INSULIN-LIKE GROWTH FACTOR (IGF) AND IGF BINDING PROTEINS DURING PREGNANCY IN THE RAT AND HUMAN

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Doctor of Philosophy

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This dissertation is dedicated to my parents

and unseen friends.

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Errata

For Figure 1.4, the correct reference is Morgan *et al.*, **1987**.

p 6, line 10 and p 88, reference 12: Helper should read **Hepler**.

p 88, reference 2 and 3: Guidice should read **Giudice**.

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STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge it contains no material that has been previously published, except where reference is made in the text. I consent to this thesis being made available for photocopying and loan if applicable.

Dated at Adelaide: 13 June 91

Sharron Erna Gargosky (BSc. Hons I).

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ABSTRACT

The insulin-like growth factors (IGF) are anabolic peptides that stimulate cellular proliferation and differentiation. The IGFs are found in association with specific IGF binding proteins (IGFBP). To date six classes, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 have been classified on the basis of sequence and immunological data. These IGFBP: modulate the biological actions of IGF, in either an inhibitory or stimulatory manner; promote the half life of IGF in the circulation; and can act as endocrine stores of IGF. In the blood there is a good correlation between the concentrations of IGF and IGFBP.

The research presented in this dissertation examined IGF and IGFBP during pregnancy in the rat and human. The metabolic adaptations during pregnancy allows mothers to meet a large demand in energy and nutrient required by the conceptus. Since the placenta expresses IGF receptors and the IGFs are regulated both developmentally and nutritionally, the IGFs were considered to play a role in this metabolic adaptation and to modulate fetal growth.

The rat was our experimental model. The IGFBP found initially in biological fluids and secreted by liver-, bone- and muscle-derived cells were characterised by size-exclusion chromatography, Western-ligand blot and Western-immunoblot analysis to identify the IGFBP produced by different tissue types and found in specific biological fluids. Adult serum and abdominal lymph contained a 200 kDa IGFBP (possibly the soluble type-II receptor) and a 150 kDa IGFBP that contained subunits of 40-50 kDa aligning with IGFBP-3 on Western-ligand blots. Both fluids also contained a 30 kDa IGFBP that was immunoreactive with an antiserum to bovine IGFBP-2, a 28 kDa IGFBP that aligned but did not cross react with an antiserum to human IGFBP-1, and a 24 kDa IGFBP, possibly IGFBP-4. Fetal serum and amniotic fluid from pups between 17 and 20 days gestation, contained a 30 kDa IGFBP-2 whereas CSF contained only a 30 kDa IGFBP that was not IGFBP-2. Several smaller molecular mass IGFBP were detected in media conditioned by liver-, bone- and muscle-derived cells, but none of the cells secreted IGFBP-3. Thus at least 6 IGFBP were found in rat fluids: a 200 kDa IGFBP, a 150 kDa complex that contained IGFBP-3, a 32 kDa IGFBP-2, and a 32 kDa, 28 kDa and 24 kDa IGFBP.

Next the IGF-I, IGF-II and IGFBP profiles were examined in sera from non-pregnant, pregnant and lactating rats. During pregnancy and lactation in the rat, IGF-I was the major IGF present in maternal serum. I was unable to detect IGF-II above the limit of the assay. In early pregnancy, IGF-I levels rose until day 13, after which both IGF-I and IGFBP-3 declined and concentrations of both remained low for the duration of pregnancy (ie., to 21 days). One day postpartum, the IGF and IGFBP pattern began to recover to non-pregnant levels. To determine the distribution of IGF-I amongst the IGFBP, sera were fractionated by size-exclusion chromatography at physiological pH and then each fraction was acidified and subjected to acid size-exclusion chromatography to separate the IGF from IGFBP. The IGF-containing region was assayed by RIA. In sera from non-pregnant rats, the 150 kDa IGFBP complex, which contained IGFBP-3, carried the majority of the circulating IGF-I whilst the smaller IGFBP carried the remainder. In contrast, during late pregnancy, the smaller molecular mass IGFBP carried the majority of the IGF-I in blood. Consistent with the observation of low IGFBP-3 and IGF-I levels, the smaller molecular mass IGFBPs carried the majority of the circulating IGF. This result was consistent with the loss of IGFBP-3 requiring the other IGFBP to play an endocrine role.

