diabetic (Binz *et al.* 1990).

IGF-I increases linear growth *in vivo* by stimulating long bone growth (Isgaard *et al.* 1986; van Buul-Offers *et al.* 1986; Guler *et al.* 1988; Behringer *et al.* 1990; Spencer *et al.* 1991). Other effects include increased tibial epiphyseal width and stimulation of incorporation of [^{3}H] thymidine into cartilage (Schoenle *et al.* 1985; Hizuka *et al.* 1986).

Continuous 14 day infusion into the hindlimb of rats results in the stimulation of osteoblast numbers and the amount of collagen in bone, but decreases the number of osteoclasts, suggesting that IGF-I is mainly involved in bone formation rather than resorption (Spencer *et al.* 1991). Bone loss in rats whose limbs have been unloaded is also partly prevented by infusion of IGF-I (Machwate *et al.* 1994). In women with osteoporosis, injection of IGF-I is able to increase the rate of bone remodelling (Ebeling *et al.* 1993).

Additionally, IGF-I or variants can increase glomerular filtration rate and blood flow in the kidney (Guler *et al.* 1987; Hirschberg & Kopple 1989, Baumann *et al.* 1992), increase forearm blood flow in humans (Copeland and Nair 1994) and increase blood flow or milk production in the mammary gland of the goat (Prosser *et al.* 1991; 1995).

1.9.5 In vivo studies: effects of IGF-I on carcass composition.

It has been established for some time that GH can increase body weight gain, the efficiency of feed utilisation and the proportional content of protein (and hence nitrogen) in the carcass while decreasing the proportion of carcass fat. Some or all of these effects have been attributed to GH in experiments involving animals such as rats (Martin *et al.* 1989; Flint & Gardner 1993), cattle (Baumann *et al.* 1985; Sandles & Peel 1987; Eisemann *et al.* 1989), pigs (Machlin 1972; Walton & Etherton 1986; Etherton *et al.* 1986; Campbell *et al.* 1988, 1991), sheep (Johnsson *et al.* 1985; Pell *et al.* 1990; Bass *et al.* 1991) and also humans (Novak *et al.* 1972). Many of the actions of GH are thought to be mediated by IGF-I and so it follows that IGF-I is likely to have similar effects to those of GH in its effects on carcass composition.

The effects of GH on muscle growth *in vivo* appear mediated by the actions of IGF-I. IGF-I stimulates both mitogenesis and protein synthesis, but inhibits protein

breakdown, in muscle cells *in vitro* (Ballard *et al.* 1986; Roeder *et al.* 1988). IGF-I can also stimulate the differentiation of myoblasts (Florini *et al.* 1991a). In chickens, breeds which have high concentrations of IGF-I in their blood also have the highest rate of carcass protein formation (Ballard *et al.* 1990b; Tomas *et al.* 1991c). Continuous infusion of IGF-I for 14 days to normal rats can increase feed conversion efficiency and the amount of carcass protein (Tomas *et al.* 1993). Nitrogen retention is also increased. IGF-I infusion into the hind limb of fed or underfed calves or lambs for 4 hours is able to inhibit muscle protein breakdown (Oddy & Owens 1994). LR^3IGF-I has a similar effect in underfed steers (Owens et al. 1994). In hypophysectomised rats, serum urea, a measure of amino acid oxidisation, is lowered by IGF-I treatment (Shaar *et al.* 1989), as it is in fasted lambs (Douglas *et al.* 1991a). IGF-I treatment of normal humans can also increase nitrogen retention (Guler *et al.* 1987). These *in vivo* results support the evidence for inhibition of muscle protein breakdown and increased muscle protein synthesis observed *in vitro*. Thus IGF-I actions are consistent with increasing muscle mass and hence nitrogen retention.

There has been some debate about whether the lipolytic and/or anti-lipogenic effects of GH can be mediated via IGF-I (eg. Goodman & Grichtig 1983; Cordido *et al.* 1991; Gertner 1992). GH treatment of growing pigs has been shown to increase IGF-I mRNA levels in subcutaneous adipose tissue (Wolverton *et al.* 1992) and *in vitro* studies of porcine preadipocytes show that an increase in IGF-I expression is related to a decrease in adipocyte development (Gaskins *et al.* 1990). In contrast, the work of Smith *et al.* (1988) indicates that IGF-I is essential for the differentiation of 3T3-L1 adipocytes to proceed. Since Flint & Gardner (1993) have shown that GH is needed to stimulate rat adipocyte differentiation *in vivo*, then there is some strong evidence that adipocyte differentiation is stimulated via the GH-IGF-I axis. However, the effects on

differentiation are not the same as effects on fat deposition, although the number of differentiated adipocytes will no doubt influence the capacity of a depot to store fat.

By treating neonatal female rats with an antibody directed against GH, Gardner & Flint (1990) have found an increase in the amount of fat in the parametrial fat depot of these animals. This suggests that GH acts to decrease fat deposition in neonatal rats, and since serum IGF-I concentrations were also lowered, this is consistent with, but not proof of, a lowering of fat deposition by IGF-I. Other evidence also supports this action by IGF-I. For instance, IGF-I treatment can reduce the proportional carcass fat content of normal rats (Tomas *et al.* 1993) and has been shown to decrease the amount of fat in the foot pad of hypophysectomised rats (Guler *et al.* 1988). IGF-I also inhibits lipogenesis in isolated rat adipocyte cells (Zapf *et al.* 1978). Treatment of normal and diabetic animals, including humans, reduces the concentration of lipids in the circulation, consistent with an inhibition of lipolysis (Schalch *et al.* 1993; Giacca *et al.* 1994). Under euglycemic clamping conditions IGF-I increased lipolysis in humans given an isocaloric diet (Hussain *et al.* 1993) yet inhibited lipolysis to the same extent as an insulin dose with the same hypoglycaemic potency, in similarly clamped individuals which had been fasted overnight (Laager *et al.* 1993). In depancreatised dogs, IGF-I was less potent than insulin in reducing lipolysis at doses with the same hypoglycaemic potency (Giacca *et al.* 1990).

Thus, in a range of species, IGF-I often, but not always, acts in a manner which is conducive to an increase in muscle mass and a but a decrease in fat reserves. The responsiveness of a tissue to IGF-I may depend on the species and, as indicated by the variable response of fat depots from different body locations to GH (Flint & Gardner 1993), the site of the target tissue within an animal.

1.9.6 In vivo studies: reversal of catabolism by IGF-I treatment.

IGF-I can improve growth and nitrogen balance of animals in catabolic states which are usually accompanied by low blood IGF-I concentrations. In rats, IGF-I treatment at least partially protects against the growth retardation and/or nitrogen losses associated with diabetes (Scheiwiller *et al.* 1986, Tomas *et al.* 1991a), dietary protein restriction (Tomas *et al.* 1991b), gut resection (Lemmey *et al.* 1991), dexamethasone treatment (Read *et al.* 1992; Tomas *et al.* 1992), cancer cachexia (Ng *et al.* 1992) and impaired kidney function (Martin *et al.* 1991, Miller *et al.* 1992; Ding *et al.* 1993; Martin *et al.* 1994). Aspects of wound healing can also be improved by IGF-I in rats treated with corticosteroids (Suh *et al.* 1992). In humans, IGF-I inhibits protein breakdown or improves nitrogen balance in individuals who have a 50% calorie restriction (Clemmons *et al.* 1992) or who suffer under various catabolic conditions (Ross *et al.* 1991; Clemmons & Underwood 1994), including GH resistance (Walker *et al.* 1991). Similar responses to IGF-I have been described in conditions of fasting or trauma in other animals such as mice (O'Sullivan *et al.* 1989) or sheep (Douglas *et al.* 1991a,b; Koea *et al.* 1992b). Also, infusion of IGF-I into pregnant sheep (pregnancy can be regarded as a form of metabolic stress) is able to lower amino nitrogen concentrations in both the maternal and fetal circulations (Liu *et al.* 1994).

1.9.7 Effects of IGF-I on glucose metabolism.

Blood glucose concentrations can be lowered by treatment with a sufficiently high dose of either IGF-I or IGF-II in a range of species including rats (Zapf *et al.* 1986; 1987; Jacob *et al.* 1989), dogs (Giacca *et al.* 1990; 1994), humans (Guler *et al.* 1987; Elahi *et al.* 1991), pigs (Ballard *et al.* 1994) and sheep (Douglas *et al.* 1991). Studies in normal rats (Jacob *et al.* 1989) and normal humans (Elahi *et al.* 1991) indicate that administration

of IGF-I, like insulin, lowers the concentration of blood glucose by stimulating the uptake of by the tissues. However, unlike insulin, glucose production by the liver in these animals does not seem to be affected. Nevertheless, IGF-I treatment can lower the higher than normal production rate of glucose by the liver in diabetic animals (Giacca *et al.* 1990; Jacob *et al.* 1991). When results from experiments examining the relative potencies of IGF-I and insulin in a range of species were compared, it was apparent that IGF-I was around 100 times less potent than insulin in rats, but only approximately 10 times less potent than insulin in dogs, humans, pigs and sheep (see Giacca *et al.* 1994 for references and discussion). Thus, at least in terms of its glucose-lowering capacity, the actions of IGF-I in the rat seem different to those in many other species.

Dimitriadis *et al.* (1992) have compared the effects of a single injection of IGF-I into rats with that of chronic administration in which IGF-I was injected twice daily into rats for 10 days. In the soleus muscle isolated from these animals, they showed that IGF-I was able to increase the rate of conversion of glucose to hexose monophosphate and the formation of lactate and glycogen, but the effects were not as great following chronic treatment. Also, the muscle became resistant to the effects of insulin following acute treatment. Thus acute or chronic administration of IGF-I can result in different effects on glucose metabolism.

The risk of hypoglycaemia limits the doses of IGF-I that can be used for improving growth and nitrogen balance. Kupfer *et al.* (1993) have demonstrated that the treatment of calorie-restricted patients with a combination of GH and IGF-I is able to maintain normal blood glucose levels and produce a positive nitrogen balance, whereas IGF-I alone did neither. Maintenance of a normal IGFBP-3 and acid-labile sub-unit concentration by the addition of GH may contribute to this.

Although studies of rats made diabetic by streptozotocin have shown that chronic IGF-I treatment can partly reverse the associated growth retardation, by improving muscle protein synthesis and nitrogen balance (Tomas *et al.* 1991a), such treatment does not significantly reverse the changes in glucose metabolism, the rate of muscle protein catabolism or the reduction in carcass fat content. Indeed IGF-I and insulin appear to have at least some distinct physiological functions *in vivo* (Scheiwiller *et al.* 1986; Tomas *et al.* 1991a; Tomas *et al.* 1993b). By administering IGF-I or insulin to fasted lambs at doses with equivalent hypoglycaemic potency, Douglas *et al.* (1991a) revealed that protein synthesis was stimulated more potently by IGF-I than by insulin and also that IGF-I influenced protein metabolism more strongly than glucose metabolism. Under euglycaemic clamp conditions in humans, IGF-I is more potent than insulin in decreasing protein catabolism (Laager *et al.* 1993).

1.10 Actions of IGF-II.

1.10.1 General description.

Like IGF-I, IGF-II is able to stimulate growth and differentiation of numerous cell lines and tissues (Cohick & Clemmons 1993; Jones & Clemmons 1995). Blood IGF-II concentrations have not been strongly linked to growth in the postnatal animal. However, a study by Buonomo & Klindt (1993) does suggest such a role in the pig. They showed IGF-II concentrations to rise at a time when reproductive organs are rapidly maturing and also found that concentrations were higher in lines of animals that were obese, suggesting a role in fat metabolism. Fasting affects plasma IGF-II concentrations much less than those of IGF-I in animals where the IGF-II concentration is normally substantial. Brief periods of fasting do not lower blood IGF-II concentration in humans (Davenport *et al.* 1988) but long periods of malnutrition can (Soliman *et al.* 1986). Even though fasting

lowers IGF-I concentrations in the plasma of the mother and fetus during rat pregnancy, IGF-II is not similarly affected in either circulation (Davenport *et al.* 1990). However, studies in fetal sheep have demonstrated that plasma concentrations of both IGF-I and IGF-II are under control of nutrition and oxygen during the latter stages of gestation, and evidence suggests that both IGFs may be similarly controlled during the final stages of human fetal development (Owens *et al.* 1994).

1.10.2 IGF-II is regarded as a fetal growth factor.

IGF-II is often regarded as a fetal growth factor. A study by Liu *et al.* (1989) showed that 10 day old rat embryos, which had been placed under the capsule of kidneys in rats, grew in response to IGF-II infusion into the renal artery of the host, but not in response to IGF-I, suggesting a stronger role for IGF-II than IGF-I during embryonic development. The gene knock-out work of DeChiara *et al.* (1991) and Filson *et al.* (1993), which was described earlier (see section 1.6.4), also established that a lack of, or overabundance of, IGF-II in the developing mouse affects its growth.

Another reason that IGF-II is considered as a fetal growth factor is that the concentration of IGF-II in blood is higher in the fetus of rats (Moses *et al.* 1980), sheep (Gluckman & Butler 1983) and guinea pigs (Daughaday *et al.* 1986) than in the corresponding postnatal animals. While concentrations remain substantial in adult sheep and guinea pigs, in rats the concentration becomes negligible at about 3 weeks after birth and remains at this level throughout adulthood (Donovan *et al.* 1989). On the other hand, in postnatal humans (Engberg & Hall 1984) and pigs (Owens *et al.* 1991) blood IGF-II does not decline, but increases. Many other species of animals also have measurable, if not high, concentrations of IGF-II in the blood postnatally (Zangger *et al.* 1987). The pubertal growth spurt of rats and humans, which is associated with elevated

blood IGF-I concentrations, is not associated with a similar increase in IGF-II in either humans (Luna *et al.* 1987) or rats (in which concentrations are at the lower limits of detection). In elderly humans, plasma IGF-II concentration does not decline with increasing age like IGF-I and GH concentrations (Abbasi *et al.* 1993). The postnatal increase in plasma IGF-II concentrations in the pig are associated with declines in IGF-II mRNA levels in many of the tissues (liver, muscle, lung and kidney), prompting Lee *et al.* (1993) to propose that increases in plasma concentrations of both IGFs during this time are a result of a lower clearance rate that stems from changes in plasma IGFBP forms and in tissue receptor levels.

IGF-II expression is high in tissues during embryonic and fetal development. The expression pattern of the IGF-II gene has been most extensively studied in rodents. Expression begins in the preimplantation embryo (Heyner *et al.* 1989), is most strongly expressed in many tissues during mid-gestation, and then declines substantially in late gestation or during the neonatal period, depending on the tissue (Brown *et al.* 1986; Graham *et al.* 1986; Lund *et al.* 1986; Stylianopoulou *et al.* 1988; Wood *et al.* 1992). IGF-II tends to be expressed in tissues of mesodermal origin, though not exclusively (Stylianopoulou *et al.* 1988; Wood *et al.* 1992). The only tissues in which IGF-II mRNA is strongly produced in the adult of rodents is in the choroid plexus epithelium and the leptomeninges of the brain (Stylianopoulou *et al.* 1988), but there are some indications that IGF-II could be weakly expressed in other tissues (Lund *et al.* 1986). The pattern of IGF-I and IGF-II gene expression is often distinct during development (Bondy *et al.* 1990; Streck *et al.* 1992). Measurement of IGF-II extracted from tissues of the rat confirms the high expression of IGF-II in fetal tissues and the subsequent postnatal fall in levels (Lee *et al.* 1991). However, Lee *et al.* (1991) were still able to detect IGF-II in most adult tissues. Also, immunocytochemistry has demonstrated that IGF-II is

produced in tissues of the thymus in 6 week old rats (Geenen *et al.* 1993). A similar developmental trend in IGF-II gene expression has been observed in humans (Brice *et al.* 1989), cattle (Boulle *et al.* 1993), pigs (Lee *et al.* 1993) and sheep (Delhanty & Han 1993), although unlike the rat, IGF-II is still expressed in the adult liver. The patterns of IGF-II expression in the tissues of these latter animals (all of which have substantial blood IGF-II concentrations in the adult) have more in common with each other than with rodents (Boulle *et al.* 1993; Delhanty & Han 1993).

Although IGF-II is regarded as a fetal growth factor, and for good reason, it must be emphasised that IGF-I should also be regarded as a fetal growth factor given that it also has a considerable influence on the growth of the fetus (Powell-Braxton *et al.* 1993).

1.10.3 Roles of IGF-II in tissue growth and differentiation/maturation.

IGF-II mRNA levels are often high and then fall in tissues of rats, humans and other animals at a time when those tissues mature/undergo terminal differentiation (Brown *et al.* 1986; Brice *et al.* 1989). Numerous studies, many of which are described below, confirm this and additionally show that IGF-II has an integral and sometimes essential part in differentiation.

Beck *et al.* (1988) had noticed that in the rat a rise in plasma cortisol concentration occurred at around the time that liver IGF-II mRNA levels fell (ie. postnatal days 18-20), and that cortisol had previously been shown to be involved in tissue maturation. They then showed that the rise in plasma cortisol was likely to shut down liver IGF-II expression, since injection of cortisone acetate into 9 day old neonatal rats terminated liver IGF-II gene expression. Levinovitz & Norstedt (1989) showed that dexamethasone (a glucocorticoid) treatment of 1 day old neonatal rats for 4 days lowered liver IGF-II mRNA levels 10-fold and also inhibited body weight gain. This was also

associated with precocious differentiation of the liver. Similarly, dihydrotestosterone inhibits growth and liver IGF-II mRNA production in neonatal rats (Martinoli & Pelletier 1991). These results indicate that liver development is controlled at least partly by IGF-II and cortisol in the rat.

IGF-II is especially important in muscle development. In addition to being a mitogen for myoblasts, autocrine production of IGF-II is necessary for the spontaneous differentiation of rat myoblasts *in vitro* (Florini *et al.* 1991b) and is implicated in myogenesis in cattle (Listrat *et al.* 1994). The level of IGF-II mRNA in rat skeletal muscle declines at a time when myoblasts cease to proliferate (Brown *et al.* 1986) and cardiac IGF-II gene expression also falls in the neonatal rat when myocyte cell division in this tissue stops (Clubb *et al.* 1987). A study by Gerrard & Grant (1994) has demonstrated that the likely reason that some calves are born with up to 40% more muscle fibres than their normal counterparts is because the peak in IGF-II gene expression in this tissue, which is associated with terminal myoblast differentiation, is delayed, allowing more time for muscle fibres to be produced. High IGF-II mRNA levels in muscle are linked with the formation of neuromuscular synapses (Ishi 1989).

Levels of IGF-II mRNA are greater than IGF-I mRNA in the adrenal of fetal sheep (Han *et al.* 1992). A fall in IGF-II gene expression in the adrenals (and other tissues) of fetal sheep in late gestation is associated with a blood cortisol and ACTH surge (Li *et al.* 1993). Lu *et al.* (1994) have shown that ACTH or cortisol treatment reduces IGF-II mRNA levels in ovine adrenals and point out that this is likely to be the cause of adrenocortical cells becoming differentiated and hence being able to increase steroid production.

Kidney development is regulated by both IGFs, as evidenced by the fact that both are produced by metanephroi and that antibodies directed against these peptides inhibit

metanephros development *in vitro* (Rogers *et al.* 1991). Studies of the cause of Wilms' tumour in children have led to the finding that the failure of kidney blastemal cells to undergo normal differentiation is because IGF-II continues to be expressed past a time when it normally declines (Drummond *et al.* 1992).

Growth and development of lungs is aided by its expansion by liquid in the developing organ. Experiments by Hooper *et al.* (1993) have demonstrated that the associated lung expansion and growth following tracheal obstruction of fetal sheep is linked to an increase in IGF-II mRNA levels in pulmonary tissue. Harding *et al.* (1993) revealed that levels of IGF-II mRNA in this tissue decreased when fetal breathing movements of sheep, thought responsible for maintaining lung volume, were abolished, resulting in a stunting of lung growth. In rats, the lungs of the fetus mature just prior to birth, a time linked to a reduction in IGF-II mRNA levels in the tissue (Brown *et al.* 1986).

IGF-I and -II have distinct patterns of expression during development of long bones in rats (Shinar *et al.* 1993). IGF-II expression is higher in the growth plate of the tibia and gradually declines throughout and past the neonatal period, whereas IGF-I expression increases throughout this time in regions where ossification occurs. Both IGFs can contribute to the maintenance of bone mass (Canalis 1994). Human bone matrix stores a number of growth factors including IGFs, but IGF-II is by far the most abundant and is believed to regulate bone density by contributing to the control of the coupling of bone resorption and formation rates (Bautista *et al.* 1991; Mohan & Baylink 1991a).

IGF-II also seems to contribute to the development of brain and neural tissues. The pattern of IGF-II gene expression in the mesenchyme of the choroid plexus of the rat suggests that it is responsible for inducing differentiation of the epithelium of this tissue (Cavallaro *et al.* 1993). Also, growth of human neuroblastomas is accounted for by the

autocrine production of IGF-II. Interferon-gamma halts the growth of this tumour and stimulates differentiation by a mechanism that involves inhibition of IGF-II gene expression (Martin *et al.* 1993).

Both IGF-I and IGF-II can stimulate differentiation and both can be regarded as fetal growth factors. However, as indicated in the preceding paragraphs, once differentiation of tissues has taken place, IGF-II expression often falls markedly or ceases, suggesting that it regulates activities that mainly occur prior to, and during, differentiation.

1.10.4 In vivo and transgenic animal studies: effects of IGF-II on postnatal growth.

Studies on the actions of IGF-II in the postnatal animal are relatively few when compared to those of IGF-I. Of the *in vivo* studies, most have involved young or adult rodents, which lack significant IGF-II concentrations in blood, and in most cases these animals have had some form of GH deficiency. Recently a number of research teams have produced transgenic mice which over-express IGF-II as an alternative to IGF-II infusion studies.

Effects that have resulted from 6 or 7 days of continuous infusion of recombinant human IGF-II into hypophysectomised rats (100g plus or 50 days old) include increased body weight gain, tibial epiphyseal width, femur alkaline phosphatase or hydroxyproline concentrations (indices of bone formation) and decreased serum urea concentrations (Schoenle *et al.* 1985; Shaar *et al.* 1989). IGF-I was more potent than IGF-II. The doses of IGF-I or IGF-II used were not able to stimulate a reappearance of the 150 kDa IGFBP complex of serum, whereas GH could (Schoenle *et al.* 1985). These doses did not induce hypoglycaemia. Effects on the growth of other tissues were not described. Continuous infusion of IGF-II into broiler chickens for 14 days at a dose of 0.5 mg/kg/day failed to

stimulate growth but increased the deposition of fat and decreased lean muscle growth (Spencer *et al.* 1994). However, passive immunisation against both IGF-I and IGF-II, but not IGF-I alone, reduced abdominal fat and body weight but increased the growth of the spleen in chickens (Spencer *et al.* 1995). The authors suggest that IGF-II may have a role in fat metabolism in chickens. Since IGF-II appears to have had opposite effects in the two chicken studies, further studies are required before any real conclusions can be drawn.

IGF-II injection, like IGF-I and insulin, can cause hypoglycaemia and stimulate liver glycogen synthesis in rats (Zapf *et al.* 1986; Hartmann *et al.* 1992; Stuempel & Hartmann 1992). Both IGFs can stimulate glucose uptake by muscle (Zierath *et al.* 1992).

A focus of IGF-II studies has been on the effects on bone formation and metabolism. This is reasonable given that IGF-II is stored in large amounts in human bone and is thought to link the rates of bone formation and resorption (Mohan & Baylink 1991a). Schiltz *et al.* (1992) gave daily injections of equimolar doses of rhIGF-II or rat GH into hypophysectomised, 6 week old rats over a period of 12 days. While GH increased body weight gain, femur alkaline phosphatase concentrations, bone cell formation, collagen formation and serum osteocalcin concentrations (a measure of osteoclast function), IGF-II did not. Thus GH has a greater ability to induce bone formation in this model. Both IGFs can stimulate the incorporation of sulphate into the cartilage of the GH-deficient Snell dwarf mice (van Buul-Offers *et al.* 1988). In newborn lambs rhIGF-II, but not rhIGF-I (even at a 20 times higher dose), can increase concentrations of osteocalcin in blood following single injections (Coxam *et al.* 1992), further supporting a more specific role for IGF-II in bone formation than for IGF-I.

Van Buul-Offers *et al.* (1995) have demonstrated that both IGFs can promote the growth of a number of tissues and also body weight gain and body length (although IGF-

II did not stimulate the latter two parameters significantly) in Snell dwarf mice. IGF-I appeared only slightly more potent than IGF-II in increasing the weights of kidneys and spleen, but was considerably more potent in increasing lung growth. Both were equally effective in stimulating growth of the salivary glands. A combination of IGF-I and -II was also able to increase the growth of kidneys and salivary glands. These authors have also shown that IGF-II, but not IGF-I or GH, was able to lower the levels of glycogen stored in the liver, suggesting specific effects on some aspect of glucose metabolism. IGF-II was a more potent inhibitor of GH-stimulated liver growth than IGF-I and also had specific effects on the size of centrilobular hepatocytes. IGFs and GH increased IGFBP-3 concentrations in blood.

Short (300 minute) infusions of rhIGF-II or rhIGF-I into fasted lambs demonstrated that IGF-II was the less potent of the two in lowering the rate of net protein loss and in stimulating plasma glucose clearance (Douglas *et al.* 1991a). In castrated male lambs the net rate of protein catabolism was significantly reduced by a 300 minute infusion of IGF-I, but this effect was abolished when the same dose of IGF-I was administered together with IGF-II (Koea *et al.* 1992a). IGF-I concentrations in blood were not changed from that of infusion of IGF-I alone. It was suggested that IGF-II acts to modulate the anti-catabolic actions of IGF-I in the postnatal animal (Koea *et al.* 1992a; Gluckman & Ambler 1993).

Flint *et al.* (1992 & 1994) have demonstrated that injections of IGF-I, IGF-I analogues, IGF-II, IGF-I pre-complexed with IGFBP-3, or a combination of IGF-I and IGF-II pre-complexed with IGFBP-3 are probably unable to mimic the effects of GH in re-initiating lactation in rats whose milk production has been suppressed by a combination of immunoneutralisation of GH and a lowering of prolactin concentrations by bromocriptine.

Elevations in plasma IGF-II concentrations in rodents, following transplantation of tumours secreting this peptide, did not result in stimulation of growth (Wilson *et al.* 1989; Ren-Qui *et al.* 1993).

The expression pattern of IGF-II in the brain of adult rats and other species has led to the suggestion of a role for IGF-II in the brain, possibly as a regulator of extracellular fluid production or substrate supply (Ayer-LeLievre *et al.* 1991). *In vivo* studies support the possibility of a role for IGF-II in the central and peripheral nervous systems. IGF-II, but not IGF-I or insulin, was able to inhibit food intake and body weight gain of adult rats in a dose-dependent manner when injected intracerebroventricularly (Lauterio *et al.* 1987). Also, IGF-II injected into the cisterna of the rat brain inhibits the stimulation of gastric acid secretion by pentagastrin, consistent with IGF-II having a function in the control of digestion (Mulholland & Debas 1988). Insulin is also thought to regulate food intake, and can regulate IGF-II expression in the rat hypothalamus (Lauterio *et al.* 1990). The rate of nerve regeneration in rats is increased by local IGF-II treatment, which it appears to do more potently than IGF-I (Near *et al.* 1992; Glazner *et al.* 1993).

The effects of IGF-II produced endogenously by over-expression in transgenic mice have varied between studies. None of these studies has demonstrated a significant increase in postnatal body weight gain, and in one case (Rogler *et al.* 1994) mice have a reduced weight after 4 months of age, probably a result of the lower muscle and fat content. The mice of that study also have a tendency towards developing cancer, consistent with a role for IGF-II in tumourigenesis. In another transgenic model, a low carcass fat content and low white and brown adipose lipid content were found in mice at different ages (Da Costa *et al.* 1994). Snell dwarf mice that express an IGF-II transgene have significantly increased thymus growth and blood IGFBP-3 concentrations (van Buul-Offers *et al.* 1995). The most consistent effect noted in these studies has been the

lowering of fat content in various depots. In another transgenic mouse model in which IGF-II is over-expressed, Wolf *et al.* (1994) have demonstrated increases in kidney and testis weights in 4-week-old mice and also increases in adrenal weights in 12-week-old mice, but body weights were not affected. The wide range of responses of mice to IGF-II over-expression in these models is likely to be a result of the different transcriptional control sequences that have been fused with IGF-II and differences in the types of tissue sites in which the transgenes are expressed.

CHAPTER 2.

DESCRIPTION OF THE PROBLEM.

IGF-I is involved in numerous aspects of growth and metabolism in both the developing fetus and the postnatal animal. During postnatal growth it is a mediator of lean growth under conditions where nutrition is sufficient to support it and has an influence on aspects of protein, carbohydrate and fat metabolism. IGF-II has a prominent role in fetal development but its actions in the postnatal animal are not well understood. The number of *in vivo* experiments involving IGF-II are few compared with IGF-I, and the several chronic infusion studies that have been reported have mainly examined the effects on rats and mice. The use of these rodents for studies of the physiological actions of IGF-II has a possible drawback. That is, the concentrations of this peptide in the adult circulation are at the lower limits of detection, whereas most other mammals have substantial circulating concentrations during adulthood. This suggests that IGF-II does not play a large part in the processes associated with the postnatal growth and metabolism of rats and mice, except perhaps for the first few weeks after birth, when IGF-II concentrations are still relatively high. Conversely, IGF-II probably does have a function in the adult of other animals. Thus it is hypothesised that the response of rats or mice to chronic IGF-II infusion will differ in many respects to the response elicited by postnatal animals with substantial circulating IGF-II. Indeed, by comparing such responses, the function(s) of IGF-II *in vivo* is likely to be made clearer.

The aim of the work described in this thesis is to further our knowledge of the actions of IGF-II in postnatal animals. Accordingly, the effects of chronic IGF-I and IGF-II treatment of animals have been compared. Firstly in the rat and then in another small animal model, but one which has a substantial concentration of IGF-II in blood throughout postnatal life, the guinea pig. There have been no previous reports of IGF-I and IGF-II treatment of guinea pigs.

CHAPTER 3.

PREPARATION OF RECOMBINANT HUMAN IGF-II.

3.1 Introduction.

In order to chronically administer IGF-II to animals it was first necessary to prepare sufficient quantities of the peptide. IGF-I was readily available.

The method for the production of recombinant human IGF-II is based on that described by Francis *et al.* (1993). That method was modified slightly for production on a larger scale and is described below.

3.2 Materials and methods.

All chemicals and reagents used in this purification were of analytical grade.

3.2.1 Production of LongIGF-II inclusion bodies.

An IGF-II fusion protein ([Met^{1}]-porcineGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-IGF-II), also referred to as LongIGF-II, was produced by fermentation of *E. coli* JM101 that had been transformed with the expression vector that was engineered by Francis *et al.* (1993). The fermentation of *E. coli* and the isolation of inclusion bodies (containing LongIGF-II) from the cells was carried out by Bresatec Ltd., Thebarton, SA, Australia.

3.2.2 Solubilisation and desalting of inclusion bodies.

Inclusion bodies (25 g wet weight, containing 1.7 g of protein) were dissolved (10 % (w:v)) in a buffer containing 8 M urea, 0.1 M Tris base, 40 mM glycine, 5 mM zinc chloride and 40 mM dithiothreitol (pH 9.1) for 30 minutes at room temperature. The mixture was centrifuged at 50,000 x *g* for 15 minutes, and the aqueous phase passed through a Whatman GMF150 glass microfibre filter with a 1 µm pore size (Whatman International Ltd., Maidstone, England). To separate inclusion body proteins from salts, the filtrate was pumped at a rate of 40 ml/minute through a column (10 cm x 30 cm) packed with Sephadex G-25 M (Pharmacia

AB, Biotechnology, Uppsala, Sweden), using the buffer described for dissolving inclusion bodies, but which had a dithiothreitol concentration of only 1.6 mM (desalt buffer). The desalting step and subsequent chromatography steps were carried out using a Pharmacia Biopilot chromatography workstation except where otherwise indicated.

3.2.3 Anion-exchange chromatography.

Proteases were removed, and the fusion protein was further purified, by anion-exchange chromatography. That is, fractions containing desalted inclusion body proteins were pooled (to give 1.7 g in 600 ml) and loaded onto a column (50 mm x 55 mm) containing Q Sepharose Fast Flow media (Pharmacia AB) at a rate of 40 ml/minute. The column had been equilibrated with desalt buffer prior to loading. LongIGF-II was eluted with a solution consisting of 8 M Urea, 0.1 M Tris base, 40 mM glycine, 1.6 mM dithiothreitol and 5 mM ethylenediaminetetraacetic acid (EDTA)(pH 2.5) at a rate of 40 ml/minute.

3.2.4 Refolding (disulphide bond formation) of LongIGF-II.

The Q Sepharose Fast Flow eluate containing LongIGF-II (1.3 g in 650 ml) was adjusted to give a solution containing 4 M urea, 0.1 M Tris base, 20 mM glycine and 6 mM EDTA (pH 9.0), and a LongIGF-II concentration of 0.1 mg/l (w/v). Formation of disulphide bonds was started by the addition of 2-hydroxyethyldisulphide to a final concentration of 10 mM. The formation of disulphide bonds, which was carried out at room temperature, was monitored by C_4 analytical H.P.L.C (see section 3.2.12). Once bond-formation had reached completion (after 20 hours), conditions conducive to the formation of these bonds were terminated by the addition of 12 µl of 10 M hydrochloric acid per ml of solution.

3.2.5 Cation-exchange chromatography.

The solution containing refolded forms of LongIGF-II (1.3 g in 650 mls) was loaded at a rate of 40 ml/minute onto an S Sepharose Fast Flow (Pharmacia AB) cation-exchange column (50 mm x 65 mm) for partial purification. Prior to loading, the column was equilibrated with a solution consisting of 8 M urea, 40 mM glycine and 50 mM ammonium acetate (pH 4.8). LongIGF-II forms were eluted with a solution containing 8 M urea, 40 mM glycine, 50 mM ammonium acetate and 1 M sodium chloride (pH 4.8) at a rate of 20 ml/minute.

3.2.6 C_{18} matrex chromatography and freeze-drying.

Peptides in the S Sepharose Fast Flow eluate (1.2 g in 280 ml) were concentrated by passage through a column (50 cm x 65 cm) containing C_{18} Matrex (Matrex, Silica LC Chromatography Packing, Amicon, Danvers, MA, USA). The S Sepharose Fast Flow eluate was adjusted to 10 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid before being loaded onto the column, which had been equilibrated with a solution of 10 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid, at a rate of 20 ml/minute. Peptides were eluted with a solution of 80 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid, using the same flow rate.

The eluate fractions containing LongIGF-II forms were pooled and freeze-dried in a glass round-bottom flask using a Dynavac Freeze-drying Unit (Dynavac Pty. Ltd, Lidcombe, NSW, Australia).

3.2.7 Cleavage of the N-terminal fusion partner of LongIGF-II from IGF-II.

The N-terminal fusion partner attached to IGF-II was removed by the use of the protease genenase (recombinant H64A subtilisin BPN, a gift from Genentech Inc., South San Francisco, CA, USA). LongIGF-II (750 mg) was dissolved in a buffer which contained 2.5 M

urea, 20 mM Tris base, 5 mM calcium chloride and 0.2 M sodium chloride (pH 8.5). The volume (3.8 litres) was adjusted to give a LongIGF-II concentration of 200 mg/l. To begin digestion, 0.033 mg of genenase was added to the buffer for each 1 mg of LongIGF-II. Digestion was carried out at 37°C and monitored by C_4 analytical H.P.L.C. Upon completion of digestion, the reaction conditions were altered by the addition of trifluoroacetic acid to a final concentration of 0.1 % (v/v).

3.2.8 Isolation of the correctly folded form of IGF-II by C_4 reverse-phase chromatography.

IGF-II from the genenase digest was loaded onto a Waters C_4 PrepPak 500 cartridge (47 mm x 300 mm) for reverse-phase chromatography, at a flow rate of 25 ml/minute, using a Waters Delta Prep 3000 chromatography system (Waters Millipore, Milford, MA, USA), to separate the differently folded (disulphide-bonded) forms and to collect the correctly folded form. A gradient was generated using the same solutions as those used for C_4 analytical H.P.L.C. (section 3.2.12.), except that in this case a gradient of 0 % to 100 % (v/v) of acetonitrile in 0.08 % (v/v) trifluoroacetic acid was run over several hours to elute individual forms of IGF-II into separate fractions. Part of this gradient involved a change from 35 % to 39 % (v/v) acetonitrile over a 100 minute period to resolve the correctly folded form of IGF-II. The fractions were analysed by C_4 analytical chromatography and those containing IGF-II which eluted at the position of the correctly folded form were pooled and then freeze dried.

3.2.9 Final desalting step.

To remove any remaining salts, IGF-II was redissolved in a sterile 0.1 M acetic acid solution and passed through a Sephadex G-25 F column (22 mm x 250 mm), which had been equilibrated with sterile 0.1 M acetic acid, at a rate of 8 ml/minute. The elution position of IGF-II was determined by monitoring the A_{215} of the eluate. IGF-II was collected as a pool.

3.2.10 Calculation of the IGF-II yield.

The amount of IGF-II was determined from the absorbance profile area of IGF-II produced by C_4 and C_{18} analytical chromatography using the method of Buck *et al.* (1989). Extinction co-efficients were calculated as described by Gill & von Hippel (1989). The final value was determined by averaging the amounts calculated from the A_{215} of three runs on the C_4 analytical system (see section 3.2.12), and from the A_{280} of three runs on another analytical system, containing instead a C_{18} reverse phase cartridge (a NOVA-PAK C_{18} Radial-PAK cartridge, 8 cm x 10 cm, from Waters Millipore). The gradient in the C_{18} system was a change from 20 % to 36 % (v/v) n-propanol in 0.13 % (v/v) heptafluorobutyric acid over 140 minutes.

3.2.11 Storage method and analysis of the purified material.

IGF-II was freeze dried in sterile vials for storage at 4°C.

The ability of the purified IGF-II to stimulate protein synthesis was determined by the method of Francis *et al.* (1986) by GroPep Pty. Ltd. Briefly, confluent monolayers of L6 myoblasts in multiwell plates were incubated with constant amounts of [^{3}H]-leucine and appropriate amounts of peptide in Dulbecco's Modified Eagles Medium (DMEM). The amount of protein synthesis stimulated by IGFs during the incubation period was calculated from the amount of [^{3}H]-leucine incorporated into total cell protein which was above that incorporated by incubation with medium and [^{3}H]-leucine alone. The bioactivity of the purified IGF-II was compared with that of a reference standard of IGF-II (GroPep Pty.Ltd., dated 15/5/92) and a standard of IGF-I (GroPep Pty. Ltd., dated 14/11/91). Values obtained were from triplicate measures.

The ability of purified IGF-II to bind to type-2 IGF receptors on confluent monolayers of rat L6 myoblast cells was compared with a reference standard of IGF-II (see above) using the method described by Rechler *et al.* (1980). Binding capacity was determined by the ability

of the IGF-II to compete with ^{125}I-IGF-II (approximately 80 Ci/g) for binding to the type-2 IGF receptor. Radioactivity bound is expressed as the percentage of the total ^{125}I-IGF-II which was bound in the absence of added competing IGF-II (ie. % B/B_0). Values were derived from triplicate measures. Again this assay was carried out by GroPep Pty. Ltd.

The correct molecular mass of the purified IGF-II was confirmed by an electrospray mass spectrometer (VG Biotech Ltd., Altrincham, Cheshire, UK) courtesy of Dr. M. Shiel (University of Wollongong, NSW, Australia).

The N-terminal amino acid sequence of the purified protein was determined by the Edman degradation method (Hunkapiller *et al.* 1983) using a gas-phase sequencer (Model 470A, Applied Biosystems, Foster City, CA, USA). The sequencing was carried out by Denise Turner (Department of Biochemistry, University of Adelaide, SA, Australia).

3.2.12 Analytical C_4 reverse-phase chromatography.

Throughout the IGF-II preparation, progress was monitored by C_4 reverse-phase high performance liquid chromatography H.P.L.C. That is, trifluoroacetic acid was added to the sample for analysis to a final concentration of 0.1 % (v/v). This was then loaded onto a C_4 column (an Aquapore Butyl column, 2.1 mm diameter x 10 cm, from Brownlee Labs Inc., Santa Clara, CA, USA) that had been equilibrated with a solution containing 15 % (v/v) acetonitrile in 0.08 % (v/v) trifluoroacetic acid. Samples were eluted by increasing the acetonitrile concentration by 1 % per minute over a 30 minute period. The flow rate was 0.5 ml/min. The A_{215} of eluted material from the column was recorded during the gradient by a computer (utilising the ICI DP800 Chromatography Data Station computer software program) linked to the absorbance monitor. Software and instruments were from ICI Australia Operations Pty. Ltd., Scientific Instruments Division, Dingley, Melbourne, Vic, Australia. The elution position of correctly folded IGF-II was determined by loading standards of rhIGF-II.

3.3 Results.

Analytical C_4 reverse-phase chromatography was carried out during most stages of the purification process to assess the amount of protein present at each stage of the method and to ensure that the expected changes in the A_{215} absorbance profile were occurring. Figure 3.1 shows the A_{215} absorbance profile following a number of key stages in the purification. The earlier retention times of peaks in Figure 3.1b as compared with those of the starting material in Figure 3.1a are indicative of disulphide bond formation of peptides having occurred as expected prior to the C_{18} matrex chromatography step. The yet earlier retention times of peaks in the analysis of sample taken following the subtilisin cleavage reaction (Figure 3.1c) indicates that the fusion partner had been successfully removed from IGF-II. A single peak of IGF-II was obtained following the whole purification procedure, at a position corresponding to the elution position of an IGF-II reference standard (Figure 3.1d).

The yield of purified protein was 140 mg.

The N-terminal sequence of the purified protein was Ala-Tyr-Arg-Pro-Ser, the same sequence described for human IGF-II (Rinderknecht & Humbel 1978a). The sequencing indicated that the IGF-II was greater than 97 % pure.

Mass spectrometry revealed that the purified material was predominantly a molecule with a molecular weight of 7471.5 Da, which matches with the minimum molecular weight of human IGF-II of 7471 Da which was calculated by Rinderknecht & Humbel 1978a.

The purified IGF-II behaved in an almost identical manner to a reference standard of IGF-II in both its capacity to stimulate protein synthesis (Figure 3.2) and bind to the type-2 IGF receptor (Figure 3.3) in L6 myoblasts. As expected (eg. see Francis *et al.* 1993), IGF-II was less potent than IGF-I in stimulating protein synthesis (Figure 3.2).

These results indicate that IGF-II had been successfully purified.

FIGURE 3.1 Absorbance profiles of samples taken at several stages of IGF-II purification following C_4 reverse-phase H.P.L.C.. Samples were analysed following: (a) solubilisation of inclusion bodies, (b) C_{18} matrex chromatography, (c) subtilisin cleavage of the fusion protein, and (d) final purification of the correctly folded form of IGF-II. The profiles are of the A_{215} of protein eluted from the C_4 column by a 15 % to 45 % (v/v) acetonitrile gradient, in 0.1 % (v/v) trifluoroacetic acid, over 30 minutes.

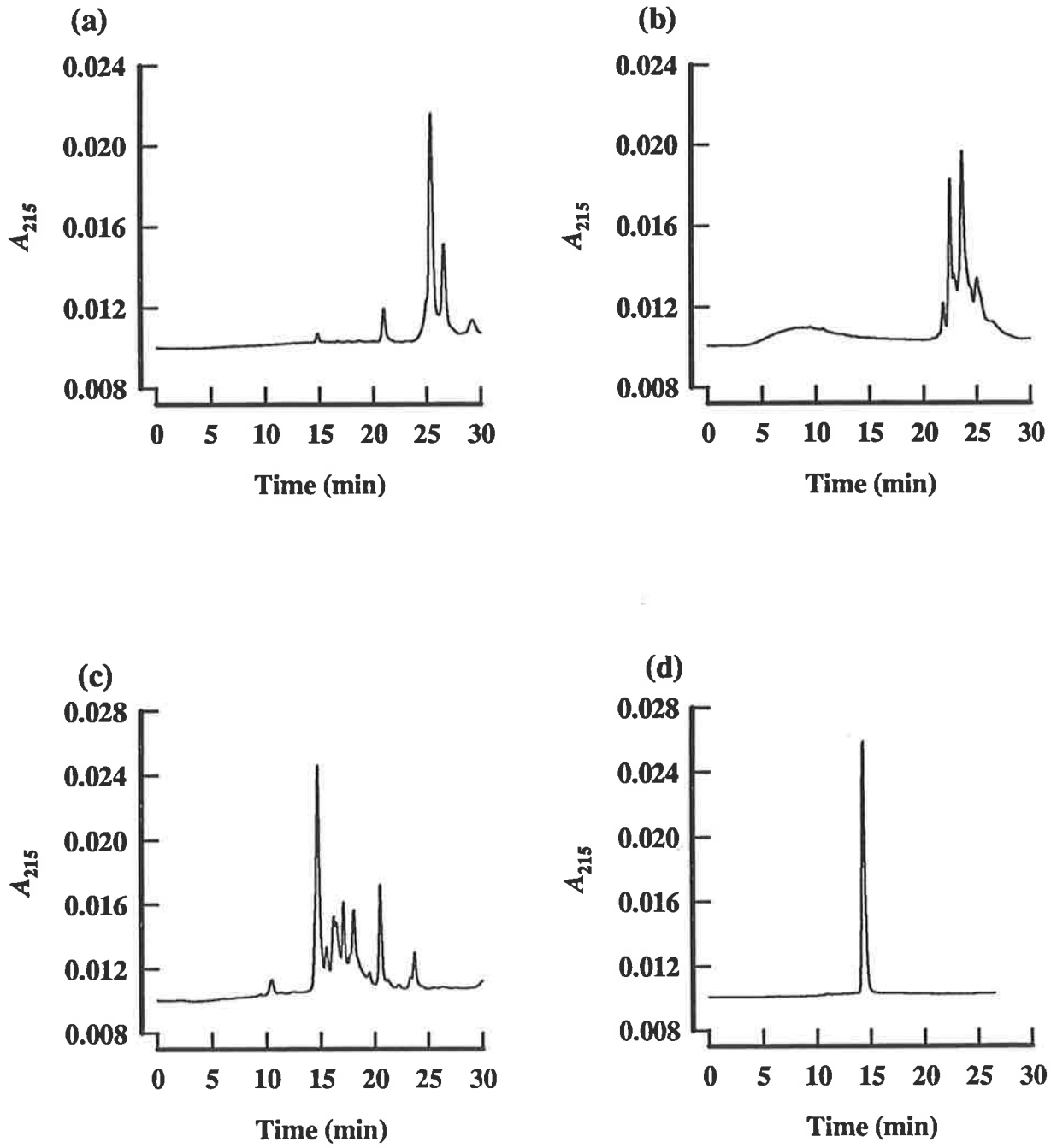

(a)
0.024
0.020
0.016
0.012
0.008
A_{215}
0 5 10 15 20 25 30
Time (min)
(b)
0.024
0.020
0.016
0.012
0.008
A_{215}
0 5 10 15 20 25 30
Time (min)
(c)
0.028
0.024
0.020
0.016
0.012
0.008
A_{215}
0 5 10 15 20 25 30
Time (min)
(d)
0.028
0.024
0.020
0.016
0.012
0.008
A_{215}
0 5 10 15 20 25 30
Time (min)

FIGURE 3.2 The ability of purified IGF-II (closed circles) and reference IGF-II (open circles) to bind to the type-2 IGF receptor of L6 myoblasts at the indicated doses. Results are shown as the amount of ^{125}I-IGF-II bound to cells in the presence of added protein expressed as a percentage of ^{125}I-IGF-II bound in the absence of added protein (ie. % B/B_0).