IGFs have been shown to promote net protein accumulation both *in vitro* and *in vivo*. Thus, it was considered that the low levels of IGF-I may promote muscle protein catabolism in the mother, so as to provide supplementary nutrients to the fetus. I endeavoured to reverse the depressed serum IGF-I and IGFBP concentrations during late pregnancy and assess the effects on maternal, fetal and placental weight gain. I chose to infuse recombinant human growth hormone (rhGH; 2.4 mg/kg per day; n=9; days 11-21) that has been shown by others to regulate the synthesis of IGF-I and IGFBP-3, and rhIGF-I (1.4 mg/kg per day; n=10; days 11-21) which will increase circulating IGF-I and IGFBP levels in rats in states of low IGF. Administration of rhGH did not significantly elevate IGF-I levels, alter maternal, fetal or placental weights, or affect the IGFBP profile. Infusion of IGF-I into pregnant rats during late pregnancy significantly increased circulating IGF-I levels and slightly induced IGFBP-3 and a 32 kDa IGFBP. Most notable was the significant gain in maternal weight during the IGF-I treatment

period, whereas neither fetal nor placental weights were influenced. This suggested a repartitioning of nutrients to maternal tissues without detrimental effects on fetal growth.

Finally, in view of the results obtained in rats, I was interested in measuring IGF and IGFBP in the sera of women before, during and after pregnancy. During pregnancy, IGF-I and IGF-II levels were significantly elevated, reaching a maximum between weeks 26-33 and declining postpartum to non-pregnant levels. The IGFBPs, as assessed following size exclusion chromatography at pH 2.8 and measured as interference in the IGF assays or characterised by Western-ligand blotting, showed an apparent decline in IGFBP-3 and the 32 kDa IGFBP after week 7 of pregnancy. The IGFBP profile recovered 3-4 days postpartum. These results were unusual as IGF and IGFBP generally show a close correlation in the circulation, yet I found elevated levels of IGF-I and IGF-II whilst the major serum carrier, IGFBP-3 levels were low. Unbound IGF is rapidly cleared from the circulation, thus higher concentrations of free IGF would be expected during pregnancy. To investigate this anomaly, the distribution of IGF amongst the IGFBP was determined by measuring the IGF concentrations in sera fractionated on a size-exclusion column at pH 7.4. The IGF-I and IGF-II concentrations in the 150 kDa IGFBP complex that contained IGFBP-3 were elevated during late pregnancy, but IGFBP-3 detected by Western-ligand blot analysis of the same fractions was diminished. I suggest that the structural and functional integrity of IGFBP-3 as part of the 150 kDa complex is maintained since most of the IGF-I and IGF-II is present in a complex of 150 kDa whose formation depends on active IGFBP-3. Techniques that dissociate the IGFBP-3 from the 150 kDa complex (acidification or electrophoresis in SDS) appear therefore to markedly reduce IGFBP-3 stability. In conclusion, IGF-I, IGF-II and IGFBP-3, as part of the 150 kDa, are elevated in the serum of women in late pregnancy.

The research in this thesis presents a comparative study of IGF and IGFBP during pregnancy in rats and human. I observed a parallelism between the changing IGF and IGFBP levels during pregnancy; in the rat both IGF-I and IGFBP-3 declined whereas in the human, IGF-I, IGF-II and IGFBP-3 concentrations increased. The physiological significance of the inter-species variation is unresolved. Possibly the energy and nutrients required by the rat to fulfil

the demands of up to 16 conceptuses demands extreme metabolic adjustments; thus the dramatic loss of IGF-I. In contrast, women with a single conceptus may be more subtly regulated, possibly by IGFBP-proteases that can alter the stability of IGFBP-3 and subsequently modify the bioavailability and bioactivity of IGF-I. Clearly much interesting work into the role of IGF and IGFBP during pregnancy remains to be done.

PUBLICATIONS arising from Thesis Research

Gargosky,S.E., Owens,P.C., Walton,P.E., Robinson,J.C., Wallace,J.C. and Ballard,F.J. (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,S.E., Walton,P.E., Owens,P.C., Wallace,J.C. and Ballard,F.J. (1990).
Insulin-like growth factor-I (IGF-I) and IGF binding proteins both decline in the rat during late pregnancy. *Journal of Endocrinology* **127,** 183-290.

Gargosky,S.E., Walton,P.E., Wallace,J.C. and Ballard,F.J. (1990).
Insulin-like growth factor binding proteins (IGFBP) in the rat: characterization of IGFBP in serum, lymph, cerebrospinal and amniotic fluids, and in media conditioned by liver, bone and muscle cells. *Journal of Endocrinology* **127,** 391-400.

Gargosky,S.E., Owens,J.A., Walton,P.E., Owens,P.C., Wallace,J.C. and Ballard,F.J. (1991). Administration of insulin-like growth factor-I, but not growth hormone, increased maternal weight gain in late pregnancy without effecting fetal or placental growth. *Journal of Endocrinology* (in press).

Gargosky,S.E., Owens,P.C., Walton,P.E., Owens,J.A., Robinson,J.S., Wallace,J.C. and Ballard,F.J. (1991).
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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,F.M., Knowles,S.E., Owens,P.C., Read,L.C., Chandler,C.S., Gargosky,S.E. and Ballard,F.J. (1991).
Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with IGF-I and especially des(1-3) IGF-I. *Biochemical Journal* (in press).

Conference Proceedings: published as Abstracts:

Gargosky,S.E., Walton,P.E., Owens,P.C., Webb,H.A., Wallace,J.C. and Ballard,F.J. (1989). Circulating insulin-like growth factor (IGF) binding protein levels vary during pregnancy. Abstract, 28th National Scientific Conference for the Australian Society for Medical Research.

Gargosky,S.E., Walton,P.E., Wallace,J.C. and Ballard,F.J. (1989). Characterization of IGF binding proteins in rat biological fluids and cell line-conditioned medium (1989). Abstract, 32nd Annual Meeting of the Endocrine Society of Australia.

Gargosky,S.E., Walton,P.E. Owens,P.C., Wallace,J.C. and Ballard,F.J. (1990). IGF-I and IGF binding proteins are dramatically reduced during late pregnancy in the rat. Abstract, 4th biennial IGF symposium, Albury, Australia.

Gargosky,S.E., Moyse,K.J., Owens,J.A., Walton,P.E., Wallace,J.C., Robinson J.C. and Owens,P.C. (1990). Pregnancy-associated changes in serum insulin-like growth factors and their binding proteins. Abstract, 33rd Annual Meeting of the Endocrine Society of Australia.

Gargosky,S.E., Walton,P.E., Owens,P.C., Moyse,K.J., Wallace,J.C. and Ballard,F.J. (1990). IGF-I and IGFBP both decline in the rat during late pregnancy. 33rd Annual Meeting of the Endocrine Society of Australia.

Gargosky,S.E., Owens,P.C., Walton,P.E., Robinson,J.C., Wallace,J.C. and Ballard,F.J. (1990). In both rat and human there is a redistribution of the higher molecular types of IGF during late pregnancy. 29th National Scientific Conference for the Australian Society for Medical Research.

Gargosky,S.E., Walton,P.E., Owens,P.C., Owens,J.A., Wallace,J.C. and Ballard,F.J. (1991) The pregnancy-associated decline in IGF-I and IGF binding proteins is altered by infusion of rhIGF--I, but not rhGH in the rat. Abstract A 45. 2nd International IGF Symposia, San Francisco.