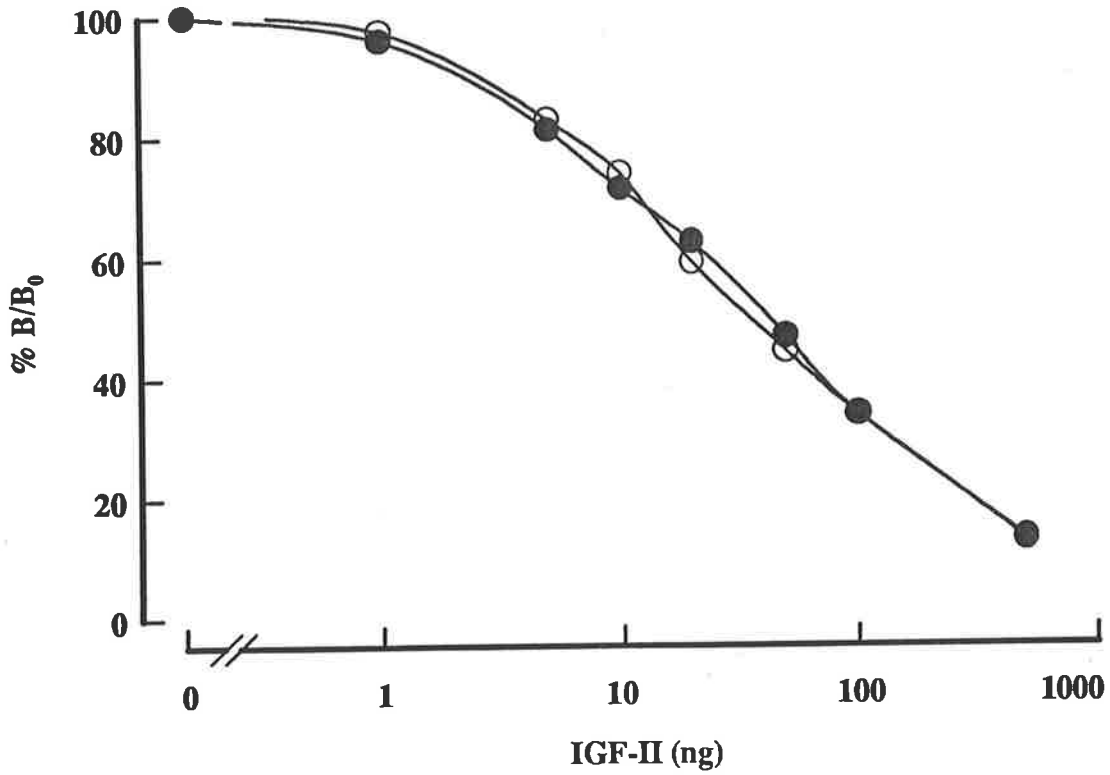
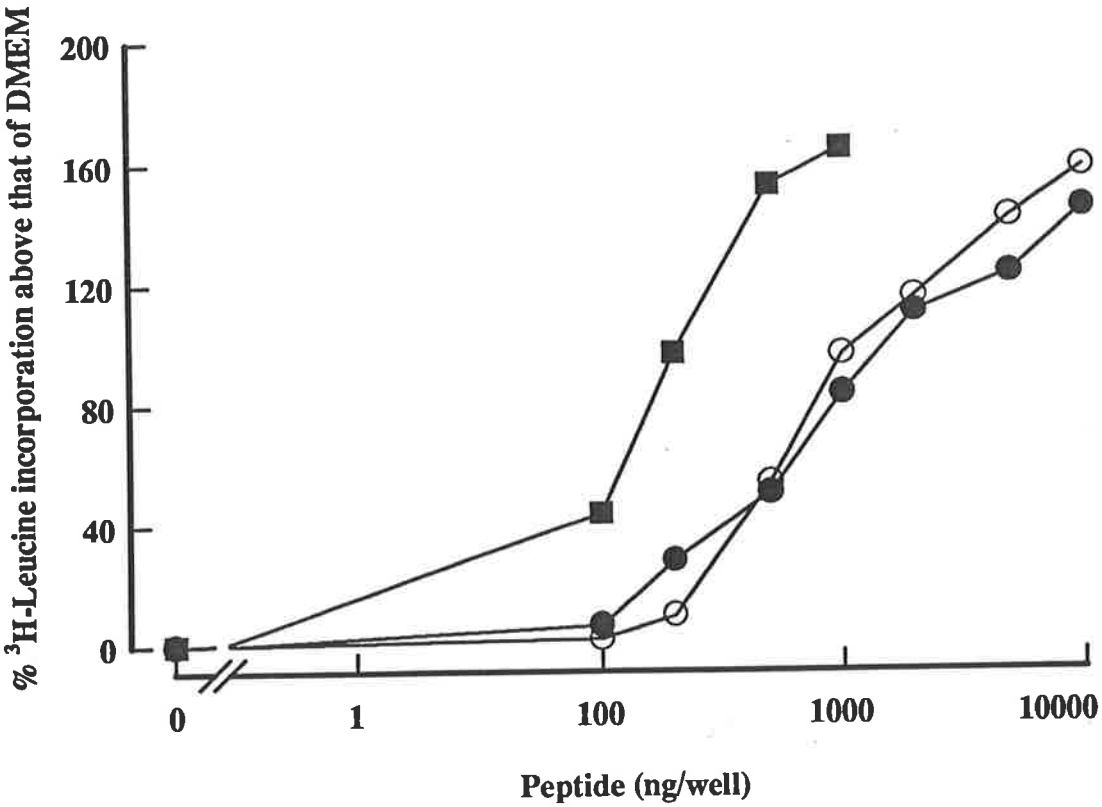

100
80
60
40
20
0
% B/B0
0
1
10
100
1000
IGF-II (ng)

FIGURE 3.3 The effects of purified IGF-II on protein synthesis. The graph shows the ability of reference IGF-II (open circles), purified IGF-II (closed circles) and reference IGF-I (squares) at the indicated doses to stimulate the incorporation of ^{3}H-leucine into L6 myoblasts in the presence of DMEM. Results are expressed as the % incorporation of ^{3}H-leucine above that achieved by DMEM.


% ³H-Leucine incorporation above that of DMEM
200
160
120
80
40
0
0
1
100
1000
10000
Peptide (ng/well)

CHAPTER 4.

CONTINUOUS 14 DAY INFUSION OF IGF-II INCREASES THE GROWTH OF NORMAL FEMALE RATS, BUT EXHIBITS A LOWER POTENCY THAN IGF-I.

4.1 Introduction.

IGF-I stimulates the growth of hypophysectomised (Schoenle *et al.* 1982) and also normal male (Hizuka *et al.* 1986) and female (Tomas *et al.* 1993) rats. Likewise, IGF-II increases body weight gain when administered to hypophysectomised rats, but IGF-II is less potent than IGF-I (Schoenle *et al.* 1985; Shaar *et al.* 1989). Effects on both linear growth and metabolism were indicated by increased tibial epiphyseal width and femur alkaline phosphatase, and reduced serum urea. More recently, mice bearing transgenes leading to overexpression and excess production of IGF-II have shown a tendency to deposit less fat than their non-transgenic counterparts, but body weight gain and most parameters of growth are not altered (van Buul-Offers *et al.* 1995; Da Costa *et al.* 1994; Rogler *et al.* 1994, Wolf *et al.* 1994). Thus the effects of IGF-II on postnatal growth of rodents remain poorly defined.

In this study, the effects of continuous 14 day infusion of a range of doses of rhIGF-I or rhIGF-II on growth, feed conversion efficiency, nitrogen balance, carcass composition, muscle protein breakdown, and plasma IGF and IGFBP levels have been compared in normal, young, female rats with a view to further clarifying the role of IGF-II in these aspects of physiology and endocrinology. Importantly, I sought to determine whether previously reported responses of hypophysectomised rats to IGF-II treatment is representative of the effects seen in a non-hypophysectomised animal.

4.2 Materials and methods.

4.2.1 Peptides.

rhIGF-I (lot HJC-102) was supplied by GroPep Pty. Ltd., Adelaide, S A, Australia and rhIGF-II was prepared as described in chapter 3.

4.2.2 Animals and animal maintenance.

Female Hooded-Wistar rats were obtained from the CSIRO Division of Human Nutrition, O'Halloran Hill, SA, Australia. Rats were housed in individual metabolism cages throughout the experiment. The cages were kept in a room with controlled temperature (25°C) and lighting (a 12 hour light/12 hour dark cycle). The rats were given free access to water and a starch/sucrose based diet that contained 180 g/kg of casein and 2.5 g/kg of methionine as sources of nitrogen (Tomas *et al.* 1984).

4.2.3 Experimental design.

Rats were placed in metabolism cages a few days prior to the start of treatment to familiarise them with the environment and diet of the experiment. Animals averaged 111 g in body weight at the start of treatment and were randomised into 7 treatment groups with similar mean body weights. Osmotic pumps were then inserted into rats and used to continuously infuse the animals with vehicle or IGFs for 14 days (see section 4.2.4.). One group of rats (pre-treatment controls) were killed at the time the other rats received pumps. Following each 24 hour period of the experiment, water and feed intakes were measured, animals were weighed and urine and faeces were collected. Urine and faeces were stored at -20°C. Blood was taken from a tail vein of each rat following 7 days of treatment. After 14 days of treatment, rats were stunned with a sharp blow to the head and then beheaded. Blood and tissues were collected for analysis (see section 4.2.5.).

Due to caging limitations, the study was split into two parts, with 3 animals per treatment in each arm of the study. There was a total of 6 animals per treatment group.

The study was approved by the Animal Care and Ethics Committee of the CSIRO Division of Human Nutrition, Adelaide, SA, Australia.

4.2.4 Filling and insertion of osmotic pumps.

Alzet model 2002 osmotic pumps (Alza, Palo Alto, CA, USA) were filled with one of the following: 0.1M acetic acid (vehicle), 9.2 mg of IGF-I or IGF-II/ml, 23.1 mg of IGF-I or IGF-II/ml, or 57.6 mg of IGF-II/ml. The mean delivery rate of pumps was 0.47 µl/hr, therefore rats were infused with IGFs at a rate of 0, 104, 260 or 650 µg/day respectively.

Pumps were implanted subcutaneously in the supra-scapular region of rats that had been anaesthetised with Halothane (May & Baker, West Footscray, Vic, Australia). Implantation involved making a small incision in the skin with a scalpel, inserting the pump into a small pocket under the skin created by separating the skin and underlying tissues, and finally sealing the pocket with wound clips. I wish to thank Kerry Wright for her assistance during the pump implantation surgery of this study and also the studies described in subsequent chapters.

4.2.5 Blood and tissue collections.

Blood was collected from the trunk of beheaded rats into ice-cold heparinised tubes and then centrifuged at 4°C to obtain plasma, which was stored at -20°C.

The pelt was removed and weighed, followed by the removal and weighing of the adrenals, brain, gut (stomach to colon inclusive; see section 4.2.6.), heart, kidneys, spleen, liver, lungs, spleen and thymus. The feet and tail were cut from the body and weighed together with the head (minus the brain). Residual viscera was removed. The remainder of the body,

defined as the carcass in this study, was also weighed. All tissues, except the gut (see section 4.2.7.), were frozen immediately in liquid nitrogen and stored at -20°C. The right femur was removed from the carcass at a later date, for weighing and length measurement with vernier callipers, but returned to the carcass prior to analysis of carcass composition. The weight measurements and other tasks performed at the time of the kill were carried out with the assistance of Dr. Frank Tomas and technicians from his laboratory (CSIRO Division of Human Nutrition, Adelaide) and gut measurements (see section 4.2.6) were performed with the help of Mr. Gordon Howarth and technicians from the Child Health Research Institute (North Adelaide).

4.2.6 Gut weight and length measurements.

After excision, the gut was immediately placed in an ice-cold saline solution. Then, on an ice-cold glass plate, it was divided into stomach, duodenum, jejunum, ileum, caecum and colon, from which weights were recorded after removal of any luminal contents. The length of the various gut segments was determined while the tissue was fully horizontally extended, but not stretched, on the glass plate.

4.2.7 Jejunum histology and morphological measurements.

A small length of tissue was excised from the jejunum and placed in Bouin's fixative for histology. The jejunal section was then dehydrated by soaking it in a series of increasing concentrations of alcohol prior to immersing it in chloroform and embedding it in paraffin wax. The embedded sample from each animal was cut at an angle perpendicular to the surface at approximately 200 µm intervals, and three 2 µm thick sections were dewaxed, stained using haematoxylin and eosin, and then mounted on slides. The preceding steps were carried out by staff of the Child Health Research Institute, but following tasks were performed by myself.

Sections were viewed using a light microscope and villus height, crypt depth and muscularis width measured by a drawing tube attachment coupled to a digitising tablet (Summasketch II, Summagraphics, Seymour, CT, USA) and a computer running the MacMeasure software program (Apple MacIntosh). On three sections from each animal, ten villus, crypt and muscularis measurements were made on typical regions of the section with a good orientation. Thus the average value for each morphological parameter was derived from thirty observations per animal. The method was derived from those of Read *et al.* (1993).

4.2.8 Nitrogen and protein determinations.

Carcasses and faeces were dried under vacuum prior to analyses. Nitrogen was measured in sub-samples of faeces, feed or carcass that had been ground and well mixed, or in urine, by using a Carlo Erba NA1500 Nitrogen Analyser (Milan, Italy). Nitrogen retention was calculated by subtracting the amount of nitrogen excreted in urine and faeces from the amount of nitrogen ingested as feed. The protein content of carcasses was calculated by multiplying nitrogen content by 6.25.

4.2.9 Carcass water and fat measurements.

The amount of water in the carcass was determined by subtracting the dry weight of the carcass from that of the wet weight measured at the time of kill.

To determine fat content, 1 g of dried, ground carcass was mixed with 10 ml of chloroform:methanol (1:1, v:v) for 3 hours, and, after centrifugation for 20 minutes at 2,000 x *g*, the supernatant was passed through Whatman Phase-Separation filter paper and collected in a glass tube. The pellet was subjected to a second chloroform:methanol extraction and the supernatant pooled with that from the first extraction. The supernatants were dried

under vacuum and the fat residue was weighed. Fat determinations were carried out in duplicate.

4.2.10 Measurement of creatinine and 3-methylhistidine (3-MH) concentrations in urine.

The concentration of creatinine in urine was measured using a Skalar continuous flow analyser by Skalar analyser method number 07907001 (Skalar Analytical B.V., Breda, The Netherlands). The urine was diluted in a solution of 1.8 % (w/v) sodium chloride and 0.03 % (v/v) Brij-35, and, following loading onto the analyser, it was mixed with 1.3 % (w/v) picric acid. The amount of creatinine present was determined by the subsequent formation of a red complex, which was measured by its absorbance at 510 nm. The concentration was calculated by comparing the absorbance with that of creatinine standards.

To measure 3-MH, urinary acetyl-3-MH was first hydrolysed and then partly purified by an ion-exchange method described by Tomas *et al.* (1984). The amount of 3-MH in the eluate was then determined using the Skalar continuous flow analyser as described by Murray *et al.* (1981). Urine and faeces were each pooled to provide day 1 to 7 and day 8 to 14 samples. Therefore, 14 day values were obtained from the sum, or if appropriate, the average, of the values from the two periods.

4.2.11 Separation of IGFs and IGFBPs in plasma by acid gel-filtration chromatography.

To prepare for chromatography, 300 µl of a solution containing plasma diluted in water was mixed with 100 µl of a solution of 0.8 M acetic acid and 0.2 M trimethylamine (pH 2.5). IGF/IGFBP complexes were dissociated by standing the acidic mixture at room temperature for approximately 30 minutes. An equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (freon) was mixed thoroughly with the acidified plasma solution in order to remove lipids. The solution was centrifuged at 10,000 x *g* for 10 minutes at 4°C to separate

the freon and plasma phases. The plasma solution was then subjected to gel-filtration chromatography under acid conditions using a Waters Protein Pak 125 column (7.8 mm x 30 cm; Waters, Melbourne, Vic, Australia), in order to separate and then collect IGFs and IGFBPs as two distinct pools. The mobile phase consisted of 0.2 M acetic acid, 50 mM trimethylamine and 0.05 % (v/v) Tween 20 (pH 2.5). The flow rate was 1ml/minute. To determine which eluate fractions to pool for IGF and IGFBP assays of plasma from individual animals, the fractions collected following chromatography of plasma pools from vehicle, IGF-I and IGF-II treated rats were assayed in each of the IGF-I and IGF-II radioimmunoassays (RIAs) (see section 4.2.12.). To ensure that there were no shifts in the positions of the elution pools throughout the period of chromatography, the elution volume of chromatographed ^{125}I-labelled IGFs was compared at regular intervals. The method has been described by Ballard *et al.* (1990b) and Owens *et al.* (1990).

4.2.12 IGF-I and IGF-II RIAs.

IGF-I and IGF-II concentrations in plasma were calculated by RIA of IGFs in the neutralised IGF pools generated by the chromatography described in section 4.2.11. Total IGF concentration was calculated from the sum of the IGF-I and IGF-II concentrations.

An anti-rhIGF-I antibody raised in rabbits (a gift from Dr. Phil Owens of the Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia), and which binds rat IGF-I about 70 % as well as rhIGF-I, was used for the IGF-I RIA. A mouse monoclonal antibody directed against rat IGF-II (Amano Pharmaceutical Co. Ltd., Nagoya, Japan), and which has 100 % crossreactivity with human IGF-II, was used for the IGF-II assay.

For RIA, tubes contained 50 µl of chromatography eluate (sample tubes) or mobile phase (standards or reference tubes), 30 µl of 0.4 M Tris, 50 µl of antibody solution, 50 µl of

^{125}I-IGF and 200 µl of RIA buffer (in the case of standards tubes this contained defined amounts of IGFs). ^{125}I-IGF was a gift from Dr. Phil Owens. IGFs had been iodinated by the Chloramine T method described by Van Obberghen-Schilling & Pouyssegur (1983) to a specific activity of between 70 to 80 Ci/g. ^{125}I-IGFs were separated from unreacted iodide and aggregates by gel-filtration chromatography using Sephadex G-50 (Pharmacia AB) and a mobile phase consisting of 50 mM sodium phosphate (pH 6.5) and 0.25 % (w/v) bovine serum albumin. The RIA buffer consisted of 0.2 M sodium phosphate, 0.5 M EDTA, 0.02 % (w/v) protamine sulphate and 0.05 % (v/v) Tween 20 (pH 7.5). RIAs were set up at room temperature and left to incubate for 18 hours at 4°C. Following incubation, IGFs bound to antibodies were precipitated by the addition of 50 µl of a second antibody (sheep, anti-rabbit for the IGF-I RIA or sheep, anti-mouse for the IGF-II RIA), which was diluted 1 in 20 using RIA buffer, and also by the addition of 10 µl of serum (rabbit or mouse for the IGF-I or IGF-II assays respectively), which was diluted 1 in 50 using RIA buffer. The second antibody and sera solutions were obtained from Silenus Laboratories, Hawthorn, Vic, Australia. Incubation proceeded for 30 minutes at 4°C before the addition of 1 ml of a 4°C solution containing 5.5 % (w/v) polyethylene glycol 5000 and 0.9 % (w/v) sodium chloride. RIA tubes were then centrifuged at 4,000 x *g* for 20 minutes at 4°C, followed by the removal of the supernatant by aspiration. Radioactivity in the pellet was determined by a gamma counter. Each sample was assayed in triplicate.

4.2.13 Measurement of IGFBP concentrations by RIA interference.

The assay was carried out as described for the IGF-I and IGF-II RIAs (see section 4.2.12), except that the chromatography elution pools containing IGFBPs were used instead of the pools containing IGFs. Measurement was facilitated by the ability of the IGFBPs to sequester ^{125}I-IGF-I or ^{125}I-IGF-II from the antibodies used in the IGF-I and IGF-II RIAs

respectively, and hence appear as competing cold IGF. That is, IGFBPs were measured as interference in the RIAs. Values obtained in the interference assays are expressed in IGF equivalents. Each sample was assayed in triplicate.

The gel-filtration method used in this thesis, as well as the methods for the IGF and IGFBP assays, have been extensively validated by the laboratory of Dr. Owens and others and have been used in previously published work (eg. Ballard *et al.* 1990b; Owens *et al.* 1990, 1991). The method has been described in fine detail only recently (Owens *et al.* 1995). Also, the method conforms to the consensus on valid means of measuring and separating IGFs and IGFBPs present in biological fluids that was reached at the 3rd International Symposium on Insulin-like Growth Factors (Bang *et al.* 1994).

4.2.14 Western ligand blotting of plasma.

Treatment pools of plasma were produced by combining equal volumes of plasma from each of the animals within a treatment group. 20 µl of plasma pool was mixed with 55 µl of water and 25 µl of loading buffer solution containing 3.02 % (w/v) Tris base, 8 % (w/v) sodium dodecyl sulphate, 20 % (w/v) glycerol and 0.004 % (w/v) bromophenol blue (pH 6.8). These plasma mixtures were heated to 65°C for 15 minutes. ^{14}C-labelled "rainbow" molecular weight markers (Amersham International plc., Buckinghamshire, UK) were also dissolved and heated in the same manner as the plasma samples. The individual IGFBPs in 10 µl of each plasma mixture was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli *et al.* 1970) using an LKB 2050 Midget electrophoresis unit (LKB Produkter AB, Bromma, Sweden). The gel consisted of a 4 % (w/v) acrylamide, 0.1 % (w/v) methyl bis-acrylamide, 0.1 % (w/v) sodium dodecyl sulphate, 0.13 M Tris-HCl (pH 8.8) stacking gel, set by the addition of 0.1 % (w/v) ammonium persulphate and 0.05 % (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED), and a 12.5 % (w/v) acrylamide, 0.31 % (w/v) methyl

bis-acrylamide, 0.1 % (w/v) sodium dodecyl sulphate, 0.38 M Tris-HCl (pH 6.8), separating gel, set by the addition of 0.04 % (w/v) ammonium persulphate and 0.05 % (v/v) TEMED. Electrophoresis was carried out at 15 mA.

Following electrophoresis, proteins were transferred from gels to nitrocellulose (Schleicher and Schuell, Dassel, West Germany) using a Multiphor II electrophoresis system (Pharmacia LKB Biotechnology, Uppsala, Sweden). This involved construction of a discontinuous buffer system as follows. 6 pieces of chromatography paper (3MM Chr, Whatman) soaked in anode solution 1 (0.3 M Tris, pH 10.4) were placed on the anode electrode plate. On top of this was placed 3 pieces of chromatography paper soaked in anode solution 2 (25 mM Tris base, pH 10.4) followed by a square of nitrocellulose that had also been soaked in anode solution 2. The gel was placed on top of the nitrocellulose. Nine pieces of chromatography paper that had been soaked in cathode solution (4 mM 6-amino-n-hexanoic acid, pH 7.6) were laid over the gel before the completion of the transfer unit by the placement of the cathode electrode plate over the top of the stack of papers. Transfer was carried out for 1 hour with a current of 0.8 mA per cm^2 of the gel surface area.

The nitrocellulose containing the transferred IGFBPs was washed by soaking it for half of an hour in a saline solution (0.15 M NaCl, 10 mM Tris base, pH 7.4) and 1 % (v/v) Triton X-100. This and subsequent steps were carried out at room temperature in a shaker. To block non-specific binding sites, the nitrocellulose was soaked for 18 hours in saline containing 0.1 % (v/v) Tween 20 and 1 % (w/v) bovine serum albumin. Nitrocellulose was placed in a fresh aliquot of the latter solution, but which contained approximately 500,000 cpm of either ^{125}I-IGF-I or ^{125}I-IGF-II, for 2 hours, and was then washed in saline containing 0.1 % (v/v) Tween 20. Once the nitrocellulose had dried it was exposed to X-ray film (Konica Corporation, Tokyo, Japan).

The Western ligand blotting method is based on that described by Hossenlopp *et al.* (1986).

4.2.15 Statistics.

Results are expressed as treatment means ± S.E.M. for 6 animals. Significant effects between treatment groups were determined by one-way analysis of variance (ANOVA) and the significance of a treatment from the vehicle-infused control was determined by Dunnett's test using the pooled estimate of the standard error. Regression analysis was also used to determine the effect of dose on nitrogen retention and the fractional weight of the thymus. Significance was deemed to be $P < 0.05$. Significant differences from the vehicle control group are denoted as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Analyses were carried out using the SYSTAT computer software program (SYSTAT Inc.; Evanston, IL, USA).

4.3 Results.

Figures and tables of parameters for which there were significant treatment effects are presented in this chapter, whereas other results that are described are included in appendix A.

The effect of the infused IGFs on the circulating levels of IGF-I, IGF-II and IGFBPs may provide clues to any potency differences between IGF-I and IGF-II in this study. Accordingly, plasma collected from individual rats at the time of kill was chromatographed using a gel-filtration column under acid conditions, to separate and then collect IGFBPs and the IGFs into separate pools for analysis. Treatment with IGF-I increased plasma IGF concentration, with a dose of 260 µg/day nearly doubling the concentration found in the vehicle control group (Figure 4.1a). Plasma IGF-II concentrations were negligible in both vehicle and IGF-I treated rats (Figure 4.1b). Treatment with IGF-II significantly increased the concentration of circulating IGF-II in a dose-dependent manner (Figure 4.1b). Administration of 260 µg/day of either IGF-I or IGF-II increased the respective plasma concentrations to the same degree. A similar comparison applied to the 104 µg/day doses. IGF-II infusion decreased plasma IGF-I concentration in a dose-dependent manner with 650 µg/day significantly reducing IGF-I concentration by 195 µg/l (Figure 4.1a). As a consequence of this depression in IGF-I concentration, the total amount of IGF in circulation (IGF-I plus IGF-II) is increased to a slightly greater degree by IGF-I than by IGF-II (Figure 4.1c).

Since the IGFBPs potentially control the tissue availability of IGFs, we estimated the relative ability of IGFBPs to sequester ^{125}I-IGFs from the antibody in the RIAs for both IGF-I and also IGF-II (Figure 4.2). This measure does not distinguish changes in individual IGFBPs, but rather represents total IGFBP content. Both RIAs showed that IGF treatment led to an increase in binding proteins with IGF-I producing larger responses than IGF-II.

To assess the possibility of changes in the types of IGFBP following IGF treatment, western ligand blotting was carried out on plasma pools from each treatment group, using ^{125}I-

IGF-I or ^{125}I-IGF-II as probes (Figure 4.3). The results of these measurements confirmed the increases in IGFBP obtained from the RIA interference measurements and demonstrated that all visible IGFBP types increased over those of the vehicle group following treatment with increasing amounts of IGF-I or IGF-II. The effects were especially pronounced with what is likely to be the IGFBP-3 doublet between 40 to 50 kDa and with an approximately 32 kDa IGFBP that may represent IGFBP-2. There appeared to be a tendency for IGF-II to increase these IGFBPs in a dose-dependent manner with the 32 kDa IGFBP prominent after the treatment of animals with 650 µg/day (Figure 4.3a; 4.3b; lane 8). The ligand blots also showed that 104 µg of IGF-I/day was more potent than 104 µg of IGF-II/day at increasing IGFBP-3 and the 32 kDa IGFBP (compare lanes 4 and 6 of Figure 4.3a; 4.3b) but that there were no

FIGURE 4.1 (a) Plasma IGF-I, (b) plasma IGF-II and (c) plasma total IGF (IGF-I plus IGF-II) concentrations in rats following treatment with: vehicle (open bars), IGF-I (solid bars) or IGF-II (hatched bars) at the indicated doses. Results are expressed as the mean ± S.E.M. for 6 animals in each group. Significance from the vehicle group is denoted by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

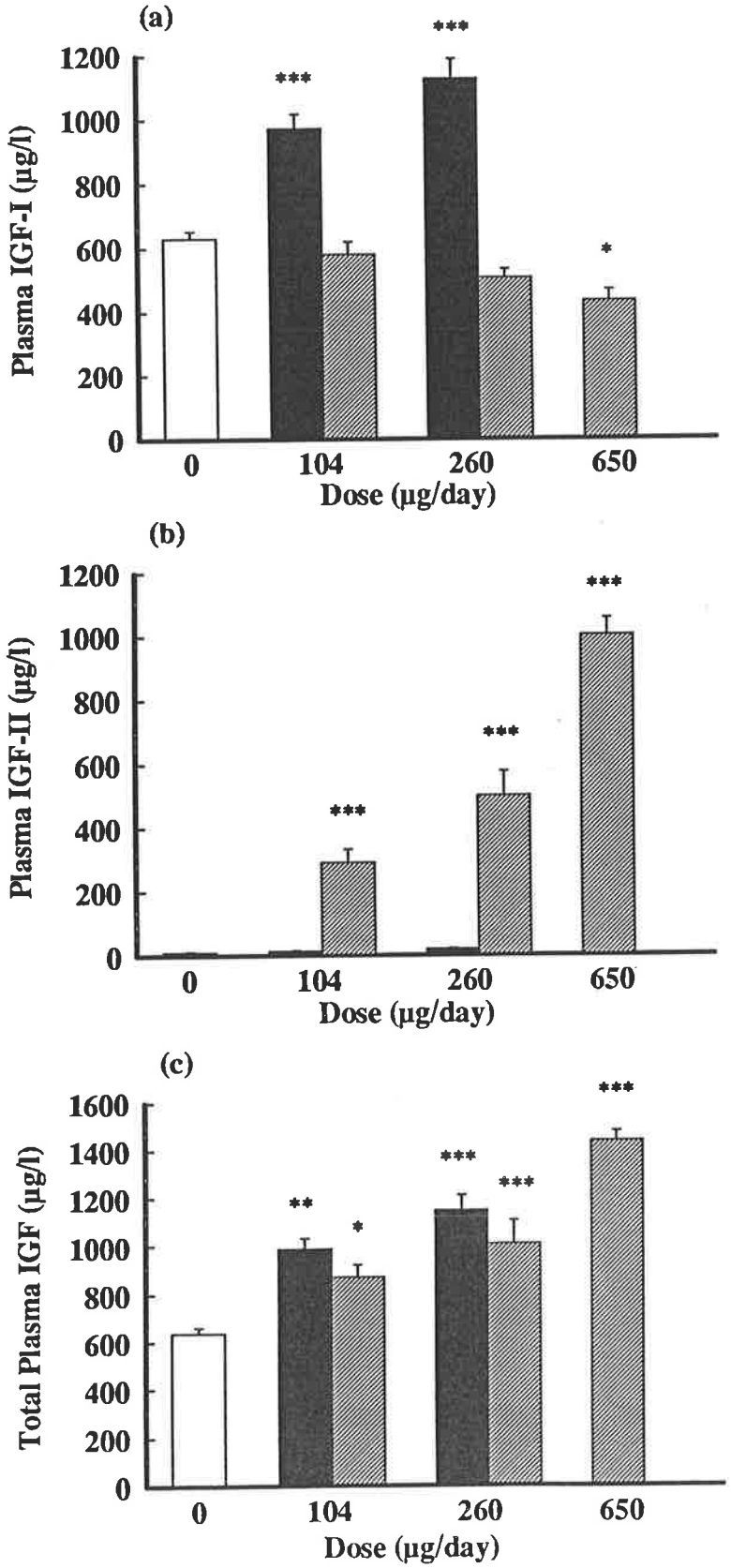
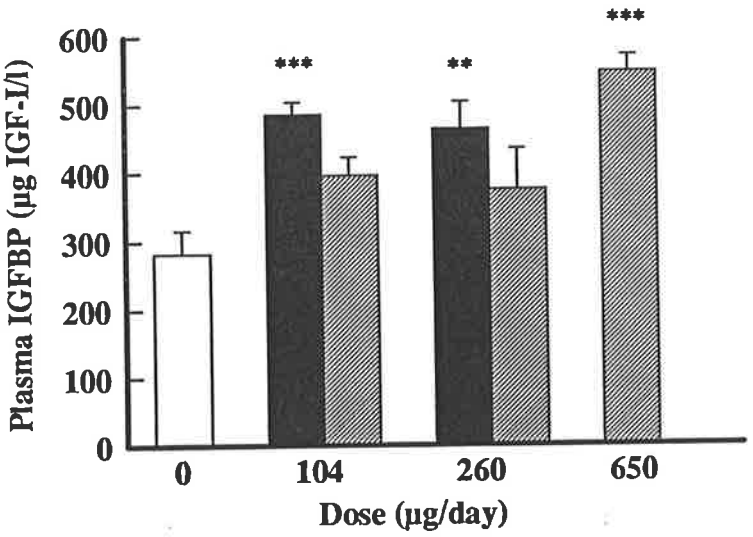

(a)
Plasma IGF-I (µg/l)
1200
1000
800
600
400
200
0
***
***
*
0
104
260
650
Dose (µg/day)
(b)
Plasma IGF-II (µg/l)
1200
1000
800
600
400
200
0
***
***
***
0
104
260
650
Dose (µg/day)
(c)
Total Plasma IGF (µg/l)
1600
1400
1200
1000
800
600
400
200
0
**
*
***
***
***
0
104
260
650
Dose (µg/day)

FIGURE 4.2 Plasma IGFBP concentrations in rats following treatment with: vehicle (open bars), IGF-I (solid bars) or IGF-II (hatched bars) at the indicated doses. Panel (a) represents the interference of IGFBPs in the IGF-I RIA and panel (b) represents the interference of IGFBPs in the IGF-II RIA. Results are expressed as the mean ± S.E.M. for 6 animals in each group. Significance from the vehicle group is denoted by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

(a)


Plasma IGFBP (µg IGF-I/l)
600
500
400
300
200
100
0
***
**
***
0
104
260
650
Dose (µg/day)

(b)

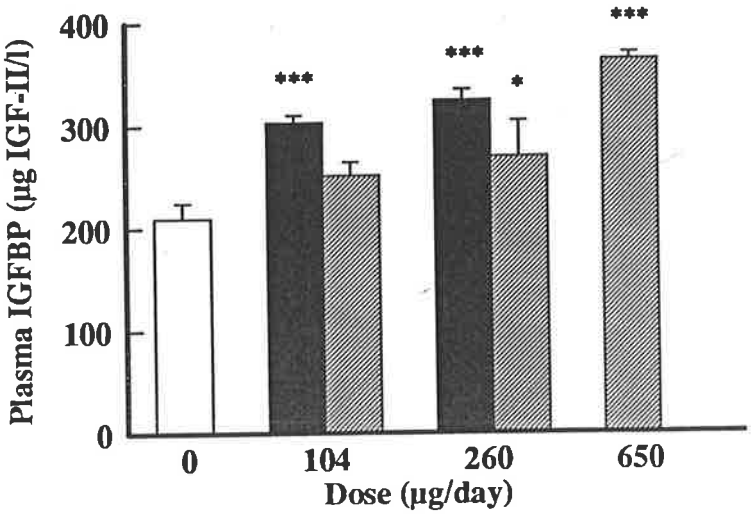
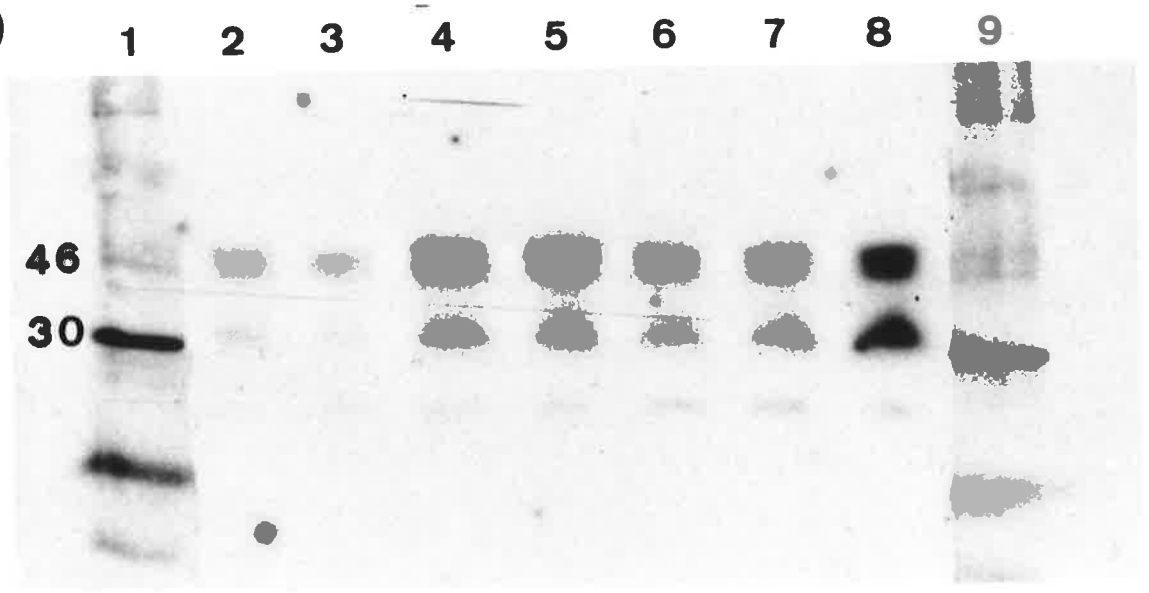
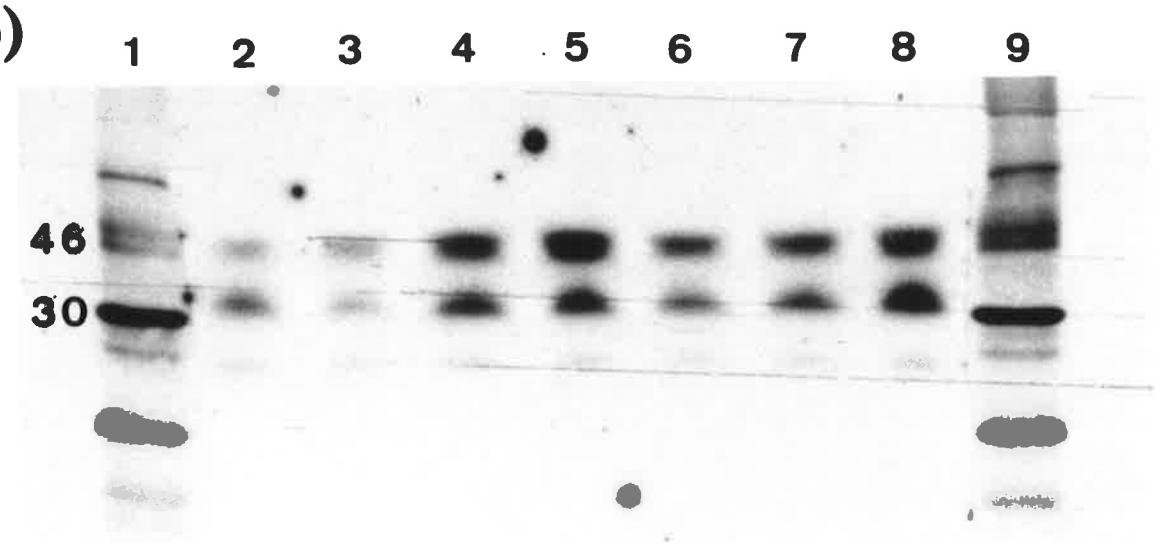

Plasma IGFBP (µg IGF-II/l)
400
300
200
100
0
***
***
*
***
0
104
260
650
Dose (µg/day)

FIGURE 4.3 Plasma IGFBP profiles as determined by Western ligand blotting of rat plasma using (a) ^{125}I-IGF-I or (b) ^{125}I-IGF-II as probe. Lanes 1 and 9 contain size markers (sizes (in kDa) are as indicated). Other lanes were loaded with treatment pools of plasma from pre-treatment controls (lane 2), or from animals treated with vehicle (lane 3), 104 µg of IGF-I/day (lane 4), 260 µg of IGF-I/day (lane 5), 104 µg of IGF-II/day (lane 6), 260 µg of IGF-II/day (lane 7) or 650 µg of IGF-II/day (lane 8).

a)



b)



obvious differences between the effects of IGF-I and IGF-II on the levels of those IGFBPs at the 260 µg/day dose (compare lanes 5 and 7 of Figure 4.3a; 4.3b).

Body weight gain over the full 14 day treatment period was significantly increased by administration of 260 µg of IGF-I/day or 650 µg of IGF-II/day, and showed that IGF-II responses were two to three fold less potent than observed with IGF-I (Figure 4.4.; Table 4.1.).

A temporary slowing of growth is evident in some treatment groups immediately following day 7 (Figure 4.4) and this is likely to result from the stress associated with tail vein bleeds. Weight gains were greater during the first week of treatment than the second in all treatment groups, although rats treated with 260 µg of IGF-I/day maintained a similar growth rate during both weeks and grew the fastest during the second week. Average daily weight gain over the 14 days was 3.0 g in vehicle treated rats and 3.7 g in the rats treated with the highest dose of IGF-I and IGF-II, an increase of 23 %. These were no significant changes in feed intakes (Table 4.2) but increases in body weight gain were associated with increased feed conversion efficiency (weight gained as a fraction of feed consumed) (Table 4.3). Regression analyses demonstrate that body weight gain was significantly correlated with both feed intake and feed conversion efficiency, but the correlation with feed conversion efficiency was much stronger (Figure 4.5).

In addition to improving body weight gain, both IGF-I and IGF-II treatment led to a significant increase in the weight, as a fraction of body weight, of the gut (stomach to colon), kidneys and spleen (Table 4.5), but not of the adrenals, brain, heart, liver, lungs, thymus, carcass, skin, or head plus tail plus paws. Although individual treatment means did not differ significantly from the control, thymus weights were significantly related to peptide dose, IGF-I being more potent than IGF-II. These results show that IGF-II

FIGURE 4.4 Body weights (g) of rats on each day of treatment. Osmotic pumps were implanted, and treatment began, immediately following weighing of animals on day 0. Treatments are represented as follows: vehicle (filled circles), 104 µg of IGF-I/day (open circles), 260 µg of IGF-I/day (squares), 104 µg of IGF-II/day (triangles), 260 µg of IGF-II/day (inverted triangles) and 650 µg of IGF-II/day (diamonds). Results are expressed as the mean for 6 animals in each treatment. S.E.M.s have not been shown for the sake of clarity. The pooled S.E.M. on day 14 of treatment was 1.4.

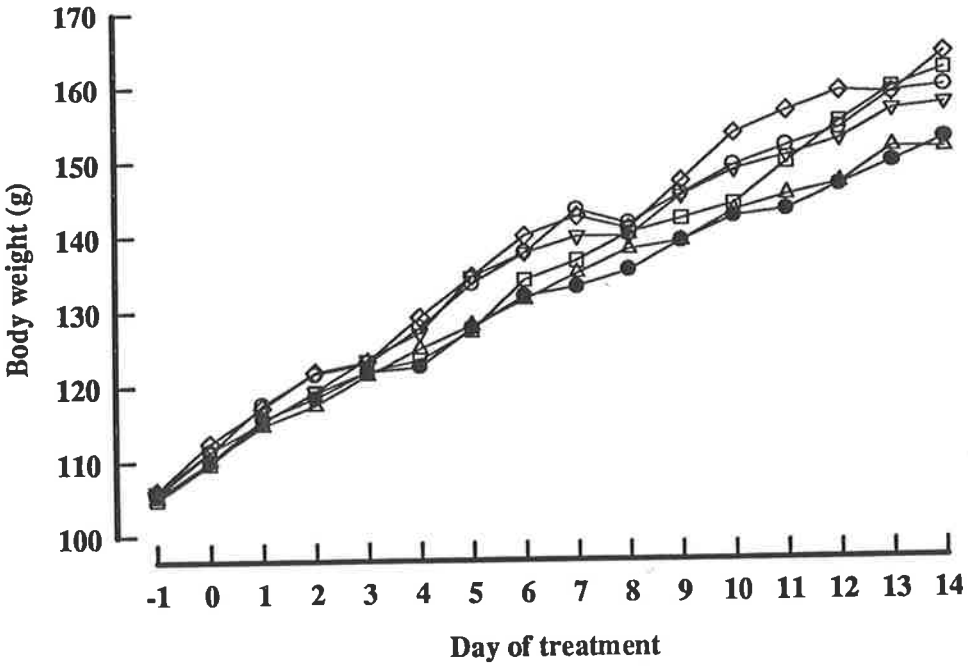

Body weight (g)
170
160
150
140
130
120
110
100
-1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day of treatment

TABLE 4.1. Body weight gains (g) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group.

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	23.0 ± 2.4	19.5 ± 2.7	42.5 ± 1.8
IGF-I (104 µg/day)	31.9 ± 2.5	16.2 ± 2.8	48.2 ± 0.9
IGF-I (260 µg/day)	26.8 ± 4.2	25.3 ± 1.7	52.1 ± 3.1*
IGF-II (104 µg/day)	24.7 ± 2.6	16.3 ± 3.2	41.0 ± 1.6
IGF-II (260 µg/day)	28.3 ± 2.3	17.5 ± 2.5	45.9 ± 3.6
IGF-II (650 µg/day)	30.0 ± 2.1	21.6 ± 2.8	51.5 ± 1.4*

* $P < 0.05$.

TABLE 4.2. Feed intakes (g) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group.

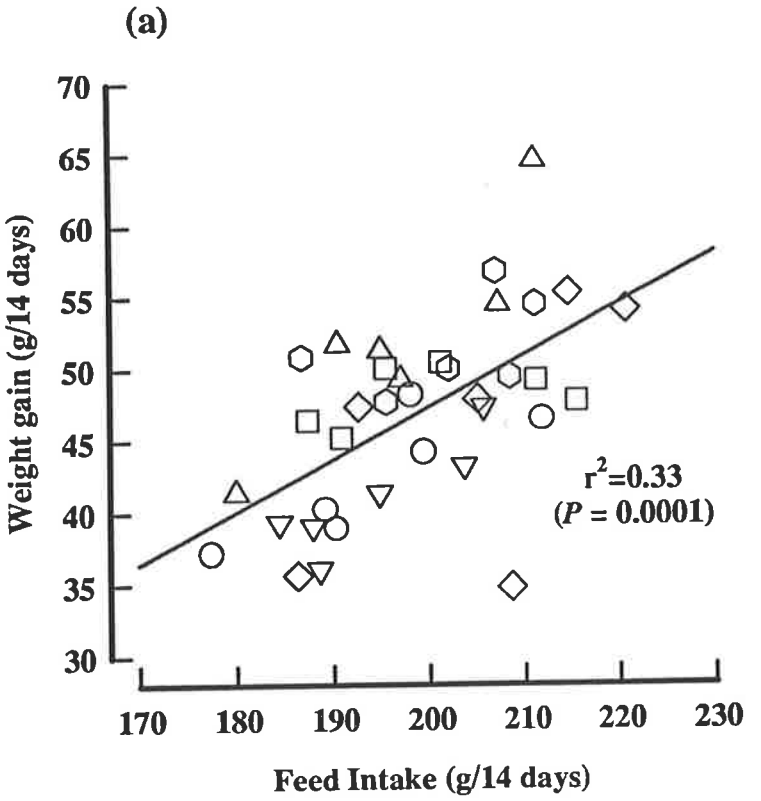
	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	96.7 ± 4.3	97.8 ± 3.0	194.4 ± 4.8
IGF-I (104 µg/day)	103.5 ± 3.7	96.9 ± 2.8	200.4 ± 4.6
IGF-I (260 µg/day)	94.5 ± 4.8	102.4 ± 2.6	196.9 ± 4.6
IGF-II (104 µg/day)	97.9 ± 1.8	96.7 ± 2.3	194.2 ± 3.6
IGF-II (260 µg/day)	103.2 ± 4.1	101.6 ± 2.8	204.7 ± 5.3
IGF-II (650 µg/day)	100.9 ± 1.0	101.0 ± 3.2	201.9 ± 3.8

TABLE 4.3. Feed conversion efficiencies (g of body weight gain/g of feed intake) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group.

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	0.236 ± 0.017	0.196 ± 0.022	0.218 ± 0.006
IGF-I (104 µg/day)	0.310 ± 0.025	0.165 ± 0.026	0.241 ± 0.005
IGF-I (260 µg/day)	0.276 ± 0.036	0.246 ± 0.013	0.264 ± 0.010*
IGF-II (104 µg/day)	0.252 ± 0.026	0.168 ± 0.031	0.211 ± 0.005
IGF-II (260 µg/day)	0.272 ± 0.012	0.174 ± 0.025	0.223 ± 0.015
IGF-II (650 µg/day)	0.298 ± 0.022	0.211 ± 0.022	0.255 ± 0.006*

* $P < 0.05$.

FIGURE 4.5 Regression analyses of (a) mean body weight gain versus mean feed intake and (b) mean body weight gain versus feed conversion efficiency, for each animal over the 14 day treatment period (n=36). Treatments are symbolised as follows: vehicle (circles), 104 µg of IGF-I/day (squares), 260 µg of IGF-I/day (triangles), 104 µg of IGF-II/day (inverted triangles), 260 µg of IGF-II/day (diamonds) and 650 µg of IGF-II/day (hexagons).


(a)
Weight gain (g/14 days)
70
65
60
55
50
45
40
35
30
170
180
190
200
210
220
230
Feed Intake (g/14 days)
r²=0.33
(P = 0.0001)

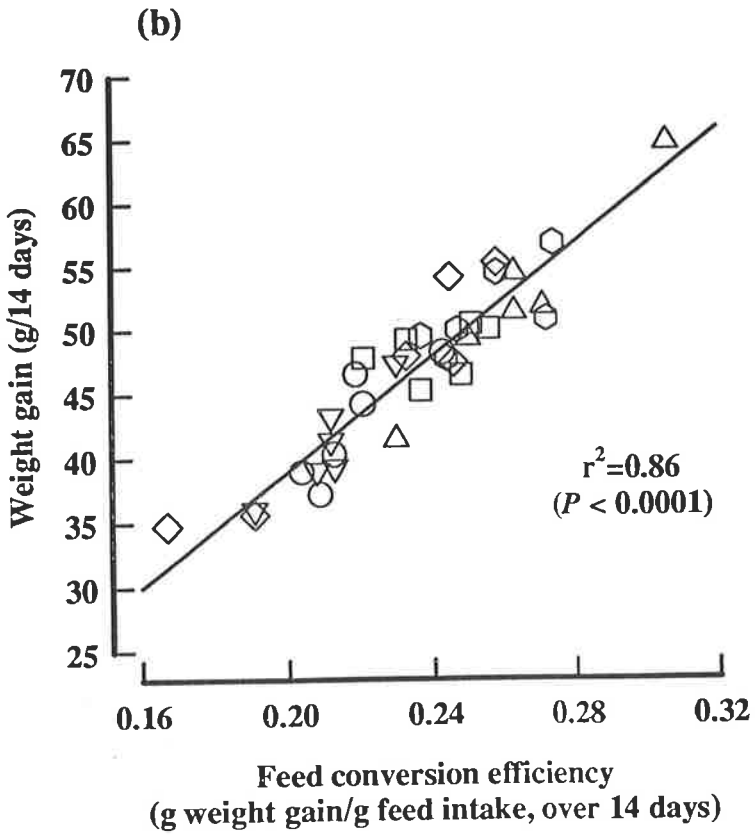

(b)
Weight gain (g/14 days)
70
65
60
55
50
45
40
35
30
25
0.16
0.20
0.24
0.28
0.32
Feed conversion efficiency
(g weight gain/g feed intake, over 14 days)
r²=0.86
(P < 0.0001)

TABLE 4.4 Organ and tissue weights expressed as a fraction of body weight (g/kg body weight). The values represent the mean ± S.E.M. for 6 animals in each group.

Treatment	Adrenals	Brain	Gut (total)	Heart
Pre-treatment control	0.22 ± 0.01	12.00 ± 0.46*	50.6 ± 1.1***	5.39 ± 0.23*
Vehicle control	0.24 ± 0.03	8.63 ± 0.57	41.1 ± 0.7	4.50 ± 0.08
IGF-I (104 µg/day)	0.25 ± 0.03	8.52 ± 0.32	44.5 ± 0.3*	4.46 ± 0.07
IGF-I (260 µg/day)	0.25 ± 0.02	8.51 ± 0.25	47.0 ± 0.6***	4.50 ± 0.11
IGF-II (104 µg/day)	0.22 ± 0.02	8.30 ± 0.82	41.2 ± 1.0	4.37 ± 0.09
IGF-II (260 µg/day)	0.20 ± 0.01	8.55 ± 0.38	42.9 ± 0.9	4.45 ± 0.11
IGF-II (650 µg/day)	0.28 ± 0.04	7.78 ± 0.54	45.2 ± 1.2*	4.43 ± 0.14

Treatment	Kidneys	Liver	Lungs	Spleen
Pre-treatment control	10.20 ± 0.15***	53.16 ± 1.14	6.66 ± 0.46	3.35 ± 0.20***
Vehicle control	9.17 ± 0.21	49.62 ± 1.17	5.92 ± 0.45	2.31 ± 0.12
IGF-I (104 µg/day)	10.25 ± 0.16**	49.64 ± 2.04	6.83 ± 0.45	2.73 ± 0.08
IGF-I (260 µg/day)	10.57 ± 0.23***	49.40 ± 1.07	6.61 ± 0.28	3.09 ± 0.12***
IGF-II (104 µg/day)	9.49 ± 0.16	47.74 ± 1.25	6.36 ± 0.13	2.40 ± 0.09
IGF-II (260 µg/day)	10.12 ± 0.16*	51.05 ± 1.34	6.32 ± 0.22	2.52 ± 0.09
IGF-II (650 µg/day)	10.98 ± 0.33***	50.87 ± 0.96	6.05 ± 0.17	2.84 ± 0.11**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 4.4 (continued):

Treatment	Thymus	Carcass	Head, tail and paws	Pelt
Pre-treatment control	2.66 ± 0.15	395 ± 9*	171 ± 4*	169 ± 3
Vehicle control	2.39 ± 0.06	466 ± 4	144 ± 3	156 ± 3
IGF-I (104 µg/day)	2.35 ± 0.07	455 ± 9	149 ± 2	155 ± 3
IGF-I (260 µg/day)	2.66 ± 0.07	444 ± 3	145 ± 4	156 ± 4
IGF-II (104 µg/day)	2.19 ± 0.13	466 ± 5	144 ± 5	160 ± 2
IGF-II (260 µg/day)	2.16 ± 0.15	451 ± 9	149 ± 4	159 ± 5
IGF-II (650 µg/day)	2.62 ± 0.04	455 ± 5	143 ± 5	157 ± 3

* $P < 0.05$.

is affecting the same set of organs as IGF-I but that IGF-II was less potent, generally requiring a dose of between 260 and 650 µg/day to achieve the same increases as obtained with 104 µg of IGF-I/day. The fractional weight of some organs was decreased in vehicle-treated animals compared with pre-treatment controls, which is a normal developmental pattern. In the tissues that were responsive to IGF treatment, the IGFs maintained the fractional weights of the tissues closer to those seen in the pre-treatment controls, apparently delaying the normal maturation changes.

The absolute weights of individual regions of the gut (Table 4.5) were also significantly increased by IGF treatment, as was total gut weight. Highly significant ($P < 0.001$) increases in the stomach and small intestine weight accompanied treatment with 260 µg of IGF-I/day or 650 µg of IGF-II/day. In each of these measurements the lower IGF-I dose also produced a significant effect. The caecum was less responsive to IGFs than the small intestine or stomach with only the higher IGF-I dose leading to a significant weight increase. There were no significant effects on colon weight and hence it was the least responsive of the gut tissues. Small intestine lengths were not altered by IGF infusion but the weight per unit length was significantly increased by the high dose of IGF-I (Table 4.6).

To determine whether the increase in feed conversion efficiency following IGF treatment may result in part from any changes in the absorptive capacity of the gut, the size of villi, crypts and muscularis lining the lumen of the jejunum was measured in four of the treatment groups (Table 4.7). The significant increase in villus height and muscularis width by 260 µg of IGF-I/day or 650 µg of IGF-II/day again reflects the pattern seen in this study of IGF-II being less potent than IGF-I, and would be consistent with the increased feed conversion efficiency being aided by an increased absorptive capacity of the gut. Interestingly, and in contrast to the other results obtained in this study, IGF-II was at least as potent as IGF-I

in its capacity to increase crypt depth in the jejunum. The villus/crypt ratio was not significantly altered by any treatment.

Water intakes (appendix A, Table A.1) were not altered by any treatment.

IGFs also had no significant effect on the fractional weight, length or weight per unit of length of femurs (appendix A, Table A.2).

Faecal nitrogen excretion as a percentage of nitrogen intake was not significantly changed by any treatment during the first or second weeks of treatment, nor over the entire treatment period (appendix A, Table A.3). This indicates that neither improved feed conversion efficiency nor the increase in the size of villi and crypts in the jejunum lumen were associated with a significant increase in the extraction of nitrogen from food in the gut.

Nitrogen retention did not differ significantly between treatment groups (appendix A, Table A.4). However, regression analysis of the data showed a significant effect of peptide dose on nitrogen retention over the 14 day treatment period.

The amount of muscle protein breakdown in the rat, as assessed by measuring the urinary 3-MH/creatinine excretion ratio, was also not affected significantly by any of the IGF treatments (appendix A, Table A.5).

No treatment by IGFs significantly changed the proportional content of protein, fat, water or residual material in the carcass from that of the vehicle control, but fat and water content were different in the carcasses from pre-treatment control animals (appendix A, Table A.6).

TABLE 4.5 Gut tissue weights (g). Values represent the mean ± S.E.M. for 6 animals in each group.

	Stomach	Small intestine	Caecum
Treatment			
Pre-treatment control	0.69 ± 0.01***	3.87 ± 0.10	0.39 ± 0.02
Vehicle control	0.82 ± 0.02	4.32 ± 0.11	0.45 ± 0.02
IGF-I (104 µg/day)	0.92 ± 0.03**	4.94 ± 0.12*	0.49 ± 0.03
IGF-I (260 µg/day)	0.97 ± 0.02***	5.39 ± 0.16***	0.51 ± 0.03*
IGF-II (104 µg/day)	0.82 ± 0.03	4.34 ± 0.17	0.46 ± 0.02
IGF-II (260 µg/day)	0.88 ± 0.02	4.67 ± 0.07	0.47 ± 0.03
IGF-II (650 µg/day)	0.99 ± 0.03***	5.17 ± 0.16***	0.50 ± 0.03

	Colon	Total gut
Treatment		
Pre-treatment control	0.65 ± 0.05	5.60 ± 0.14*
Vehicle control	0.71 ± 0.04	6.30 ± 0.14
IGF-I (104 µg/day)	0.79 ± 0.03	7.14 ± 0.17**
IGF-I (260 µg/day)	0.77 ± 0.02	7.64 ± 0.19***
IGF-II (104 µg/day)	0.64 ± 0.03	6.27 ± 0.21
IGF-II (260 µg/day)	0.74 ± 0.05	6.76 ± 0.15
IGF-II (650 µg/day)	0.79 ± 0.04	7.45 ± 0.21***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 4.6 Small intestine lengths (cm) and weights per unit length (g/cm). Weights (g) are included for comparison. Values represent the mean ± S.E.M. for 6 animals in each group.

Treatment	Weight	Length	Weight/length
Pre-treatment control	3.87 ± 0.10	69.8 ± 2.6	0.056 ± 0.002
Vehicle Control	4.32 ± 0.11	74.4 ± 2.2	0.058 ± 0.002
IGF-I (104 µg/day)	4.94 ± 0.12*	77.5 ± 1.8	0.064 ± 0.003
IGF-I (260 µg/day)	5.39 ± 0.16***	79.3 ± 2.9	0.069 ± 0.004*
IGF-II (104 µg/day)	4.34 ± 0.17	72.7 ± 3.1	0.060 ± 0.003
IGF-II (260 µg/day)	4.67 ± 0.07	74.5 ± 2.2	0.063 ± 0.002
IGF-II (650 µg/day)	5.17 ± 0.16***	78.3 ± 2.5	0.066 ± 0.003

* $P < 0.05$; *** $P < 0.001$.

TABLE 4.7 Morphological measurements (µm) taken from jejunum sections. Values represent the mean ± S.E.M. for 6 animals in each group.

	Villus height	Crypt depth	Villus/crypt ratio	Muscularis width
Treatment				
Vehicle control	538.0 ± 15.1	184.9 ± 3.8	2.91 ± 0.06	69.3 ± 4.4
IGF-I (260 µg/day)	609.0 ± 15.2*	201.9 ± 5.0*	3.02 ± 0.08	80.4 ± 2.5*
IGF-II (260 µg/day)	570.9 ± 23.2	207.1 ± 5.0**	2.76 ± 0.13	73.6 ± 3.0
IGF-II (650 µg/day)	601.4 ± 13.2*	204.4 ± 4.7**	2.94 ± 0.05	80.5 ± 2.3*

* $P < 0.05$; ** $P < 0.01$.

4.4 Discussion.

I have demonstrated that continuous infusion of IGF-II promotes the growth of young female rats. These findings also confirm and extend the findings of Schoenle *et al.* (1985), who used hypophysectomised rats, and found IGF-II to be less potent than IGF-I. Moreover, in both studies equivalent doses of either IGF give similar increases in circulating levels of the respective IGF. Additionally, it has been demonstrated that IGF-II infusion leads to a significant reduction in endogenous plasma IGF-I concentrations in normal intact rats.

In an earlier experiment, Tomas *et al.* (1993) showed that treatment of young female rats for 14 days with 278 µg of IGF-I/day significantly increased body weight gain, feed conversion efficiency, 14 day nitrogen retention, fractional weights of gut, kidney, spleen and thymus, and carcass water over the control. Carcass fat was significantly decreased. Although I attempted to mimic the IGF-I component of that study, including the use of female rats, I found that 260 µg of IGF-I/day led to similar trends in carcass composition, but the changes failed to reach statistical significance. The reason for the difference is unknown but may be due to variation in the rats. Nitrogen retention was related to IGF dose, but muscle protein breakdown, as assessed by creatinine corrected urinary 3-methylhistidine excretion, was not significantly altered. Therefore, increases in protein associated with increased body weight gain following IGF treatment are likely to be a result of increased protein synthesis and not decreased protein degradation.

Previous studies in which IGF-I has been administered to young or adult rats and mice has shown that the gut, kidneys, spleen and thymus consistently grow the most in response to this growth factor irrespective of whether the animal is normal or whether it has some form of growth-retardation or trauma (Guler *et al.* 1988; Skottner *et al.* 1989; Thissen *et al.* 1991; Pell & Bates *et al.* 1992; Read *et al.* 1992; Tomas *et al.* 1993). This study has confirmed that

IGF-I selectively increases the growth of those organs in the normal rat but additionally has shown that IGF-II also stimulates the same set of organs.

The lesser potency of IGF-II compared with IGF-I that has been observed *in vivo* also occurs *in vitro*. By using structural analogues of both IGF-I and IGF-II, Francis *et al.* (1993) have demonstrated that the type-2 IGF receptor, to which IGF-II but not IGF-I can bind, was a major contributing factor to the reduced biological potency of IGF-II *in vitro* by reducing the amount of IGF-II available for binding to the type-1 receptor. It is generally considered that most of the biological activities of IGF-II occur through binding to the type-1 IGF receptor (Czech *et al.* 1989).

A number of other factors may contribute to the relatively low potency of IGF-II. For example, the reduction in plasma IGF-I levels by IGF-II infusion would play a part, and the lesser ability of IGF-II infusion to induce total plasma IGFBP may also be relevant. Other studies have demonstrated a reciprocal relationship between concentrations of IGF-I and IGF-II in plasma including experiments with rats, humans and pigs (Daughaday *et al.* 1988; Owens *et al.* 1990; Wilson *et al.* 1989). In the study by Wilson *et al.* (1989) increased serum IGF-II was achieved by transplanting IGF-II-secreting tumours into immunodeficient rats, and this then led to reduced serum IGF-I levels. They demonstrated that the reduction in serum IGF-I was not associated with a reduction in liver IGF-I mRNA levels. Presumably it reflects a displacement of IGF-I by IGF-II on circulating IGFBPs.

The clearance of ^{125}I-IGF-II is faster than that of ^{125}I-IGF-I in young male rats (Ballard *et al.* 1991). In the present study steady state concentrations of IGF-I and IGF-II were increased to a similar extent by infusion of the same amount of each growth factor. This may reflect the altered IGFBP, and possibly receptor binding sites, of the IGFs in the circulation following chronic administration.

One of the findings of this study was that increases in growth resulting from treatment with either IGF-I or IGF-II were accompanied by parallel increases in feed conversion efficiency and the size of villi, crypts and muscularis lining the jejunum. This prompted an examination of whether the absorptive capacity of the gut was increased, thereby facilitating an increased feed conversion efficiency. Nitrogen absorption and the surface area of the duodenal mucosa have been shown to increase following infusion of IGF-I or LR^3IGF-I into normal female rats (Steeb *et al.* 1994). Also, treatment of neonatal rats with IGF-I has also been known to change the activity of brush border enzymes in the jejunum (Young *et al.* 1990), indicating that metabolic changes in the small intestine are possible. In the present experiment the percentage of ingested nitrogen excreted in faeces was measured, but there were no significant treatment effects, which indicated that nitrogen absorption via the gut also was not significantly changed. This does not rule out the possibility of an increase in the absorption of other components of food. Baumrucker *et al.* (1994) have shown that dietary rhIGF-I can influence the ability of the gut of neonatal calves to absorb sugars and deliver it to the circulation. It is feasible that IGFs in circulation could do the same. However, the data from the current rat study is consistent with an increase in feed conversion efficiency resulting from metabolic changes, leading to more efficient utilisation of absorbed nutrients.

The rat has been used here to aid our understanding of the *in vivo* actions of IGF-II even though it normally has negligible amounts of IGF-II in circulation. Because of the lack of IGF-II, results obtained in this study may not be representative of effects that may be seen in an animal that has substantial concentrations of IGF-I and IGF-II in blood. However, it can be argued that the lack of IGF-II is advantageous. That is, a lack of circulating endogenous blood IGF-II may cause the rat to be more responsive to IGF-II infusion than animals that do have substantial circulating endogenous IGF-II, thereby making detection of IGF-II induced effects easier.

In this study I found only one aspect of growth for which IGF-II is as potent as IGF-I, the increase in the depth of jejunal crypts (see Table 3). Crypts are a site of production of cells which replace those continually lost by the villi, and so increased crypt depth probably reflects an increase in the number of crypt cells and hence an increase in the availability of cells for growth of the villi. In normal rats, IGF-I infusion can increase the number of cells in the crypt compartment of the duodenum epithelium, thereby apparently increasing crypt depth, and this parallels the increase in the height of villi (Steeb *et al.* 1994). The ratio between villus height and crypt depth has not been significantly altered by either IGF-I or IGF-II treatment in this study either. It is not surprising that the crypts should be responsive to IGF treatment since the tissue contains both type-1 and -2 IGF receptors, which it has in greater abundance than in the villi (Laburthe *et al.* 1988). The question remains as to how IGF-II has a higher than usual potency in stimulating growth in the crypts. Since IGF-II, but not IGF-I, binds to the type-2 receptor, differences in the concentration or even function of this receptor relative to other rat tissues could conceivably be responsible for the increased potency. IGFBPs with high affinity for IGF-II, if produced in the vicinity of the crypts, might also potentiate IGF-II action.

There are likely to be other examples where IGF-II is either more potent than IGF-I or produces unique effects. In this context IGF-II, but not IGF-I, has been shown to increase serum osteocalcin levels in newborn lambs (Coxam, *et al.* 1992), and there is evidence of specific roles for IGF-II in immunity (Geenen *et al.* 1993), muscle development (Florini *et al.* 1991b) and nerve growth (Near *et al.* 1992).

In summary, I have shown for the first time that IGF-II can promote the growth of normal postnatal rats and, by comparing the effects of several doses of IGFs, I have shown that IGF-II has a lower overall potency than IGF-I. Further studies are now required to determine whether such effects will occur in postnatal animals that have substantial concentrations of IGF-II in circulation.

CHAPTER 5.

IGF-I OR LONG R^3 IGF-I INFUSION CAN STIMULATE ORGAN GROWTH IN THE GUINEA PIG.

5.1 Introduction.

Chronic administration of rhIGF-I or rhIGF-II to rats promotes the growth of hypophysectomised (Schoenle *et al.* 1982; Schoenle *et al.* 1985) and normal animals (Hizuka *et al.* 1986; Tomas *et al.* 1993; also see chapter 4), and in most respects IGF-I is more potent than IGF-II. Thus IGF-II is acting like a weak form of IGF-I in an animal lacking circulating IGF-II. It is not known whether animals with substantial circulating concentrations of both IGFs during adulthood will grow in response to chronic IGF-II treatment in a manner similar to rats.

The guinea pig, because of its relatively small size, is often a useful model for metabolic studies, a situation that may be more so with IGF research because guinea pig IGF-I (Bell *et al.* 1990) and IGF-II (Levinovitz *et al.* 1992) are identical to human IGF-I and IGF-II respectively. Also, both IGF-I and IGF-II circulate in the blood of the adult guinea pig (Daughaday *et al.* 1986). Unlike rats and mice, but like humans, IGF-II continues to be expressed by the liver in the adult (Levinovitz *et al.* 1992). As in other species, blood IGF-I concentration, but not IGF-II concentration, is lowered by undernutrition in the adult guinea pig (Palka *et al.* 1989; Dwyer & Stickland 1992). The guinea pig releases GH into circulation from the pituitary in an episodic fashion like most animals (Gabrielsson *et al.* 1990), yet hypophysectomy does not retard growth substantially (Mitchell *et al.* 1954; Clayton & Worden 1960) and the guinea pig does not grow in response to GH (Knobil & Greep 1959). Also, passive immunoneutralisation of IGF-I in the guinea pig fails to slow growth (Kerr *et al.* 1990). These findings suggest a less active role for the GH-IGF-I axis in promoting the growth of the guinea pig, even though both IGF-I and IGF-II can stimulate neurite outgrowth of cultured neurons derived from neonatal guinea pigs (Mulholland *et al.* 1992). Also, as in many other animals, the IGFBPs appear to regulate IGF actions in guinea pigs (Peterkofsky *et al.* 1991 & 1994; Gosiewska *et al.* 1994).

This study was designed to determine whether IGF-I or IGF-II can promote the growth of guinea pigs, and if so, whether IGF-II is more or less potent than IGF-I. LR^3IGF-I, a potent analogue of IGF-I that binds poorly to IGFBPs (Francis *et al.* 1992), has also been included in the comparison to provide information on the role of IGFBPs in IGF action.

5.2 Materials and methods.

5.2.1 Peptides.

rhIGF-I (lot EJD-IO1) and rhIGF-II (lot JJD-O01), as well as LongR^{3}IGF-I (referred to as LR^{3}IGF-I) (lot BJB-AO1), were supplied by GroPep Pty. Ltd., Adelaide, SA, Australia. LR^{3}IGF-I is an analogue of rhIGF-I that was derived by substitution of the glutamic acid at position 3 with arginine and the addition of 13 amino acids to the N-terminus (Francis *et al.* 1992).

5.2.2 Animals and animal maintenance.

Female guinea pigs, of the IMVS coloured strain, were obtained from the Gilles Plains Animal Resource Centre, Gilles Plains, SA, Australia. Guinea pigs were kept in individual metabolism cages during the experiment in a room maintained at 25°C with a 12 hour light/12 hour dark cycle. Animals were fed *ad libitum* on a standard animal-care facility pellet diet for guinea pigs (Rabbit Pellets, Milling Industries Stock Feeds, Murray Bridge, SA, Australia) which had been finely milled, and were allowed free access to water which contained 0.5 mg of vitamin C/ml (Golden Life, Dee Why, NSW, Australia). In each 24 hour period, water and food intakes were measured, animals were weighed and urine and faeces were collected.

5.2.3 Experimental design.

Animals were placed in individual metabolism cages and given access to the diet of the experiment four days prior to the start of IGF administration. On the day that treatment began, guinea pigs were randomised into treatment groups with average body weights of approximately 350 g. Osmotic pumps were implanted into guinea pigs, allowing continuous infusion of vehicle or IGFs into the animals for 7 days (see section 5.2.4). After 7 days of

treatment, guinea pigs were anaesthetised with halothane and then beheaded. Blood and tissues were collected for analysis (see section 5.2.5).

The study was done in 4 stages, with 2 animals in each treatment group per stage. Therefore there was a total of 8 animals per treatment group.

This study was approved by the Animal Ethics Committee of the Women's and Children's Hospital, North Adelaide, SA, Australia.

5.2.4 Filling and insertion of osmotic pumps.

Alzet model 2001 osmotic pumps (Alza, Palo Alto, CA, USA) were filled with one of the following solutions: 0.1M acetic acid (vehicle), 4.72 mg of IGF-I, IGF-II or LR^3IGF-I/ml, or 14.2 mg of IGF-I or IGF-II/ml. The pump delivery rate was 1.06 µl/hr. Hence the animals were infused with IGFs at rates of 0, 120 or 360 µg/day.

Pumps were inserted subcutaneously into the upper dorsal region of the guinea pig using the same method as described for rats (see section 4.2.4).

5.2.5 Blood and tissue collections.

The details are essentially the same as described in section 4.2.5 with the exception that the combined head and feet weights (there was no tail) included the weight of the brain. The thymus was not weighed because it was not detectable in guinea pigs of this age. Blood was collected for obtaining plasma when animals were killed following 7 days of treatment. I wish to acknowledge the assistance of Kerry Wright (CSIRO) and technicians from CHRI during the collection and weighing of tissues. Gut measurements were carried out by the CHRI technicians. Carcass fat content was later measured by Kerry Wright.

5.2.6 Plasma IGF and IGFBP measurements, and Western ligand blotting.

IGFs and IGFBPs, in plasma of individual animals, were collected as separate pools following gel-filtration chromatography (see section 4.2.11). The pools were then used to determine the plasma concentrations of IGFs or IGFBPs by RIA according to the methods described in sections 4.2.12 and 4.2.13. For the IGF-II RIA, the same mouse monoclonal antibody directed against rat IGF-II, that was used in the study of chapter 4, has again been used, but in this case it was a generous gift from Dr. Nishikawa of Kanaza Medical Centre, Ishikawa, Japan. The plasma LR^3IGF-I concentration was measured in the same manner, and hence in the same neutralised chromatographic elution pools, as those described for the IGF-I and IGF-II RIAs. The LR^3IGF-I RIA utilised affinity purified rabbit anti-LR^3IGF-I antibody (lot EJD-PAB1) supplied by GroPep Pty. Ltd., Adelaide, SA, Australia.), ^{125}I-LR^3IGF-I as tracer (a gift from Dr. Phil Owens) and LR^3IGF-I for the standard curve. The LR^3IGF-I RIA has been validated by GroPep Pty. Ltd. Total IGF refers to the sum of the IGF-I and IGF-II concentrations, or in the case of the LR^3IGF-I-treated animals it refers to IGF-I plus IGF-II plus LR^3IGF-I concentrations, obtained from the respective RIAs.

Western ligand blotting of treatment pools of plasma was performed as described in section 4.2.14.

5.2.7. Statistics.

Analyses were carried out as previously described (see section 4.2.15). Results are expressed as the mean ± S.E.M. for 8 animals in each treatment group.

5.3 Results.

Some data for which there were no significant effects, most of which is described in the final paragraph, can be found in figures and tables contained within appendix B.

To explain any differences in the effects of IGF treatment on the growth of guinea pigs and rats, animals with and without substantial IGF-II in blood respectively, a knowledge of the effects on the concentrations of IGFs and IGFBPs in plasma is required. Therefore plasma, collected from guinea pigs after 7 days of treatment, was passed through a gel-filtration column under acid conditions, to separate IGFs and IGFBPs. Analyses of these eluates showed that treatment with IGF-I at 120 or 360 μg/day led to mean increases in plasma IGF-I concentration of 149 and 293 μg/l respectively over the concentration of vehicle-infused animals (Figure 5.1a). However, the highest dose of IGF-II, 360 μg/day, led to an increase in plasma IGF-II concentration of 638 μg/l (Figure 5.1b). In keeping with a rapid clearance of LR^3IGF-I from blood, LR^3IGF-I was detectable at low concentrations (37 ± 4 μg/l) in plasma of guinea pigs treated with this analogue. Treatment with 360 μg of IGF-II/day significantly reduced plasma IGF-I concentration (Figure 5.1a), and IGF-I treatment reduced plasma IGF-II concentrations (Figure 5.1b). However, LR^3IGF-I reduced both plasma IGF-I and IGF-II concentrations and was more potent in this activity than either IGF-I or IGF-II (Figure 5.1a; 5.1b). As a consequence of these changes, there was little difference in total IGF concentration relative to vehicle-infused animals following IGF-I treatment, although total IGF was increased following treatment with the high dose of IGF-II (Figure 5.1c). LR^3IGF-I substantially reduced total IGF concentrations ($P < 0.001$).

The relative concentrations of IGFBPs in neutralised acid-column pools from individual guinea pigs were measured by their capacity to sequester ^{125}I-IGFs from the antibody used in the RIAs for IGF-I and IGF-II. By this technique, it was shown that total plasma IGFBP was only affected by treatment with LR^3IGF-I, which reduced IGFBP

concentrations relative to vehicle-treated animals (Figure 5.2a; 5.2b). This conclusion is supported by Western ligand blot (Figure 5.3), which demonstrates a substantial reduction in the IGFBP-3 doublet at 40 to 50 kDa in a plasma pool from LR^3IGF-I treated animals. LR^3IGF-I also led to an increase in a 28 kDa binding protein.

Guinea pigs lost an average of 24 g in weight during the 4 days of acclimatisation in metabolic cages, although weights were more stable in the 2 days immediately prior to pump implantation. As a result, animals in all treatment groups were in a catch-up growth phase throughout much of the 7 day treatment period.

Body weight gain (Table 5.1; see appendix B, Figure B.1, for a diagram showing daily weights) was not significantly increased over 7 days by any treatment. Feed intake, feed conversion efficiency and water intake were also not affected (Table 5.1). The fractional weight of adrenals, gut (stomach to colon), kidneys and spleen were significantly increased by LR^3IGF-I and the fractional weight of adrenals was also increased by 360 μg of IGF-I/day (Table 5.2). IGF-II did not have any effect on these organs. There were no effects of treatment on the fractional weights of heart, liver, lungs, carcass, head plus paws or pelt (Table 5.2). Of the individual regions of the gut that were analysed, a significant difference in weight from that of the vehicle-infused animals was observed only for the stomach of LR^3IGF-I treated animals, although the trend was for an effect of LR^3IGF-I on most regions of the gut, as evidenced by the significant change in total gut weight (Table 5.3).

The fractional weight, length and weight per unit length of femurs was unaffected by any treatment (appendix B, Table B.1) as was the length and weight per unit length of the small intestine (appendix B, Table B.2). The proportion of fat and water in the carcass was not changed by any treatment, and hence the proportion of the remaining constituents, which includes protein, was also not changed (appendix B, Table B.3).

FIGURE 5.1 (a) Plasma IGF-I, (b) plasma IGF-II and (c) plasma total IGF (IGF-I plus IGF-II, or, in the case of LR^3IGF-I treated animals, IGF-I plus IGF-II plus LR^3IGF-I) concentrations in guinea pigs following treatment with: vehicle (open bars), IGF-I (solid bars), IGF-II (hatched bars) and LR^3IGF-I (cross-hatched bars) at the indicated doses. Results are expressed as the mean ± S.E.M. for 8 animals in each group. Significance from the vehicle group is denoted by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

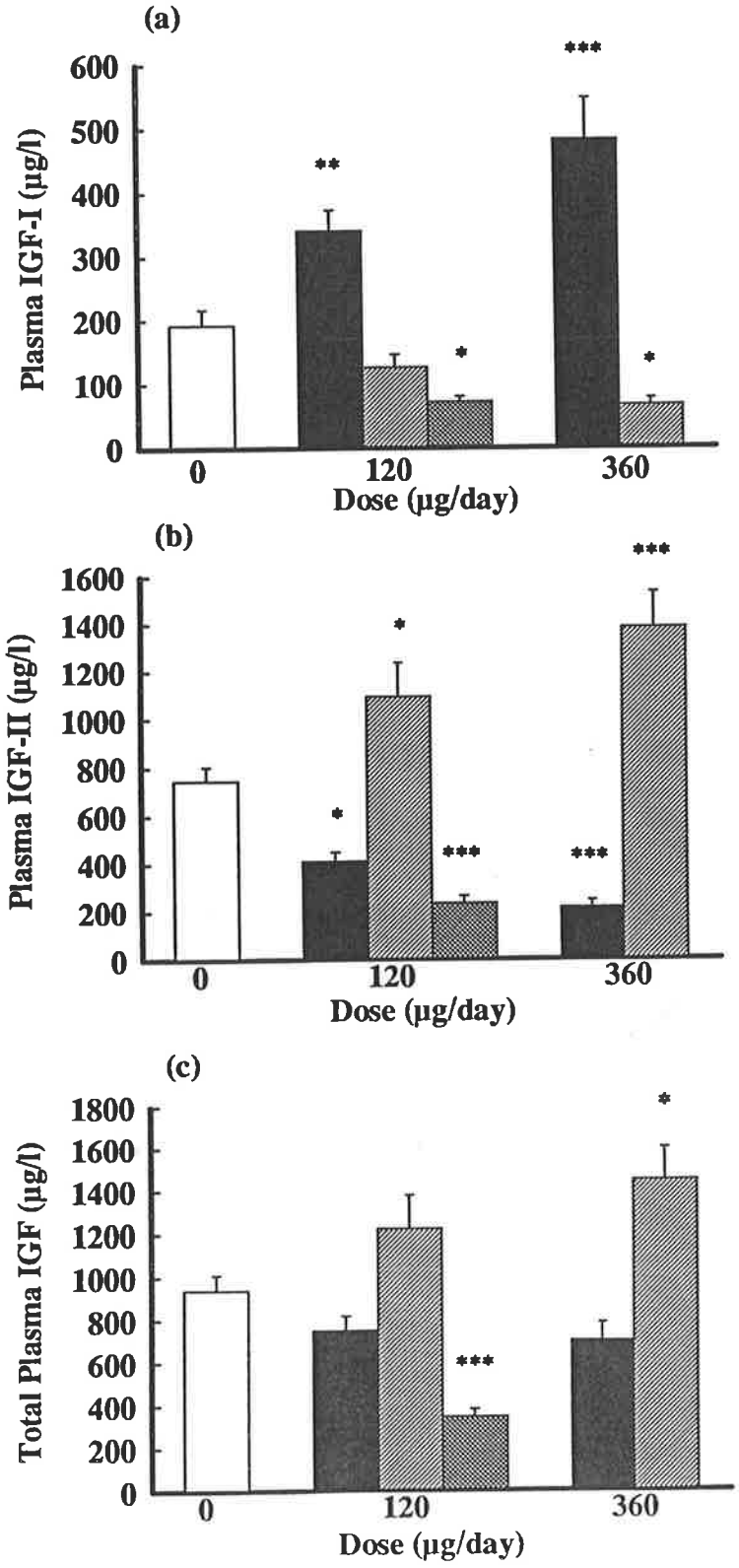
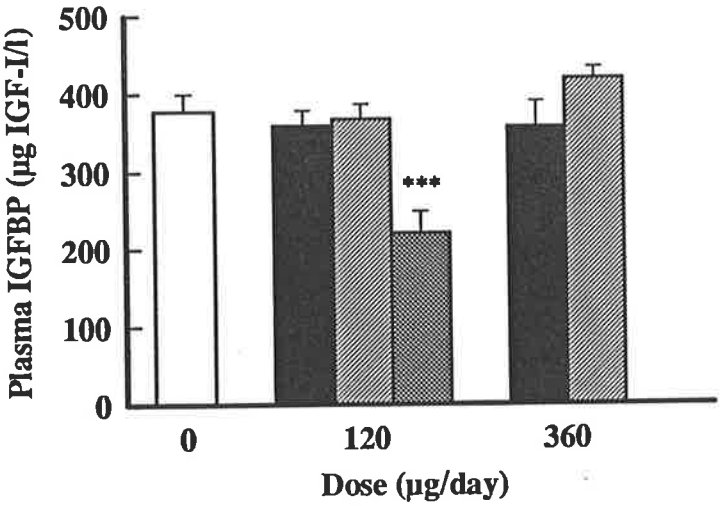

(a)
Plasma IGF-I (µg/l)
600
500
400
300
200
100
0
**
***
*
*
0
120
360
Dose (µg/day)
(b)
Plasma IGF-II (µg/l)
1600
1400
1200
1000
800
600
400
200
0
*
*
***
***
***
0
120
360
Dose (µg/day)
(c)
Total Plasma IGF (µg/l)
1800
1600
1400
1200
1000
800
600
400
200
0
***
*
0
120
360
Dose (µg/day)

FIGURE 5.2 Plasma IGFBP concentrations in guinea pigs following treatment with: vehicle (open bars), IGF-I (solid bars), IGF-II (hatched bars) and LR^3IGF-I (cross-hatched bars) at the indicated doses. Panel (a) represents the interference of IGFBPs in the IGF-I RIA and panel (b) represents the interference of IGFBPs in the IGF-II RIA. Results are expressed as the mean ± S.E.M. for 8 animals in each group. Significance from the vehicle group is denoted by *** $P < 0.001$.

(a)


Plasma IGFBP (µg IGF-I/l)
500
400
300
200
100
0
***
0
120
360
Dose (µg/day)

(b)

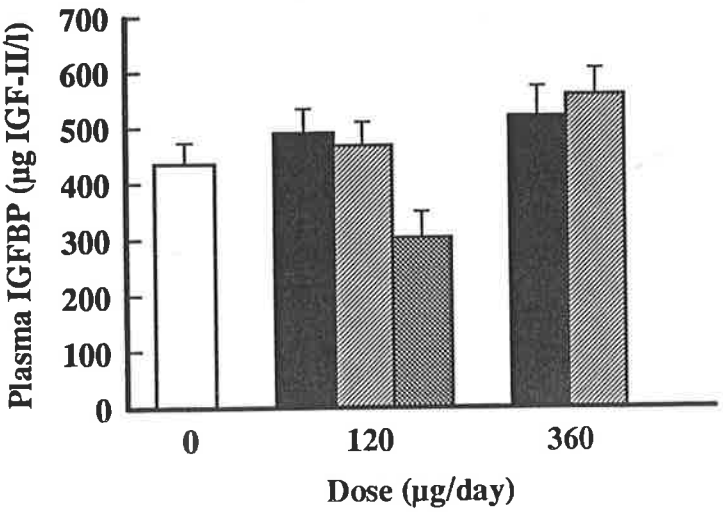

Plasma IGFBP (µg IGF-II/l)
700
600
500
400
300
200
100
0
0
120
360
Dose (µg/day)

FIGURE 5.3 Plasma IGFBP profiles as determined by Western ligand blotting of guinea pig plasma using ^{125}I-IGF-I as probe. Lane 1 contains size markers (sizes (in kDa) are as indicated). Other lanes were loaded with treatment pools of plasma from animals treated with vehicle (lane 2), 120 µg of IGF-I/day (lane 3), 360 µg of IGF-I/day (lane 4), 120 µg of IGF-II/day (lane 5), 360 µg of IGF-II/day (lane 6) or 120 µg of LR^{3}IGF-I/day (lane 7).

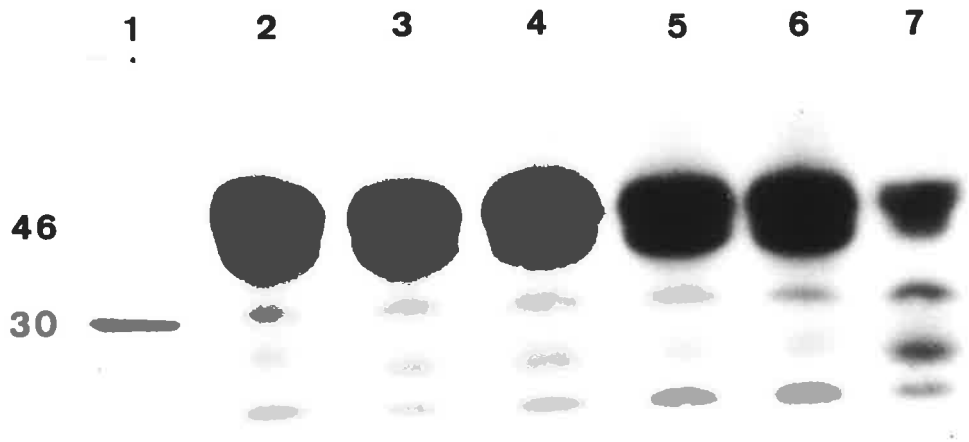

1
2
3
4
5
6
7
46
30

TABLE 5.1 Body weight gains (g), feed intakes (g), feed conversion efficiencies (weight gain (g)/feed intake (g)) and water intakes (mls) during 7 days of treatment. The values represent the mean ± S.E.M. for 8 animals in each group.

	Body weight gain	Feed intake	Feed conversion efficiency	Water intake
Treatment				
Vehicle control	16.4 ± 3.8	162 ± 5	0.099 ± 0.022	378 ± 25
IGF-I (120 µg/day)	16.3 ± 6.9	170 ± 4	0.095 ± 0.038	651 ± 144
IGF-I (360 µg/day)	18.0 ± 6.5	154 ± 11	0.101 ± 0.031	468 ± 61
IGF-II (120 µg/day)	25.6 ± 11.0	170 ± 8	0.138 ± 0.059	490 ± 62
IGF-II (360 µg/day)	23.6 ± 6.1	167 ± 5	0.142 ± 0.038	549 ± 82
LR^3IGF-I (120 µg/day)	20.4 ± 4.4	162 ± 8	0.122 ± 0.024	532 ± 90

TABLE 5.2 Organ and tissue weights, expressed as a fraction of body weight (g/kg body weight). The values represent the mean ± S.E.M. for 8 animals in each group.

	Adrenals	Gut (total)	Heart	Kidneys
Treatment				
Vehicle control	0.54 ± 0.02	56.14 ± 2.02	3.97 ± 0.10	9.21 ± 0.21
IGF-I (120 µg/day)	0.62 ± 0.03	61.19 ± 1.77	4.08 ± 0.21	9.44 ± 0.31
IGF-I (360 µg/day)	0.64 ± 0.03*	59.79 ± 1.20	3.98 ± 0.14	10.04 ± 0.26
IGF-II (120 µg/day)	0.57 ± 0.04	58.34 ± 1.16	3.98 ± 0.12	9.13 ± 0.32
IGF-II (360 µg/day)	0.58 ± 0.02	59.23 ± 1.64	4.11 ± 0.22	9.14 ± 0.24
LR^3IGF-I (120 µg/day)	0.68 ± 0.02**	63.50 ± 1.72**	4.38 ± 0.09	11.20 ± 0.25***

	Liver	Lungs	Spleen	Carcass
Treatment				
Vehicle control	34.18 ± 1.62	7.07 ± 0.19	1.70 ± 0.10	376 ± 9
IGF-I (120 µg/day)	35.04 ± 0.87	7.68 ± 0.32	1.64 ± 0.06	374 ± 9
IGF-I (360 µg/day)	32.08 ± 0.96	7.45 ± 0.28	1.99 ± 0.08	371 ± 4
IGF-II (120 µg/day)	35.38 ± 2.14	7.11 ± 0.33	1.78 ± 0.08	374 ± 11
IGF-II (360 µg/day)	37.83 ± 1.58	7.19 ± 0.38	1.77 ± 0.10	368 ± 12
LR^3IGF-I (120 µg/day)	29.43 ± 1.34	7.93 ± 0.58	2.86 ± 0.31***	363 ± 5

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 5.2 (continued):

	Head (with brain) plus paws.	Pelt
Treatment		
Vehicle control	166 ± 3	147 ± 6
IGF-I (120 µg/day)	166 ± 3	140 ± 4
IGF-I (360 µg/day)	165 ± 3	146 ± 5
IGF-II (120 µg/day)	166 ± 4	146 ± 7
IGF-II (360 µg/day)	165 ± 2	144 ± 5
LR^3IGF-I (120 µg/day)	170 ± 3	147 ± 7

TABLE 5.3 Gut tissue weights (g). The values represent the mean ± S.E.M. for 8 animals in each group.