ABBREVIATIONS

AF	amniotic fluid
ALS	acid labile subunit of the 150 kDa IGFBP complex
BP	binding protein
BSA	bovine serum albumin
C-terminus	carboxyl terminus
cDNA	complementary deoxyribonucleic acid
c.p.m.	counts per minute
CSF	cerebrospinal fluid
GH	growth hormone
hypox.	hypophysectomy
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
kb	kilobase
kDa	kilo dalton
mRNA	messenger ribonucleic acid
N-terminus	amino terminus
P	probability
RIA	radioimmunoassay
RRA	radioreceptor assay
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SE	standard error of the mean

CHAPTER ONE: GENERAL INTRODUCTION

GENERAL INTRODUCTION

This chapter presents a brief review of the insulin-like growth factors (IGF), their receptors and binding proteins (IGFBP). Topics include: the isolation and characterization of IGF and IGFBP as both proteins and the genes that encode them; the variation in IGF and IGFBP concentrations in different metabolic conditions; and the endocrine and molecular regulation of IGF and IGFBP.

1.1 THE INSULIN-LIKE GROWTH FACTORS

Three independent areas of research led to the discovery of the insulin-like growth factors (IGF): 1) the formation of the somatomedin hypothesis; 2) the demonstration of nonsuppressible insulin-like activity; and 3) the characterization of serum growth promoters.

1.1.1 SOMATOMEDIN HYPOTHESIS

In 1931, Evans and Simpson documented the growth-promoting properties of the anterior hypophysis. Later, growth hormone (GH) was purified from the pituitary and injected into hypophysectomized rats: this resulted in increased skeletal growth (Becks *et al.,* 1949). These observations were extended by Denko and Bergenstal (1955) who found that *in vitro* incorporation of $^{35}SO_4$ into proteoglycans of costal cartilage was reduced in hypophysectomized rats, but elevated 3 to 4 fold in cartilage from GH-treated hypophysectomized rats. However, it was Salmon and Daughaday (1957) who reported that GH (somatotrophin) stimulated $^{35}SO_4$ incorporation *in vivo* but not *in vitro*. They proposed a "sulfation factor" that was GH inducible, as it appeared in the plasma of hypophysectomized rats following GH administration. This "sulfation factor" or "thymidine factor" was renamed "somatomedin" by Daughaday *et al.,* (1972). "Somato" indicated its hormonal relevance to somatotrophin and "medin" referred to the intermediate role in somatotrophin action. The "Somatomedin Hypothesis" was thus formulated.

1.1.2 NONSUPPRESSIBLE INSULIN-LIKE ACTIVITY

Molecules similar to insulin that did not bind to anti-insulin antibodies were termed nonsuppressible insulin-like activity (NSILA) (Slatter *et al.*, 1961; Froesch *et al.*, 1963). NSILA in human serum was mostly insoluble in acid-ethanol and chromatographed with a molecular weight of 100-150,000. The acid soluble portion had a molecular weight of 6-10,000. These two activities were called NSILA-P and NSILA-S, respectively (Burgi *et al.*, 1966; Jakob *et al.*, 1968). The NSILA-S fraction was found to contain two peptides, NSILA-I and NSILA-II, (Rinderknecht and Humbel, 1976) which were subsequently purified and completely sequenced from Cohn fractions of plasma. These polypeptides were then named IGF-I and IGF-II (Rinderknecht and Humbel, 1978a,b).

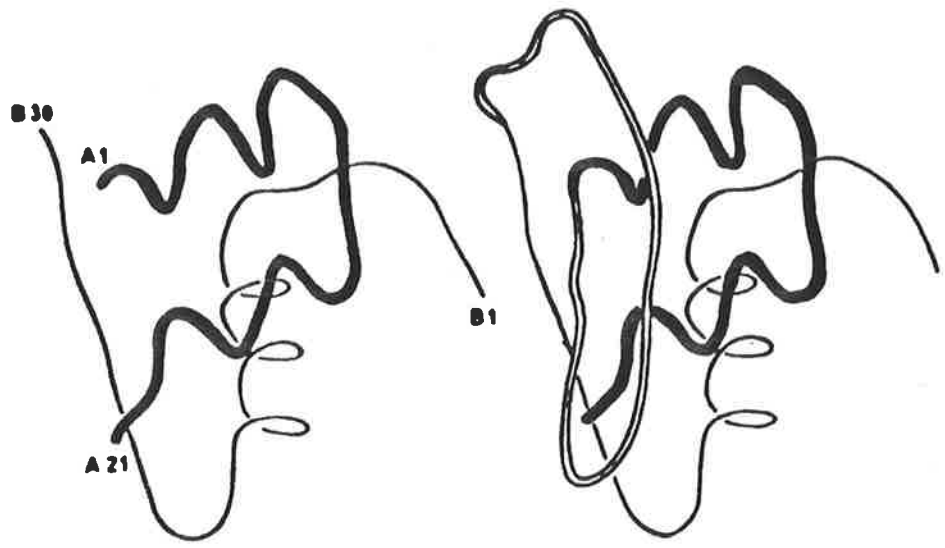
1.1.3 MULTIPLICATION-STIMULATING ACTIVITY (MSA)