Treatment	Stomach	Small intestine	Caecum	Colon	Total gut
Vehicle control	2.63 ± 0.08	8.56 ± 0.32	4.96 ± 0.18	4.47 ± 0.09	20.61 ± 0.39
IGF-I (120 µg/day)	2.72 ± 0.11	8.83 ± 0.23	5.47 ± 0.25	4.88 ± 0.22	21.89 ± 0.63
IGF-I (360 µg/day)	2.87 ± 0.03	9.08 ± 0.29	5.29 ± 0.26	4.73 ± 0.21	21.96 ± 0.68
IGF-I (120 µg/day)	2.72 ± 0.09	8.97 ± 0.12	5.29 ± 0.24	4.73 ± 0.19	21.71 ± 0.45
IGF-II (360 µg/day)	2.74 ± 0.07	8.87 ± 0.21	5.33 ± 0.34	4.68 ± 0.11	21.62 ± 0.43
LR^3IGF-I (120 µg/day)	3.01 ± 0.09**	9.43 ± 0.32	5.49 ± 0.22	5.11 ± 0.31	23.05 ± 0.78*

* $P < 0.05$; ** $P < 0.01$.

5.4 Discussion.

Studies in normal young female rats have shown that IGF-I or IGF-II infusion can increase weight gain, feed intake, feed conversion efficiency and the fractional weights of the gut, kidneys, spleen and thymus, with IGF-I being more potent than IGF-II (Tomas *et al.* 1993; also see chapter 4). Despite their potency differences, the same doses of IGF-I or IGF-II result in similar increases in total plasma IGF concentration. Plasma IGFBP concentrations are increased by both peptides. Several differences to these responses to IGF-I or IGF-II have been noted in the guinea pig. Firstly, the only aspect of growth that was affected was an increase in the fractional weight of adrenals following treatment with 360 μg of IGF-I/day (Table 5.1). Adrenals are increased in a transgenic mouse model in which blood IGF-II concentrations are raised (Wolf *et al.* 1994) and growth of the adrenals in response to IGF treatment has been observed previously in neonatal rats (Glasscock *et al.*1992), although not when normal growing rats are treated with IGF-I (see Tomas *et al.* 1993 and chapter 4). Another difference to the rat is that IGF-I infusion did not result in an increase in total plasma IGF concentration, whereas IGF-II infusion did. Furthermore, plasma IGFBP concentrations were not increased by these treatments in the guinea pig.

Administration of IGF-I to guinea pigs results in an increase in plasma IGF-I concentrations and a decrease in plasma IGF-II concentrations presumably because the infused IGF-I displaces endogenous IGFs from their binding proteins. This response would also explain why IGF-I failed to increase total plasma IGF concentrations. Similarly, IGF-II treatment resulted in a reduction in plasma IGF-I concentration. In this case, the rise in plasma IGF-II was greater than the fall in plasma IGF-I, resulting in an increase in total IGF concentrations. The reciprocal relationship between plasma IGF-I and IGF-II observed in the guinea pig has also been noted in other species with circulating IGF-II. These include humans, pigs and sheep (Guler *et al.* 1989b; Owens *et al.* 1990; Gluckman & Ambler 1993).

One possible reason for not finding the effects on growth in the guinea pig that have been noted previously in the rat is that the doses used were lower when expressed relative to body weight. Significant body weight gain in the rat required doses of at least 2.6 or 6.5 mg/kg/day of IGF-I or IGF-II respectively (see chapter 4). The highest dose administered to the guinea pig, 360 μg/day, is approximately 1 mg/kg/day. At such a dose rate in the rat, there was no significant effect on body weight gain by either IGF-I or IGF-II, although IGF-I did increase the fractional weights of the gut and kidneys. Consistent with the notion that higher doses of IGF-I or IGF-II may be needed to stimulate growth of the guinea pig, is the fact that the potent analogue LR^3IGF-I was able to increase the fractional weights of a number of organs at a dose of 120 μg/day.

The reduction in plasma IGF-I, IGF-II and IGFBP-3 concentrations that followed LR^3IGF-I infusion may contribute to its inability to promote body weight gain. These effects are similar to those occurring in pigs (Walton *et al.* 1994) but unlike those seen in female rats (Tomas *et al.* 1993a) and those treated with dexamethasone (Tomas *et al.* 1992). A fall in IGFBP-3 has also been reported in humans following IGF-I treatment (Baxter *et al.* 1993). Such an effect will reduce the capacity of plasma to carry IGFs, and could explain why the IGF concentrations are decreased by LR^3IGF-I treatment in guinea pigs. A possible mechanism responsible for the reduction in the plasma concentration of IGFBP-3 is a feed-back inhibition of GH production and release from the pituitary (Tannenbaum *et al.* 1983). If this mechanism does explain the reduction in IGFBP-3 in guinea pigs or other species, it is difficult to understand why plasma IGFBP-3 concentration is not also decreased by IGF-I treatment in rats (eg. see chapter 4).

The ability of LR^3IGF-I to stimulate growth of certain organs but not overall body growth in guinea pigs indicates a differential sensitivity of body tissues to the analogue. Since LR^3IGF-I binds poorly to IGFBPs, the response might be a consequence of free IGF being

required for growth of those organs, with IGFBPs largely preventing a similar response to IGF-I or IGF-II.

Although total IGFBP concentrations, and especially IGFBP-3 concentrations, are shown to have decreased by LR^{3}IGF-I treatment when they are assessed by Western ligand blotting or by interference in the IGF RIAs, it cannot be certain that IGFBP-3 concentrations have decreased. The ability of ^{125}I-IGFs to bind plasma IGFBPs during pregnancy has been shown to decrease substantially due to modification of the IGFBPs, thus giving the appearance of a fall in concentrations by Western ligand blotting (Gargosky *et al.* 1990; Suikkari & Baxter 1991). Therefore IGFBPs may simply have been modified by LR^{3}IGF-I treatment in the guinea pig. Nevertheless, even such a modification is likely to affect IGF actions in some way.

Western ligand blotting indicated that there was a clear increase in the concentration of an approximately 28 kDa IGFBP, and a possible increase in the concentration of an approximately 32 kDa IGFBP, following LR^{3}IGF-I administration. These may represent the 29 kDa and 34 kDa IGFBPs which have been reported to increase in concentration in the blood of guinea pigs which have scurvy or which have been fasted (Peterkofsky *et al.* 1991, 1994; Gosiewska *et al.* 1994). These IGFBPs were identified as IGFBP-1 and IGFBP-2 respectively. In the latter guinea pig studies, the concentrations of IGFBP-1 were increased compared to normal animals and appeared to be responsible for the inhibition of collagen gene expression in connective tissues by inhibiting IGF-I action. In the current study, I have been able to increase the growth of some guinea pig organs with LR^{3}IGF-I, an analogue which has poor affinity for IGFBPs (Francis *et al.* 1992). This growth occurred despite an increase in the concentration of what is probably IGFBP-1. Thus the growth stimulation which I have observed in the guinea pig is probably related to the ability of the analogue to reach tissue receptors without being sequestered by the IGFBPs.

Guinea pigs are not as suited to metabolic cages as rats. Guinea pigs have a timid nature and they have responded to placement in individual cages by going off their food and water for one or two days. Gabrielsson *et al.* (1990) have reported that guinea pigs reacted in a similar way to metabolic cages in their study. Hence the animals lost some weight during the 4 days of acclimatisation, and this probably resulted in the guinea pigs being in some form of catch-up growth during IGF infusions. A longer acclimatisation period in metabolic cages may minimise this effect in future studies.

This study has shown that the guinea pig is likely to prove useful as an alternative small animal model to the rat in defining the role of IGFs in the regulation of growth and metabolism. We have identified some differences between the two species which contribute to our understanding of that role. Further studies are required to gain a deeper understanding of these differences.

CHAPTER 6.

THE EFFECTS OF HIGH DOSES OF IGF-I, LONG R^3 IGF-I, IGF-II, OR A COMBINATION OF IGF-I AND IGF-II ON THE GROWTH OF THE GUINEA PIG.

6.1 Introduction.

In chapter 5 of this thesis, the guinea pig was used to study the effects of IGFs in a postnatal animal which has both IGFs in circulation. In that study, treatment with IGF-I, IGF-II or LR^3IGF-I was unable to significantly increase body weight gain or a number of other parameters, but the effects on organ growth indicated that guinea pigs are responsive to IGF treatment and suggested that higher doses of IGFs may need to be administered to effect further changes.

IGF-I and IGF-II have been reported to act synergistically in stimulating cell growth *in vitro* (Conover *et al.* 1994). In an experiment using Snell dwarf mice, an animal that lacks substantial circulating IGF-II, daily co-injection of IGF-I and IGF-II stimulated growth, but there was no obvious synergism between the two growth factors (van Buul-Offers *et al.* 1994). It is possible that IGF-I and IGF-II are more likely to exert synergistic effects in an animal where both IGFs are already present in blood, although no such effects were reported following short-term co-infusion of the IGFs into castrated lambs (Koea *et al.* 1992a).

In the current study IGF-I, IGF-II or LR^3IGF-I were infused into guinea pigs at doses which were double those of the previous experiment, in order to establish the effects of IGFs in these animals and to more completely understand any differences in the actions of IGFs between guinea pigs and rats. The treatment period was also increased to 14 days, and the time given to animals to acclimatise themselves to metabolic cages was increased to 7 days. Also, IGF-I and IGF-II were co-infused into guinea pigs to determine whether IGF-I and IGF-II can act synergistically *in vivo*.

6.2 Materials and methods.

The materials and methods are essentially the same as in chapter 5 but differ as described below. Those who assisted me in the study of chapter 5 again carried out the same tasks in this study.

6.2.1 Peptides.

Lot numbers of peptides purchased from GroPep Pty. Ltd. were as follows: rhIGF-I (EJE-102), rhIGF-II (JJD-O01) and LR^3IGF-I (BJB-A03).

6.2.2 Experimental design.

Details are similar to those for chapter 5, but differ as follows. Female guinea pigs were placed in individual metabolism cages 7 days prior to the start of treatment and were then infused continuously for 14 days. On the day that treatment started, animals were randomised into treatment groups with average body weights of about 370 g. The study was done in 3 stages, with 2 animals from each treatment group per stage. There was a total of 6 animals per treatment group. Animals were killed at the end of the 14 days of treatment, at which time blood and tissues were collected for analysis.

6.2.3 Osmotic pump filling.

Alzet model 2002 osmotic pumps (Alza, Palo Alto, CA, U.S.A.) were filled with one of the following: 0.1M acetic acid (vehicle), 19.6 mg of LR^3IGF-I/ml, 58.8 mg of IGF-I or IGF-II/ml, or a mixture of IGF-I and IGF-II consisting of 29.4 mg of IGF-I/ml plus 29.4 mg of IGF-II/ml (a total of 58.8 mg/ml). The mean delivery rate of the pumps was 0.51 µl/hr. The animals were therefore infused with IGFs at rates of 0, 240 (for LR^3IGF-I) or 720 µg/day.

6.2.4 Plasma glucose and urea measurements.

Plasma glucose and urea concentrations in plasma from individual animals were measured in duplicate using a COBAS MIRA automated analyser (Roche Diagnostic Systems), measurements which were kindly carried out by the laboratory of Dr. Julie Owens of the Department of Obstetrics and Gynaecology, the School of Medicine, Adelaide University, Adelaide, SA, Australia.

6.2.5 Statistics.

Values are presented as the mean ± S.E.M. for 6 animals in each treatment group, except that the mean of the values from the LR^3IGF-I treatment group was calculated from the 3 animals which survived a full 14 days of treatment. Results from animals infused with LR^3IGF-I were not included in statistical analyses. Significance values shown in figures and tables were derived by ANOVA followed by Dunnett's test. Significance was calculated by, and denoted in, the same manner as described in section 4.2.15.

6.3 Results.

As in previous chapters some information (where indicated in the text) has been placed in an appendix (C).

Three guinea pigs treated with 240 µg of LR^3IGF-I/day, one in each stage of the experiment, developed physical signs suggestive of hypoglycaemia mid-way through the treatment period and were consequently killed before 14 days of treatment had been completed. These signs included tremors and periods of malaise or unconsciousness. Therefore, data from the LR^3IGF-I treatment group was not included with data from the other groups during statistical analyses even though results from the surviving guinea pigs of this group have been displayed along with the rest of the data in figures and tables.

Administration of 720 µg of IGF-I/day increased the mean IGF-I concentration to 646 µg/l in plasma collected at the end of 14 days of treatment, a value which is 458 µg/l greater than in plasma derived from animals treated with vehicle (Figure 6.1a). IGF-II concentration was significantly decreased, relative to that of the vehicle group, by 389 µg/l following IGF-I treatment (Figure 6.1b). The equivalent dose of IGF-II increased the mean plasma IGF-II concentration by 235 µg/l (Figure 6.1b) and lowered the mean IGF-I concentration by 91 µg/l (Figure 6.1a). The combination of 360 µg of IGF-I plus 360 µg of IGF-II/day increased plasma IGF-I by 214 µg/l, approximately half of the increase produced by 720 µg of IGF-I/day of IGF-I (Figure 6.1a). The total plasma IGF concentration did not differ significantly between treatment groups (Figure 6.1c). The mean concentrations of IGF-I, IGF-II and total IGF in the plasma of guinea pigs that survived 14 days of LR^3IGF-I treatment were lower than the respective mean concentrations in vehicle treated animals (Figure 6.1), although this was not tested statistically. These concentrations were also low in the animals that had to be killed prematurely (Table 6.1). LR^3IGF-I concentrations were very low in guinea pigs treated with the analogue (Table 6.1), as they were in the previous study.

The relative concentrations of total IGFBP in plasma were determined by interference of the neutralised gel filtration chromatography IGFBP pools in the IGF-I and IGF-II RIAs (Figure 6.2). IGFBP concentrations were increased by IGF-I and the IGF combination when assessed by IGF-I RIA. LR^3IGF-I treatment lowered the mean concentration of IGFBPs in plasma relative to the concentration in animals administered the vehicle, although this was more pronounced in the IGF-II RIA (Figure 6.2). There was no consistent difference between the IGFBP concentrations of LR^3IGF-I-treated guinea pigs which survived 14 days of treatment and those which did not (Table 6.2).

The different types of IGFBPs in plasma do not appear to have changed in concentration following treatment with IGF-I, IGF-II or the IGF combination when assessed by Western ligand blotting (Figure 6.3). IGFBPs in plasma taken from individual animals treated with LR^3IGF-I were also assessed by Western ligand blotting (Figure 6.4). As in the previous guinea pig study, the intensity of bands in the 40 to 50 kDa range, likely to represent IGFBP-3, are lower in animals treated with LR^3IGF-I than in those treated with the vehicle. Also, in the plasma of animals killed after less than 14 days of infusion, some lower molecular weight bands were more intense.

Low concentrations of glucose (0.73 and 1.34 mM) in the plasma of two of the LR^3IGF-I-treated guinea pigs that were culled (Table 6.3), supports the suspicion that the condition of these animals was contributed to by hypoglycaemia. The mean concentration of glucose in the three guinea pigs treated with the analogue and which were treated for a full 14 days also had a mean glucose concentration which was lower than that of animals from other groups. This is a result of one of these animals having a very low blood glucose concentration (2.03 mM), whereas the concentration in the other two animals was close to normal. Glucose concentrations in other treatment groups were not significantly different from those in guinea pigs treated with the vehicle.

FIGURE 6.1 (a) Plasma IGF-I, (b) plasma IGF-II and (c) plasma total IGF (IGF-I plus IGF-II) concentrations in guinea pigs following treatment with: vehicle (open bars), LR^3IGF-I (cross-hatched bars), IGF-I (solid bars), IGF-II (hatched bars) or IGF-I plus IGF-II (horizontal bars) at the indicated doses. Results are expressed as the mean ± S.E.M. for 6 animals in each group, or, in the case of guinea pigs treated with LR^3IGF-I, as the mean of 3 animals (only those surviving 14 days of treatment are included). Values obtained from animals treated with LR^3IGF-I were not included in statistical analyses. Significance from the vehicle group is denoted by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

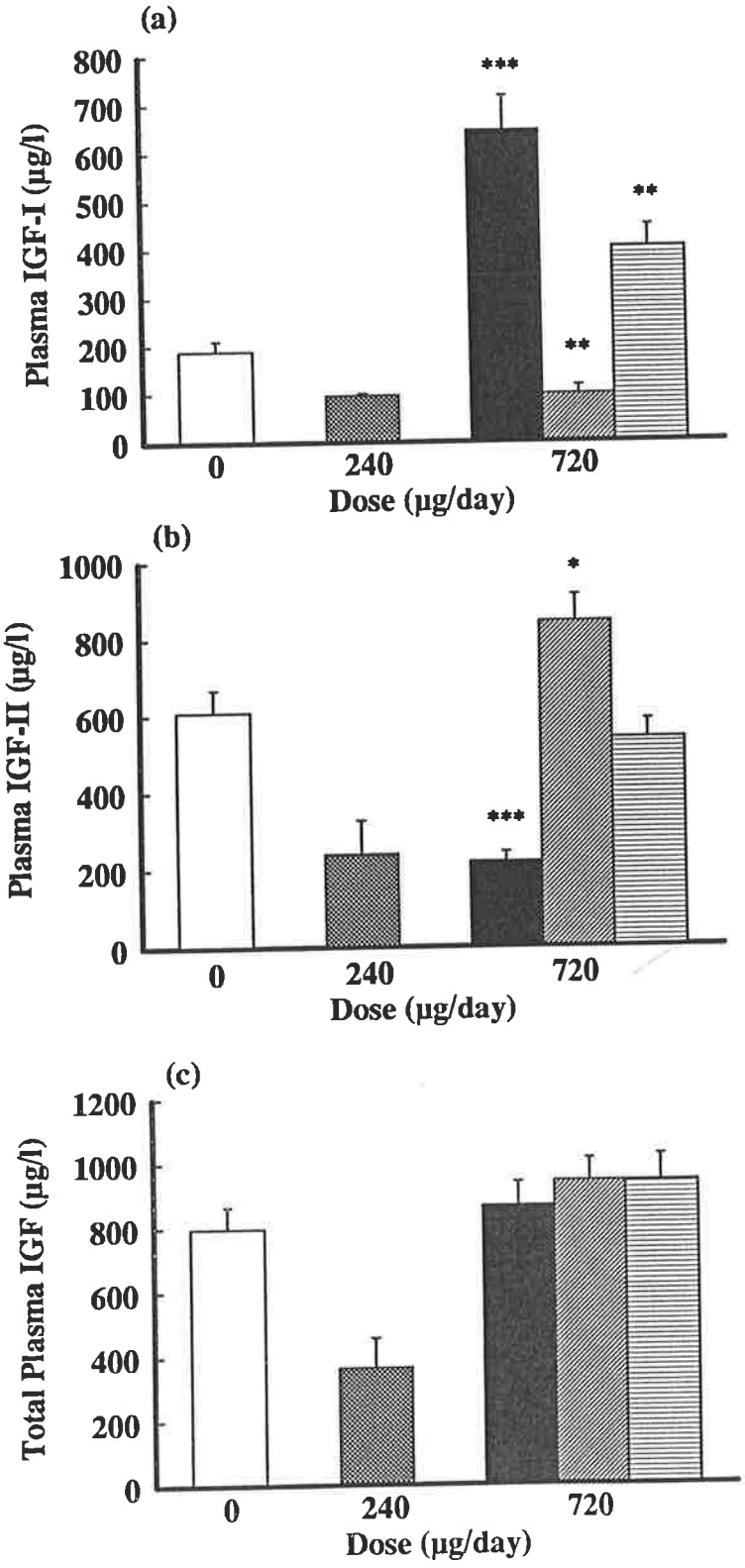

(a)
Plasma IGF-I (µg/l)
800
700
600
500
400
300
200
100
0
***
**
**
0
240
720
Dose (µg/day)
(b)
Plasma IGF-II (µg/l)
1000
800
600
400
200
0
*
***
0
240
720
Dose (µg/day)
(c)
Total Plasma IGF (µg/l)
1200
1000
800
600
400
200
0
0
240
720
Dose (µg/day)

TABLE 6.1 The concentrations (µg/l) of IGF-I, IGF-II, LR^3IGF-I and total IGF, in the plasma of guinea pigs treated with LR^3IGF-I (plasma was not available from one of the guinea pigs). Plasma was collected at the time of kill. Values are compared with concentrations in guinea pigs treated with vehicle (shown as the mean ± S.E.M. for 6 animals). NA means not applicable. * denotes animals that were killed before14 days of treatment had been completed.

	IGF-I	IGF-II	LR^3IGF-I	Total IGF
Treatment				
Vehicle control	188 ± 21	607 ± 60	NA	795 ± 68
LR^3IGF-I:				
Animal 2	95	124	26	245
Animal 4	86	183	25	294
Animal 5	103	408	34	545
Animal 3*	74	92	21	187
Animal 6*	55	62	18	135

FIGURE 6.2 Plasma IGFBP concentrations in guinea pigs following treatment with: vehicle (open bars), LR^3IGF-I (cross-hatched bars), IGF-I (solid bars), IGF-II (hatched bars) or IGF-I plus IGF-II (horizontal bars) at the indicated doses. Panel (a) represents the interference of IGFBPs in the IGF-I RIA and panel (b) represents the interference of IGFBPs in the IGF-II RIA. Results are expressed as the mean ± S.E.M. for 6 animals in each group or, in the case of guinea pigs treated with LR^3IGF-I, as the mean of 3 animals (only those surviving 14 days of treatment are included). Values obtained from animals treated with LR^3IGF-I were not included in statistical analyses. Significance from the vehicle group is denoted by * $P < 0.05$.

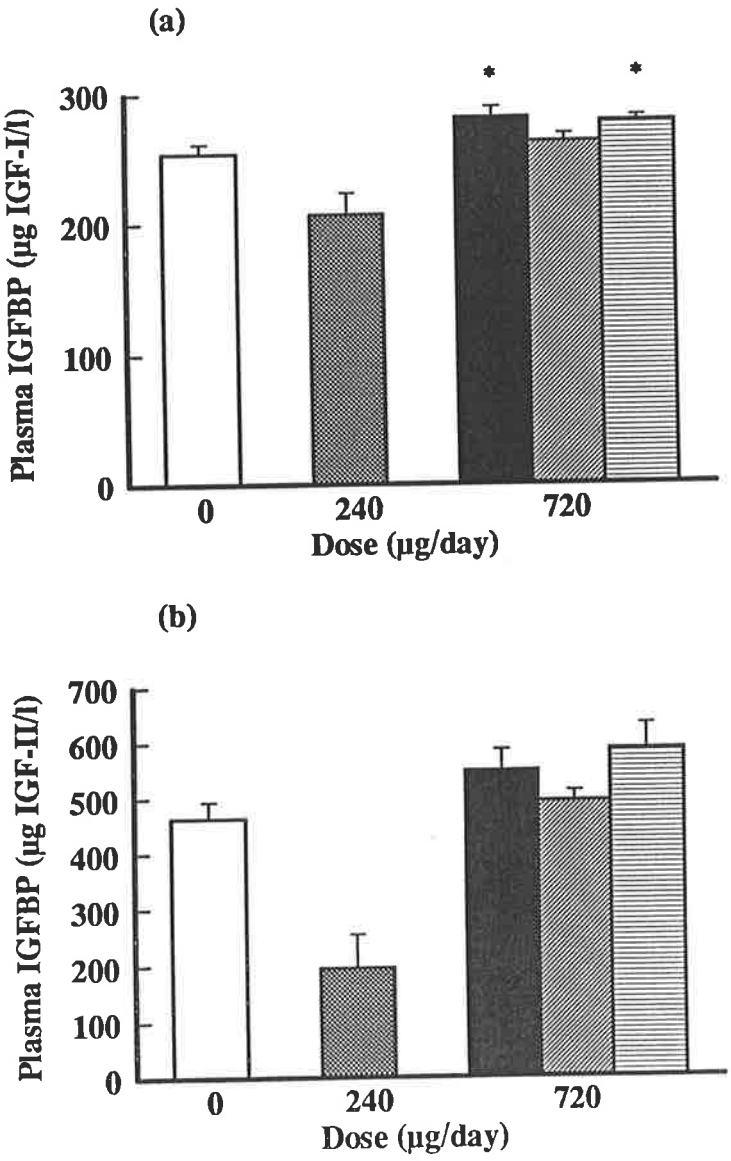

(a)
Plasma IGFBP (µg IGF-I/l)
300
200
100
0
*
*
0
240
720
Dose (µg/day)
(b)
Plasma IGFBP (µg IGF-II/l)
700
600
500
400
300
200
100
0
0
240
720
Dose (µg/day)

TABLE 6.2 The concentrations (µg of IGF-I or IGF-II equivalents/l) of IGFBPs, as assessed by interference in the IGF-I and IGF-II RIAs, in the plasma of guinea pigs treated with LR^3IGF-I (plasma was not available from one of the guinea pigs). Plasma was collected at the time of kill. Values are compared with concentrations in guinea pigs treated with the vehicle (shown as the mean ± S.E.M. for 6 animals). * denotes animals that were killed before14 days of treatment had been completed.

	IGFBP (IGF-I RIA)	IGFBP (IGF-II RIA)
Treatment		
Vehicle control	254 ± 7	464 ± 30
LR^3IGF-I:		
Animal 2	178	87
Animal 4	204	198
Animal 5	237	295
Animal 3*	191	89
Animal 6*	178	110

FIGURE 6.3 Plasma IGFBP profiles as determined by Western ligand blotting of guinea pig plasma using (a) ^{125}I-IGF-I or (b) ^{125}I-IGF-II as probe. Lane 1 contains size markers (sizes (kDa) are as indicated). Other lanes were loaded with treatment pools of plasma from animals treated with vehicle (lane 2), 720 µg of IGF-I/day (lane 3), 720 µg of IGF-II/day (lane 4) and 360 µg of IGF-I/day plus 360 µg of IGF-II/day (lane 5).

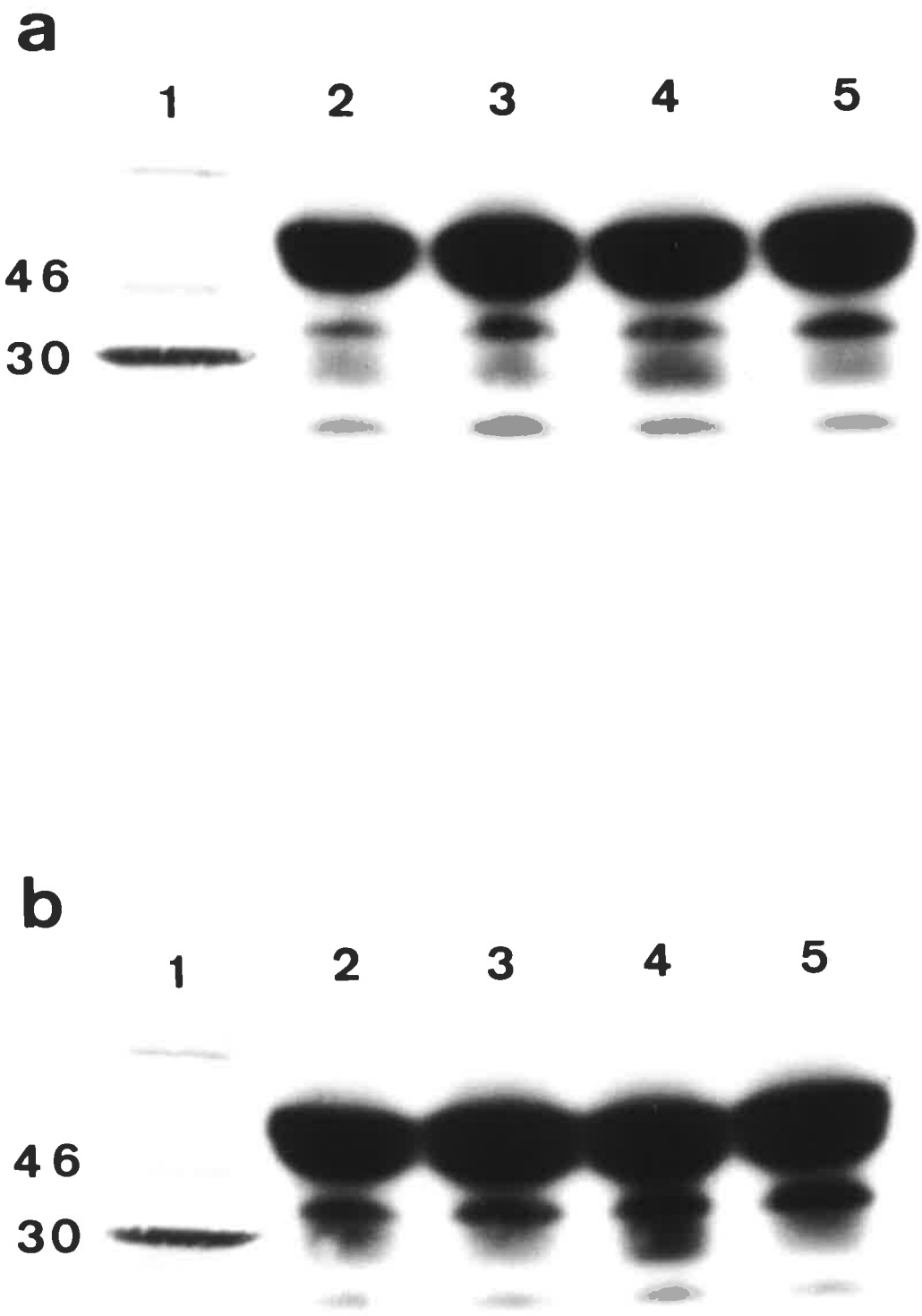

a
1
2
3
4
5
46
30
b
1
2
3
4
5
46
30

FIGURE 6.4 A comparison of plasma IGFBP profiles of individual guinea pigs treated with vehicle and 240 µg of LR^3IGF-I/day by Western ligand blotting using ^{125}I-IGF-I as probe. Lane 1 contains size markers (sizes (kDa) are as indicated). Other lanes were loaded with plasma from individual animals treated with vehicle (lanes 2 to 7), or with LR^3IGF-I (lanes 8 to 12). LR^3IGF-I treated animals were loaded as follows: animal 2 (lane 8), animal 4 (lane 9), animal 5 (lane 10), animal 3 (lane 11) and animal 6 (lane 12). The latter two animals were hypoglycaemic.

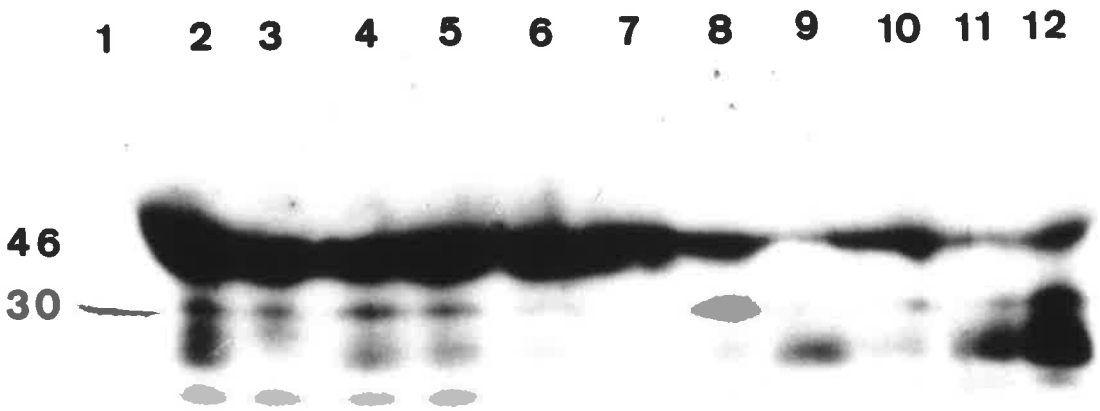

1
2
3
4
5
6
7
8
9
10
11
12
46
30

TABLE 6.3 Glucose concentrations (mM) in plasma collected at the time of kill. Results are expressed as the mean ± S.E.M. for 6 animals or, in the case of guinea pigs treated with LR^3IGF-I, as the mean of 3 animals (those surviving 14 days of treatment). Also included in the table are the concentrations of glucose in individual animals treated with LR^3IGF-I. Plasma was not available from one of the LR^3IGF-I-treated animals. * denotes animals that were killed before14 days of treatment had been completed.

Treatment	
Vehicle Control	8.26 ± 0.34
IGF-I (720 µg/day)	7.82 ± 0.21
IGF-II (720 µg/day)	7.45 ± 0.40
IGF-I + IGF-II (360 µg/day of each)	7.26 ± 0.70
LR^3IGF-I (240 µg/day)	5.27 ± 1.62
Individual animals treated with LR^3IGF-I:	
Animal 2	7.03
Animal 4	2.03
Animal 5	6.75
Animal 3*	0.73
Animal 6*	1.34

Animals were placed in metabolism cages 7 days prior to pump implantation to give them a longer period of acclimatisation than those used for the study of chapter 5. The mean weight loss of guinea pigs one day after placement in cages was 32 g (from a mean starting weight of 385 g). By the time of pump implantation they had regained 18 g of the weight loss. Thus the guinea pigs are likely to have been in catch-up growth during part of the infusion period.

None of the treatments significantly increased body weight gain over that which was achieved with the vehicle (Figure 6.5; Table 6.3). The mean body weight gain over 14 days, for the 3 animals in the LR^{3}IGF-I treatment group which survived that period, was similar to that for the vehicle-infused guinea pigs (Table 6.3). The body weight changes during treatment are plotted for each of the LR^{3}IGF-I treated animals in Figure 6.6. and show that two animals which had to be killed following day 6 of infusion had relatively large falls in body weight in the preceding day or days. These falls were associated with negligible feed and water intakes.

Feed intakes (Table 6.4) and feed conversion efficiencies (Table 6.5) of animals in the IGF treatment groups did not differ significantly from those of guinea pigs infused with the vehicle. Nevertheless, regression analysis revealed that there was a significant association between feed intake and body weight gain over 14 days of treatment ($r^2 = 0.57$; $P < 0.0001$) and also a significant association between feed conversion efficiency and body weight gain over the same period of treatment ($r^2 = 0.94$; $P < 0.0001$). The average feed intake and feed conversion efficiency for the guinea pigs that survived 14 days of LR^{3}IGF-I administration was similar to that for animals treated with the vehicle.

The fractional weight of lungs was significantly increased by IGF-II but not by any other treatment (Table 6.6). The growth of other tissues, including adrenals, gut, kidneys and spleen, organs which have previously grown in response to IGF infusion in rats or guinea pigs, were not significantly altered by any treatment. The growth of adrenals, gut, kidneys and

spleen appear to have increased in guinea pigs treated with LR^3IGF-I and the fractional weight of the liver may have decreased in these animals (Table 6.6 and Table 6.7). There was no indication of an increase in the fractional weight of lungs by LR^3IGF-I infusion (Table 6.6 and Table 6.7).

Water intakes did not differ significantly between treatment groups (appendix C, Table C.1). The fractional weights of the carcass, head plus paws and the pelt (appendix C, Table C.2) were unaffected by IGF treatment, as was the growth of the femurs (appendix C, Table C.3). There were no significant treatment effects on the weights of individual regions of the gut (appendix C, Table C.4) nor on the length or weight per unit length of the small intestine (appendix C, Table C.5). There were no differences in the proportions of fat, water and the remainder (of which protein is a part) between the carcasses of any treatment group (appendix C, Table C.6). Since the proportion of carcass protein is therefore unlikely to have changed, it was not measured. Plasma urea was measured as an indicator of protein utilisation, but again there were no significant effects of treatment (appendix C, Table C.7) and there were also no significant differences in nitrogen retention between the treatment groups over 14 days of treatment (appendix C, Table C.8).

FIGURE 6.5. Mean body weights (g) of guinea pigs on each day of treatment. Osmotic pumps were implanted, and treatment began, immediately following weighing of animals on day 0. Treatments are represented as follows: vehicle (filled circles), 720 µg of IGF-I/day (open circles), 720 µg of IGF-II/day (squares), 360 µg of IGF-I/day plus 360 µg of IGF-II/day (triangles) and 240 µg of LR^3IGF-I/day (inverted triangles). Values are expressed as the mean for 6 animals in each treatment group, or, in the case of guinea pigs treated with LR^3IGF-I, as the mean of 3 animals (only those surviving 14 days of treatment are included). S.E.M.s have been omitted for the sake of clarity. The pooled S.E.M. on day 14 of treatment was 7.1.

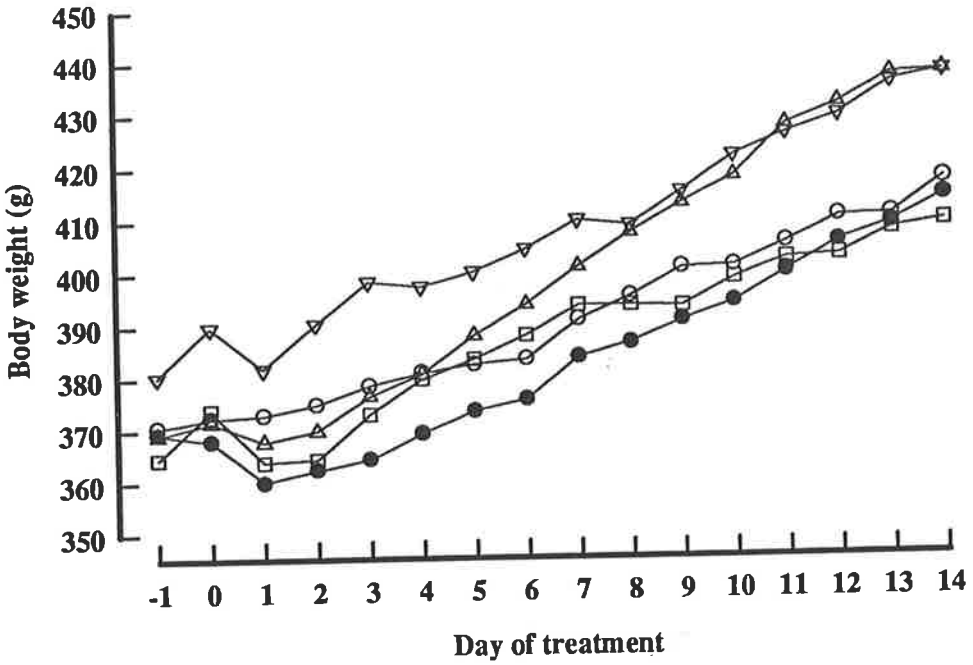

450
440
430
420
410
400
390
380
370
360
350
Body weight (g)
-1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day of treatment

FIGURE 6.6. Daily body weights (g) of each guinea pig administered 240 µg of LR^3IGF-I/day. Osmotic pumps were implanted, and treatment began, immediately following weighing of animals on day 0. Each animal is represented as follows: animal 1 (filled circles), animal 2 (open hexagons), animal 3 (filled squares), animal 4 (open triangles), animal 5 (open inverted triangles) and animal 6 (filled diamonds). Filled symbols represent animals that were killed prior to completion of 14 days of treatment due to poor health.

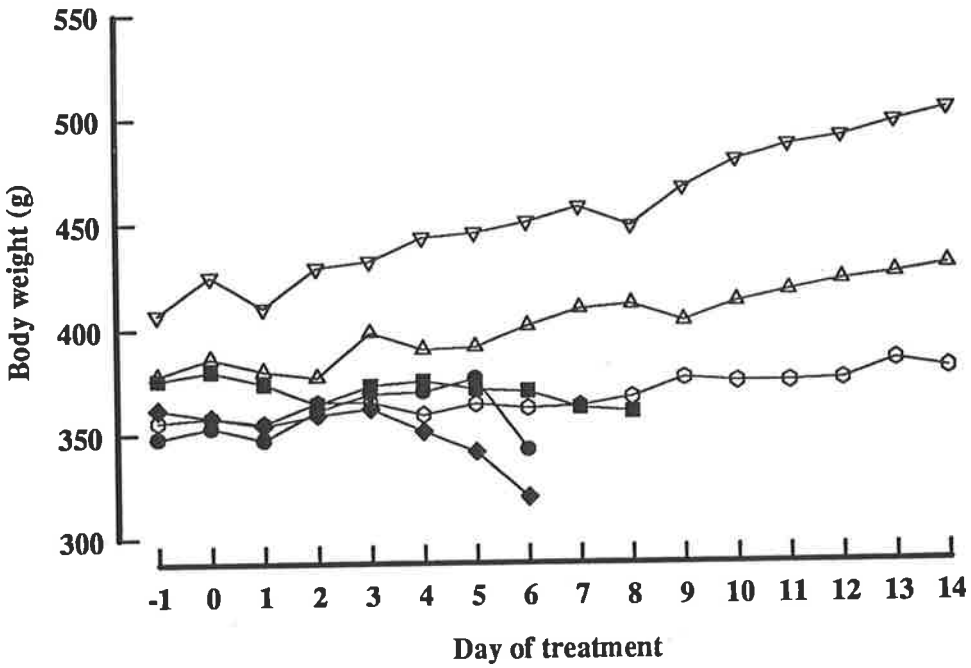

550
500
450
400
350
300
Body weight (g)
-1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day of treatment

TABLE 6.4. Body weight gains (g) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group (in the case of the $LR^3IGF\text{-}I$ treatment group values are derived from the 3 animals that survived 14 days of infusion).

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	15.5 ± 6.3	30.3 ± 4.8	45.8 ± 4.4
IGF-I (720 µg/day)	18.7 ± 9.2	26.2 ± 9.3	44.8 ± 12.4
IGF-II (720 µg/day)	19.8 ± 6.1	15.5 ± 6.0	35.3 ± 5.5
IGF-I + IGF-II (360 µg/day of each)	29.3 ± 5.7	36.5 ± 3.8	65.8 ± 5.4
$LR^3IGF\text{-}I$ (240 µg/day)	20.0 ± 7.9	27.7 ± 9.2	47.7 ± 16.3

TABLE 6.5. Feed intakes (g) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group (in the case of the $LR^3IGF\text{-}I$ treatment group values are derived from the 3 animals that survived 14 days of infusion).

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	158 ± 3	187 ± 8	344 ± 9
IGF-I (720 µg/day)	160 ± 13	181 ± 11	341 ± 21
IGF-II (720 µg/day)	176 ± 7	182 ± 6	358 ± 10
IGF-I + IGF-II (360 µg/day of each)	175 ± 11	211 ± 10	386 ± 20
$LR^3IGF\text{-}I$ (240 µg/day)	161 ± 15	184 ± 19	346 ± 33

TABLE 6.6. Feed conversion efficiencies (g of body weight gain/g of feed intake) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group (in the case of the LR^3IGF-I treatment group values are derived from the 3 animals that survived 14 days of infusion).

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	0.096 ± 0.040	0.160 ± 0.020	0.133 ± 0.011
IGF-I (720 µg/day)	0.098 ± 0.048	0.135 ± 0.049	0.125 ± 0.027
IGF-II (720 µg/day)	0.107 ± 0.033	0.084 ± 0.033	0.098 ± 0.015
IGF-I + IGF-II (360 µg/day of each)	0.162 ± 0.029	0.174 ± 0.018	0.170 ± 0.009
LR^3IGF-I (240 µg/day)	0.116 ± 0.042	0.144 ± 0.034	0.132 ± 0.035

FIGURE 6.7 Regression analyses of (a) mean body weight gain versus mean feed intake and (b) mean body weight gain versus feed conversion efficiency, for each animal over the 14 day treatment period (n = 27). Treatments are symbolised as follows: vehicle (circles), 720 µg of IGF-I/day (squares), 720 µg of IGF-II/day (triangles), 360 µg of IGF-I/day plus 360 µg of IGF-II/day (inverted triangles) and 240 µg of LR^3IGF-I/day (diamonds). Animals that did not survive a full 14 days of treatment have not been included.

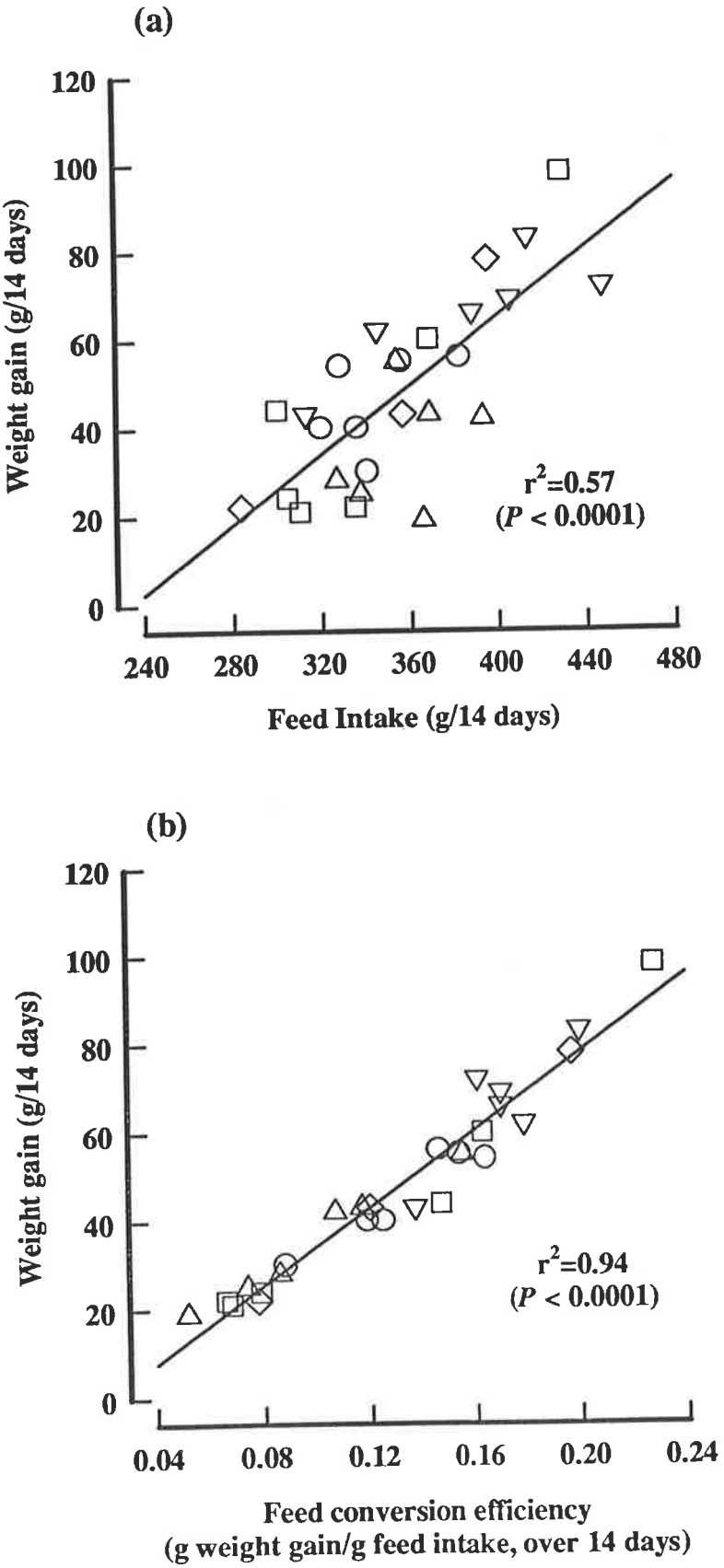

(a)
120
100
80
60
40
20
0
Weight gain (g/14 days)
r^2=0.57
(P < 0.0001)
240
280
320
360
400
440
480
Feed Intake (g/14 days)
(b)
120
100
80
60
40
20
0
Weight gain (g/14 days)
r^2=0.94
(P < 0.0001)
0.04
0.08
0.12
0.16
0.20
0.24
Feed conversion efficiency
(g weight gain/g feed intake, over 14 days)

TABLE 6.7. Organ and tissue weights, expressed as a fraction of body weight (g/kg body weight). The values represent the mean ± S.E.M. for 6 animals in each group (or in the case of the LR^3IGF-I treatment group values are derived from the 3 animals that survived 14 days of infusion).