The realisation that serum was an essential constituent for mammalian cell culture led to the investigation of serum mitogens (Temin, 1971). A Buffalo rat liver-derived cell line was found to grow in the absence of serum. Temin showed that conditioned media from these cells could stimulate DNA synthesis in chick embryo fibroblasts. This activity was partially purified and called multiplication-stimulating activity (MSA) (Dulak and Temin, 1973a,b).

1.1.4 NOMENCLATURE

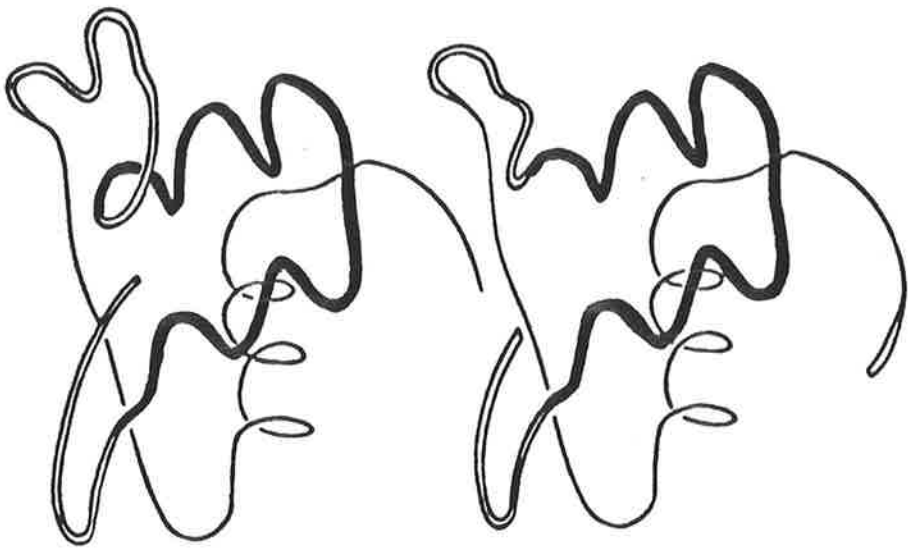
The nomenclature of the somatomedins was clarified when somatomedin-C, somatomedin-A and NSILA-I/ IGF-I were found to be identical (Hintz *et al.*, 1980; Van Wyk *et al.*, 1980; Klapper *et al.*, 1983; Enberg *et al.*, 1984) while human IGF-II/ NSILA-II and MSA were found to be 93% homologous. MSA was clearly rat IGF-II (Rinderknecht and Humbel, 1978b; Marquardt and Todaro, 1981). Thus, the nomenclature was resolved for the insulin-like growth factors (IGF) (Daughaday *et al.*, 1987).

Figure 1.1: Insulin and IGF structural relationship (Blundell *et al.*, 1978).