	Adrenals	Brain	Gut (total)	Heart
Treatment				
Vehicle control	0.55 ± 0.02	9.02 ± 0.38	54.35 ± 1.81	4.05 ± 0.23
IGF-I (720 µg/day)	0.69 ± 0.10	8.51 ± 0.20	55.84 ± 0.78	3.91 ± 0.11
IGF-II (720 µg/day)	0.54 ± 0.03	9.03 ± 0.31	55.74 ± 0.70	3.78 ± 0.10
IGF-I + IGF-II (360 µg/day of each)	0.55 ± 0.03	8.48 ± 0.41	57.37 ± 0.79	4.15 ± 0.20
LR^3IGF-I (240 µg/day)	0.85 ± 0.09	8.35 ± 0.57	65.00 ± 1.30	3.83 ± 0.13

	Kidneys	Liver	Lungs	Spleen
Treatment				
Vehicle control	8.15 ± 0.42	32.25 ± 1.44	7.00 ± 0.37	1.56 ± 0.17
IGF-I (720 µg/day)	9.31 ± 0.44	31.88 ± 1.11	7.29 ± 0.20	2.12 ± 0.15
IGF-II (720 µg/day)	8.73 ± 0.37	31.62 ± 1.42	8.39 ± 0.30**	1.51 ± 0.08
IGF-I + IGF-II (360 µg/day of each)	9.41 ± 0.42	34.03 ± 1.62	6.91 ± 0.25	2.14 ± 0.26
LR^3IGF-I (240 µg/day)	10.32 ± 0.60	27.46 ± 1.27	7.32 ± 0.38	2.74 ± 0.35

** $P < 0.01$.

TABLE 6.8 Organ weights of individual guinea pigs treated with LR^3IGF-I. Weights are expressed as a fraction of body weight (g/kg body weight). The fractional weights of organs in animals which were treated with vehicle have been included for comparison and are expressed as the mean ± S.E.M. for 6 animals in each group. * denotes animals that were killed before 14 days of treatment had been completed. -- indicates that the value was not available.

	Adrenals	Gut	Kidneys	Liver	Lungs	Spleen
Treatment						
Vehicle control	0.55±0.02	55.38±1.38	8.15±0.42	32.25±1.44	7.00±0.37	1.56±0.17
LR^3IGF-I:						
Animal 2	1.03	62.84	11.43	25.00	8.08	3.40
Animal 4	0.71	67.24	10.14	28.13	6.88	2.58
Animal 5	0.82	64.86	9.39	29.26	6.99	2.23
Animal 1*	0.83	-----	11.18	26.27	7.67	1.41
Animal 3*	0.83	69.24	10.25	24.82	7.53	2.44
Animal 6*	0.97	59.02	11.41	27.03	8.64	1.79

6.4 Discussion.

This study has confirmed that rats and guinea pigs respond differently to IGF infusion in a number of ways. This includes apparent differences in the way that infused IGFs are maintained in blood and the way in which they control endogenous IGF and IGFBP concentrations. Of particular note is an effect of IGF-II on lung growth, an effect not manifested in the rat study. Also, LR^3IGF-I has a glucose-lowering potency which seems higher than that recorded for rats. The fall in IGFBP concentrations following LR^3IGF-I treatment in guinea pigs, demonstrated here and in chapter 5, could contribute to the development of hypoglycaemia. I have been unable to demonstrate a significantly greater improvement in growth by infusing a combination of IGF-I and IGF-II than by treatment with IGF-I or IGF-II alone.

The IGFs are known to lower blood glucose concentrations when administered in a high enough dose to a range of animals including rats, humans, sheep, dogs and pigs (Zapf *et al.* 1986; Guler *et al.* 1987; Douglas *et al.* 1991a; Giacca *et al.* 1990; Ballard *et al.* 1994). Most of the IGFs in circulation are bound to IGFBPs and are prevented from being cleared/transported to the tissues where they can exert biological actions. The IGFs in blood, if not restrained, would probably cause a large drop in blood glucose concentration (Lewitt & Baxter 1991). LR^3IGF-I can stimulate greater anabolic responses than IGF-I in rats (Tomas *et al.* 1993a), probably because its low affinity for IGFBPs (Francis *et al.* 1992) allows it be cleared more rapidly from the blood to the tissues (Bastian *et al.* 1993). In the previous guinea pig study (chapter 5) I demonstrated that this analogue was more potent than IGF-I and IGF-II in stimulating the growth of several tissues. Again in the current study, LR^3IGF-I seems to have increased the growth of those organs more potently than either IGF-I or IGF-II, but has also lowered blood glucose more potently. This indicates that at least one of the roles of IGFBPs in the guinea pig is to protect against hypoglycaemia by retaining the IGFs in blood.

I have been unable to significantly increase the weight gain of guinea pigs with IGFs infused at almost 2 mg/kg/day (based on weights at the start of treatment). In the previous study involving female rats (chapter 4) a dose of about 2.6, but not 1, mg of IGF-I/kg/day stimulated body weight gain while a dose of about 6.5 mg of IGF-II/kg/day was needed to obtain the same effects elicited by the high dose of IGF-I. Other published work shows significant body weight gains in normal rats with 2.4 mg of IGF-I/kg/day over 14 days (Tomas *et al.* 1993a). Thus the IGFs appear to have a lower capacity for growth promotion in the guinea pig than in the rat. It is possible that the guinea pig may respond in a manner more akin to the rat at some other stage of development. It is interesting to note that IGF-I treatment did not lead to a significant increase in the fractional weights of several organs that were clearly responsive to such treatment in rats, although there was an indication of such a trend for the kidneys and spleen. In the previous guinea pig study LR^3IGF-I significantly increased the growth of adrenals, gut, kidneys and spleen. In this experiment, LR^3IGF-I appeared to stimulate growth of those same organs and, additionally, IGF-II was able to increase the growth of lungs. Thus with the appropriate treatment significant growth of guinea pig tissues is possible. In all it appears that the guinea pig is generally more resistant to growth stimulation by IGF treatment than the rat.

One possible reason for not obtaining more significant effects on growth with IGF-I or IGF-II is that we still have not used a high enough dose. However, in light of the consequences of LR^3IGF-I administration, it cannot be certain that such doses are attainable without the development of hypoglycaemia. For example, LR^3IGF-I was able to increase fractional organ weights at a dose of approximately 0.33 mg/kg/day (chapter 5) but in the current study, at about 0.66 mg/kg/day, half of the animals had to be killed because of sudden weight loss and other physical signs of stress associated with hypoglycaemia. In female rats, the analogue has been able to stimulate gains in body weight and tissue weight at doses of

approximately 1 or 2.4 mg/kg/day, without signs of stress being reported (Tomas *et al.* 1993a; Steeb *et al.* 1994). Similar doses in male rats do not alter plasma glucose concentrations (Dr. Frank Tomas, personal communication). It seems that a more potent insulin-like effect of IGFs in guinea pigs could prevent these peptides from stimulating growth to the extent that they do in rats. Indeed, the countering of the insulin-like effects of IGF-I can improve its anabolic actions, as has been demonstrated by coinfusion of GH and IGF-I into calorie-restricted patients (Kupfer *et al.* 1993). However, blood glucose concentrations in guinea pigs treated with IGF-I or IGF-II were not significantly lowered. Thus negative insulin-like effects are unlikely to explain why IGF-I in particular has not stimulated growth in this study.

Another novel finding here is that IGF-II infusion into guinea pigs led to an increase in the fractional weight of the lungs, a response not observed in our rat study. This confirms and extends the report that lung growth was increased by treatment of hypophysectomised neonatal rats with 1.9 mg of IGF-II/kg/day (Glasscock *et al.* 1992), a dose similar to that used in this study. Also, as in the guinea pig, IGF-II did not appear to increase other parameters of growth. However, unlike the growing guinea pig, lung growth in the hypophysectomised neonatal rat was also stimulated by IGF-I. In Snell dwarf mice, treatment with IGF-I, but not IGF-II, increased lung weight (van Buul-Offers *et al.* 1994). Experiments have demonstrated that lung growth in fetal sheep is stimulated by increasing lung distension and slowed by a reduction in distension. In those studies the level of growth was closely associated with the level of IGF-II gene expression (Harding *et al.* 1993; Hooper *et al.* 1993). The failure of the combination of IGF-I and IGF-II to increase lung growth may simply be a result of having infused half of the amount of IGF-II, given that IGF-II and not IGF-I seems to be the growth promoter for this tissue.

In the rat study of chapter 4, similar doses of IGF-I or IGF-II resulted in similar increases in the respective peptides in blood. In the first guinea pig study (chapter 5), IGF-II

infusion increased plasma IGF-II concentrations twice as much as IGF-I infusion increased plasma IGF-I. Yet with the higher doses used in the current experiment, IGF-II was increased by only half as much as IGF-I was following infusion of IGF-II and IGF-I respectively. Even though IGF-II was being infused at a dose of 360 µg/day, along with IGF-I, in the combination treatment, it failed to increase plasma concentrations of IGF-II. The ability of the co-infused IGF-I to compete with IGF-II for binding to IGFBPs in blood may contribute to this. Administration of 720 µg of IGF-II/day increased IGF-II concentrations by 235 µg/l. This falls short of the 638 µg/l increase that followed infusion of IGF-II alone at a dose of 360 µg/l (see chapter 5). Perhaps the clearance rate of circulating IGF-II has been increased at the higher dose. Thus the manner in which infused IGFs are maintained in circulation is complex and seems different for IGF-I and IGF-II and also for rats and guinea pigs.

In summary, this study has further confirmed that rats and guinea pigs can respond differently to IGF treatment. Although differences in the response of rats and guinea pigs to IGFs has been focused upon, there are nevertheless many strong similarities in the way that these animals respond. This is highlighted by the fact that in both animals, the gut, kidneys and spleen are especially responsive to the IGFs and also by the greater potency of LR^3IGF-I, indicating that IGFBPs inhibit IGF actions in the guinea pig as they do in the rat.

CHAPTER 7.

A COMPARISON OF THE CAPACITIES OF RAT AND GUINEA PIG LIVER MEMBRANES TO BIND IGF-I AND IGF-II.

7.1 Introduction.

The IGFs can lower blood glucose concentration when administered to a range of species including rats (Zapf *et al.* 1986, Jacob *et al.*1989), dogs (Giacca *et al.* 1990 & 1994), humans (Guler *et al.* 1987; Elahi *et al.* 1991), pigs (Ballard *et al.* 1994) and sheep (Douglas *et al.* 1991a), albeit between 10 to 100 times less potently than insulin. In normal adult rats the fall in blood glucose concentration which follows IGF-I administration is associated with increased uptake of glucose by the tissues (Jacob *et al.* 1989). Unlike the effects elicited by insulin, IGF-I does not inhibit glucose production by the liver in these animals (Jacob *et al.* 1989). IGF-I also has a far greater effect on glucose disposal by the peripheral tissues than it does on hepatic glucose production in normal human adults (Elahi *et al.* 1991). However, in diabetic dogs and rats, the high rate of hepatic glucose production can be reduced by IGF-I infusion (Giacca *et al.* 1990; Jacob *et al.* 1991). Thus IGF-I treatment can influence liver glucose metabolism *in vivo*. This is supported by *in vitro* studies which show that IGF-I can suppress glycogenolysis and stimulate production of lactate from glucose in primary cultures of adult rat hepatocytes, but with a potency which is only 2 to 4 % of that achieved by insulin (Hartmann *et al.* 1990). Similar effects occur following IGF-II treatment (Hartmann *et al.* 1992).

The actions of the IGFs are primarily mediated via high affinity binding to the type-1 IGF receptor but the IGFs can also bind with low affinity to the insulin receptor (Massague & Czech 1982). The specific binding of ^{125}I-IGF-I to liver cells or membranes of adult rats, humans and chickens is very low in comparison with other tissues, as a result of a low type-1 IGF receptor concentration (Rechler *et al.* 1980; Caro *et al.* 1988; Duclos & Goddard 1990). The relative concentration of insulin receptors in liver cells is much higher. Similarly a low type-1 IGF receptor, but high insulin receptor concentration, is found in adipose tissue (Zapf *et al.* 1981), but in other tissues such as muscle the concentration of both receptors is high (Poggi

et al. 1979). At a sufficiently high concentration, IGF-I can elicit effects such as the stimulation of growth and metabolism of liver cells (Gunn *et al.* 1977; Mottola & Czech 1984; Hartmann *et al.* 1990, 1992) and the stimulation of glucose uptake in rat and human adipocytes (Zapf *et al.* 1978; Bolinder *et al.* 1987) *in vitro*. Since the type-1 IGF receptor concentration is very low in these cells, the actions of IGF-I are thought to be mediated via cross-reactivity with the insulin receptor, and this explains why such high concentrations of IGFs are required to exert insulin-like actions in these cells. The type-2 IGF receptor, which is abundant in adult rat livers (Bryson & Baxter 1987), does not mediate the actions of IGF-II in adult rat hepatocytes, since antibodies which prevent IGF-II binding to the receptor do not inhibit the actions of IGF-II (Hartmann *et al.* 1992).

The type-1 IGF receptor may be able to mediate the insulin-like effects of the IGFs independently of the insulin receptor, since the two receptors, which are members of the tyrosine kinase receptor family, share some signalling pathways (Nissley *et al.* 1985; McLain *et al.* 1987; Heyner *et al.* 1989; Pessin 1994). A study by Quin *et al.* (1990) gives support to this possibility. They showed that IGF-I injection could lower blood glucose in a patient with Mendenhall syndrome even though insulin was unable to do so, suggesting actions independent of those mediated via the insulin receptor. Therefore, under normal physiological conditions, the relatively low number of type-1 IGF receptors in the liver may limit the extent to which the IGFs can affect the carbohydrate metabolism of the liver, since supraphysiological doses of IGFs would be required to exert actions via the insulin receptor.

Doses of LR^3IGF-I which are similar to those resulting in hypoglycaemia in guinea pigs (see chapter 6) have been infused into normal rats of similar weight to those of the chapter 4 experiment and did not cause hypoglycaemia (Dr. Frank Tomas, personal communication). One possible explanation is that there is a greater concentration of type-1 IGF receptors in the liver of guinea pigs than rats, perhaps resulting in a greater inhibition of glucose production or

stimulation of glucose uptake in the liver following IGF administration. To test this, the IGF-I binding capacity of liver membranes from the two species has been compared. The IGF-II binding capacity has also been examined.

7.2 Materials and methods.

7.2.1 Preparation of liver membranes.

Liver membranes were prepared from livers taken from rats of approximately 100 g in weight and guinea pigs of approximately 350 g in weight, weights approximating those used in the studies of the previous chapters. Rat livers were obtained from freshly killed animals whereas the livers from guinea pigs had been frozen in liquid nitrogen upon excision and stored frozen at -80°C. Livers were homogenised on ice for several minutes in 0.3 M sucrose containing 500 kallikrein inhibitor units/ml of aprotinin. The homogenate was centrifuged at 4,000 x *g* for 10 minutes at 4°C. The resultant supernatant was centrifuged at 30,000 x *g* for 15 minutes and then the supernatant from this centrifuged at 100,000 x *g* for 1 hour. Each centrifugation was carried out at 4°C. The pellet was resuspended in 4 M magnesium chloride (1:2 ; w:v) to displace ligands bound to membrane receptors. This suspension was centrifuged at 100,000 x *g* for 1 hour at 4°C. The membrane preparation was based on a method described by Shiu *et al.* (1973). The pellet was resuspended in receptor binding assay (RBA) buffer A (25 mM Tris base, 10 mM calcium chloride, 0.05 % (v:v) Tween 20, pH 7.6). The protein concentrations of the membrane preparations were determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, Illinois, USA).

7.2.2 Measurement of specific binding to liver membranes.

Binding assays were carried out in a total volume of 350 µl of RBA buffer A that contained 30,000 c.p.m. of ^{125}I-IGF (a gift from Dr. Phil Owens; iodination and specific activity details are as described in chapter 4), varying amounts of liver membranes (see section 7.2.1) and, as appropriate, 1 µg of IGF. Tubes were incubated for 18 hours at 4°C, a period previously demonstrated to achieve close to optimal binding in studies involving liver membranes (Duclos & Goddard 1990). Radioactivity bound to membranes was precipitated

by the addition of 1 ml of a cold solution of 10 mM Tris base, 100 mM calcium chloride, 0.05% (v:v) Tween 20 (pH 7.6) to the tube, followed by centrifugation at 4,000 x *g* for 20 minutes at 4°C. The supernatant was removed by aspiration and the radioactivity of the pellet measured in a gamma counter. The membrane binding assay buffers and method are based on those descibed by Owens *et al.* (1990)

Non-specific binding of ^{125}I-rhIGF-I or ^{125}I-rhIGF-II to the membrane preparations was calculated as the percentage of radioactivity bound in the presence of an excess (1 µg) of cold IGF-I or IGF-II respectively. Specific binding was determined by subtracting the percentage of non-specifically bound ^{125}I-IGF from the percentage bound in the absence of competing non-radioactive IGF. Binding estimations were carried out in triplicate.

7.3 Results.

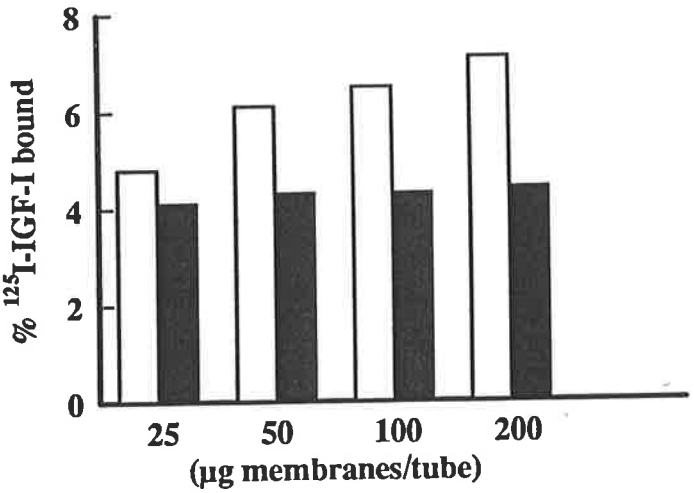
Total ^{125}I-IGF-I binding to rat liver membranes was fairly constant across the range of 25 µg to 200 µg of membranes per tube, increasing from 4.1 % to 4.4 % of total c.p.m. added (Figure 7.1a). This contrasted with the binding of ^{125}I-IGF-I to guinea pig membranes, which increased from 4.8 % to 7.1 % of total c.p.m. across the same range of membrane content per tube (Figure 7.1a). The total binding of ^{125}I-IGF-II to rat and guinea pig liver membranes exhibited a fairly similar pattern across the range of 25 µg to 200 µg of membranes per tube, increasing from 16.9 % to 26.6 % for the rat and increasing from 14.2 % to 28.3 % for guinea pigs (Figure 7.1b). ^{125}I-IGF-II binding to rat membranes appears to have peaked with 100 µg per tube, whereas binding to 200 µg of guinea pig membranes (28.3 %) was close to a maximum since the total binding to 300 µg and 400 µg of membranes was 29.8 % and 30.9 % respectively (data not included in Figure 7.1b).

Specific binding was calculated by subtracting the non-specific binding of radiolabelled IGFs from the total binding of the radiolabelled IGFs to membranes. The maximum specific binding of ^{125}I-IGF-I to rat and guinea pig liver membranes was 1.4 % and 3.1 % respectively (Figure 7.2a). In both cases this was achieved by using 50 µg of membrane protein per tube. The maximum specific binding of ^{125}I-IGF-II was higher; 14.4 % for membranes of the rat and 8.2 % for those of the guinea pig (Figure 7.2b). These were achieved with 100 and 200 µg of membrane protein per tube respectively. The binding of ^{125}I-IGF-II to 300 µg and 400 µg of guinea pig membranes was not included in Figure 7.2 but was 6.6 % and 6.5 % respectively.

The specific binding of IGF-II was expressed relative to IGF-I binding (Figure 7.3). There was 2 to 3 times greater binding of IGF-II than IGF-I to guinea pig liver membranes, but for rat membranes IGF-II binding was at least 8 times higher than for IGF-I.

FIGURE 7.1. A comparison of the total amount of (a) ^{125}I-IGF-I and (b) ^{125}I-IGF-II bound to the indicated amounts of guinea pig (open bars) and rat (filled bars) liver membranes. Results are expressed as a % of total c.p.m. added.

(a)


8
6
4
2
0
% 125I-IGF-I bound
25
50
100
200
(µg membranes/tube)

(b)

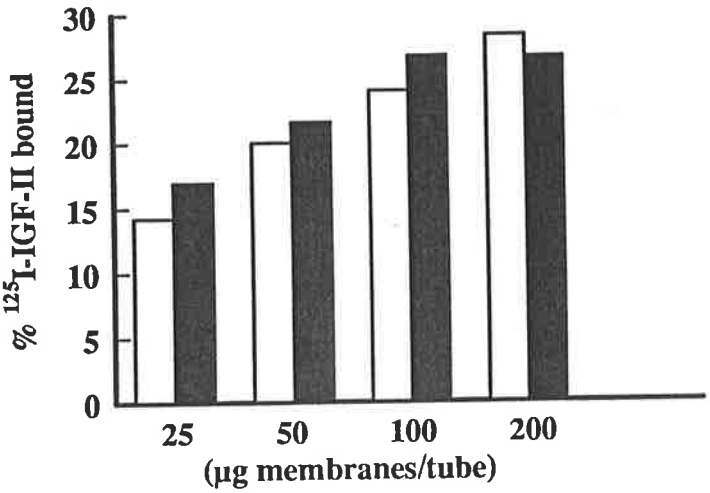
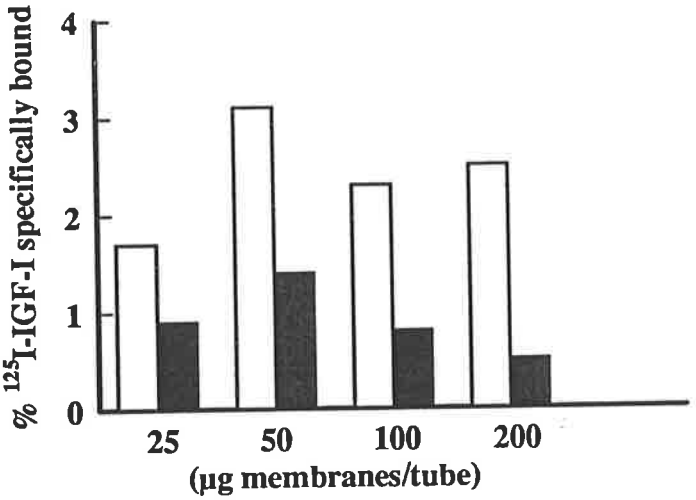

30
25
20
15
10
5
0
% 125I-IGF-II bound
25
50
100
200
(µg membranes/tube)

FIGURE 7.2. A comparison of the specific binding of (a) ^{125}I-IGF-I and (b) ^{125}I-IGF-II to the indicated amounts of guinea pig (open bars) and rat (filled bars) liver membranes. Specific binding was calculated by subtracting the amount of ^{125}I-IGF bound to membranes in the absence of competing IGF from that bound in the presence of an excess (1µg) of the appropriate cold IGF. Results are expressed as a % of total c.p.m. added.

(a)


% 125I-IGF-I specifically bound
4
3
2
1
0
25
50
100
200
(µg membranes/tube)

(b)

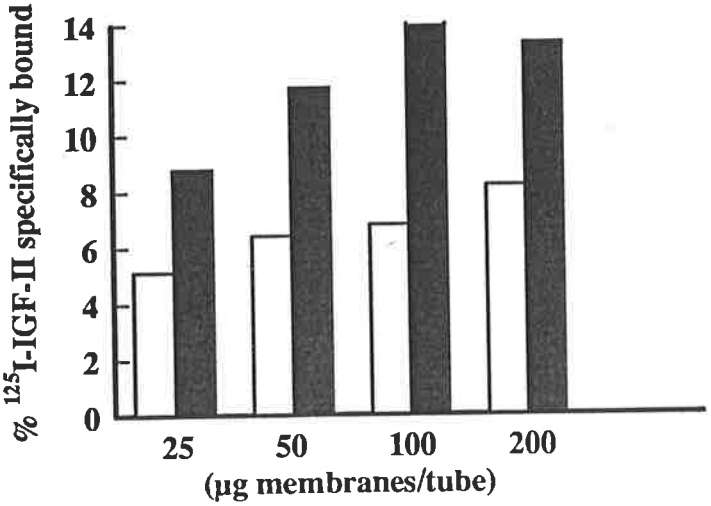
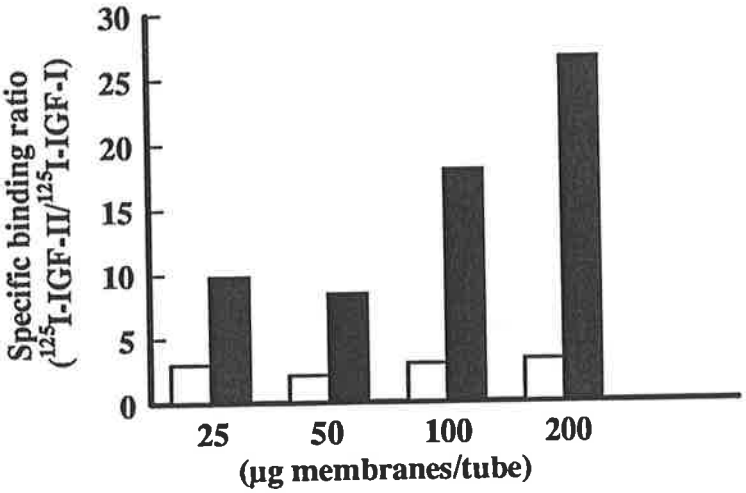

% 125I-IGF-II specifically bound
14
12
10
8
6
4
2
0
25
50
100
200
(µg membranes/tube)

FIGURE 7.3. The specific binding of ^{125}I-IGF-II to liver membranes expressed as a fraction of ^{125}I-IGF-I binding, using the indicated amounts of guinea pig (open bars) and rat (filled bars) membranes. The data was calculated from values shown in Figure 7.2.


Specific binding ratio
(^{125}I-IGF-II/^{125}I-IGF-I)
30
25
20
15
10
5
0
25
50
100
200
(µg membranes/tube)

7.4 Discussion.

The maximum specific binding of ^{125}I-IGF-I to membranes was 1.4 % and 3.1 % for rat and guinea pig membranes respectively, similar to the relatively low levels reported for the liver cells or membranes of adult rats, humans and chickens (ie. relative to other tissues)(Rechler *et al.* 1980; Caro *et al.* 1988; Duclos & Goddard 1990). The higher capacity of the guinea pig membranes to bind IGF-I indicates that membrane type-1 IGF receptor concentrations are probably higher, since IGF-I (and also LR^{3}IGF-I) binds relatively poorly to insulin and type-2 IGF receptors in other species including rats (Massague & Czech 1982; Czech *et al.* 1989; Ballard *et al.* 1994). Information on the cross-specificity of IGFs between the receptor types of guinea pigs is not known. Since LR^{3}IGF-I binds to the type-1 IGF receptor, but with 3 to 4 times lower affinity than IGF-I in cells from other species (Francis *et al.* 1992), the small increase in the concentration of these receptors in liver membranes, even at the low levels described here, could contribute to the higher proclivity of this animal to LR^{3}IGF-I-induced hypoglycaemia. That is, LR^{3}IGF-I could inhibit glucose production by the liver by inhibiting glycogenolysis or gluconeogenesis, or may stimulate glucose uptake by this tissue. LR^{3}IGF-I could also have a greater hypoglycaemic potency in the guinea pig as a result of increased glucose uptake by the peripheral tissues, as demonstrated for IGF-I in normal rats and humans (Jacob *et al.* 1989; Elahi *et al.* 1991). If this is the case then it is speculated that tissues such as muscle, which take up large amounts of glucose, would have a greater IGF-I binding capacity in the guinea pig compared with the rat.

It is also possible that the number of insulin receptors or the affinity of insulin receptors for LR^{3}IGF-I is higher in the liver of guinea pigs compared to the rat and that this explains the differences that I have observed. The possibility deserves further investigation. This is in light of the findings by Horuk *et al.* (1979) who have examined the potency of triphthaloyl bovine insulin in a range of species. They showed that this insulin has at least a 3 times higher potency

in lowering blood glucose in the guinea pig compared with the mouse, rabbit and dog. Also, the effects of the insulin were 7 to 8 times greater on fat cells derived from the guinea pig than on fat cells from the mouse. The authors conclude that the properties of the insulin receptor in the guinea pig are quite different from those of the receptor in many other species.

Since IGF-II can bind to both type-1 and type-2 IGF receptors, then the type-2 receptors can compete with the type-1 receptors for binding IGF-II. Thus, the potency of IGF-II actions in a given tissue could partly depend on the number of type-2 receptors relative to the type-1 receptor, since the latter is the primary mediator of IGF actions (Ewton *et al.* 1987; Kiess *et al.* 1987a; Ballard *et al.* 1988; Adashi *et al.* 1990). The specific binding of IGF-II relative to that for IGF-I was measured here and indicated that in the liver membranes of both rats and guinea pigs there are probably more binding sites for IGF-II than for IGF-I. The maximum specific binding of IGF-I to membranes is an indicator of type-1 receptor binding. So for the guinea pig, where there was at least two times greater binding of IGF-II to membranes than for IGF-I, the number of type-2 receptors is probably at least twice that of the number of type-1 receptors. This ratio was much greater in the rat, where binding capacity was at least 8 times greater for IGF-II than for IGF-I. Other studies have also indicated that the number of type-2 IGF receptors in the rat liver are relatively high (Bryson & Baxter 1987). The significance of this difference between the rat and guinea pig will require further investigation. However, it is likely that the potency of IGF-II actions relative to those of IGF-I, will be less in livers of rats than guinea pigs. This is because the type-2 IGF receptor does not appear to mediate IGF-II actions in adult rat hepatocytes (Hartmann *et al.* 1992) and may instead act as a sink for IGF-II, thereby minimising type-1 IGF or insulin receptor interactions (Kiess *et al.* 1987a; Haig & Graham 1991; Francis *et al.* 1993). An obvious question relates to why the rat should have a relatively higher number of type-2 IGF receptors when it lacks substantial circulating IGF-II. Perhaps the number of these receptors is more

closely linked to functions involving ligands that contain mannose 6-phosphate moieties, which also bind to the receptor (Morgan *et al.* 1987; Kiess *et al.* 1988; Braulke *et al.* 1990). Although increased membrane binding points to an increase in receptor numbers, changes in receptor affinities could also explain such differences.

In summary, there is a higher IGF-I binding capacity in the liver membranes of guinea pigs compared with rats, which may be responsible for the difference in the ability of $LR^3IGF\text{-}I$ to induce hypoglycaemia in these animals. Further studies are required to determine if the analogue can substantially alter the carbohydrate metabolism of the guinea pig liver.

CHAPTER 8.

GENERAL DISCUSSION.

The primary objective of this study has been to gain a greater understanding of the actions of IGF-II in postnatal animals. There have been few studies, relative to those using IGF-I, that have examined the effects of IGF-II infusion on growth and metabolism. Schoenle *et al.* (1985) were first able to demonstrate, by continuous 6 day infusion of IGFs into rats whose growth rates were impaired due to hypophysectomy, that human IGF-II was less potent than human IGF-I in stimulating body weight gain, the widening of the tibial epiphysis and the incorporation of ^{3}H-thymidine into costal cartilage. At a dose of a little over 1 mg/kg/day, IGF-II did not significantly increase body weight gain in these animals. On the other hand, Shaar *et al.* (1989) were able to significantly increase body weight gain of hypophysectomised rats of about 50 days of age (weights were not given) when continuously infused with 155 µg of rhIGF-II/day over 7 days. Again using hypophysectomised rats, Schiltz *et al.* (1992) demonstrated that rat GH was more potent than rhIGF-II in stimulating weight gain and osteogenesis when injected daily over 12 days, but IGF-II had no significant effects on body weight gain. More recently, van Buul-Offers *et al.* (1994) have compared the effects of injecting IGF-I and IGF-II into Snell dwarf mice over several weeks and have been able to demonstrate effects of IGF-II on some organs, including kidneys, spleen, lungs and salivary glands. Although body weight gain and body length were elevated by IGF-II treatment, the effects were not deemed statistically significant. Thus, of these chronic treatment experiments, all involving rats or mice (which lack substantial circulating IGF-II) and all of which have some form of growth deficiency, only one has clearly indicated that IGF-II can stimulate body weight gain in the postnatal animal. Female broiler chickens which were continuously infused with 0.5 mg of IGF-II/kg/day over a two week period did not grow in response to this treatment, although there was an increase in the weight of the abdominal fat pad and a decrease in the weight of some muscle (Spencer *et al.* 1994). In other work,

increasing blood IGF-II concentrations by transplanting IGF-II secreting tumours into rodents, has also failed to stimulate growth (Wilson *et al.* 1989; Ren-Qui *et al.* 1993).

Another strategy to examine the effects of IGF-II on postnatal growth has been to produce transgenic mice that over-express IGF-II. Once again there have been no significant increases in body weight gain, but the animals have usually had a lower fat content and in some cases organs have grown or become cancerous (DaCosta *et al.* 1994; Rogler *et al.* 1994; Wolf *et al.* 1994; van Buul-Offers *et al.* 1995). Van Buul-Offers *et al.* 1995 have even concluded, based on results from these transgenic animal models, that pharmacological concentrations of IGF-II have little effect on whole body growth and that it cannot replace IGF-I in this action.

In this thesis I have demonstrated for the first time that IGF-II can significantly stimulate body weight gain of normal (non-hypophysectomised) rats. This is only the second time that IGF-II has been demonstrated to significantly increase the body weight gain of any animal. Additionally, I have been able to increase the growth of the gut, kidneys, spleen and thymus, as well as weight gain and feed conversion efficiency, by IGF-I and also IGF-II in the same study. This has enabled me to show that IGF-II is generally less potent than IGF-I in its ability to stimulate the growth of normal young rats. In most respects IGF-II is acting like a weak form of IGF-I. That is, IGF-II is doing what IGF-I is doing, but less potently. Therefore, a sufficiently high dose of IGF-II can take the place of IGF-I in stimulating the postnatal growth of rats. Although the potency difference may apply in a general sense, other studies indicate that IGF-II may be more potent than IGF-I in affecting some aspects of growth and metabolism. IGF-II appears to more potently lower liver glycogen stores in Snell dwarf mice (van Buul-Offers *et al.* 1994) and IGF-II seems more potent than IGF-I in stimulating nerve growth (Near *et al.*

1992) and muscle development (Florini *et al.* 1991b). Injection of IGF-II into lambs also affects the function of osteoclasts in bone more potently than IGF-I (Coxam *et al.* 1992).

The studies of this thesis involving guinea pigs represent the first comparisons of the effects of chronic infusion of IGF-I and IGF-II on the growth of animals that have both IGF-I and IGF-II in circulation. I have been unable to significantly stimulate body weight gain with either of these peptides, nor with a combination of the two nor with LR^3IGF-I. A dose of approximately 6 mg/kg/day of IGF-II was required to stimulate body weight gain and organ growth in normal rats, and since IGF-II was only infused at up to 2 mg/kg/day in the guinea pig, a yet higher dose of IGF-II may be required to prove whether or not these animals can grow in response to this peptide. Hence, it is not possible to say whether IGF-II is generally less potent than IGF-I in the guinea pig. Nevertheless, IGF-II was more potent than IGF-I in increasing the fractional weight of the lungs. This represents a further example of actions which may be peculiar to IGF-II, at least in the guinea pig. Although some studies implicate IGF-II rather than IGF-I in the growth and development of the lungs in sheep (Harding *et al.* 1993; Hooper *et al.* 1993), IGF-I was more potent than IGF-II in increasing the growth of the lungs in Snell dwarf mice (van Buul-Offers *et al.* 1994). The potency of the response of a tissue to IGF-I or IGF-II may therefore differ between the species and also the stage of development.

The cause of the relatively lower potency of IGF-II compared with IGF-I in the rat is not known, but as discussed in earlier chapters, the most probable cause is the presence of type-2 IGF receptors in the tissues acting as sinks for IGF-II (Kiess *et al.* 1987a; Haig & Graham 1991; Filson *et al.* 1993; Francis *et al.* 1993), reducing the amount of IGF-II available for type-1 receptor binding. There have been no *in vivo* experiments designed to specifically test this hypothesis as yet. By comparing the relative potencies of IGF-I, IGF-II and IGF-II analogues such as [Leu^{27}]IGF-II (Rosenfeld *et al.* 1991), which bind well to

the type-2 IGF receptor and poorly to the type-1 receptor, as well as analogues which bind poorly to the type-2 IGF receptor but well to the type-1 receptor (eg. [Thr^{48} Ser^{49} Ile^{50}]IGF-II, (Sakano *et al.* 1991)), it should be possible to prove or disprove the hypothesis. If the type-2 IGF receptor is acting as a sink, it would be expected that [Thr^{48} Ser^{49} Ile^{50}]IGF-II will have a potency which is close to that of IGF-I but greater than that of IGF-II in stimulating the growth of the rat, whereas [Leu^{27}]IGF-II will probably have no effect on growth.

The expression of type-2 IGF receptor levels in the tissues of rats roughly follows that of IGF-II (Kiess *et al.* 1987b; Sklar *et al.* 1989; Tollefsen *et al.*1989) and type-2 IGF receptors are high in human cancers over-expressing IGF-II (Raile *et al.* 1994; Hoeflich *et al.* 1994). Therefore it is feasible that infusion of IGF-II into an animal will stimulate tissue type-2 IGF receptor expression, perhaps as a means of reducing the interaction of IGF-II with the type-1 IGF receptor. The study of Filson *et al.* (1993) has already implied that the survival of fetal mice is probably dependent on such a mechanism. That is, mice that do not express the type-2 IGF receptor die prematurely, but if they also do not express IGF-II during development the fetus survives. Perhaps the type-2 IGF receptor can be used to control the concentration of IGF-II in blood and/or in extracellular spaces, by increasing the uptake of IGF-II by the tissues. If this mechanism does exist, it might explain why infusion of IGF-II at 360 µg/day for 7 days into guinea pigs increased plasma IGF-II by 638 µg/l, but 720 µg of IGF-II/day over 14 days increased plasma concentrations by only 235 µg/l. That is, the higher doses or longer treatment period might have enabled the development of a response designed to minimise the actions of IGF-II. To test this it is envisaged that guinea pigs will need to be treated with at least 720 µg of IGF-II/day and some killed every few days over a 14 day period to determine if

changes in blood IGF-II concentrations and tissue type-2 IGF receptor levels are taking place during the treatment period.

If the type-2 IGF receptor has evolved as a means to decrease the binding of IGF-II to the type-1 IGF receptor (or insulin receptor), even though evidence suggests that it may not be its sole function (Tally *et al.* 1987; Jirtle *et al.* 1991; Minniti *et al.* 1992; Rosenthal *et al.* 1994), the question remains as to the advantage to be gained in doing so. Haig & Graham (1991) propose that the uptake of IGF-II by the receptor in tissues of the developing rodent will reduce the size of the fetus, based on the fact that mice that lack IGF-II during this stage are stunted in growth (DeChiara *et al.* 1990). They argue that because the type-2 IGF receptor gene is maternally imprinted in these animals, the receptor may have evolved as a mechanism to reduce the size of individual animals in a litter, thereby enhancing the survival of the mother. This has further cemented the view of IGF-II as a *fetal* growth factor. Nevertheless, it is possible that the type-2 IGF receptor still acts to modulate the postnatal growth of tissues in other animals, particularly since the presence of IGF-II in blood may mean that IGF-II is still contributing to growth. There may also be other reasons for ensuring that the interaction of IGF-II with type-1 IGF or insulin receptors is minimised. For example, it is possible that IGF-II has a particularly strong influence on insulin or carbohydrate metabolism, and clearance via the type-2 receptor may be a means of keeping such influences in check. It is interesting to note that van Buul-Offers *et al.* (1994) have found that IGF-II, but not IGF-I, lowers the amount of glycogen stored in the liver of mice, which points to effects on carbohydrate metabolism. In contrast, Jones *et al.* (1990) have speculated that IGF-II may have a role in the deposition of glycogen in the liver of fetal guinea pigs, since the peak in blood IGF-II concentration in the developing fetus occurs at the time of maximum liver glycogen deposition. Also, some transgenic mice that overexpress IGF-II gradually

develop hypoglycaemia along with declines in body weight and feed intake (Rogler *et al.* 1994).

The increased potency of naturally occurring variants and analogues of the IGFs which have reduced affinity for the IGFBPs has demonstrated that one of the primary roles of the IGFBPs is to inhibit the actions of the IGFs by preventing their access to receptors. This has been demonstrated by numerous *in vitro* studies (Carlsson-Skwirut *et al.* 1986; Francis *et al.* 1986; Sara *et al.* 1986; Ballard *et al.* 1987; Bayne *et al.* 1988; Forbes *et al.* 1988; Francis *et al.* 1988b; Bagley *et al.* 1989; Carlsson-Skwirut *et al.* 1989; Ross *et al.* 1989; King *et al.* 1992; Francis *et al.* 1992; Oh *et al.* 1993; Francis *et al.* 1993). This has also been confirmed by *in vivo* studies, which have been carried out largely in rats. These experiments have shown that des(1-3)IGF-I, a natural variant, is cleared from blood at a faster rate than IGF-I or IGF-II when injected into normal young rats (Ballard *et al.* 1991). Cascieri *et al.* (1988) also demonstrated that analogues of IGF-I with reduced affinity for the IGFBPs have a reduced half-life in blood. The [Gln^3 Ala^4 Tyr^{15} Leu^{16}]IGF-I analogue was more effective than IGF-I in stimulating the incorporation of glucose into the glycogen of the diaphragm when injected into rats. Bastian *et al.* (1993) have shown that due to modifications to IGFBPs in the plasma of pregnant rats, which are thought to reduce the affinity of IGFBPs for IGFs, the rate of clearance of IGF-I from blood was increased compared with the rate in non-pregnant counterparts, a rate closer to that achieved with LR^3IGF-I, an analogue which has limited interaction with the IGFBPs (Francis *et al.* 1992). The clearance rate of des(1-3)IGF-I from blood is also faster than IGF-I following injection into lambs (Francis *et al.* 1988a). However, Prosser *et al.* (1995) were unable to demonstrate a significant difference in the clearance rate of IGF-I and des(1-3)IGF-I from blood when infused over 24 hours into an artery leading to the mammary gland of lactating goats. Despite this, they were able to

show that mammary blood flow was significantly greater in the goats treated with the truncated form of IGF-I. Other studies, involving chronic infusion of des(1-3)IGF-I or LR^3IGF-I into rats, have demonstrated that the variants are more potent than IGF-I in stimulating numerous aspects of growth and metabolism (Gillespie *et al.* 1990; Lemmey *et al.* 1991; Martin *et al.* 1991; Tomas *et al.* 1991a,b; Vanderhoof *et al.* 1992; Tomas *et al.* 1992; 1993a). The increased potency is likely to be related to their quicker clearance from the blood. Further evidence that circulating IGFBPs inhibit the actions of the IGFs by limiting their transfer from the blood to the tissues comes from the work of Zapf *et al.* (1995). Injection of IGF-I/IGFBP-3 complexes into hypophysectomised rats was able to stimulate glycogen synthesis in the diaphragm and lower blood glucose, but these effects did not occur in non-hypophysectomised animals. This was probably because the IGF-I/IGFBP-3 complexes were unable to form part of the 150 kDa complex in the blood of hypophysectomised animals and so were able to leave blood and reach the tissues more readily. IGF-I bound to IGFBPs in an approximately 50 kDa pool is quicker to leave blood than when in the 150 kDa complex of a number of species including lambs, humans and rats (Francis *et al.* 1988a; Davis *et al.* 1989; Guler *et al.* 1989b; Lewitt *et al.* 1994). This further highlights the importance of the IGFBPs in controlling the potency of the IGFs *in vivo*.