INSULIN

PROINSULIN



IGF (1)

IGF (2)

1.2 CHEMICAL CHARACTERIZATION OF IGF

1.2.1 STRUCTURE of IGF

Human IGF-I and IGF-II are single polypeptides chains of molecular weight 7649 and 7471 with three intramolecular disulfide bonds, respectively. The two IGF peptides are 65% homologous with each other and 43% homologous with insulin. The A and B chains of IGF contain the homologous regions to proinsulin. The connecting peptides of IGF-I and IGF-II are shorter (12 and 8 amino acids, respectively) and show no sequence homology to the 35 amino acid C-peptide of proinsulin. In addition, IGF-I and IGF-II have an 8 and 6 amino acid extension at the carboxyl terminal or D-domain (Humbel and Rinderknecht, 1978a,b). Both peptides are synthesised in precursor forms that include signal peptides and carboxyl terminal extensions: a region called the E domain (Janson *et al.*, 1983).

Based on the known X-ray crystallographic analysis of insulin, three-dimensional models of IGF-I and II were constructed (Blundell *et al.*, 1978) (Fig. 1.1).

1.2.2 ISOLATION of IGF

Many IGF peptides have been isolated (Table 1.1) and show extensive sequence homology between species. In addition, the amino acid sequence for Xenopus IGF-I has been inferred from the cDNA sequence (Shuldiner *et al.*, 1990) and immunoreactive IGF-I material has been reported in *Bufo woodhousi* (toad), *Bombyx mori* (silkworm), and Hagfish (Thornedyke *et al.*, 1989; Maruyama *et al.*, 1990; Pancak-Roessler and Lee, 1990).

1.2.3 VARIANTS of IGF

Although IGF-I and IGF-II are the principal somatomedins, variants have been isolated. A form of IGF-I was isolated from adult and fetal human brain and bovine colostrum that lacked the N-terminal tripeptide Gly-Pro-Glu (Carlsson-Skwirut *et al.*, 1986; Francis *et al.*, 1986; Sara *et al.*, 1986; Carlsson-Skwirut *et al.*, 1987; Francis *et al.*, 1988). Another line of evidence suggests that IGF-I exists as a heterogeneous group of peptides with each subform displaying varying affinities for the IGF receptors (Blum *et al.*, 1986; Rosenfeld *et al.*, 1987).

Table 1.1: Isolation of IGF in biological fluids and in media conditioned by cells.

This table is not complete but contains many of the isolates purified to date (1991).

Species	Source	IGF	References
Human	Plasma	IGF-I	Rinderknecht & Humbel, 1978a
		IGF-II	Rinderknecht & Humbel, 1978b
Rat	Sera	IGF-I	Rubin *et al.*, 1982
	BRL3A cells	IGF-II	Marquardt *et al.*, 1981
Bovine	Colostrum	IGF-I	Francis *et al.*, 1986
	Sera	IGF-I/II	Hoggner & Humbel, 1986
Avian	Sera	IGF-I/II	Dawe *et al.*, 1988
		IGF-II	Kallincos *et al.*, 1990
Ovine	Plasma	IGF-I/II	Francis *et al.*, 1989a
	Sera	IGF-I/II	Hey *et al.*, 1990
Porcine	Sera	IGF-I/II	Francis *et al.*, 1989b

Rinderknecht and Humbel found that 25% of the IGF-II purified from human serum lacked the N-terminal Ala (Rinderknecht and Humbel, 1978b). Another human IGF-II variant detected at both the RNA and protein level had the Ser residue 29 in the B domain replaced by Arg-Leu-Pro-Gly. This variant could arise from an alternative splice site (Jansen *et al.*, 1985; Hampton *et al.*, 1989). Larger variants of IGF-II have also been isolated in human serum: Zumstein *et al.* reported a pro-form of IGF-II of 10 kDa with a substitution of Ser 33 with Cys-Gly-Asp, and a carboxy terminal extension of 21 amino acid (E domain) (Zumstein *et al.*, 1985); Haselbacher *et al.* reported a 13 kDa species of IGF-II in cerebrospinal fluid (Haselbacher *et al.*, 1985); and Gowan *et al.* purified a 15 kDa species with 28 identical N-terminal amino acids to IGF-II, but a different carboxyl end (Gowan *et al.*, 1987). Normally, these large forms of IGF-II contribute less than 10% of the total IGF-II in human plasma, although tumours associated with hypoglycemia have up to 75% of the plasma IGF-II as a higher molecular mass (Daughaday and Rotwein, 1989).