Although there is a paucity of knowledge regarding the actions of the IGFs in guinea pigs, studies by Peterkofsky *et al.* (1991, 1994) and Gosiewska *et al.* (1994) have demonstrated that the IGFBPs are likely to play a part in the control of the IGFs in these animals during conditions of fasting or vitamin C deficiency. Specifically, they have shown that the increased concentration of IGFBP-1 and IGFBP-2 in these animals is probably responsible for the subsequent inhibition of tissue collagen gene expression (and possibly weight loss) in these animals. That is, by sequestering IGF-I and inhibiting its

actions in the tissues. In the guinea pig studies of this thesis I have shown, by the generally higher potency of LR^{3}IGF-I compared with IGF-I or IGF-II, that the IGFBPs act to inhibit the anabolic actions and hypoglycaemic potential of the IGFs in this species. In the guinea pig study of chapter 5, 120 µg of LR^{3}IGF-I/day increased the fractional weights of adrenals, gut, kidneys and spleen, whereas 360 µg of IGF-I/day was required to increase the fractional weight of the adrenals, the only tissue to respond significantly to IGF-I. IGF-II had no effects on growth. In the experiment of chapter 6, administration of 240 µg of LR^{3}IGF-I/day appeared to be more potent than infusion of IGF-I, IGF-II or a combination of IGF-I and IGF-II at a dose of 720 µg/day, although this was not tested statistically. That is, the fractional weights of organs which were increased by 120 µg of LR^{3}IGF-I /day in the first study were also elevated at the higher dose used in the second study. The greater potency of LR^{3}IGF-I compared to IGF-I and IGF-II is supported by the hypoglycaemia induced in many of the animals following infusion of the analogue, but not in the animals of the other treatment groups. Irrespective of the generally higher potency of LR^{3}IGF-I in the guinea pig, IGF-II was more potent than LR^{3}IGF-I, IGF-I or a combination of IGF-I and IGF-II in stimulating the growth of the lungs. In all, the most likely explanation for the greater stimulation of organ growth and the lowering of blood glucose by LR^{3}IGF-I is its reduced affinity for the IGFBPs, enabling it to be more rapidly cleared from the circulation to the tissues. Thus, as in other species, IGFBPs in the guinea pig may serve to protect against insulin-like actions, especially the lowering of blood glucose, that may result from excessive amounts of free IGF in circulation.

The potency with which LR^{3}IGF-I caused hypoglycaemia in the guinea pig appears to be greater than in rats. I have shown that the reason for this may be due to a greater number of liver IGF-I binding sites in the guinea pig compared with the rat. Therefore, LR^{3}IGF-I might inhibit hepatic glucose production or uptake to a greater

degree in the guinea pig. Studies in non-diabetic rats and humans indicates that IGF-I treatment has little influence on hepatic glucose production, but it can lower blood glucose by stimulating uptake of glucose by peripheral tissues such as muscle (Zapf *et al.* 1986; Jacob *et al.* 1989; Elahi *et al.* 1991; Dimitriadis *et al.* 1992). Therefore, it is likely that the greater ability of LR^3IGF-I to lower blood glucose in the guinea pig compared to the rat may also be due to differences in the rate of glucose uptake by the tissues.

The effect of LR^3IGF-I on circulating IGFBP concentrations in the guinea pig may contribute to its enhanced hypoglycaemic potency in this animal. Chronic IGF-I administration increases blood IGFBP-3 concentrations in rats (Zapf *et al.* 1989; Tomas *et al.* (1992) and chapter 4) and can also do so in humans (Zapf *et al.* 1990), although Baxter *et al.* (1993) noted a fall in IGFBP-3 concentration. IGF-I and IGF-II tended to increase or have no effect on IGFBP concentrations in the blood of the guinea pig, whereas LR^3IGF-I decreased IGFBP-3 and the overall IGFBP concentration. A lowering of blood IGFBP-3 concentration by infusion of LR^3IGF-I into an animal has been reported only once before, and that was a study involving pigs (Walton *et al.* 1994). LR^3IGF-I has reduced affinity for IGFBPs and this is thought to explain its increased potency (Francis *et al.* 1992). However, the analogue, like des(1-3)IGF-I when infused into goats (Prosser *et al.* 1995), can still bind to IGFBPs in the blood of a range of species, albeit weakly (Lord *et al.* 1994). Therefore, in the case of guinea pigs, not only would the analogue have reduced affinity for IGFBPs but its action of lowering IGFBP concentrations could further reduce the capacity of blood to retain LR^3IGF-I, increasing its potency. The lowering of IGFBP concentrations would probably free large amounts of endogenous IGF-I and IGF-II that would be cleared via the tissues. It is likely that the freeing of these IGFs will have a strong insulin-like action resulting in hypoglycaemia (see Lewitt & Baxter 1991). Increased clearance of endogenous IGFs is supported by lower IGF concentrations in the

analogue-treated animals, although decreased production of these peptides by the tissues cannot be ruled out.

A comparison of the abilities of IGF-I and insulin to lower blood glucose in a number of species reveals that IGF-I is least hypoglycaemic in the rat (see Giacca *et al.* 1994). In rats, IGF-I is approximately 100 times less potent than insulin (Zapf *et al.* 1986, Jacob *et al.* 1989; Moxley *et al.* 1990; Rosetti *et al.* 1991), whereas in other species such as dogs, humans, pigs and sheep, IGF-I is closer to 10 times less potent (Shojaee-Moradie *et al.* 1991; Guler *et al.* 1987; Douglas *et al.* 1991a; Ballard *et al.* 1994; Giacca *et al.* 1994). The glucose-lowering potency of IGFs in the guinea pig may therefore be more akin to that seen in the latter animals than in the rat. However, the carbohydrate metabolism of guinea pigs appears quite different to most other species. Insulin circulates at a concentration in the guinea pig which is about 10 times the concentration found in rats and humans, and this thought to compensate for the fact that guinea pig insulin has an unusual structure and a low hypoglycaemic potency relative to insulins from other species (Zimmerman *et al.* 1974; Horuk *et al.* 1979; Gorray & Fujimoto 1980). The guinea pig insulin receptor binds insulin from other species with a higher affinity than guinea pig insulin (Zimmerman *et al.* 1974). Hence, IGFs might cross-react more potently with the guinea pig insulin receptor than the receptor from other species. This might also explain why LR^3IGF-I is so potent in lowering blood glucose in the guinea pig.

The guinea pig seems to be more resistant to the stimulation of weight gain by the IGFs than rats. Firstly, hypophysectomy in this species does not significantly affect growth rate (Clayton & Worden 1960) even though GH is released in an episodic fashion from the pituitary as in other animals (Gabrielsson *et al.* 1990). Fairhill *et al.* (1990) suggested that one possibility is that GH is playing more of a metabolic rather than a growth-promoting role in the guinea pig. Many of the growth-promoting effects of GH

are mediated by IGF-I. Possible explanations for an apparent failure of GH to affect growth could be an inability of GH to stimulate IGF-I production or a failure of the guinea pig to respond to IGF-I. In this context, Kerr *et al.* (1990) failed to demonstrate changes in the growth of guinea pigs that were passively immunised against IGF-I, supporting a lack of growth-responsiveness to circulating IGF-I. The lack of responsiveness to IGF-I cannot apply to all tissues in the guinea pig because the experiments of this thesis have shown that certain organs grow in response to IGFs and Mulholland *et al.* (1992) demonstrated that both IGF-I and IGF-II can stimulate guinea pig neurite outgrowth in culture. Also, as mentioned earlier, Gosiewska *et al.* (1994) have strong evidence that IGF-I is likely to be responsible for stimulating growth and/or collagen production in the connective tissues of these animals. Beaune *et al.* (1992) found that as with rats, humans and some other animals, plasma IGF-I concentrations increased about the time of puberty in guinea pigs, but they were unable to unequivocally attribute the concomitant increase in body weight to the IGF changes. I have been unable to stimulate body weight gain or change the composition of the carcass by treatment of guinea pigs with IGF-I, IGF-II or a combination of the two, at a dose of up to nearly 2 mg/kg/day, although the growth of some organs are increased at the same or lower doses. Despite the more potent stimulation of organ growth by LR^3IGF-I treatment, body weight gain was not increased. Hence, there appears to be an underlying resistance to weight gain, at least by GH and the IGFs, in these animals. This is not to suggest that these peptides do not normally contribute to the growth of these animals during development, but the contributions to that growth, if small, may be difficult to measure and prove. The guinea pig model may be useful in shedding new light on the contribution of IGFs to states of growth resistance that occur in other animals including humans.

Higher than normal concentrations of phosphorylated IGFBP-1 have been associated with resistance to the anabolic actions of IGF-I or GH in patients with some diseases (Frost *et al.* 1994). Close examination of Western ligand blots from the guinea pig studies reveals that a band approximating 28 kDa (in plasma pools of vehicle-treated animals) was slightly lower in size in plasma from IGF-I and LR^3IGF-I treated animals (Figure 5.3). The position of this band relative to the other IGFBPs that were visualised by Western ligand blotting, together with its size, suggests that it is probably IGFBP-1. Also, it is very close to the size of a 29 kDa IGFBP from guinea pig plasma that has previously been identified as IGFBP-1 (Peterkofsky *et al.* 1991, 1994; Gosiewska *et al.* 1994). Assuming that there is a real difference in the size of bands, the changes would be consistent with a difference in the level of IGFBP-1 phosphorylation. Highly phosphorylated IGFBP-1 appears lower in size than non-phosphorylated forms following Western ligand blotting (Frost *et al.* 1994). Thus guinea pigs might normally have non-phosphorylated or less phosphorylated forms of IGFBP-1 in circulation and IGF-I or LR^3IGF-I might increase production of highly phosphorylated forms. The high affinity that phosphorylated IGFBP-1 has for IGF-I (Jones *et al.* 1991) may be contributing to a resistance to both GH and IGF-I actions in the guinea pig. That blood IGFBP-1 concentration may also be a substantial determinant of resistance to growth in the guinea pig is supported by the work of Palka *et al.* (1989), Peterkofsky *et al.* (1991) and Gosiewska *et al.* (1994). Through a series of experiments they have shown that blood IGFBP-1 is probably the most potent inhibitor of IGF-I-stimulated collagen gene expression in the guinea pig during conditions of fasting or scurvy. In the future, assays using antibodies directed against phosphorylated IGFBP-1 isoforms could be used to determine whether this hypothesis is plausible.

In the studies of this thesis involving either rats or guinea pigs I have shown that there is an inverse relationship between the concentrations of IGF-I and IGF-II in blood. That is, infusion of IGF-I results in a fall in IGF-II concentrations and IGF-II infusion causes a lowering of IGF-I concentration. It was not possible to show that IGF-I lowers blood IGF-II concentrations in the rat because it lacks IGF-II in circulation. The inverse relationship between the IGFs has been demonstrated previously in a range of species including rats (Wilson *et al.* 1989), humans (Guler *et al.* 1989a), pigs (Owens *et al.* 1990) and sheep (Gluckman & Ambler 1993). It is likely that infusion of IGFs results in displacement of IGFs from the IGFBPs in blood, and probably does not inhibit IGF expression in the tissues. Since the increased IGF-II but lowered IGF-I concentrations in the blood of rats were not accompanied by changes in liver IGF gene expression in the study by Wilson *et al.* (1989), then it is likely that infusion of IGFs into animals results in displacement of IGFs from the IGFBPs in blood. Nevertheless, further experiments will be required to demonstrate whether tissue expression of IGFs is being altered in a range of species. A knowledge of the mechanism by which the inverse relationship operates will be required before its physiological significance can be ascertained. However, the overall effect may simply be to buffer against changes in total IGF concentration. Since both IGF-I and IGF-II exert biological actions primarily via the type-1 IGF receptor, the total IGF concentration in blood may in many instances be a better indicator of the capacity to stimulate the growth and/or metabolism of an animal than either IGF-I or IGF-II alone. In the study of chapter 5, the failure of exogenous IGF-I to exert much of an influence on growth in the guinea pig, despite significantly increased IGF-I concentrations in blood, could be due to the fact that the concomitant lowering of blood IGF-II concentrations resulted in insignificant changes in the total IGF concentration. However, total IGF concentrations were significantly increased by IGF-II treatment, yet did not significantly

increase growth in the same study. Also, if IGFs are being displaced from IGFBPs by the exogenously infused peptides, they should be free to be cleared to the tissues and exert biological actions. Thus it is not yet clear if the total circulating IGF concentration is of any real consequence in determining the growth response of an animal.

This thesis has contributed some valuable information on the actions of the IGFs *in vivo* and has raised some issues which need to be more fully examined, particularly those relating to the response of animals to LR^3IGF-I. The role of IGF-II in postnatal growth remains unresolved, particularly with regard to functions which are separate to those of IGF-I, although there is growing evidence, such as the stimulation of lung growth in guinea pigs, to suggest that there may be such functions. Further experiments are sorely needed, especially in species other than rats and mice, to understand the role of IGF-II in animal growth.

CHAPTER 8.

REFERENCES

Abbasi AA, Drinka PJ, Mattson DE & Rudman D 1993. Low circulating levels of insulin -like growth factors and testosterone in chronically institutionalized elderly men. *Journal of the American Geriatrics Society* **41** 975-982.

Adamo ML, Neuenschwander S, LeRoith D & Roberts CT 1994. Structure, expression, and regulation of the IGF-I gene. In: Current Directions in Insulin-Like Growth Factor Research p.1-11. Le Roith D & Raizada MK (Eds). Plenum Press, New York.

Adams SO, Nissley SP, Handwerger S & Rechler MM 1983. Developmental patterns of insulin-like growth factor-I and -II synthesis and regulation in rat fibroblasts. *Nature* **302** 150-152.

Adashi EY, Resnick CE, Ricciarelli E, Hurwitz A, Kokia E, Tedeschi C, Botero L, Hernandez ER, Rosenfeld RG, Carlsson-Skwirut E & Francis GL 1992. Granulosa cell-derived insulin-like growth factor (IGF) binding proteins are inhibitory to IGF-I hormonal action; Evidence derived from the use of a truncated IGF-I analogue. *Journal of Clinical Investigation* **90** 1593-1599.

Adashi EY, Resnick CE & Rosenfeld RG 1990. Insulin-like growth factor-I (IGF-I) and IGF-II. Hormonal action in cultured rat granulosa cells: mediation via type I but not type II IGF receptors. *Endocrinology* **126** 216-222.

Andress DL & Birnbaum RS 1992. Human osteoblast-derived insulin-like growth factor (IGF) binding protein-5 stimulates osteoblast mitogenesis and potentiates IGF action. *Journal of Biological Chemistry* **267** 22467-22472.

Argente J, Barrios V, Pozo J, Munoz MT, Hervas F, Stene M & Hernandez M 1993. Normative data for insulin-like growth factors (IGFs), IGF-binding proteins, and growth hormone-binding protein in a healthy spanish pediatric population: age- and sex-related changes. *Journal of Clinical Endocrinology and Metabolism* **77** 1522-1528.

Ayer-Le Lievre C, Stahlbom P-A & Sara VR 1991. Expression of IGF-I and -II mRNA in the brain and craniofacial region of the rat fetus. Development 111 105-115.

Bach MA, Chin E & Bondy CA 1994. The effects of subcutaneous insulin-like growth factor-I infusion in insulin-dependent diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism* **79** 1040-1045.

Bach MA, Chin E & Bondy CA 1994. The effects of subcutaneous insulin-like growth factor-I infusion in insulin-dependent diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism* **79** 1040-1045.

Bagley CJ, May BL, Szabo L, McNamara PJ, Ross M, Francis GL, Ballard FJ and Wallace JC 1989. A key functional role for the insulin-like growth factor-I N-terminal pentapeptide. *Biochemical Journal* **259** 665-671.

Ballard F, Baxter R, Binoux M, Clemmons D, Drop S, Hall K, Hintz R, Rechler M, Rutanen E & Schwander J 1990a. On the nomenclature of the IGF binding proteins. *Acta Endocrinologica* **121** 751-752.

Ballard FJ, Francis GL, Ross M, Bagley CJ, May B & Wallace JC 1987. Natural and synthetic forms of insulin-like growth factor-1 (IGF-I) and the potent derivative, destripeptide IGF-I: Biological activities and receptor binding. *Biochemical and Biophysical Research Communications* **149** 398-404.

Ballard FJ, Knowles SE, Walton PE, Edson K, Owens PC, Mohler MA & Ferraiolo BL 1991. Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I), IGF-II and des (1-3) IGF-I in rats. *Journal of Endocrinology* **128** 197-204.

Ballard FJ, Johnson RJ, Owens PC, Francis GL, Upton FM, McMurtry JP & Wallace JC 1990b. Chicken insulin-like growth factor I: amino acid sequence, radioimmunoassay and plasma levels between strains and during growth. *General and Comparative Endocrinology* **79** 459-468.

Ballard FJ, Read LC, Francis GL, Bagley CJ & Wallace JC 1986. Binding properties and biological potencies of insulin-like growth factors in L6 myoblasts. *Biochemical Journal* **233** 223-230.

Ballard FJ, Ross M, Upton FM & Francis GL 1988. Specific binding of insulin-like growth factors 1 and 2 to the type 1 and type 2 receptors respectively. *Biochemical Journal* **249** 721-726.

Ballard FJ, Walton PE, Dunshea FR, Francis GL & Tomas FM 1994. Does IGF-I ever act through the insulin receptor? In: The insulin-like growth factors and their regulatory proteins. p131-138. Baxter RC, Gluckman PD & Rosenfeld RG (Eds). Elsevier Science BV, Amsterdam, The Netherlands.

Bang P, Baxter RC, Blum WF, Breier BH, Clemmons DR, Hall K, Hintz RL, Holly JMP, Rosenfeld RG & Zapf J 1994. Consensus statement: Valid measurements of total IGF concentrations in biological fluids. Recommendations from the 3rd international symposium on insulin-like growth factors. *Journal of Endocrinology* **143** C1-C2.

Bar RS, Boes M, ClemmonsDR, Busby WH, Sandra A, Dake BL & Booth BA 1990. Insulin differentially alters transcapillary movement of intravascular IGFBP-1, IGFBP-2 and endothelial cell IGF-binding proteins in the rat heart. *Endocrinology* **127** 497-499.

Barlow DP, Stoger R, Herrmann BG, Saito K & Schweifer N 1991. The mouse insulin-like growth factor type 2 receptor is imprinted and closely linked to the Tme locus. *Nature* **349** 84-87.

Bass JJ, Oldham JM, Hodgkinson SC, Fowke PJ, Sauerwein H, Molan P, Breier BH & Gluckman PD 1991. Influences of nutrition and bovine growth hormone (GH) on hepatic GH binding, insulin-like growth factor-I and growth of lambs. *Journal of Endocrinology* **128** 181-186.

Bastian SEP, Walton PE, Wallace JC & Ballard FJ 1993. Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I) and an analogue LR^3IGF-I in pregnant rats. *Journal of Endocrinology* **138** 327-336.

Bauman DE, McCutcheon SN, Steinhour WD, Eppard PJ & Sechen SJ 1985. Sources of variation and prospects for improvement of productive efficiency in the dairy cow: a review. *Journal of Animal Science* **63** 583.

Baumann U, Eisenhauer T & Hartmann H 1992. Increase of glomerular filtration rate and renal plasma flow by insulin-like growth factor-I during euglycaemic clamping in anaesthetized rats. *European Journal of Clinical Investigation* **22** 204-209.

Baumrucker CR, Green MH & Blum JW 1994. Effects of dietary rhIGF-I in neonatal calves on the appearance of glucose, insulin, D-xylose, globulins and gamma-glutamyl transferase in blood. *Domestic Animal Endocrinology* **11** 393-403.

Bautista CM, Baylink DJ & Mohan S 1991. Isolation of a novel insulin-like growth factor (IGF) binding protein from human bone: a potential candidate for fixing IGF-II in human bone. *Biochemical and Biophysical Research Communications* **176** 756-763.

Baxter RC 1988. The insulin-like growth factors and their binding proteins. *Comparative Biochemistry and Physiology* **91B** 229-235.

Baxter RC, Hizuka N, Takano K, Holman SR & Asakawa K 1993. Responses of insulin-like growth factor binding protein-1 (IGFBP-1) and the IGFBP-3 complex to administration of insulin-like growth factor-I. *Acta Endocrinologica* **128** 101-108.

Baxter RC & Martin JL 1989a. Structure of the Mr 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity labeling. *Proceedings of the National Academy of Sciences USA* **86** 6898-6902.

Baxter RC & Martin JL 1989b. Binding proteins for the insulin-like growth factors: structure, regulation and function. *Progress in Growth Factor Research* **1** 49-68.

Baxter RC, Martin JL & Wood MH 1987. Two immunoreactive binding proteins for insulin-like growth factors in human amniotic fluid: relationship to fetal maturity. *Journal of Clinical Endocrinology and Metabolism* **65** 423-31.

Bayne ML, Applebaum J, Chicchi GG, Haynes NS, Green BG & Cascieri MA 1988. Structural analogs of human insulin-like growth factor I with reduced affinity for serum binding proteins and the type 2 insulin-like growth factor receptor. *Journal of Biological Chemistry* **263** 6233-6239.

Beaune B, Fellman N, Delaitre M & Coudert J 1992. Interrelationships between plasma insulin-like growth factor-I (IGF-I) concentrations and plasma sex hormone levels in the growing male guinea pig. *Journal of Developmental Physiology* **17** 189-194.

Beck F, Samani NJ, Senior P, Byrne S, Morgan K, Gebhard R & Brammar WJ 1988. Control of IGF-II mRNA levels by glucocorticoids in the neonatal rat. *Journal of Molecular Endocrinology* **1** R5-R8.

Behringer RR, Lewin TM, Quaife CJ, Palmitter RD, Brinster RL & D'Ercole AJ 1990. Expression of insulin-like growth factor I stimulates normal somatic growth hormone-deficient transgenic mice. *Endocrinology* **127** 1033-1040.

Bell GI, Stempien MM, Fong NM & Seino S 1990. Sequence of a cDNA encoding guinea pig IGF-I. *Nucleic Acids Research* **18** 4275

Beukers MW, Oh Y, Zhang H, Ling N & Rosenfeld RG 1991. [Leu^{27}] Insulin-like growth factor II is highly selective for the type-II receptor in binding, cross-linking and thymidine incorporation experiments. *Endocrinology* **128** 1201-1203.

Binoux M, Roghani M, Hossenlopp P, Hardouin S & Gourmelen M 1991. Molecular forms of human IGF binding proteins: physiological implications. *Acta Endocrinologica* **124** Suppl. 2 41-47.

Binz K, Joller P, Froesch P, Binz H, Zapf J & Froesch ER 1990. Repopulation of the atrophied thymus in diabetic rats by insulin-like growth factor I. *Proceedings of the National Academy of Sciences USA* **87** 3690-3694.

Blum WF, Albertsson-Wikland K, Rosberg S & Ranke MB 1993. Serum levels of insulin-like growth factor I (IGF-I) and IGF binding protein 3 reflect spontaneous growth hormone secretion. *Journal of Clinical Endocrinology and Metabolism* **76** 1610-1616.

Blum WF, Jenne EW, Reppin F, Kietzmann K, Ranke MB & Bierich JR 1989. Insulin-like growth factor I (IGF-I)-binding protein complex is a better mitogen than free IGF-I. *Endocrinology* **125** 766-772.

Bolinder J, Lindblad A, Engfeldt P & Arner P 1987. Studies of acute effects of insulin-like growth factors I and II in human fat cells. *Journal of Clinical Endocrinology and Metabolism* **65** 732-737.

Bondy CA, Werner H, Roberts CT Jr. & LeRoith D 1990. Cellular pattern of insulin-like growth factor-I (IGF-I) and type I IGF receptor gene expression in early organogenesis: comparison with IGF-II gene expression. *Molecular Endocrinology* **4** 1386-1398.

Boni-Schnetzler M, Binz K, Mary JL, Schmid C, Schwander J & Froesch ER 1989. Regulation of hepatic expression of IGF-I and fetal IGF binding protein mRNA in streptozotocin diabetic rats. *FEBS Letters* **251** 253-256.

Boni-Schnetzler M, Schmid C, Mary J-L, Zimmerli B, Meier PJ, Zapf J, Schwander J & Froesch ER 1990. Insulin regulates the expression of the insulin-like growth factor binding protein 2 mRNA in rat hepatocytes. *Molecular Endocrinology* **4** 1320-1326.

Boulle N, Schneid H, Listrat A, Holthuizen P, Binoux M & Groyer A 1993. Developmental regulation of bovine insulin-like growth factor-II (IGF-II) gene expression: homology between bovine transcripts and human IGF-II exons. Journal of *Molecular Endocrinology* **11** 117-128.

Braulke T, Bresciani R, Buergisser DM & von Figura K 1991. Insulin-like growth factor II overexpression does not affect sorting of lysosomal enzymes in NIH-3T3 cells. *Biochemical and Biophysical Research Communications* **179** 108-115.

Braulke T, Tippmer S, Chao H-J & von Figura K 1990. Regulation of mannose 6-phosphate/insulin-like growth factor II receptor distribution by activators and inhibitors of protein kinase C. *European Journal of Biochemistry* **189** 609-616.

Braulke T, Tippmer S, Neher E & von Figura K 1989. Regulation of the mannose 6-phosphate/IGF II receptor expression at the cell surface by mannose 6-phosphate, insulin like growth factors and epidermal growth factor. *EMBO Journal* **8** 681-686.

Breier BH, Bass JJ, Butler JH & Gluckman PD 1986. The somatotrophic axis in young steers: influence of nutritional status on pulsatile release of growth hormone and circulating concentrations of insulin-like growth factor 1. *Journal of Endocrinology* **111** 209-215.

Brice AL, Checthan JE, Bolton VN, Hill NCW & Schofield PN 1989. Temporal changes in the expression of the insulin-like growth factor II gene associated with tissue maturation in the human fetus. *Development* **106** 543-554.

Brown AL, Chiariotti L, Orlowski CC, Mehlem T, Burgess WH, Ackerman EJ, Bruni CB & Rechler MM 1989. Nucleotide sequence and expression of a cDNA clone encoding a fetal rat binding protein for insulin-like growth factors. *Journal of Biological Chemistry* **264** 5148-5154.

Brown LB, Graham DE, Nissley SP, Hill DJ, Strain AJ & Rechler MM 1986. Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. *Journal of Biological Chemistry* **261** 13144-13150.

Bryson JM & Baxter RC 1987. High-affinity receptor for insulin-like growth factor-II in rat liver: properties and regulation. *Journal of Endocrinology* **113** 27-35.

Buck MA, Olah TA, Weitzmann CJ & Cooperman BS 1989. Protein estimation by the product of integrated peak area and flow rate. *Analytical Biochemistry* **182** 295-299.

Buonomo FC & Klindt J 1993. Ontogeny of growth hormone (GH), insulin-like growth factors (IGF-I and IGF-II) and IGF binding protein-2 (IGFBP-2) in genetically lean and obese swine. *Domestic Animal Endocrinology* **10** 257-265.

Burgi H, Muller WA, Humbel RE, Labhart A & Froesch ER 1966. Nonsuppressible insulin-like activity of human serum. I. Physiochemical properties, extraction and partial purification. *Biochemica et Biophysica Acta* **121** 349-359.

Burgisser DM, Roth BV, Giger R, Luthi C, Weigl S, Zarn J & Humbel RE 1991. Mutants of human insulin-like growth factor II with altered affinities for the type 1 and type 2 insulin-like growth factor receptor. *Journal of Biological Chemistry* **266** 1029-1033.

Campbell RG, Johnson RJ, Taverner MR & King RH 1991. Interrelationships between exogenous porcine somatotropin (PST) administration and dietary protein and energy intake on protein deposition capacity and energy metabolism of pigs. *Journal of Animal Science* **69** 1522-1531.

Campbell RG, Steele NC, Caperna TJ, McMurtry JP, Solomon MB & Mitchell AD 1988. Interrelationships between energy intake and endogenous porcine growth hormone administration on the performance, body composition and protein and energy metabolism of growing pigs weighing 25 to 55 kg live weight. *Journal of Animal Science* **66** 1643-1655.

Canalis E 1994. Skeletal growth factors and aging. *Journal of Clinical Endocrinology and Metabolism* **78** 1009-1010.

Canalis E, Centrella M & McCarthy TL 1991. Regulation of insulin-like growth factor-II production in bone cultures. *Endocrinology* **129** 2457-2462.

Cara JF, Nagakawa SH & Tager SH 1988. Structural determinants of ligand recognition by type-I insulin-like growth factor receptors: use of semisynthetic insulin analog probes. *Endocrinology* **122** 2881-2887.

Carlsson-Skwirut C, Jornvall H, Holmgren A, Andersson C, Bergman J, Lundquist G, Sjogren B & Sara VR 1986. Isolation and characterization of variant IGF-1 as well as IGF-2 from adult human brain. *FEBS Letters* **201** 46-50.

Carlsson-Skwirut C, Lake M, Hartmanis M, Hall K & Sara VR 1989. A comparison of the biological activity of the recombinant intact and truncated insulin-like growth factor 1 (IGF-1). *Biochemica et Biophysica Acta* **1011** 192-197.

Caro JF, Poulos J, Ittoop O, Pories WJ, Flickinger EG & Sinha MK 1988. Insulin-like growth factor I binding in hepatocytes from human liver, human hepatoma, and normal, regenerating, and fetal rat liver. *Journal of Clinical Investigation* **81** 976-981.

Carr JM, Grant PA, Francis GL, Owens JA, Wallace JC & Walton PE 1994. Isolation andcharacterization of ovine IGFBP-4: protein purification and cDNA sequence. *Journal of Molecular Endocrinology* **13** 219-236.

Carson MJ, Behringer RR, Brinster RL & McMorris FA 1993. Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. *Neuron* **10** 729-740.

Cascieri MA, Chicchi GG, Applebaum J, Green BG, Hayes NS & Bayne ML 1989. Structural analogs of human insulin-like growth factor (IGF) I with altered affinity for type-2 IGF receptors. *Journal of Biological Chemistry* **264** 2199-2202.

Cascieri MA, Saperstein R, Hayes NS, Green BG, Chicchi GG, Applebaum J & Bayne ML 1988. Serum half-life and biological activity of mutants of human insulin-like growth factor I which do not bind to serum binding proteins. *Endocrinology* **123** 373-381.

Cavallaro T, Martone RL, Stylianopoulou F & Herbert J 1993. Differential expression of the insulin-like growth factor II and transthyretin genes in the developing rat choroid plexus. *Journal of Neuropathology and Experimental Neurology* **52** 153-162.

Chan KC & Nicoll CS 1994. Characterization of rat serum insulin-like growth factor-binding proteins by two-dimensional gel electrophoresis: identification of a potentially novel form. *Endocrinology* **134** 2574-2580.

Chard T 1989. Hormonal control of growth in the human fetus. *Journal of Endocrinology* **123** 3-9.

Chard T, Blum WF, Brunjes J, Campbell DJ & Wathen NC 1994. Levels of insulin-like growth factor-binding protein-2 and insulin-like growth factor-II in maternal serum, amniotic fluid and extraembryonic coelomic fluid at 9-20 weeks of pregnancy. *Journal of Endocrinology* **142** 379-383.

Cheetham TD, Taylor A, Holly JMP, Clayton K, Cwyfan-Hughes S & Dunger DB 1994. The effects of recombinant human insulin-like growth factor-I (IGF-I) administration on the levels of IGF-I, IGF-II and IGF-binding proteins in adolescents with insulin-dependent diabetes mellitus. *Journal of Endocrinology* **142** 367-374.

Chernausek SD, Dickson BA, Smith EP & Hoath SB 1991. Suppression of insulin-like growth factor-I during epidermal growth factor-induced growth retardation. *American Journal of Physiology* **260** E416-E421.

Chin E, Zhou J, Dai J, Baxter RC & Bondy CA 1994. Cellular localization and regulation of gene expression for components of the insulin-like growth factor ternary binding protein complex. *Endocrinology* **134** 2498-2504.

Christoffersen CT, Bornfeldt KE, Rotella CM, Gonzales N, Vissing H, Shymko RM, ten Hoeves J, Groffen J, Heisterkamp N & DeMeyts P 1994. Negative cooperativity in the insulin-like growth factor-I receptor and a chimeric IGF-I/insulin receptor. *Endocrinology* **135** 472-475.

Clairmont KB & Czech MP 1989. Chicken and Xenopus mannose 6-phosphate receptor lacks the high affinity binding site for insulin-like growth factor II. *Journal of Biological Chemistry* **264** 16390-16392.

Clayton BE & Worden JM 1960. Growth in young hypophysectomized guinea pigs. *Journal of Endocrinology* **20** 30-47.

Claussen M, Zapf J & Braulke T 1994. Proteolysis of insulin-like growth factor binding protein-5 by pregnancy serum and amniotic fluid. *Endocrinology* **134** 1964-1966.

Clemmons DR, Seek MM & Underwood LE 1985. Supplementary essential amino acids augment the somatomedin C/ insulin-like growth factor I response to refeeding after fasting. *Metabolism* **34** 391-395.

Clemmons DR, Smith-Banks A, Celniker AC & Underwood LE 1992. Reversal of diet-induced catabolism by infusion of recombinant insulin-like growth factor-I (IGF-I) in humans. *Journal of Clinical Endocrinology and Metabolism* **75** 234-238.

Clemmons DR, Snyder DK & Busby WH 1991. Variables controlling the secretion of insulin-like growth factor binding protein-2 in normal human subjects. *Journal of Clinical Endocrinology and Metabolism* **73** 727-733.

Clemmons DR, Thissen JP, Maes M, Ketelslegers JM & Underwood LE 1989. Insulin-like growth factor-I (IGF-I) infusions into hypophysectomized or protein-deprived rats induces specific IGF-binding proteins in serum. *Endocrinology* **125** 2967-2972.

Clemmons DR & Underwood LE 1994. Uses of human insulin-like growth factor-I in clinical conditions. *Journal of Clinical Endocrinology and Metabolism* **79** 4-6.

Clubb FJ Jr., Bell PD, Kriseman JD & Bishop SP 1987. Myocardial cell growth and blood pressure development in neonatal spontaneously hypertensive rats. *Laboratory Investigation* **56** 189-197.

Cohick WS & Clemmons DR 1993. The insulin-like growth factors. *Annual Reviews of Physiology* **55** 131-153.

Coleman ME & Etherton TD 1991. effects of exogenous porcine growth hormone on serum insulin-like growth factor-binding proteins in growing pigs. *Journal of Endocrinology* **128** 175-180.

Conover CA, Clarkson JT & Bale LK 1994. Insulin-like growth factor-II enhancement of human fibroblast growth via a nonreceptor-mediated mechanism. *Endocrinology* **135** 76-82.

Conover CA, Divertie GD & Lee PDK 1993. Cortisol increases plasma insulin-like growth factor binding protein-1 in humans. *Acta Endocrinologica* **128** 140-143.

Copeland KC & Nair 1994. Recombinant human insulin-like growth factor-I increases forearm blood flow. *Journal of Clinical Endocrinology and Metabolism* **79** 230-232.

Cotterill AM, Cowell CT, Baxter RC, McNeil D & Silink M 1988. Regulation of the growth hormone independent insulin-like growth factor binding protein in children. *Journal of Clinical Endocrinology and Metabolism* **67** 882-887.

Cordido F, Casanueva FF, Vidal JI & Dieguez C 1991. Study of insulin-like growth factor I in human obesity. *Hormone Research* **36** 187-191.

Coxam V, Davicco M-J, Pastoureau P, Delmas PD & Barlet J-P 1992. Insulin-like growth factor-II increases plasma osteocalcin concentration in newborn lambs. *Bone and Mineral* **17** 177-186.

Cwyfan-Hughes SC, CotterillAM, Molloy AR, Cassell TB, Braude N, Hinds JC, Wass JA & Holly JM 1992. The induction of s pecific proteases for insulin-like growth factor-binding proteins following major heart surgery. *Journal of Endocrinology* **135** 135-145.

Czech MP 1989. Signal transmission by the insulin-like growth factors. *Cell* **59** 235-238.

Da Costa THM, Williamson DH, Ward A, Bates P, Fisher R, Richardson L, Hill DJ, Robinson ICAF & Graham CF 1994. High plasma insulin-like growth factor-II and low lipid content in transgenic mice: measurements of lipid metabolism. *Journal of Endocrinology* **143** 433-439.

Davis SR, Hodgkinson SC, Moore LG & Gluckman PD 1989. Improved estimates of clearance of ^{125}I-labelled insulin-like growth factor-I carrier protein complexes from blood plasma of sheep. *Journal of Endocrinology* **123** 469-475.

Davenport ML, Isley WL, Pucilowska JB, Pemberton LB, Lyman B, Underwood LE & Clemmons DR 1992. Insulin-like growth factor-binding protein-3 proteolysis is induced after elective surgery. *Journal of Clinical Endocrinology and Metabolism* **75** 590-595.

Daughaday WH 1989. A personal history of the origin of the somatomedin hypothesis and recent challenges to its validity. *Perspectives in Biology and Medicine* **32** 194-211.

Daughaday WH, Emanuele MA, Brooks MH, Barbato AL, Kapadia M & Rotwein P 1988. Synthesis and secretion of insulin-like growth factor II by a leiomyosarcoma with associated hypoglycemia. *New England Journal Medicine* **319** 1434-1440.

Daughaday WH, Ward AP, Goldberg AC, Trivedi B & Kapadia M 1982. Characterization of somatomedin binding in human serum by ultracentrifugation and gel-filtration. *Journal of Clinical Endocrinology and Metabolism* **55** 916-921.

Daughaday WH, Wu J-C, Lee S-D & Kapadia M 1990. Abnormal processing of pro-IGF-II in patients with hepatoma and in some hepatitis B virus antibody-positive asymptomatic individuals. *Journal of Laboratory and Clinical Medicine* **116** 555-562.

Daughaday WH & Rotwein P 1989. Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocrine Reviews* **10** 68-91.

Daughaday WH, Yanow CE & Kapadia M 1986. Insulin-like growth factors I and II in maternal and fetal guinea pig serum. *Endocrinology* **119** 490-494.

Davenport ML, D'Ercole AJ & Underwood LE 1990. Effect of maternal fasting on fetal growth, serum insulin-like growth factors (IGFs), and tissue IGF messenger ribonucleic acids. *Endocrinology* **126** 2062-2067.

Davenport ML, Svoboda ME, Koerber KL, Van Wyk JJ, Clemmons DR & Underwood LE 1988. Serum concentrations of insulin-like growth factor II are not changed by short term fasting and refeeding. *Journal of Clinical Endocrinology and Metabolism* **67** 1231- 1236.

DeChiara TM, Robertson EJ & Efstratiadis A 1991. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64** 849-859.

Delhanty PJD & Han VKM 1993. The expression of insulin-like growth factor (IGF) binding protein-2 and IGF-II genes in the tissues of the developing ovine fetus. *Endocrinology* **132** 41-52.

Denver RJ & Nicoll CS 1994. Pancreatic hormones differentially regulate insulin-like growth factor (IGF)-I and IGF-binding protein production by primary rat hepatocytes. *Journal of Endocrinology* **142** 299-310.

D'Ercole AJ, Wilson DF & Underwood LE 1980. Changes in the circulating form of serum somatomedin-C during fetal life. *Journal of Clinical Endocrinology and Metabolism* **51** 674-676.

DeVol DL, Rotwein P, Sadow JL, Novakofski J & Bechtel PJ 1990. Activation of insulin-like growth factor gene expression during work-induced skeletal muscle growth. *American Journal of Physiology* **259** E89-E95.

Dimitriadis G, Parry-Billings M, Dunger D, Bevan S, Colquhoun A, Taylor A, Calder P, Krause U, Wegener G & Newsholme EA 1992. Effects of in-vivo administration of insulin-like growth factor-I on the rate of glucose utilization in the soleus muscle of the rat. *Journal of Endocrinology* **133** 37-43.

Ding H, Kopple JD, Cohen A & Hirschberg R 1993. Recombinant human insulin-like growth factor-I accelerates recovery and reduces catabolism in rats with ischemic acute renal failure. *Journal of Clinical Investigation* **91** 2281-2287.

Donovan SM, Oh Y, Pham H & Rosenfeld RG 1989. Ontogeny of serum insulin-like growth factor binding proteins in the rat. *Endocrinology* **125** 2621-2627.

Douglas RG, Gluckman PD, Ball K, Breier B & Shaw JHF 1991a. The effects of infusion of insulinlike growth factor (IGF)-I, IGF-II and insulin on glucose and protein metabolism in fasted lambs. *Journal of Clinical Investigation* **88** 614-622.

Douglas RG, Gluckman PD, Breier BH, McCall JL, Parry B & Shaw JHF 1991b. Effects of recombinant IGF-I on protein and glucose metabolism in rTNF-infused lambs. *American Journal of Physiology* **261** 606-612.

Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP & Rauscher FJ 1992. Repression of the insulin-like growth factor II gene by the wilms tumor suppressor WT1. *Science* **257** 674-678.

Duclos MJ & Goddard C 1990. Insulin-like growth factor receptors in chicken liver membranes: binding properties, specificity, developmental pattern and evidence for a single receptor type. *Journal of Endocrinology* **125** 199-206.

Dwyer CM & Stickland NC 1992. The effects of maternal undernutrition on maternal and fasted serum insulin-like growth factors, thyroid hormones and cortisol in the guinea pig. *Journal of Developmental Physiology* **18** 303-313.

Ebeling PR, Jones JD, O'Fallon WM, Jones CH & Riggs BL 1993. Short-term effects of recombinant human insulin-like growth factor I on bone turnover in normal women. *Journal of Clinical Endocrinology and Metabolism* **77** 1384-1387.

Eigenmann JE, Patterson DF & Froesch ER 1984. Body size parallels insulin-like growth factor I levels but not growth hormone secretory capacity. *Acta Endocrinologica* **106** 448-453.

Eisemann JH, Hammond AC, Rumsey TS & Bauman DE 1989. Nitrogen and protein metabolism and metabolites in plasma and urine of beef steers treated with somatotropin. *Journal of Animal Science* **67** 105-115.

Elahi D, McAloon-Dyke M, Fukagawa NK, Wong G, Minaker KL, Seaman JJ, Good WR, Vandepol CG, Shannon RS, Miles JM & Wolfe RR 1991. Hemodynamic and metabolic responses to human insulin-like growth factor I (IGF-I) in men. In: Modern concepts of insulin-like growth factors, pp219-224. Spencer EM (Ed.). Elsevier, New York.

Elgin RG, Busby WH & Clemmons DR 1987. An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. *Proceedings of the National Academy of Sciences USA* **84** 3254-3258.

Elsasser TH, Caperna TJ & Rumsey TS 1995. Endotoxin administration decreases plasma insulin-like growth factor (IGF)-I and IGF-binding protein-2 in Angus x Hereford steers independent of changes in nutritional intake. *Journal of Endocrinology* **144** 109-117.

Elsasser TH, Rumsey TS & Hammond AC 1989. Influence of diet on basal and growth hormone-stimulated plasma concentrations of IGF-I in beef cattle. *Journal of Animal Science* **67** 128-137.

Engberg G & Hall K 1984. Immunoreactive IGF-II in serum of healthy subjects and patients with growth hormone disturbances and uraemia. *Acta Endocrinologica* **107** 164-170.

Etherton TD, Wiggins JP, Chung CS, Evock CM, Rebhun JF & Walton PE 1986. Stimulation of pig growth performance by porcine growth hormone and growth hormone-releasing factor. *Journal of Animal Science* **64** 433.

Ewton DZ, Falen SL & Florini JR 1987. The type II insulin-like growth factor (IGF) receptor has low affinity for IGF-I analogues: Pleiotrophic actions of IGFs on myoblasts are apparently mediated by the type I receptor. *Endocrinology* **120** 115-123.

Fairhill KM, Gabrielson BG & Robinson ICAF 1990. Effect of food withdrawal and insulin on growth hormone secretion in the guinea pig. *Endocrinology* **127** 716-723.

Feige JJ & Baird A 1991. Growth factor regulation of adrenal cortex growth and function. *Progress in Growth Factor Research* **3** 103-113.

Filson AJ, Louvi A, Efstratiadis A & Robertson EJ 1993. Rescue of the T-associated maternal effect in mice carrying null mutations in igf-2 and igf2r, two reciprocally imprinted genes. *Development* **118** 731-736.

Flint DJ & Gardner MJ 1993. Influence of growth hormone deficiency on growth and body composition in rats: site-specific effects upon adipose tissue development. *Journal of Endocrinology* **137** 203-211.

Flint DJ, Tonner E, Beattie J & Gardner M 1994. Several insulin-like growth factor-I analogues and complexes of insulin-like growth factors-I and -II with insulin-like growth factor-binding protein-3 fail to mimic the effect of growth hormone upon lactation in the rat. *Journal of Endocrinology* **140** 211-216.

Flint DJ, Tonner E, Beattie J & Panton D 1992. Investigation of the mechanism of action of growth hormone in stimulating lactation in the rat. *Journal of Endocrinology* **134** 377-383.

Florini JR, Ewton DZ & Roof SL 1991a. Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression. *Molecular Endocrinology* **5** 718-724.

Florini JR, Magri KA, Ewton DZ, James PL, Grindstaff K & Rotwein PS 1991b. "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. *Journal of Biological Chemistry* **266** 15917-15923.

Forbes B, Szabo L, Baxter RC, Ballard FJ & Wallace JC 1988. Classification of the insulin-like growth factor binding proteins into three distinct categories according to their binding specificities. *Biochemical and Biophysical Research Communications* **157** 196-202.

Fowlkes JL, Suzuki K, Nagase H & Thrailkill KM 1994. Proteolysis of insulin-like growth factor binding protein-3 during rat pregnancy: a role for matrix metalloproteinases. *Endocrinology* **135** 2810-2813.

Francis GL, Aplin SE, Milner SJ, McNeil KA, Ballard FJ & Wallace JC 1993. Insulin-like growth factor (IGF)-II binding to IGF-binding proteins and IGF receptors is modified by deletion of the N-terminal hexapeptide or substitution of arginine for glutamine-6 in IGF-II. *Biochemical Journal* **293** 713-719.

Francis GL, McNamara PJ, Filsell OM & Ballard FJ 1988a. Plasma half-lives of native and modified insulin-like growth factor-I in lambs. *Journal of Endocrinology* **117** 183-189.

Francis GL, Read LC, Ballard FJ, Bagley CJ, Upton FM, Gravestock PM & Wallace JC 1986. Purification and partial sequence analysis of insulin-like growth factor-I from bovine colostrum. *Biochemical Journal* **233** 207-213.

Francis GL, Ross M, Ballard FJ, Milner SJ, Senn C, McNeil KA, Wallace JC, King R & Wells JRE 1992. Novel recombinant fusion protein analogues of insulin-like growth factor (IGF)-I indicate the relative importance of IGF-binding protein and receptor binding for enhanced biological potency. *Journal of Molecular Endocrinology* **8** 213-223.

Francis GL, Upton FM, Ballard FJ, McNeil KA & Wallace JC 1988b. Insulin-like growth factors 1 and 2 in bovine colostrum. Sequences and biological activities compared with those of a potent truncated form. *Biochemical Journal* **251** 95-103.

Froesch ER, Burgi H, Ramseier EB, Bally P & Labhart A 1963. Antibody suppressible and nonsuppressible insulin-like activities in human serum and their physiological significance. An insulin assay with adipose tissue of increased precision and specificity. *Journal of Clinical Investigation* **42** 1816-1834.

Frost RA, Bereket A, Wilson TA, Wojnar MM, Lang CH and Gelato MC 1994. Phosphorylation of insulin-like growth factor (IGF) binding protein-1 in patients with insulin-dependent diabetes mellitus and severe trauma. *Journal of Clinical Endocrinology and Metabolism* **78** 1533-1535.

Funk B, Kessler U, Eisenmenger W, Hansmann A, Kolb HJ & Kiess W 1992. The expression of insulin-like growth factor binding proteins is tissue specific during human fetal life and early infancy. *Acta Endocrinologica* **127** 107-114.

Gabrielson B, Fairhill KM & Robinson ICAF 1990. Growth hormone secretion in the guinea pig. *Journal of Endocrinology* **124** 371-380.

Gardner MJ & Flint DJ 1990. Long-term reductions in GH, insulin-like growth factor-I and body weight gain in rats treated neonatally with antibodies to rat GH. *Journal of Endocrinology* 124 381-386.

Gargosky SE, Moyse KJ, Walton PE, Owens JA, Wallace JC, Robinson JS and Owens PC 1990. Circulating levels of insulin-like growth factors increase and molecular forms of their serum binding proteins change with human pregnancy. *Biochemical and Biophysical Research Communications* **170** 1157-1163.

Gargosky SE, Wilson KF, Fielder PJ, Vaccarello MA, Guevara-Aguirre J, Diamond FB, Baxter RC, Rosenbloom AL & Rosenfeld RG 1993. The composition and distribution of insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) in the serum of growth hormone receptor-deficient patients: effects of IGF-I therapy on IGFBP-3. *Journal of Clinical Endocrinology and Metabolism* 77 1683-1689.

Gaskins HR, Kim J-W, Wright JT, Rund LA & Hausman GJ 1990. Regulation of insulin-like growth factor-I ribonucleic acid expression, polypeptide secretion, and binding protein activity by growth hormone in porcine preadipocyte cultures. *Endocrinology* **126** 622-630.

Geenen V, Achour I, Robert F, Vandersmissen E, Sodoyez J-C, Defresne M-P, Boniver J, Lefebvre PJ & Franchimont P 1993. Evidence that insulin-like growth factor 2 (IGF2) is the dominant thymic peptide of the insulin superfamily. *Thymus* **21** 115-127.

Geier A, Haimshon M, Beery R, Hemi R & Lunenfeld B 1993. Insulin-like growth factor-I inhibits cell death induced by cycloheximide in MCF-7 cells: a model system for analyzing control of cell death. *In Vitro Cellular and Developmental Biology* **28 A** 725-729.

Gelato MC, Kiess W, Lee L, Malozowski S & Rechler MM 1988. The IGF-II/M6P receptor is present in monkey serum. *Journal of Clinical Endocrinology and Metabolism* **67** 669-675.

Gelato MC, Rutherford C, Stark RI & Daniel SS 1989. The insulin-like growth factor II/mannose-6-phosphate receptor is present in fetal and maternal sheep serum. *Endocrinology* **124** 1-9.

Gerrard DE & Grant AL 1994. Insulin-like growth factor-II expression in developing skeletal muscle of double muscled and normal cattle. *Domestic Animal Endocrinology* **11** 339-347.

Gertner JM 1992. Growth hormone actions on fat distribution and metabolism. *Hormone Research* **38** Suppl.2 41-43.

Giacca A, Fisher SJ, SHI ZQ, Gupta R, Lickley HLA, Skottner A, Anderson GH, Efendic S & Vranic M 1994. Insulin-like growth factor-I and insulin have no differential effects on glucose production and utilization under conditions of hyperglycemia. *Endocrinology* **134** 2251-2258.

Giacca A, Gupta R, Efendic S, Hall K Skottner A, Lickley L & Vranic M 1990. Differential effects of IGF-I and insulin on glucoregulation and fat metabolism in depancreatized dogs. *Diabetes* **39** 340-347.

Gilhar A, Ish-Shalom S, Pillar T, Etzioni A & Silbermann M 1994. Effect of antiinsulin-like growth factor I on epidermal proliferation of human skin transplanted onto nude mice treated with growth hormone. *Endocrinology* **134** 229-232.

Gill SC & von Hippel PH 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Analytical Biochemistry* **182** 319-326.

Gillespie C, Read LC, Bagley CJ & Ballard FJ 1990. Enhanced potency of truncated insulin-like growth factor-I (des(1-3)IGF-I) relative to IGF-I in *lit/lit* mice. *Journal of Endocrinology* **127** 401-405.

Glasscock GF, Gelber SE, Lamson G, McGee-Tekula R & Rosenfeld RG 1990. Pituitary control of growth in the neonatal rat: effects of neonatal hypophysectomy on somatic and organ growth, serum insulin-like growth factors (IGF)-I and -II levels, and expression of IGF binding proteins. *Endocrinology* **127** 1792-1803.

Glasscock GF, Hein AN, Miller JA, Hintz RL & Rosenfeld RG 1992. Effects of continuous infusion of insulin-like growth factor I and II, alone and in combination with thyroxine or growth hormone, on the neonatal hypophysectomized rat. *Endocrinology* **130** 203-210.

Glazner GW, Lupien S, Miller JA & Ishii DN 1993. Insulin-like growth factor II increases the rate of sciatic nerve regeneration in rats. *Neuroscience* **54** 791-797.

Gluckman PD & Ambler GR 1993. At the cutting edge. What is the function of circulating insulin-like growth factor-2 in postnatal life? *Molecular and Cellular Endocrinology* **92** C1-C3.

Gluckman PD & Butler JH 1983. Parturition-related changes in insulin-like growth factor-I and -II in the perinatal lamb. *Journal of Endocrinology* **99** 223-232.

Goodman HM & Grichtig G 1983. Growth hormone and lipolysis: a reevaluation. *Endocrinology* **113** 1697-1702.

Gorray KC & Fujimoto WY 1980. "Micro"-insulin radioimmunoassay: measurement of the insulin response during glucose tolerance tests in guinea pigs. *Proceedings of the Society for Experimental Biology and Medicine* **163** 388-392.

Gosiewska A, Wilson S, Kwon D and Peterkofsky 1994. Evidence for an in vivo role of insulin-like growth factor binding protein-1 and -2 as inhibitors of collagen gene expression in vitamin C-deficient and fasted guinea pigs. *Endocrinology* **134** 1329-1339.

Gosteli-Peter MA, Winterhalter KH, Schmid c, Froesch ER and Zapf J 1994. Expression and regulation of insulin-like growth factor-I (IGF-I) and IGF-binding protein messenger ribonucleic acid levels in tissues of hypophysectomized rats infused with IGF-I and growth hormone. *Endocrinology* **135** 2558-2567.

Gowan LK, Hampton B, Hill DJ, Schlueter RJ & Perdue JF 1987. Purification and characterization of a unique high molecular weight form of insulin-like growth factor II. *Endocrinology* **121** 449-458.

Graham DE, Rechler MM, Brown AL, Frunzio R, Romanus JA, Bruni CB, Whitfield HJ, Nissley SP, Seelig S & Berry S 1986. Coordinate developmental regulation of high and low molecular weight mRNAs for rat insulin-like growth factor II. *Proceedings of the National Academy of Sciences USA* **83** 4519-4523.

Green BN, Jones SB, Streck,RD, Wood TL, Rotwein P & Pintar JE 1994. Distinct expression patterns of insulin-like growth factor binding proteins 2 and 5 during fetal and postnatal development. *Endocrinology* **134** 954-962.

Guler H-P, Binz K, Eigenmann F, Jaggi S, Zimmermann D, Zapf J & Froesch ER 1989a. Small stature and insulin-like growth factors: prolonged treatment of mini-poodles with recombinant human insulin-like growth factor I. *Acta Endocrinologica* **121** 456-464.

Guler H-P, Zapf J, Scheiwiller E & Froesch ER 1988. Recombinant human insulin-like growth factor I stimulates growth and has distinct effects on organ size in hypophysectomized rats. *Proceedings of the National Academy of Sciences USA* **85** 4889-4893.

Guler H-P, Zapf J & Froesch ER 1987. Short-term metabolic effects of recombinant human insulin-like growth factor-I in healthy adults. *New England Journal of Medicine* **317** 137-140.

Guler H-P, Zapf J, Schmid C and Froesch ER 1989b. Insulin-like growth factors I and II in healthy man. Estimation of half-lives and production rates. *Acta Endocrinologica* **121** 753-758.

Gunn JM, Clark MG, Knowles SE, Hopgood MF & Ballard FJ 1977. Reduced rates of proteolysis in transformed cells. *Nature* **266** 58-60.

Hahnel A & Schultz GA 1994. Insulin-like growth factor binding proteins are transcribed by preimplantation mouse embryos. *Endocrinology* **134** 1956-1959.

Haig D & Graham C 1991. Genomic imprinting and the strange case of the insulin-like growth factor II receptor. *Cell* **64** 1045-1046.

Han VKM, Lu F, Bassett N, Yang K, Delhanty PJD & Challis JRG 1992. Insulin-like growth factor-II messenger ribonucleic acid is expressed in steroidogenic cells of the developing ovine adrenal gland: evidence of an autocrine/paracrine role for IGF-II. *Endocrinology* **131** 3100-3109.

Harding R, Hooper SB & Han VKM 1993. Abolition of fetal breathing movements by spinal cord transection leads to reductions in fetal lung liquid volume, lung growth, and IGF-II gene expression. *Pediatric Research* **34** 148-153.

Hartmann H, Meyer-Alber A & Braulke T 1992. Metabolic actions of insulin-like growth factor II in cultured adult rat hepatocytes are not mediated through the insulin-like growth factor II receptor. *Diabetologia* **35** 216-223.

Hartmann H, Scmitz F, Christ B, Jungermann K & Creutzfeldt W 1990. Metabolic actions of insulin-like growth factor-I in cultured hepatocytes from adult rats. *Hepatology* **12** 1139-1143.

Haselbacher G & Humbel R 1982. Evidence for two species of insulin-like growth factorII (IGF-II and 'big' IGF-II) in human spinal fluid. *Endocrinology* **110** 1822-1824.

Haskell JF & Tucker DC 1994. Binding of insulin-like growth factors (IGF-I and IGF-II) to the IGF-II/mannose 6-phosphate receptor in fetal rat myocardium. *Endocrinology* **135** 231-239.

Hayden JM, Marten NW, Burke EJ & Straus DS 1994. The effect of fasting on insulin-like growth factor-I nuclear transcript abundance in rat liver. *Endocrinology* **134** 760-768.

Heyner S, Smith RM & Schultz GA 1989. Temporally regulated expression of insulin and insulin-like growth fctors and their receptors in early mammalian development. *BioEssays* **11** 171-176.

Hill DJ, Clemmons DR, Wison S, Han VKM, Strain AJ & Milner RDG 1989a. Immunological distribution of one form of insulin-like growth factor (IGF)-binding protein and IGF peptides in human fetal tissues. *Journal of Molecular Endocrinology* **2** 31-38.

Hill WC, Pelle-Day G, Kitzmiller JL & Spencer EM 1989b. Insulin-like growth factors in fetal macrosomia with and without maternal diabetes. *Hormone Research* **32** 178-182.

Hintz RL 1984. Plasma forms of somatomedin and the binding protein phenomenon. *Clinical Endocrinology and Metabolism* **13** 31-42.

Hintz RL & Liu F 1977. Demonstration of specific plasma protein binding sites for somatomedin. *Journal of Clinical Endocrinology and Metabolism* **45** 988-995.

Hirschberg R & Kopple JD 1989. Evidence that insulin-like growth factor I increases renal plasma flow and glomerular filtration rate in fasted rats. *Journal of Clinical Investigation* **83** 326-330.

Hizuka N, Takano K, Shizume K, Asakawa K, Miyakawa M, Tanaka I & Horikawa R 1986. Insulin-like growth factor I stimulates growth in normal growing rats. *European Journal of Pharmacology* **125** 143-146.

Hodgkinson SC, Davis SR, Burleigh DD, Henderson HV & Gluckman PD 1987. Metabolic clearance of protein bound and free insulin-like growth factor-I in fed and starved sheep. *Journal of Endocrinology* **115** 223-240.

Hodgkinson SC, Napier JR, Spencer GSG & Bass JJ 1994. Glycosaminoglycan binding characteristics of the insulin-like growth factor-binding proteins. *Journal of Molecular Endocrinology* **13** 105-112.

Hoeflich A, Yang Y, Kessler U, Heinz-Erian P, Kolb H & Kiess W 1994. Human colon carcinoma cells (CaCo-2) synthesize IGF-II and express IGF-I receptors and IGF-II/M6P receptors. *Molecular and Cellular Endocrinology* **101** 141-150.

Hogg J, Hill DJ & Han VKM 1994. The ontogeny of insulin-like growth factor (IGF) and IGF-binding protein gene expression in the rat pancreas. *Journal of Molecular Endocrinology* **13** 49-58.

Holly JMP, Dunger DB, Edge JA, Smith CP, Chard T & Wass JAH 1990. Insulin-like growth factor binding protein-I levels in diabetic adolescents and their relationship to metabolic control. *Diabetic Medicine* **7** 618-623.

Hooper SB, Han VKM & Harding R 1993. Changes in lung expansion alter pulmonary DNA synthesis and IGF-II gene expression in fetal sheep. *American Journal of Physiology* **265** L403-L409.

Horuk R, Goodwin P, O'Connor K, Neville RWJ, Lazarus NR & Stone D 1979. Evolutionary changes in the insulin receptors of hystricomorph rodents. *Nature* **279** 439-440.

Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S & Binoux M 1986. Analysis of serum insulin-like growth factor binding proteins using Western ligand blotting: use of the method for titration of the binding proteins and competitive binding studies. *Analytical Biochemistry* **154** 138-143.

Hsu CJ & Hammond JM 1987. Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor I by porcine granulosa cells in vitro. *Endocrinology* **120** 198-207.

Hu KQ, Backer JM, Sahagiani G, Feener E & King GL 1990. Modulation of the insulin-like growth factor II/manose 6 phosphate receptor in microvascular endothelial cells by phorbol ester via protein kinase C. *Journal of Biological Chemistry* **265** 13864-13870.

Hunkapiller MW, Hewick RM, Dreyer WJ & Hood LE 1983. High-sensitivity sequencing with a gas-phase sequencer. *Methods in Enzymology* **91** 399-413.

Hussain MA, Schmitz O, Mengel A, Keller A, Christiansen JS, Zapf J & Froesch ER 1993. Insulin-like growth factor I stimulates lipid oxidation, reduces protein oxidation, and enhances insulin sensitivity in humans. *Journal of Clinical Investigation* **92** 2249-2256.

Isgaard J, Nilsson A, Lindahl A, Jansson J-O & Isaakson OGP 1986. Effects of local administration of GH and IGF-I on longitudinal bone growth in rats. *American Journal of Physiology* **250** E367-E372.

Ishi DN 1989. Relationship of insulin-like growth factor II gene expression in muscle to synaptogenesis. *Proceedings of the National Academy of Sciences USA* **86** 2898-2902.

Isley WL, Underwood LE & Clemmons DR 1983. Dietary components that regulate serum somatomedin-C concentrations in humans. *Journal of Clinical Investigation* **71** 175-182.

Jacob R, Barrett E, Plewe G, Fagin KD & Sherwin RS 1989. Acute effects of insulin-like growth factor I on glucose and amino acid metabolism in the awake fasted rat. *Journal of Clinical Investigation* **83** 1717-1723.

Jacob RJ, Sherwin RS, Bowen L, Fryburg D, Fagin KD, Tamborlane WV & Shulman GI 1991. Metabolic effects of IGF-I and insulin in spontaneously diabetic BB/w rats. *American Journal of Physiology* **260** E262-E268.

Jakob A, Hauri CH & Froesch ER 1968. Nonsuppressible insulin-like activity in human serum. III. Differentiation of two distinct molecules with nonsuppressible ILA. *Journal of Clinical Investigation* **47** 2678-2688.

Jansen M, van Schaik FMA, Ricker AT, Bullock B, Woods DE, Gabbay KH, Nussbaum AL, Sussenbach JS & Van den Brande JL 1983. Sequence of cDNA encoding human insulin-like growth factor I precursor. *Nature* **306** 609.

Jirtle RL, Carr BI & Scott CD 1991. Modulation of insulin-like growth factor-II/mannose 6-phosphate receptors and transforming growth factor-beta 1 during liver regeneration. *Journal of Biological Chemistry* **266** 22444-22450.

Johnsson ID, Hart IC & Butler-Hogg BW 1985. Effects of exogenous bovine growth hormone and bromocryptine on growth, body development, fleece weight and plasma concentrations of growth hormone, insulin and prolactin in female lambs. *Animal Production* **41** 207-217.

Jones CT, Lafeber HN, Rolph TP & Parer JT 1990. Studies on the growth of the fetal guinea pig. The effects of nutritional manipulation on prenatal growth and plasma somatomedin activity and IGF concentrations. *Journal of Developmental Physiology* **13** 189-197.

Jones JI & Clemmons DR 1995. Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* **16** 3-34.

Jones JI, D'Ercole AJ, Camacho-Hubner C and Clemmons DR 1991. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: effects on affinity for IGF-I. *Proceedings of the National Academy of Sciences USA* **88** 7481-7485.

Jorgensen JOL, Blum WF, Horn N, Moller N, Moller J, Ranke MB & Christiansen JS 1993. Insulin-like growth factors (IGF)I and II and IGF binding proteins 1, 2 and 3 during low-dose growth hormone (GH) infusion and sequential euglycemic and hypoglycemic glucose clamps: studies in GH-deficient patients. *Acta Endocrinologica* **128** 513-520.

Kachra Z, Yang C-R & Posner BI 1994. The augmentation of insulin-like growth factor-I messenger ribonucleic acid in cultured rat hepatocytes: activation of protein kinase-A and -C is necessary, but not sufficient. *Endocrinology* **134** 702-708.

Kajimoto Y & Rotwein P 1990. Evolution of insulin-like growth factor I (IGF-I): structure and expression of an IGF-I precursor from Xenopus laevis. *Molecular Endocrinology* **4** 217-226.

Kanzaki S, Hilliker S, Baylink DJ & Mohan S 1994. Evidence that human bone cells in culture produce insulin-like growth factor-binding-4 and -5 proteases. *Endocrinology* **134** 383-392.

Kar S, Chabot J-G & Quirion R 1993. Quantitative autoradiographic localization of [^{125}I] Insulin-like growth factor I, [^{125}I] Insulin-like growth factor II, and [^{125}I] Insulin receptor binding sites in developing and adult rat brain. *Journal of Comparative Neurology* **333** 375-397.

Kasuga M, Van Oberghen E, Nissley SP & Rechler MM 1981. Demonstration of two subtypes of insulin-like growth factor receptors by affinity cross-linking. *Journal of Biological Chemistry* **256** 5305-5308.

Kerr DE, Laarveld B & Manns JG 1990. Effects of passive immunization of growing guinea pigs with an insulin-like growth factor-I monoclonal antibody. *Journal of Endocrinology* **124** 403-415.

Kiess W, Haskell JF, Lee L, Greenstein LA, Miller BE, Aarons AL, Rechler MM & Nissley SP 1987a. An antibody that blocks insulin-like growth factor (IGF) binding to the type II IGF receptor is neither an agonist nor an inhibitor of IGF-stimulated biologic responses in L6 myoblasts. *Journal of Biological Chemistry* **262** 12745-12751.

Kiess W, Blickenstaff GD, Sklar MM, Thomas CL, Nissley SP & Sahagian GG 1988. Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor. *Journal of Biological Chemistry* **263** 9339-9344.

Kiess W, Greenstein LA, White RM, Lee L, Rechler MM & Nissley SP 1987b. TypeII insulin-like growth factor receptor is present in rat serum. *Proceedings of the National Academy of Sciences USA* **84** 7720-7724.

King R, Wells JRE, Krieg P, Snoswell M, Brazier J, Bagley CJ, Wallace JCW, Ballard FJ, Ross M & Francis GL 1992. Production and characterization of recombinant insulin-like growth factor-I (IGF-I) and potent analogues of IGF-I, with Gly or Arg substituted for Glu^3, following their expression in *Escherichia coli* as fusion proteins. *Journal of Molecular Endocrinology* **8** 29-41.

Knobil E & Greep RO 1959. The physiology of growth hormone with particular reference to its action in the rhesus monkey and the 'species-specificity' problem. *Recent Progress in Hormone Research* **15** 1-69.

Koea JB, Breier BH, Shaw JHF & Gluckman PD 1992a. A possible role for IGF-II: evidence in sheep for in vivo regulation of IGF-I mediated protein anabolism. *Endocrinology* **130** 2423-2425.

Koea JB, Douglas RG, Breier BH, Shaw JHF Gluckman PD 1992b. Synergistic effect of insulin-like growth factor-I administration on the protein sparing effects of total parenteral nutrition in fasted lambs. *Endocrinology* **131** 643-648.

Koistinen R, Itkinen P, Selenius P & Seppala M 1990. Insulin-like growth factor binding protein-1 inhibits binding of IGF-I on fetal skin fibroblasts but stimulates their DNA synthesis. *Biochemical and Biophysical Research Communications* **173** 408-415.

Kriel GV, Bryant MJ & Lomax MA 1992. Effect of dietary protein intake and intravenous glucose infusion on plasma concentrations of insulin-like growth factor-I in lambs. *Journal of Endocrinology* **132** 195-199.

Kroonsberg C, McCutcheon SN, Siddiqui RA, MacKenzie DDS, Blair HT, Ormsby JE, Breier BH & Gluckman PD 1989. Reproductive performance and fetal growth in female mice from lines divergently selected on the basis of plasma IGF-I concentrations. *Journal of Reproduction and Fertility* **87** 349-353.

Kupfer SR, Underwood LE, Baxter RC & Clemmons DR 1993. Enhancement of the anabolic effects of growth hormone and insulin-like growth factor I by the use of both agents simultaneously. *Journal of Clinical Investigation* **91** 391-397.

Laager R, Ninnis R & Keller U 1993. Comparison of the effects of recombinant human insulin-like growth factor-I and insulin on glucose and leucine kinetics in humans. *Journal of Clinical Endocrinology* **92** 1903-1909.

Laburthe M, Rouyer-Fressard C & Gammeltoft S 1988. Receptors for insulin-like growth factor I and II in rat gastrointestinal epithelium. *American Journal of Physiology* **254** G457-G462.

Laemmli UK 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227** 680-685.

Lalou C, Silve C, Rosato R, Segovia B & Binoux M 1994. Interactions between insulin-like growth factor-I (IGF-I) and the system of plasminogen activators and their inhibitors in the control of IGF-binding protein-3 production and proteolysis in human osteosarcoma cells. *Endocrinology* **135** 2318-2326.

Lamson G, Pham H, Oh Y, Ocrant I, Schwander J and Rosenfeld RG 1989. Expression of the BRL-3A insulin-like growth factor binding protein (rBP-30) in the rat central nervous system. *Endocrinology* **123** 1100-1102.

Laron Z 1993. Somatomedin-1 (insulin-like growth factor-I) in clinical use. Facts and potential. *Drugs* **45** 1-8.

Lassarre C, Hardouin S, Daffos F, Forestier F, Frankenne F, Binoux M 1991. Serum insulin-like growth factors and insulin-like growth factor binding proteins in the human fetus. Relationships with growth in normal subjects and in those with intrauterine growth retardation. *Pediatric Research* **29** 219-225.

Lauterio TJ, Aravich PF & Rotwein P 1990. Divergent effects of insulin on insulin-like growth factor-II gene expression in the rat hypothalamus. *Endocrinology* **126** 392-398.

Lauterio TJ, Marson L, Daughaday WH & Baile CA 1987. Evidence for the role of insulin-like growth factor II (IGF-II) in the control of food intake. *Physiology of Behaviour* **40** 755-758.

Lee CY, Chung CS & Simmen FA 1993. Ontogeny of the porcine insulin-like growth factor system. *Molecular and Cellular Endocrinology* **93** 71-80.

Lee W-H, Bowsher RR, Apathy JM, Smith MC & Henry DP 1991. Measurement of insulin-like growth factor-II in physiological fluids and tissues. II. Extraction and quantification in rat tissues. *Endocrinology* **128** 815-822.

Lemmey AB, Martin AA, Read LC, Tomas FM, Owens PC & Ballard FJ 1991. IGF-I and the truncated analogue des-(1-3)IGF-I enhance growth in rats after gut resection. *American Journal of Physiology* **260** E213-E219.

Lemozy S, Pucilowska JB & Underwood LE 1994. Reduction of insulin-like growth factor-I (IGF-I) in protein-restricted rats is associated with differential regulation of IGF-binding protein messenger ribonucleic acids in liver and kidney, and peptides in liver and serum. *Endocrinology* **135** 617-623.

LeRoith D, Adamo M, Werner H & Roberts CT Jr. 1991. Insulinlike growth factors and their receptors as growth regulators in normal physiology and pathologic states. *Trends in Endocrinology and Metabolism* **2** 134-139.

Levinovitz A & Norstedt G 1989. Developmental and steroid hormonal regulation of insulin-like growth factor II expression. *Molecular Endocrinology* **3** 797-804.

Levinovitz A, Norstedt G, van den Berg S, Robinson ICAF & Ekstrom TJ 1992. Isolation of an insulin-like growth factor-II cDNA from guinea pig liver: expression and developmental regulation. *Molecular and Cellular Endocrinology* **89** 105-110.

Lewitt MS & Baxter RC 1989. Regulation of growth hormone independent insulin-like growth factor binding protein (BP-28) in cultured human fetal liver explants. *Journal of Clinical Endocrinology and Metabolism* **69** 246-252.

Lewitt MS & Baxter RC 1991. At the cutting edge. Insulin-like growth factor-binding protein-1: a role in glucose counterregulation. *Molecular and Cellular Endocrinology* **79** C147-C152.

Lewitt MS, Denyer GS, Cooney GJ & Baxter RC 1991. Insulin-like growth factor binding protein-1 modulates blood glucose levels. *Endocrinology* **129** 2254-2256.

Lewitt MS, Saunders SH, Cooney GJ & Baxter RC 1993. Effect of human insulin-like growth factor-binding protein-1 on the half-life and action of administered insulin-like growth factor-I in rats. *Journal of Endocrinology* **136** 253-260.

Lewitt MS, Saunders H, Phuyal JL & Baxter RC 1994. Complex formation by human insulin-like growth factor-binding protein-3 and human acid-labile sub-unit in growth hormone-deficient rats. *Endocrinology* **134** 2404-2409.

Li J, Saunders JC, Gilmour RS, Silver M & Fowden AL 1993. Insulin-like growth factor-II messenger ribonucleic acid expression in fetal tissues of the sheep during late gestation: effects of cortisol. *Endocrinology* **132** 2083-2089.

Lienhard GE 1994. Life without the IRS. *Nature* **372** 128-129.

Listrat A, Gerrard DE, Boulle N, Groyer A & Robelin J 1994. In situ localization of muscle insulin-like growth factor-II mRNA in developing bovine fetuses. *Journal of Endocrinology* **140** 179-187.

Liu LL, Greenberg S, Russell SM & Nicoll CS 1989. Effects of insulin-like growth factors I and II on growth and differentiation of transplanted rat embryos and fetal tissues. *Endocrinology* **124** 3077-3082.

Liu F, Baker BK, Powell DR & Hintz RL 1993a. Characterization of proinsulin-like growth factor-II E region immunoreactivity in serum and other biological fluids. *Journal of Clinical Endocrinology and Metabolism* **76** 1095-1100.

Liu L, Harding JE, Evans PC & Gluckman PD 1994. Maternal insulin-like growth factor-I infusion alters feto-placental carbohydrate and protein metabolism in pregnant sheep. *Endocrinology* **135** 895-900.

Liu J-P, Baker J, Perkins AS, Robertson EJ & Efstratiadis A 1993b. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* **75** 73-82.

Lord APD, Bastian SEP, Read LC, Walton PE & Ballard FJ 1994. Differences in the association of insulin-like growth factor-I (IGF-I) and IGF-I variants with rat, sheep, pig, human and chicken plasma-binding proteins. *Journal of Endocrinology* **140** 475-482.

Lu F, Han VKM, Milne WK, Fraser M, Carter AM, Berdusco ETM & Challis JRG 1994. Regulation of insulin-like growth factor-II gene expression in the ovine fetal adrenal gland by adrenocorticotropic hormone and cortisol. *Endocrinology* **134** 2628-2635.

Luna AM, Wilson DM, Wibblesman CJ, Brown R, Nagashima RJ, Hintz RL & Rosenfeld RG 1987. Somatomedins in adolescence: a cross-sectional study of the effect of puberty on plasma insulin-like growth factor I and II levels. *Journal of Clinical Endocrinology and Metabolism* **57** 268-271.

Lund PK, Moats-Staats BM, Hynes MA, Simmons JG, Jansen M, D'Ercole AJ & Van Wyk JJ 1986. Somatomedin-C/insulin-like growth factor-I and insulin-like growth factor-II mRNAs in rat fetal and adult tissues. *Journal of Biological Chemistry* **261** 14539-14544.

Luo J, Reid RE & Murphy LJ 1990. Dexamethasone increases hepatic insulin-like growth factor binding protein-1 (IGFBP-1) mRNA and serum IGFBP-1 concentrations in the rat. *Endocrinology* **127** 1456-1462.

Luthi C, Roth BV & Humbel RE 1992. Mutants of human insulin-like growth factor II (IGF-II). Expression and characterization of truncated IGF-II and of two naturally occurring variants. *European Journal of Biochemistry* **205** 483-490.

MacDonald RG, Pfeffer SR, Coussens L, Tepper MA, Brocklebank CM, Mole JE, Anderson JK, Chen E, Czech MP & Ullrich A 1988. A single receptor binds both insulin-like growth factor II and mannose-6-phosphate. *Science* **239** 1134-1137.

MacDonald RG, Tepper MA, Clairmont KB, Perregaux SB & Czech MP 1989. Serum form of the rat insulin-like growth factor II/mannose 6-phosphate receptor is truncated in the carboxy-terminal domain. *Journal of Biological Chemistry* **264** 3256-3261.

Machlin LJ 1972. Effect of porcine growth hormone on growth and carcass composition of the pig. *Journal of Animal Science* **35** 794.

Machwate M, Zerath E, Holy X, Pastoreau P & Marie PJ 1994. Insulin-like growth factor-I increases trabecular bone formation and osteoblastic cell proliferation in unloaded rats. *Endocrinology* **134** 1031-1038.

Maes M, Underwood LE & Ketelslegers J-M 1983. Plasma somatomedin-C in fasted and refed rats: close relationship with changes in liver somatogenic but not lactogenic binding sites. *Journal of Endocrinology* **97** 243-252.

Martin AA, Gillespie CM, Moore L, Ballard FJ & Read LC 1994. Effects of insulin-like growth factor-I peptides in rats with acute renal failure. *Journal of Endocrinology* **140** 23-32.

Martin AA, Tomas FM, Owens PC, Knowles SE, Ballard FJ & Read LC 1991. IGF-I and its variant, des-(1-3)IGF-I, enhance growth in rats with reduced renal mass. *American Journal of Physiology* **261** F626-F633.

Martin DM, Carlson RO & Feldman EL 1993. Interferon-gamma inhibits DNA synthesis and insulin-like growth factor-II expression in human neuroblastoma cells. *Journal of Neuroscience Research* **34** 489-501.

Martin JL & Baxter RC 1986. Insulin-like growth factor binding protein from human plasma: purification and characterization. *Journal of Biological Chemistry* **261** 8754-8760.

Martin RJ, Drewry M, Jewell D, Harris RBS, Young R & Patton JS 1989. Growth hormone treatment reduces total body fat accumulation in Zucker obese rats. *International Journal of Obesity* **13** 327-335.

Martinoli MG & Pelletier G 1991. Dihydrotestosterone (DHT) regulation of insulin-like growth factor II mRNA in neonatal rats. *Peptide* **12** 1267-1271.

Massague J & Czech MP 1982. The subunit structures of two distinct receptors for insulin-like growth factor I and II and their relationship to the insulin receptors. *Journal of Biological Chemistry* **257** 5038-5045.

Mathews LS, Hammer RE, Behringer RR, D'Ercole AJ, Bell GI, Brinster RL & Palmiter RD 1988. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* **123** 2827-2833.

McCarthy TL, Centrella M & Canalis E 1990. Cortisol inhibits the synthesis of insulin-like growth factor-I in skeletal cells. *Endocrinology* **126** 1569-1575.

McClain DA, Maegawa H, Lee J, Dull TJ, Ulrich A & Olefsky JM 1987. A mutant insulin receptor with defective tyrosine kinase displays no biologic activity and does not undergo endocytosis. *Journal of Biological Chemistry* **262** 14663-14671.

McCusker RH & Clemmons DR 1994. Effects on insulin-like growth factor-binding protein secretion by muscle cells in vitro. *Endocrinology* **134** 2095-2102.

McMurtry JP, Francis GL, Upton FZ, Rosselot G & Brocht DM 1994. Developmental changes in chicken and turkey insulin-like growth factor-I (IGF-I) studied with a homologous radioimmunoassay for chicken IGF-I. *Journal of Endocrinology* **142** 225-234.

Merimee TJ, Zapf J & Froesch ER 1981. Dwarfism in the pygmy: an isolated deficiency of insulin-like growth factor I. *New England Journal of Medicine* **305** 965-968.

Miller SB, Martin DR, Kisanne J & Hammerman MR 1992. Insulin-like growth factor I accelerates recovery from ischemic acute tubular necrosis in the rat. *Proceedings of the National Academy of Sciences USA* **89** 11876-11880.

Minniti CP, Kohn EC, Grubb JH, Sly WS, Oh Y, Muller HL, Rosenfeld RG & Helman LJ 1992. The insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor mediates IGF-II-induced motility in human rhabdomyosarcoma cells. *Journal of Biological Chemistry* **267** 9000-9004.

Mitchell ML, Guillemin R & Selye H 1954. The effect of somatotropic hormone in the growth of normal and hypophysectomized guinea-pigs. *Endocrinology* **54** 111-114.

Moats-Staats BM, Price WA, Xu L, Jarvis HW & Stiles AD 1995. Regulation of the insulin-like growth factor system during normal rat lung development. *American Journal of Respiratory and Cellular Molecular Biology* **12** 56 -64.

Mohan S & Baylink DJ 1991. The role of insulin-like growth factor-II in the coupling of bone formation to resorption. In: Modern Concepts of Insulin-like Growth Factors pp 169-184. Spencer EM (Ed.), Elsevier, New York.

Molnar P & Murphy LJ 1994. Effects of oestrogen on rat uterine expression of insulin-like growth factor-binding proteins. *Journal of Molecular Endocrinology* **13** 59-67.

Mondschein JS & Hammond JM 1988. Growth factors regulate immunoreactive insulin-like growth factor-I production by cultured porcine granulosa cells. *Endocrinology* **123** 463-468.

Morell B & Froesch ER 1973. Fibroblasts as an experimental tool in metabolic and hormone studies. II. Effects of insulin and nonsuppressible insulin-like activity (NSILAs) on fibroblasts in culture. *European Journal of Clinical Investigation* **3** 119-123.

Morgan DO, Edman JC, Standring DN, Fried VA, Smith MC, Roth RR & Rutter WJ 1987. Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature* **329** 301-307.

Moses AC, Nissley SP, Short PA, Rechler MM, White RM, Knight AB & Higa OZ 1980. Increased levels of multiplication-stimulating activity, an insulin-like growth factor, in fetal rat serum. *Proceedings of the National Academy of Sciences USA* **77** 3649-3653.

Mottola C & Czech MP 1984. The type II insulin-like growth factor receptor does not mediate increased DNA synthesis in H-35 hepatoma cells. *Journal of Biological Chemistry* **259** 12705-12713.

Mottola C, MacDonald RG, Brackett JL, Mole JE, Anderson JK & Czech MP 1986. Purification and amino-terminal sequence of an insulin-like growth factor-binding protein secreted by rat liver BRL-3A cells. *Journal of Biological Chemistry* **261** 11180- 11188.

Moxley RT, Amer P, Moss A, Skottner A, Fox M, James D & Livingston JN 1990. Acute effects of insulin-like growth factor I and insulin metabolism *in vivo*. *American Journal of Physiology* **259** E561-E567.

Mulholland MW & Debas HT 1988. Central nervous system inhibition of pentagastrin-stimulated acid secretion by insulin-like growth factor II. *Life Sciences* **42** 2091-2096.

Mulholland MW, Romanchuk G, Simeone DM & Flowe K 1992. Stimulation of myenteric plexus neurite outgrowth by insulin and insulin-like growth factors -I and -II. *Life Sciences* **51** 1789-1796.

Murayama Y, Okamoto T, Ogata E, Asano T, Iiri T, Katada T, Ui M, Grubb JH, Sly WS & Nishimoto I 1990. Distinctive regulation of the functional linkage between the human cation-independent mannose 6-phosphate receptor and GTP-binding proteins by insulin-like growth factor II and mannose 6-phosphate. *Journal of Biological Chemistry* 265 17456-17462.

Murphy LJ, Murphy LC & Friesen HG 1988. Estrogen induces insulin-like growth factor-I expression in the rat uterus. *Molecular Endocrinolgy* **1** 445

Murphy LJ, Seneviratne C, Ballejo G, Croze F & Kennedy TG 1990. Identification and characterization of a rat decidual insulin-like growth factor-binding protein complementary DNA. *Molecular Endocrinology* **4** 329-336.

Murray AJ, Ballard FJ & Tomas FM 1981. A rapid method for the analysis of N-methylhistidine in human urine. *Analytical Biochemistry* **116** 537-544.

Murray-Rust J, McLeod AN, Blundell TL & Wood SP 1992. Structure and evolution of insulins: implications for receptor binding. *Bioessays* **14** 325-331.

Muta K & Krantz 1993. Apoptosis of human erythroid colony forming cells is decreased by stem cell factor and insulin-like growth factor I as well as erythropoietin. *Journal of Cellular Physiology* **156** 264-271.

Nanto-Salonen K, Muller HL, Hoffman AR, Vu TH and Rosenfeld RG 1993. Mechanisms of thyroid hormone action on the insulin-like growth factor system: all thyroid hormone effects are not growth hormone mediated. *Endocrinology* **132** 781-788.

Nap RC, Mol JA & Hazelwinkel HAW 1993. Age-related plasma concentrations of growth hormone (GH) and insulin-like growth factor I (IGF-I) in great dane pups fed different dietary levels of protein. *Domestic Animal Endocrinology* **10** 237-247.

Near SL, Whalen LR, Miller JA & Ishii DN 1992. Insulin-like growth factor II stimulates motor nerve regeneration. *Proceedings of the National Academy of Sciences USA* **89** 11716-11720.

Ng E-H, Rock CS, Lazarus DD, Stiaino-Coico L, Moldawer LL & Lowry SF 1992. Insulin-like growth factor I preserves host lean tissue mass in cancer cachexia. *American Journal of Physiology* **262** R426-R431.

Nishimoto I, Hata Y, Ogata E & Kojima I 1987. Insulin-like growth factor II stimulates calcium influx in competent BALB/c 3T3 cells primed with epidermal growth factor. *Journal of Biological Chemistry* **262** 12120-12126.

Nissley SP, Haskell JF, Sasaki N, DeVroede MA & Rechler MM 1985. Insulin-like growth factor receptors. *Journal of Cell Science* suppl. **3** 39-51.

Novak LP, Hayles AB & Cloutier MD 1972. Effect of hGH on body composition of hypopituitary dwarfs. *Mayo Clinic Proceedings* **47** 241-246.

Ocrant I, Fay CT and Parmelee JT 1990. Characterization of insulin-like growth factor binding proteins produced in the rat central nervous system. *Endocrinology* **127** 1260-1267.

Oddy VH & Owens PC 1994. IGF-I inhibits muscle protein breakdown in fed and underfed lambs and calves. *Growth Regulation* **4** suppl. 1 abstract II312.

Ogawa O, McNoe LA, Eccles MR, Morison IM & Reeve AE 1993. Human insulin-like growth factor type I and type II receptors are not imprinted. *Human Molecular Genetics* **2** 2163-2165.

Oh Y, Muller HL, Lee D-Y, Fielder PJ & Rosenfeld RG 1993. Characterization of the affinities of insulin-like growth factor (IGF)-binding proteins 1-4 for IGF-I, IGF-II, IGF-I/insulin hybrid, and IGF-I analogs. *Endocrinology* **132** 1337-1344.

Ohlsson R, Nystrom A, Pfeifer-Ohlsson S, Tohonen V, Hedborg F, Schofield P, Flam F & Ekstrom TJ 1993. IGF 2 is parentally imprinted during human embryogenesis and in the Beckwith-Weidemann syndrome. *Nature Genetics* **4** 94-97.

Ooi GT, Orlowski CC, Brown AL, Becker RE, Unterman TG and Rechler MM 1990. Different tissue distribution and hormonal regulation of messenger RNAs encoding rat insulin-like growth factor-binding proteins-1 and -2. *Molecular Endocrinology* **4** 321-328.

O'Sullivan U, Gluckman PD, Breier BH, Woodall S, Siddiqui RA & McCutcheon SN 1989. Insulin-like growth factor-I (IGF-I) in mice reduces weight loss during starvation. *Endocrinology* **125** 2793-2794.

Owens JA, Kind KL, Carbone F, Robinson JS & Owens PC 1994. Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *Journal of Endocrinology* **140** 5-13.

Owens PC, Conlon MA, Campbell RG, Johnson RJ, King R & Ballard FJ 1991. Developmental changes in growth hormone, insulin-like growth factors (IGF-I and IGF-II) and IGF binding proteins in plasma of young growing pigs. *Journal of Endocrinology* **128** 439-447.

Owens PC, Johnson RJ, Campbell RG & Ballard FJ 1990. Growth hormone increases insulin-like growth factor-I (IGF-I) and decreases IGF-II in plasma of growing pigs. *Journal of Endocrinology* **124** 269-275.

Owens PC, Lindsay DB & Hunter RA 1994. Long-Arg^{3}IGF-I inhibits catabolism of muscle protein in underfed steers. *Growth Regulation* **4** suppl. 1 abstract II299.

Owens PC, Quinn KJ & Wallace SM 1995. Measurement of insulin-like growth factors in blood serum or plasma and other biological fluids: a fast size-exclusion chromatography HPLC acid extraction of IGFs before their estimation by radioimmunoassay and related techniques. *GroPep Marketing Technical Bulletin* **006** 1-4.

Pahlman S, Meyerson G, Lindgren E, Schalling M & Johansson I 1991. Insulin-like growth factor I shifts from promoting cell division to potentiating maturation during neuronal differentiation. *Proceedings of the National Academy of Sciences USA* **88** 9994-9998.

Palka J, Bird TA, Oyamada I & Peterkofsky B 1989. Similar hormonal changes in sera from scorbutic and fasted (vitamin C-supplemented) guinea pigs, including decreased IGF-I and appearance of an IGF-I reversible mitogenic inhibitor. *Growth Factors* **1** 147-156.

Palmiter RD, Norstedt G, Gelinas RE, Hammer RE & Brinster RL 1983. Metallothionein-human GH fusion genes stimulate growth of mice. *Science* **222** 809-814.

Parker MW, Johanson AJ, Rogol AD, Kaiser DL & Blizzard RM 1984. Effect of testosterone on somatomedin-C concentrations in prepubertal boys. *Journal of Clinical Endocrinology and Metabolism* **58** 87-90.

Pell JM & Bates PC 1992. Differential actions of growth hormone and insulin-like growth factor-I on tissue protein metabolism in dwarf mice. *Endocrinology* **130** 1942-1950.

Pell JM, Elcock C, Harding RL, Morrell DJ, Simmonds AD & Wallis M 1990. Growth, body composition, hormonal and metabolic status in lambs treated long-term with growth hormone. *British Journal of Nutrition* **63** 431-445.

Pessin JE 1994. Transmembrane signaling properties of the insulin and IGF-I tyrosine kinase receptors. In: The insulin-like growth factors and their regulatory proteins. p87-94. Baxter RC, Gluckman PD & Rosenfeld RG (Eds). Elsevier Science BV, Amsterdam, The Netherlands.

Peterkofsky B, Gosiewska A, Kipp DE, Shah V & Wilson S 1994. Circulating insulin-like growth factor binding proteins (IGFBPs) 1 and 2 induced in vitamin C-deficient or fasted guinea pigs inhibit IGF-I action in cultured cells. *Growth Factors* **10** 229-241.

Peterkofsky B, Palka J, Wilson S, Takeda K & Shah V 1991. Elevated activity of low molecular weight insulin-like growth factor-binding proteins in sera of vitamin C-deficient and fasted guinea pigs. *Endocrinology* **128** 1769-1779.

Philipps AF, Peisson B, Hall K, Lake M, Skottner A, Sanengen T & Sara VR 1988. The effects of biosynthetic insulin-like growth factor-1 supplementation on somatic growth, maturation, and erythropoiesis on the neonatal rat. *Pediatric Research* **23** 298-305.

Poggi C, LeMarchand-Brustel Y, Zapf J , Froesch ER & Freychet P 1979. Effects and binding of insulin-like growth factor in the isolated soleus muscle of lean and obese mice. *Endocrinology* **105** 723-730.

Powell DR, Sanwanichkal A, Cubbage ML, DePaolis LA, Smuggs MB & Lee PDK 1992. Insulin inhibits transcription of the human gene for insulin-like growth factor binding protein-1. *Journal of Biological Chemistry* **266** 18868- 18876.

Powell-Braxton L, Hollingshead P, Warburton C, Dowd M, Pitts-Meek S, Dalton D, Gillett N & Stewart TA 1993. IGF-I is required for normal embryonic growth in mice. *Genes and Development* **7** 2609-2617.

Prewitt TE, D'Ercole AJ, Switser BR & Van Wijk JJ 1982. Relationship of serum immunoreactive somatomedin-C to dietary protein and energy in growing rats. *Journal of Nutrition* **112** 144-150.

Prosser CG, Davis SR, Hodgkinson SC & Mohler MA 1995. Pharmacokinetics and bioactivity of intact versus truncated IGF-I during a 24-h infusion into lactating goats. *Journal of Endocrinology* **144** 99-107.

Prosser CG, Royle C, Fleet IR & Mcpham TB 1991. The galactopoietic effect of bovine growth hormone in goats is associated with increased concentrations of insulin-like growth factor-I in milk and mammary tissue. *Journal of Endocrinology* **128** 457-463.

Quin JD, Fisher BM, Paterson KR, Inoue A, Beastall GH & MacCuish AC 1990. Acute responses to recombinant insulin-like growth factor I in a patient with Mendenhall's syndrome. *New England Journal of Medicine* **323** 1425-1426.

Raile K, Hoeflich A, Kessler U, Yang Y, Pfuender M, Blum WF, Kolb H, Schwarz HP & Kiess W 1994. Human osteosarcoma cells express both IGF-I receptors and IGF-II/M6P receptors and synthesize IGF-II: Autocrine growth stimulation by IGF-II via the IGF-I receptor. *Journal of Cellular Physiology* **159** 531-541.

Read LC, Tomas FM, Howarth GS, Martin AA, Edson KJ, Gillespie CM, Owens PC & Ballard FJ 1992. Insulin-like growth factor-I and its N-terminal modified analogues induce marked gut growth in dexamethasone-treated rats. *Journal of Endocrinology* **133** 421-431.

Rechler MM 1993. Insulin-like growth factor binding proteins. In: Vitamins and Hormones **47** p1-14. Academic Press, Inc.

Rechler MM, Zapf J, Nissley SP, Froesch ER, Moses AC, Podskalny JM, Schilling EE & Humbel RE 1980. Interactions of insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins. *Endocrinology* **107** 1451-1459.

Ren-Qui Q, Ruelicke T, Hassam S, Haselbacher GK & Schoenle EJ 1993. Systemic effects of insulin-like growth factor-II produced and released from Wilms tumour tissue. *European Journal of Pediatrics* **152** 102-106.

Rinderknecht E & Humbel RE 1976. Polypeptides with nonsuppressible insulin-like cell growth-promoting activities in human serum: isolation, chemical characterization and some biological properties of forms I and II. *Proceedings of the National Academy of Sciences USA* **73** 2365-2369.

Rinderknecht E & Humbel RE 1978a. Primary structure of human insulin-like growth factor II. *FEBS Letters* **89** 283-286.

Rinderknecht E & Humbel RE 1978b. The amino-acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *Journal of Biological Chemistry* **253** 2769-2776.

Ritvos O, Ranta T, Jalkanen J, Suikkari A-M, Voutilainen R, Bohn H & Rutanen E-M 1988. Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells. *Endocrinology* **122** 2150-2157.

Rodriguez Tarduchy G, Collins MK, Garcia I & Lopez Rivas A 1992. Insulin-like growth factor-I inhibits apoptosis in IL-3-dependent hemopoictic cells. *Journal of Immunology* **149** 535-540.

Roeder RA, Hossner KL, Sasser RG & Gunn JM 1988. Regulation of protein turnover by recombinant human insulin-like growth factor-I in L6 myotube cultures. *Hormones and Metabolic Research* **20** 699-700.

Rogers SA, Purchio AF & Hammerman MR 1990. Mannose 6-phosphate-containing peptides activate phospholipase C in proximal tubular basolateral membranes from canine kidney. *Journal of Biological Chemistry* **265** 9722-9727.

Rogers SA, Ryan G & Hammerman MR 1991. Insulin-like growth factors I and II are produced in the metanephros and are required for growth and development in vitro. *Journal of Cell Biology* **113** 1447-1454.

Rogler CE, Yang D, Rossetti L, Donohoe J, Alt E, Chang CJ, Rosenfeld R, Neely K & Hintz R 1994. Altered body composition and increased frequency of diverse malignancies in IGF-II transgenic mice. *Journal of Biological Chemistry* **269** 13779-13784.

Romanus JA, Terrell JE, Yang YWH, Nissley SP & Rechler MM 1986. Insulin-like growth factor carrier proteins in neonatal and adult rat serum are immunologically different: demonstration using a new radioimmunoassay for the carrier protein from BRL-3A rat liver cells. *Endocrinology* **118** 1743-1758.

Rosenfeld RG, Beukers MW, Oh Y, Zhang H & Ling N 1991. Insulin-like growth factor receptor physiology: use of IGF-II analogs as probes of receptor function. In: Modern concepts of insulin-like growth factors; p 439- 448. Spencer EM (Ed.).

Rosenfield RI, Furlanetto R & Bock D 1983. Relationship of somatomedin-C concentrations to pubertal changes. *Journal of Pediatrics* **103** 723-728.

Rosenthal SM, Hsiao D & Silverman LA 1994. An insulin-like growth factor-II (IGF-II) analog with highly selective affinity for IGF-II receptors stimulates differentiation, but not IGF-I receptor down-regulation in muscle cells. *Endocrinology* **135** 38-44.

Ross M, Francis GL, Szabo L, Wallace JC & Ballard FJ 1989. Insulin-like growth factor (IGF)-binding proteins inhibit the biological activities of IGF-1 and IGF-2 but not des (1-3) IGF-I. *Biochemical Journal* **258** 267-272.

Ross RJM, Miell JP & Buchanen 1991. Avoiding autocannabalism. *British Medical Journal* **303** 1147-1148.

Rossetti L, Frontoni S, DiMarchi R, DeFronzo RA & Giaccari A 1991. Metabolic effects of insulin-like growth factor-I indiabetic rats. *Diabetes* **40** 444-448.

Roth BV, Burgisser DM, Luthi C & Humbel RE 1991. Mutants of human insulin-like growth factor II: expression and characterization of analogs with a substitution of Tyr^{27} and/or a deletion of residues 62-67. *Biochemical and Biophysical Research Communications* **181** 907-914.

Rotwein P 1986. Two insulin-like growth factor I messenger RNAs are expressed in human liver. *Proceedings of the National Academy of Sciences USA* **83** 77.

Ruoslahti E and Pierschbacher MD 1987. New perspectives in cell adhesion: RGD and integrins. *Science* **238** 491-497.

Sakano K, Enjoh T, Numata F, Fujiwara H, Marumoto Y, Higashihashi N, Sato Y, Perdue JF & Fujita-Yamaguchi Y 1991. The design, expression, and characterization of human insulin-like growth factor II (IGF-II) mutants specific for either the IGF-II/cation-independent mannose 6-phosphate receptor or IGF-I receptor. *Journal of Biological Chemistry* **266** 20626-20635.

Salmon WD Jr. & Daughaday WH 1957. A hormonal controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *Journal of Laboratory and Clinical Medicine* **49** 825-836.

Sandles LD & Peel CJ 1987. Growth and carcass composition of prepubertal dairy heifers treated with bovine growth hormone. *Animal Production* **44** 21-27.

Sara VR, Carlsson-Skwirut C, Andersson C, Hall K, Sjogren B, Holmgren A & Jornvall H 1986. Characterization of somatomedins from human fetal brain: identification of a variant form of insulin-like growth factor I. *Proceedings of the National Academy of Sciences USA* **83** 4904-4907.

Schalch DS, Turman NJ, Marcsisin VS, Heffernan M & Guler H-P 1993. Short-term effects of recombinant human insulin-like growth factor I on metabolic control of patients with type II diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism* **77** 1563-1568.

Scheiwiller E, Guler H-P, Merryweather J, Scandella C, Maerki W, Zapf J & Froesch ER 1986. Growth restoration of insulin-deficient diabetic rats by recombinant human insulin-like growth factor I. *Nature* **323** 169-171.

Schiltz PM, Ohta T, Glass D, Mohan S & Baylink DJ 1992. Growth hormone stimulates cortical bone formation in immature hypophysectomized rats. *Endocrine Research* **18** 19-30.

Schmid C, Schlapfer I, Futo E, Waldvogel M, Schwander J, Zapf J & Froesch ER 1992. Triiodothyronine (T3) stimulates insulin-like growth factor (IGF)-I and IGF binding protein (IGFBP)-2 production by rat osteoblasts in vitro. *Acta Endocrinologica* **126** 467-473.

Schoenle E, Zapf J, Hauri C, Steiner T & Froesch ER 1985. Comparison of in vivo effects of insulin-like growth factors I and II and of growth hormone in hypophysectomized rats. *Acta Endocrinologica* **108** 167-174.

Schoenle E, Zapf J, Humbel RE & Froesch ER 1982. Insulin-like growth factor I stimulates growth in hypophysectomized rats. *Nature* **296** 252-253.

Schwander J, Hauri C, Zapf J & Froesch ER 1983. Synthesis and secretion of insulin-like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. *Endocrinology* **113** 297-305.

Scott CD & Baxter RC 1987. Purification and immunological characterization of the rat liver insulin-like growth factor-II receptor. *Endocrinology* **120** 1-9.

Seneviratne C, Luo J & Murphy LJ 1990. Transcriptional regulation of rat insulin-like growth factor binding protein-1 expression by growth hormone. *Molecular Endocrinology* **4** 1199-1204.

Shaar CJ, Tinsley FC, Smith MC, Clemens JA & Neubauer BL 1989. Recombinant DNA-derived human insulin-like growth factor II (hIGF-II) stimulates growth in hypophysectomized rats. *Endocrine Research* **15** 403-411.

Shao Z-M, Sheikh MS, Ordonez JV, Feng P, Kute T, Chen J-C, Aisner S, Schnaper L, LeRoith D, Roberts CT & Fontana J 1992. IGFBP-3 gene expression and estrogen receptor status in human breast carcinoma. *Cancer Research* **52** 5100-5103.

Shinar DM, Endo N, Halperin D, Rodan GA & Weinreb M 1993. Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology* **132** 1158-1167.

Shiu RPC, Kelly PA & Friesen HG 1973. Radioreceptor assay for prolactin and other lactogenic hormones. *Science* **180** 968-971.

Shojee-Moradie F, Thomason M, Skottner A, Jones RH, Umpleby AM 1991. The effect of IGF1 and insulin on glucose and leucine metabolism in dogs. *Diabetes* suppl.1 **40** abstract 172A.

Simes JM, Wallace JC & Walton PE 1991. The effects of insulin-like growth factor-I (IGF-I), IGF-II and des(1-3)IGF-I, a potent analogue, on growth hormone and IGF-binding protein secretion from cultured rat anterior pituitary cells. *Journal of Endocrinology* **130** 93-99.

Singh JS, Rall LB & Styne DM 1991. Insulin-like growth factor I and II gene expression in Balb/C mouse liver during postnatal development. *Biology of the Neonate* **60** 7-18.

Sklar MM, Kiess W, Thomas CL & Nissley SP 1989. Developmental expression of the tissue insulin-like growth factor II/ mannose 6-phosphate receptor in the rat. Measurement by quantitative immunoblotting. *Journal of Biological Chemistry* **264** 16733-16738.

Skottner A, Clark RG, Fryklund L & Robinson IC 1989. Growth responses in a mutant dwarf rat to growth hormone and recombinant human insulin-like growth factor I. *Endocrinology* **124** 2519-2526.

Smit AB, van Marle A, van Elk R, Bogerd J, van Heerikhuizen H & Geraerts WPM 1993. Evolutionary conservation of the insulin gene structure in invertebrates: cloning of the gene encoding molluscan insulin-related peptide III from Lymnaea stagnalis. *Journal of Molecular Endocrinology* **11** 103-113.

Smith PJ, Wise LS, Berkowitz R, Wan C & Rubin CS 1988. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *Journal of Biological Chemistry* **263** 9402-9408.

Soliman AT, Hassam AEHI, Aref MK, Hintz RL, Rosenfeld RG & Rogol AD 1986. Serum insulin-like growth factor I and II concentrations and growth hormone and insulin responses to arginine infusion in children with protein-energy malnutrition before and after nutritional rehabilitation. *Pediatric Research* **20** 1122-1130.

Spencer EM, Liu CC, Si ECC & Howard GA 1991. In vivo actions of insulin-like growth factor-I (IGF-I) on bone formation and resorption in rats. *Bone* **12** 21-26.

Spencer GSG, Decuypere E & Buyse J 1994. Effect of recombinant insulin-like growth factor-2 on weight gain and body composition in chickens. *Growth Regulation* **4** suppl. 1 abstract II286.

Spencer GSG, Decuypere E, Buyse J, Hodgkinson SC, Bass JJ & Zeman M 1995. Passive immunization of insulin-like growth factor (IGF)-1 and of IGF-1 and IGF-2 in chickens. *Comparative Biochemistry & Physiology. Pharmacology, Toxicology & Endocrinology* **110** 29-33.

Steeb C-B, Trahair JF, Tomas FM & Read LC 1994. Prolonged administration of insulin-like growth factor peptides (IGFs) enhances growth of gastro-intestinal tissues in normal rats. *American Journal of Physiology* **266** G1090-G1098.

Straus DS, Ooi GT, Orlowski CC & Rechler MM 1991. Expression of the genes forinsulin-like growth factor I (IGF-I), IGF-II, and IGF-binding proteins-1 and -2 in fetal rat under conditions of intrauterine growth retardation caused by maternal fasting. *Endocrinology* **128** 518-525.

Straus DS & takemoto CD 1990. Effect of dietary protein deprivation on insulin-like growth factor (IGF)-I and -II, IGF binding protein-2, and serum albumin gene expression in rat. *Endocrinology* **127** 1849-1860.

Streck RD, Wood TL, Hsu M-S & Pintar JE 1992. Insulin-like growth factor I and II and insulin-like growth factor binding protein-2 RNAs are expressed in adjacent tissues within rat embryonic and fetal limbs. *Developmental Biology* **151** 586-596.

Stuempel & Hartmann H 1992. Acute actions of insulin-like growth factor II on glucose metabolism in adult rats. *Diabetologia* **34** 144-149.

Stylianopoulou F, Herbert J, Soares MB & Efstratiadis A 1988. Expression of the insulin-like growth factor II gene in the choroid plexus and the leptomeninges of the adult rat central nervous system. *Proceedings of the National Academy of Sciences USA* **85** 141-145.

Suh DY, Hunt TK & Spencer EM 1992. Insulin-like growth factor-I reverses the impairment of wound healing induced by corticosteroids in rats. *Endocrinology* **131** 2399-2403.

Suikkari A-M & Baxter RC 1991. Insulin-like growth factor (IGF) binding protein-3 in pregnancy serum binds native IGF-I but not iodo-IGF-I. *Journal of Clinical Endocrinology and Metabolism* **73** 1377-1379.

Suikkari AM, Koivisto VA, Rutanen EM, Yki-Jarvinen H, Karonen SL & Seppala M 1988. Insulin regulates the serum levels of low molecular weight insulin-like growth factor binding protein. *Journal of Clinical Endocrinology Metabolism* **66** 266-272.

Sullivan KA & Feldman EL 1994. Immunohistochemical localization of insulin-like growth factor-II (IGF-II) and IGF-binding protein-2 during development in the rat brain. *Endocrinology* **135** 540-547.

Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ & White MF 1991. The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* **352** 73-77.

Suttie JM, Fennessy PF, Corson ID, Laas FJ, Crosbie SF, Butler JH & Gluckman PD 1989. Pulsatile growth hormone, insulin-like growth factors and antler development in red deer (Cervus elaphus scotius) stags. *Journal of Endocrinology* **121** 351-360.

Takahashi S, Kajikawa M, Umezawa T, Takahashi S-I, Kato H, Miura Y, Nam TJ, Noguchi T & Naito H 1990. Effect of dietary proteins on the plasma immunoreactive insulin-like growth factor-1/somatomedin C concentration in the rat. *British Journal of Nutrition* **63** 521-534.

Tally M, Li CH & Hall K 1987. IGF-2 stimulated growth mediated by the somatomedin type 2 receptor. *Biochemical and Biophysical Research Communications* **148** 811-816.

Tapanainen JT, Ronnberg L, Martilainen H, Renila M, Koistinen R & Seppala M 1991. Short and long term effects of growth hormone on circulating levels of insulin-like growth factor-I (IGF-I), IGF binding protein-1, and insulin: a placebo-controlled study. *Journal of Clinical Endocrinology and Metabolism* **73** 71-74.

Thissen J-P, Pucilowska JB & Underwood LE 1994. Differential regulation of insulin-like growth factor I (IGF-I) and IGF binding protein-1 messenger ribonucleic acids by amino acid availability and growth hormone in rat hepatocyte primary culture. *Endocrinology* **134** 1570-1576.

Thissen J-P, Underwood LE, Maiter D, Maes M, Clemmons DR & Ketelslegers J-M 1991. Failure of insulin-like growth factor-I (IGF-I) infusion to promote growth in protein-restricted rats despite normalization of serum IGF-I concentrations. *Endocrinology* **128** 885-890.

Thomas MR, Miell JP, Taylor AM, Ross RJM, Arnao JR, Jewitt DE & McGregor AM 1993. Endocrine and cardiac paracrine actions of insulin-like growth factor-I (IGF-I) during thyroid dysfunction in the rat: is IGF-I implicated in the mechanism of heart weight/body weight change during abnormal thyroid function? *Journal of Molecular Endocrinology* **10** 313-323.

Tixier-Boichard M, Huybrechts LM, Decuypere E, Kuhn ER, Monvoisin J-L, Coquerelle G, Charrier J & Simon J 1992. Effects of insulin-like growth factor-I (IGF-I) infusion and dietary tri-iodothyronine (T_3) supplementation on growth, body composition and plasma hormone levels in sex-linked dwarf mutant and normal chickens. *Journal of Endocrinology* **133** 101-110.

Tollefsen SE, Sadow JL & Rotwein P 1989. Coordinate expression of IGF-II and its receptor during muscle differentiation. *Proceedings of the National Academy of Sciences USA* **86** 1543-1547.

Tomas FM, Knowles SE, Chandler CS, Francis GL, Owens PC & Ballard FJ 1993a. Anabolic effects of insulin-like growth factor-I (IGF-I) and an IGF-I variant in normal female rats. *Journal of Endocrinology* **137** 413-421.

Tomas FM, Knowles SE, Owens PC, Chandler CS, Francis GL & Ballard FJ 1993b. IGF-I and more potent variants restore growth of diabetic rats without inducing all characteristic insulin effects. *Biochemical Journal* **291** 781-786.

Tomas FM, Knowles SE, Owens PC, Chandler CS, Francis GL, Read LC & Ballard FJ 1992. Insulin-like growth factor-I (IGF-I) and especially IGF-I variants are anabolic in dexamethasone treated rats. *Biochemical Journal* **282** 91-97.

Tomas FM, Knowles SE, Owens PC, Read LC, Chandler CS, Gargosky SE & Ballard FJ 1991a. Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with insulin-like growth factor (IGF)-I and des (1-3) IGF-I. *Biochemical Journal* **276** 547-554.

Tomas FM, Knowles SE, Owens PC, Read LC, Chandler CS, Gargosky SE & Ballard FJ 1991b. Effects of full length and truncated insulin-like growth factor-I on nitrogen balance and muscle protein metabolism in nitrogen-restricted rats. Journal of *Endocrinology* **128** 97-105.

Tomas FM, Murray AJ & Jones LM 1984. Modification of glucocorticoid-induced changes in myofibrillar protein turn-over in rats by protein and energy deficiency as assessed by urinary excretion of N-methylhistidine. *British Journal of Nutrition* **51** 323-337.

Tomas FM, Pym A & Johnson RJ 1991c. Muscle protein turnover in chickens selected for increased growth rate, food consumption or efficiency of food utilization: effects of genotype and relationship to plasma IGF-I and growth hormone. *British Poultry Science* **32** 363-376.

Tremollieres FA, Strong DD, Baylink DJ & Mohan S 1991. Insulin-like growth factor II and transforming growth factor beta 1 regulate insulin-like growth factor I secretion in mouse bone cells. *Acta Endocrinologica* **125** 538-546.

Unterman TG, Jentel JJ, Oehler DT, Lacson RG & Hofert JF 1993a. Effects of glucocorticoids on circulating levels and hepatic expression of insulin-like growth factor (IGF)-binding proteins and IGF-I in the adrenalectomized streptozotocin-diabetic rat. *Endocrinology* **133** 2531-2539.

Unterman TG, Simmons RA, Glick RP & Ogata ES 1993b. Circulating levels of insulin, insulin-like growth factor-I (IGF-I), IGF-II, and IGF-binding proteins in the small for gestational age fetal rat. *Endocrinology* **132** 327-336.

Vaccarello MA, Diamond FB, Guevara-Aguirre J, Rosenbloom AL, Fielder PJ, Gargosky S, Cohen P, Wilson K & Rosenfeld RG 1993. Hormonal and metabolic effects and pharmacokinetics of recombinant insulin-like growth factor-I in growth hormone receptor deficiency/laron syndrome. *Journal of Clinical Endocrinology and Metabolism* **77** 273-280.

Van Buul-Offers SC, de Haan K, Reijnen-Gresnigt, Meinsma D, Jansen M, Oei SL, Bonte EJ, Sussenbach JS & Van Den Brande JL 1995. Overexpression of human insulin-like growth factor-II in transgenic mice causes increased growth of the thymus. *Journal of Endocrinology* **144** 491-502.

Van Buul-Offers SC, Hoogerbrugge CM, Branger J, Feijlbrief M & Van den Brande JL 1988. Growth-stimulating effects of somatomedin-/insulin-like peptides in Snell dwarf mice. *Hormone Research* **29** 229-236.

Van Buul-Offers SC, Reijnen-Gresnigt, Hoogerbrugge CM, Bloemen RJ, Kuper CF & Van den Brande JL 1994. Recombinant insulin-like growth factor-II inhibits the growth-stimulating effect of growth hormone on the liver of Snell dwarf mice. *Endocrinology* **135** 977-985.

Van Buul-Offers S, Ueda I & Van den Brande JL 1986. Biosynthetic somatomedin C (SM-C/IGF-I) increases the length and weight of Snell dwarf mice. *Pediatric Research* **20** 825-827.

Vanderhoof JA, McCusker RH, Clark R, Mohammadpour H, Blackwood DJ, Harty RF & Park JHY 1992. Truncated and native insulin-like growth factor-I enhance mucosal adaptation after jejunoileal resection. *Gastroenterology* **102** 1949-1956.

Van Obberghen-Schilling E & Pouyssegur J 1983. Mitogen-potentiating action and binding characteristics of insulin and insulin-like growth factors in Chinese hamster fibroblasts. *Experimental Cell Research* **147** 369-378.

Vileisis RA & D'Ercole AJ 1986. Tissue and serum concentrations of somatomedin-C/insulin-like growth factor I in fetal rats made growth retarded by uterine artery ligation. *Pediatric Research* **20** 126-130.

Walker JL, Ginalska-Malinowska M, Romer TE, Pucilowska JB and Underwood LE 1991. Effects of the infusion of insulin-like growth factor I in a child with growth hormone insensitivity syndrome. *New England Journal of Medicine* **324** 1483-1488.

Walton PE & Etherton TD 1986. Antagonism of insulin action in cultured pig adipose tissue by pituitary and recombinant porcine GH: potentiation by hydrocortisone. *Endocrinology* **118** 2577-2581.

Walton PE, Grant PA, Seamark RF, francis GL, Ross M, Wallace JC and Ballard FJ 1991. Characterization and binding properties of the 24/29 kDa IGF binding protein and IGFBP-3 from ovine plasma. Second International Symposium on Insulin-like Growth Factors/Somatomedins, San Francisco, CA, USA. Elsevier, New York. Abstract C56.

Walton PE, Owens PC, Knowles SE, Dunaiski V, Grant PA, Tomas FM & Dunshea FR 1994. Effects of IGF-I analogues on growth and metabolism in pigs. *Growth Regulation* **4** suppl.1 86.

Wang HS, Lim J, English J, Irvine L & Chard T 1991. The concentration of insulin-like growth factor-I and insulin-like growth factor binding protein-1 in human umbilical cord serum at delivery: relation to fetal weight. *Journal of Endocrinology* **129** 459-464.

Werner H, Woloschak M, Adamo M, Shen-Orr Z, Roberts CT Jr. & LeRoith D 1989. Developmental regulation of the rat insulin-like growth factor I receptor gene. *Proceedings of the National Academy of Science USA* **86** 7451-7455.

Werther GA, Haynes K & Waters MJ 1993. Growth hormone (GH) receptors are expressed on human fetal mesenchymal tissues-identification of messenger ribonucleic acid and GH-binding protein. *Journal of Clinical Endocrinology and Metabolism* **76** 1638-1646.

Whitfield HJ, Bruni CB, Frunzio R, Terrell JE, Nissley SP & Rechler MM 1984. Isolation of a cDNA clone encoding rat insulin-like growth factor-II precursor. *Nature* **312** 277.

Wilson DM, Perkins SN, Thomas JA, Scelig S, Berry SA, Hamm TE Jr., Hoffman AR, Hintz RL & Rosenfeld RG 1989. Effects of elevated serum insulinlike growth factor-II on growth hormone and insulinlike growth factor-I mRNA and secretion. *Metabolism* **38** 57-62.

Wilson DM, Stene MA, Killen JD, Hammer LD, Litt IF, Hayward C & Barr-Taylor C 1992. Insulin-like growth factor binding protein-3 in normal pubertal girls. *Acta Endocrinologica* **126** 381-386.

Wolf E, Kramer R, Blum WF, Foll J & Brem G 1994. Consequences of postnatally elevated insulin-like growth factor-II in transgenic mice: endocrine changes and effects on body and organ growth. *Endocrinology* **135** 1877-1886.

Wolf M, Ingbar SH & Moses AC 1989. Thyroid hormone and growth hormone interact to regulate insulin-like growth factor I messenger ribonucleic acid and circulating levels in the rat. *Endocrinology* **125** 2905-2914.

Wolverton CK, Azain MJ, Duffy JY, White ME & Ramsay TG 1992. Influence of somatotropin on lipid metabolism and IGF gene expression in porcine adipose tissue. *American Journal of Physiology* **263** E637-E645.

Wood TL, Streck RD & Pintar JE 1992. Expression of the IGFBP-2 gene in post-implantation rat embryos. *Development* **114** 59-66.

Yoshimura Y, Iwashita M, Karube M, Oda T, Akiba M, Shiokawa S, Ando M, Yoshinaga A and Nakamura Y 1994. Growth hormone stimulates follicular development by stimulating ovarian production of insulin-like growth factor-I. *Endocrinology* **135** 887-894.

Young GP, Taranto TM, Jonas HA, Cox AJ, Hogg A & Werther GA 1990. Insulin-like growth factor and the developing and mature rat small intestine: receptors and biological actions. *Digestion* **46** 240-252.

Zangger I, Zapf J & Froesch ER 1987. Insulin-like growth factor I and II in 14 animal species and man as determined by three radioligand assays and two bioassays. *Acta Endocrinologica* **114** 107-112.

Zapf J, Hauri C, Waldvogel M & Froesch ER 1986. Acute metabolic effects and half-lives of intravenously administered insulin-like growth factors I and II in normal and hypophysectomized rats. *Journal of Clinical Investigation* **77** 1768-1775.

Zapf J, Hauri C, Waldvogel M, Futo E, Hasler H, Binz K, Guler HP, Schmid C & Froesch ER 1989. Recombinant human insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats. *Proceedings of the National Academy of Science USA* **86** 3813-3817.

Zapf J, Hauri C, Futo E, Hussain M, Rutishauser J, Maack Ca & Froesch ER 1995. Intravenously injected insulin-like growth factor (IGF)I/IGF binding protein-3 complex exerts insulin-like effects in hypophysectomized, but not in normal rats. *Journal of Clinical Investigation* **95** 179-186.

Zapf J, Schmid C, Guler HP, Waldvogel M, Hauri C, Futo E, Hossenlopp P, Binoux M & Froesch ER 1990. Regulation of binding proteins for insulin-like growth factor (IGF) in humans. *Journal of Clinical Investigation* **86** 952-961.

Zapf J, Schoenle E & Froesch ER 1978. Insulin-like growth factors I and II: some biological actions and receptor binding characteristics of two purified constituents of non-suppressible insulin-like activity of human serum. *European Journal of Biochemistry* **87** 285-296.

Zapf J, Schoenle E, Waldvogel M, Sand I & Froesch ER 1981. Effect of trypsin treatment of rat adipoytes on biological effects and binding of insulin and insulin-like growth factors: further evidence for the action of insulin-like growth factors through the insulin receptor. *European Journal of Biochemistry* **113** 605-609.

Zapf J, Waldvogel M & Froesch ER 1975. Binding of non-suppressible insulin-like activity to human serum. Evidence for a carrier protein. *Archives of Biochemistry and Biophysics* **168** 638-645.

Zenobi PD, Guler H-P, Zapf J & Froesch ER 1988. Insulin-like growth factors in the Gottinger miniature-pig. *Acta Endocrinologica* **117** 343-352.

Zhou J & Bondy C 1993. Anatomy of the insulin-like growth factor system in the human testis. *Fertility and Sterility* **60** 897-904.

Zierath JR, Bang P, Galuska D, Hall K & Wallberg-Henriksson H 1992. Insulin-like growth factor II stimulates glucose transport in human skeletal muscle. *FEBS* **307** 379-382.

Zimmerman AE, Moule ML & Yip CC 1974. Guinea pig insulin. II. Biological activity. *Journal of Biological Chemistry* **249** 4026-4029.

Zumstein PP, Luthi C & Humbel R 1985. Amino acid sequence of a variant pro-form of insulin-like growth factor II. *Proceedings of the National Academy of Sciences USA* **82** 3169.

APPENDICES.

Data contained within the following appendices were not included within chapters 4 to 6 in order to improve their readability. The results included here show insignificant effects or are not considered essential to the discussions of those chapters.

APPENDIX A.

Supporting data for chapter 4.

TABLE A.1. Water intakes (ml) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group.

Treatment	Days 1 to 7	Days 8 to 14	Days 1 to 14
Vehicle Control	201 ± 18	200 ± 19	401 ± 37
IGF-I (104 µg/day)	248 ± 27	237 ± 18	485 ± 44
IGF-I (260 µg/day)	227 ± 31	239 ± 28	466 ± 58
IGF-II (104 µg/day)	213 ± 13	221 ± 13	434 ± 25
IGF-II (260 µg/day)	230 ± 20	234 ± 19	465 ± 39
IGF-II (650 µg/day)	218 ± 19	238 ± 21	456 ± 40

TABLE A.2. Femur weights (g), fractional weights (g/kg body weight), lengths (cm) and weights per unit of length (g/cm). The values represent the mean ± S.E.M. for 6 animals in each group.

	Weight	Fractional weight	Length	Weight/length
Treatment				
Vehicle control	0.372 ± 0.007	2.42 ± 0.02	2.62 ± 0.01	0.142 ± 0.002
IGF-I (104 µg/day)	0.388 ± 0.008	2.42 ± 0.06	2.65 ± 0.01	0.146 ± 0.003
IGF-I (260 µg/day)	0.397 ± 0.007	2.44 ± 0.03	2.63 ± 0.01	0.151 ± 0.002
IGF-II (104 µg/day)	0.380 ± 0.010	2.50 ± 0.04	2.63 ± 0.01	0.144 ± 0.004
IGF-II (260 µg/day)	0.385 ± 0.006	2.45 ± 0.08	2.62 ± 0.01	0.147 ± 0.002
IGF-II (650 µg/day)	0.402 ± 0.006*	2.44 ± 0.05	2.66 ± 0.01	0.151 ± 0.002

*$P < 0.05$.

TABLE A.3. Faecal nitrogen expressed as a percentage of nitrogen intake during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group.

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	5.52 ± 0.26	5.65 ± 0.24	5.57 ± 0.19
IGF-I (104 µg/day)	5.53 ± 0.20	5.88 ± 0.27	5.58 ± 0.15
IGF-I (260 µg/day)	5.19 ± 0.32	5.89 ± 0.22	5.54 ± 0.14
IGF-II (104 µg/day)	5.78 ± 0.30	6.11 ± 0.21	5.94 ± 0.17
IGF-II (260 µg/day)	5.78 ± 0.27	5.82 ± 0.22	5.80 ± 0.19
IGF-II (650 µg/day)	5.58 ± 0.35	5.73 ± 0.19	5.65 ± 0.27

TABLE A.4. Nitrogen retentions (g) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each group.

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	1.43 ± 0.10	1.57 ± 0.06	3.00 ± 0.13
IGF-I (104 µg/day)	1.60 ± 0.07	1.42 ± 0.06	3.02 ± 0.07
IGF-I (260 µg/day)	1.53 ± 0.08	1.67 ± 0.07	3.19 ± 0.04
IGF-II (104 µg/day)	1.50 ± 0.05	1.46 ± 0.11	2.95 ± 0.15
IGF-II (260 µg/day)	1.57 ± 0.06	1.65 ± 0.15	3.22 ± 0.15
IGF-II (650 µg/day)	1.65 ± 0.07	1.64 ± 0.13	3.28 ± 0.19

TABLE A.5. Urinary 3-methylhistidine concentrations (mM) expressed as a fraction of urinary creatinine concentrations (mM) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals in each treatment group.

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	43.75 ± 0.68	43.44 ± 2.96	43.45 ± 1.44
IGF-I (104 µg/day)	45.28 ± 1.74	42.01 ± 2.69	43.32 ± 1.53
IGF-I (260 µg/day)	45.51 ± 1.73	42.64 ± 2.51	43.79 ± 1.07
IGF-II (104 µg/day)	44.21 ± 1.98	43.20 ± 3.82	43.54 ± 2.66
IGF-II (260 µg/day)	47.48 ± 2.83	40.89 ± 3.09	44.00 ± 2.65
IGF-II (650 µg/day)	45.33 ± 2.17	42.66 ± 1.55	43.99 ± 1.49

TABLE A.6. Carcass composition expressed as g/100g of carcass weight. Values represent the mean ± S.E.M. for 6 animals in each group.

	Protein	Fat	Water	Residue
Treatment				
Pre-treatment control	18.28 ± 0.25	6.10 ± 0.40*	71.13 ± 0.37**	4.49 ± 0.15
Vehicle control	18.91 ± 0.18	7.97 ± 0.16	68.52 ± 0.37	4.60 ± 0.35
IGF-I (104 µg/day)	18.66 ± 0.21	8.04 ± 0.49	68.63 ± 0.42	4.68 ± 0.21
IGF-I (260 µg/day)	18.85 ± 0.12	7.12 ± 0.32	69.24 ± 0.40	4.79 ± 0.25
IGF-II (104 µg/day)	18.93 ± 0.17	7.78 ± 0.30	69.00 ± 0.49	4.29 ± 0.46
IGF-II (260 µg/day)	19.30 ± 0.26	7.93 ± 0.65	68.02 ± 0.59	4.74 ± 0.43
IGF-II (650 µg/day)	19.12 ± 0.17	7.20 ± 0.51	69.27 ± 0.56	4.41 ± 0.14

APPENDIX B.

Supporting data for chapter 5.

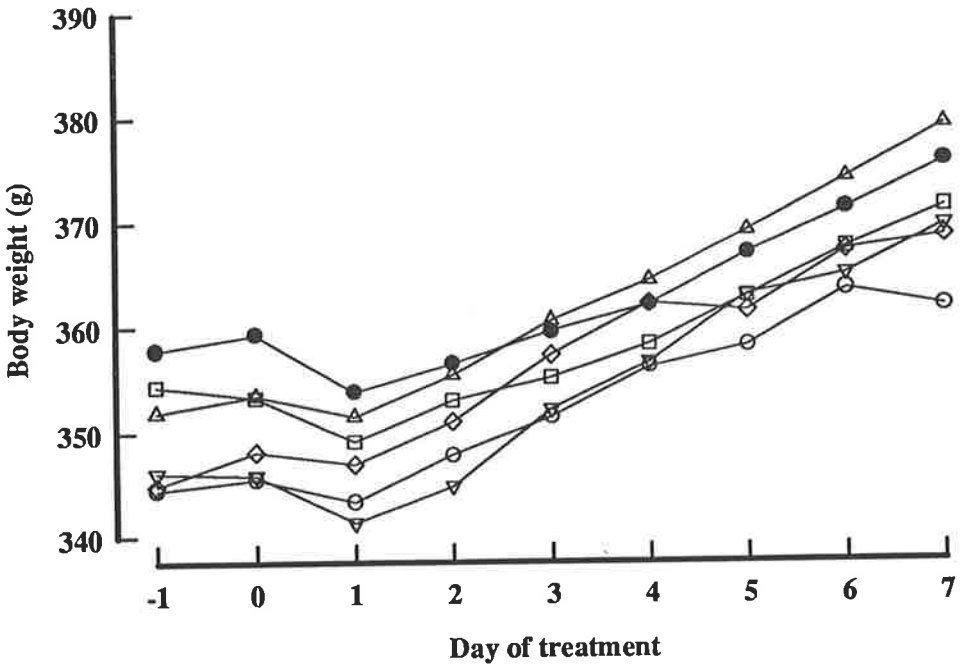


FIGURE B.1. Mean body weights (g) of guinea pigs on each day of treatment. Osmotic pumps were implanted, and treatment began, immediately following weighing of animals on day 0. Treatments are represented as follows: vehicle (filled circles), 120 µg of IGF-I/day (open circles), 360 µg of IGF-I/day (squares), 120 µg of IGF-II/day (triangles), 360 µg of IGF-II/day (inverted triangles) and 120 µg of LR^3IGF-I/day (diamonds). Results are expressed as the mean for 8 animals in each treatment. S.E.M.s have not been shown for the sake of clarity. The pooled S.E.M. on day 7 of treatment was 3.6.

TABLE B.1. Femur weights (g), fractional weights (g/kg body weight), lengths (cm) and weights per unit length (g/cm). The values represent the mean ± S.E.M. for 8 animals in each group.

	Weight	Fractional weight	Length	Weight/length
Treatment				
Vehicle control	0.94 ± 0.02	2.54 ± 0.04	3.38 ± 0.03	0.28 ± 0.01
IGF-I (120 µg/day)	0.92 ± 0.02	2.58 ± 0.05	3.36 ± 0.04	0.28 ± 0.01
IGF-I (360 µg/day)	0.90 ± 0.01	2.46 ± 0.05	3.36 ± 0.03	0.27 ± 0.01
IGF-II (120 µg/day)	0.93 ± 0.02	2.50 ± 0.08	3.35 ± 0.03	0.28 ± 0.01
IGF-II (360 µg/day)	0.92 ± 0.02	2.51 ± 0.05	3.37 ± 0.03	0.27 ± 0.01
LR^3IGF-I (120 µg/day)	0.89 ± 0.02	2.46 ± 0.05	3.35 ± 0.04	0.27 ± 0.01

TABLE B.2. Small intestine lengths (cm) and weights per unit length (g/cm). Weights (g) are included for comparison. Values represent the mean ± S.E.M. for 8 animals in each group.

Treatment	Weight	Length	Weight/length
Vehicle control	8.56 ± 0.32	134 ± 3	0.064 ± 0.003
IGF-I (120 µg/day)	8.83 ± 0.22	132 ± 3	0.067 ± 0.002
IGF-I (360 µg/day)	9.08 ± 0.29	137 ± 2	0.066 ± 0.002
IGF-II (120 µg/day)	8.97 ± 0.12	136 ± 1	0.066 ± 0.001
IGF-II (360 µg/day)	8.87 ± 0.21	136 ± 3	0.065 ± 0.002
LR^3IGF-I (120 µg/day)	9.43 ± 0.32	137 ± 2	0.069 ± 0.002

TABLE B.3. Carcass compositions (g/100g carcass). Values represent the mean ± S.E.M. for 8 animals in each group.

Treatment	Fat	Water	Remainder
Vehicle control	9.33 ± 0.53	67.74 ± 0.72	24.38 ± 0.41
IGF-I (120 µg/day)	8.66 ± 0.88	68.43 ± 0.89	23.97 ± 0.53
IGF-I (360 µg/day)	9.29 ± 0.46	67.88 ± 0.48	24.29 ± 0.27
IGF-II (120 µg/day)	9.11 ± 0.59	68.05 ± 0.85	24.23 ± 0.49
IGF-II (360 µg/day)	9.04 ± 0.44	68.45 ± 0.60	23.83 ± 0.36
LR^3IGF-I (120 µg/day)	8.30 ± 0.22	68.64 ± 0.49	24.11 ± 0.26

APPENDIX C.

Supporting data for chapter 6.

TABLE C.1. Water intakes (ml) during the indicated periods of treatment. Values represent the mean ± S.E.M. for 6 animals or, in the case of guinea pigs treated with $LR^3IGF\text{-}I$, as the mean of 3 animals (only those surviving 14 days of treatment are included).

	Days 1 to 7	Days 8 to 14	Days 1 to 14
Treatment			
Vehicle Control	522 ± 79	511 ± 59	1033 ± 133
IGF-I (720 µg/day)	332 ± 28	345 ± 48	677 ± 63
IGF-II (720 µg/day)	525 ± 44	412 ± 62	937 ± 87
IGF-I + IGF-II (360 µg/day of each)	573 ± 147	511 ± 128	1083 ± 272
$LR^3IGF\text{-}I$ (240 µg/day)	484 ± 104	431 ± 57	915 ± 159

TABLE C.2. Carcass, head plus paws and pelt weights expressed as a fraction of body weight (g/kg body weight). The values represent the mean ± S.E.M. for 6 animals in each group or, in the case of guinea pigs treated with $LR^3IGF\text{-}I$, as the mean of 3 animals (only those surviving 14 days of treatment are included).

	Carcass	Head plus paws	Pelt
Treatment			
Vehicle control	395 ± 8	159 ± 3	137 ± 3
IGF-I (720 µg/day)	386 ± 4	155 ± 2	142 ± 2
IGF-II (720 µg/day)	397 ± 8	158 ± 3	134 ± 2
IGF-I + IGF-II (360 µg/day of each)	385 ± 10	153 ± 3	135 ± 4
$LR^3IGF\text{-}I$ (240 µg/day)	363 ± 14	154 ± 7	149 ± 10

TABLE C.3. Femur weights (g), fractional weights (g/kg body weight), lengths (cm) and weights per unit length (g/cm). The values represent the mean ± S.E.M. for 6 animals in each group or, in the case of guinea pigs treated with $LR^3IGF\text{-}I$, as the mean of 3 animals (only those surviving 14 days of treatment are included).

Treatment	Weight	Fractional weight	Length	Weight/length
Vehicle control	0.98 ± 0.03	2.41 ± 0.05	3.46 ± 0.05	0.28 ± 0.01
IGF-I (720 µg/day)	0.99 ± 0.02	2.41 ± 0.08	3.45 ± 0.03	0.29 ± 0.01
IGF-II (720 µg/day)	0.94 ± 0.03	2.33 ± 0.05	3.43 ± 0.04	0.28 ± 0.01
IGF-I + IGF-II (360 µg/day of each)	1.00 ± 0.03	2.31 ± 0.03	3.46 ± 0.03	0.29 ± 0.01
$LR^3IGF\text{-}I$ (240 µg/day)	1.01 ± 0.08	2.35 ± 0.02	3.40 ± 0.04	0.30 ± 0.03

TABLE C.4. Gut tissue weights (g). The values represent the mean ± S.E.M. for 6 animals in each group or, in the case of guinea pigs treated with LR^3IGF-I, as the mean of 3 animals (only those surviving 14 days of treatment are included).

Treatment	Stomach	Small intestine	Caecum	Colon	Total gut
Vehicle control	2.79 ± 0.19	9.58 ± 0.34	5.21 ± 0.27	4.58 ± 0.21	22.17 ± 0.92
IGF-I (720 µg/day)	2.98 ± 0.17	9.58 ± 0.42	5.39 ± 0.33	5.03 ± 0.24	22.98 ± 1.07
IGF-II (720 µg/day)	2.82 ± 0.12	9.35 ± 0.41	5.58 ± 0.24	4.89 ± 0.34	22.64 ± 1.03
IGF-I + IGF-II (360 µg/day of each)	3.05 ± 0.15	10.29 ± 0.27	5.90 ± 0.19	5.47 ± 0.22	24.71 ± 0.68
LR^3IGF-I (240 µg/day)	2.92 ± 0.29	9.73 ± 0.70	5.43 ± 0.86	6.07 ± 0.33	24.20 ± 2.10

TABLE C.5. Small intestine lengths (cm) and weights per unit length (g/cm). Weights (g) are included for comparison. Values represent the mean ± S.E.M. for 6 animals in each group or, in the case of guinea pigs treated with $LR^3IGF\text{-}I$, as the mean of 3 animals (only those surviving 14 days of treatment are included).

Treatment	Weight	Length	Weight /length
Vehicle Control	9.58 ± 0.34	141 ± 5	0.068 ± 0.002
IGF-I (720 µg/day)	9.58 ± 0.42	137 ± 4	0.070 ± 0.003
IGF-II (720 µg/day)	9.35 ± 0.41	135 ± 3	0.069 ± 0.003
IGF-I + IGF-II (360 µg/day of each)	10.29 ± 0.27	145 ± 2	0.071 ± 0.002
$LR^3IGF\text{-}I$ (240 µg/day)	9.73 ± 0.70	146 ± 6	0.066 ± 0.003

TABLE C.6. Carcass compositions (g/100g carcass). Values represent the mean ± S.E.M. for 6 animals in each group or, in the case of guinea pigs treated with $LR^3IGF\text{-}I$, as the mean of 3 animals (only those surviving 14 days of treatment are included).

	Water	Fat	Remainder
Treatment			
Vehicle Control	67.77 ± 0.67	9.35 ± 0.62	22.88 ± 0.36
IGF-I (720 µg/day)	67.64 ± 0.42	9.09 ± 0.42	23.26 ± 0.37
IGF-II (720 µg/day)	68.42 ± 0.44	9.05 ± 0.24	22.53 ± 0.31
IGF-I + IGF-II (360 µg/day of each)	68.42 ± 0.47	8.86 ± 0.59	22.73 ± 0.28
$LR^3IGF\text{-}I$ (240 µg/day)	68.78 ± 0.88	8.28 ± 0.59	22.92 ± 0.42

TABLE C.7. Urea concentrations (mM) in plasma taken from animals at the end of the treatment period. Values represent the mean ± S.E.M. for 6 animals in each group or, in the case of guinea pigs treated with $LR^3IGF\text{-}I$, as the mean of 3 animals (only those surviving 14 days of treatment are included).

Treatment	
Vehicle Control	6.72 ± 0.32
IGF-I (720 µg/day)	5.96 ± 0.32
IGF-II (720 µg/day)	6.94 ± 0.33
IGF-I + IGF-II (360 µg/day of each)	6.64 ± 0.42
$LR^3IGF\text{-}I$ (240 µg/day)	6.38 ± 0.72

TABLE C.8. Nitrogen retentions (g) during 14 days of treatment. Values represent the mean ± S.E.M. for 6 animals in each group or, in the case of guinea pigs treated with LR^3IGF-I, as the mean of 3 animals (only those surviving 14 days of treatment are included).

Treatment	
Vehicle Control	4.09 ± 0.56
IGF-I (720 µg/day)	4.81 ± 0.44
IGF-II (720 µg/day)	4.56 ± 0.32
IGF-I + IGF-II (360 µg/day of each)	5.13 ± 0.35
LR^3IGF-I (240 µg/day)	5.14 ± 0.43