Several biologically active polypeptides for MSA, considered to be the rat homologue of IGF-II, have been purified and biochemically characterised (Moses *et al.*, 1980). The peptides are designated MSA-I, -II and -III. The molecular sizes differ when the different species are reduced, yet the amino acid compositions are similar. The binding affinity of MSA-III is greater than MSA-II in selective conditions. The relevance physiological significance of these MSA forms is uncertain.

1.3 MOLECULAR BIOLOGY of IGF

1.3.1 cDNA ISOLATION

The first cDNA for IGF-I was isolated in 1983 by Jansen and colleagues from a human adult liver library (Jansen *et al.*, 1983). Subsequently, additional IGF-I cDNAs were reported (Ullrich *et al.*, 1984; Le Bouc *et al.*, 1986; Rotwein, 1986) and employing the specific human IGF-I cDNA, human IGF-II cDNA clones were isolated (Bell *et al.*, 1984; Jansen *et al.*, 1985; Le Bouc *et al.*, 1987; Shen *et al.*, 1986; DePagter-Holtuizen *et al.*, 1987,1988). To date numerous cDNA species have been described (Table 1.2) leading to a greater understanding of the IGF

Table 1.2: Isolation of cDNA clones for IGF.

SPECIES	IGF	REFERENCE
Human	IGF-I	Jansen *et al.*, 1983
		Ullrich *et al.*, 1984
		Le Bouc *et al.*, 1986
		Rotwein, 1986
	IGF-II	Bell etal., 1984
		Jansen *et al.*, 1985
		Shen *et al.*, 1986
		Le Bouc *et al.*, 1987
		DePagter-Holtuizen *et al.*, 1987,1988
Rat	IGF-I	Roberts *et al.*, 1987 a,b
		Casella *et al.*, 1987
		Murphy *et al.*, 1987
	IGF-II	Whitfield *et al.*, 1984
Mouse	IGF-I	Bell *et al.*, 1986
	IGF-II	Stempien *et al.*, 1986
Bovine	IGF-II	Brown *et al.*, 1990
Porcine	IGF-I	Tavakkol *et al.*, 1988
Ovine	IGF-I	Wong *et al.*, 1989
Salmon *Oncorhynchus kitsutch*	IGF-I	Cao *et al.*, 1989
Chicken	IGF-I	Fawcett & Bulfield, 1990
Xenopus laevis	IGF-I	Kajimoto & Rotwein, 1990

precursor structure and messenger RNA processing.

1.3.2 IGF-I GENE

1.3.2 a) Precursors

Analysis of the cDNA data indicates that human, rat and mouse IGF-I are synthesised as larger precursor molecules of approximately 130 amino acids that undergo processing at both the signal peptide (N terminus) and the E domain (C terminus) to produce mature peptides of 70 amino acids (Sussenbach, 1989). Two cDNA encoding different carboxy terminal extensions have been isolated with E domains of different lengths: IGF-Ia with 35 amino acids and IGF-Ib with 77 amino acids (Jansen *et al.,* 1983; Rotwein, 1986).

1.3.2 b) Gene structure

The human IGF-I gene contains 3 initiation codons (-48, -25,-22). Comparative studies infer the -25 and -22 positions as initiation codons, but *in vitro* systems indicate position -48 as the preferred initiation site (Rotwein, 1986). The human IGF-I gene is located on chromosome 12, region 12q22 (Brissenden *et al.,* 1984; Tricoli *et al.,* 1984) and spans more than 95 kb of chromosomal DNA. The IGF-I gene for rat and human is divided into five exons divided by four introns (Fig. 1.2). Exons 1 and 2 code for the signal peptide, exons 2 and 3 contain the mature IGF peptide and exons 3, 4 and 5 contain the E domain of IGF-Ia and IGF-Ib and 3' untranslated sequences (Rotwein *et al.,* 1986; Shimatsu *et al.,* 1989). In the human, the primary transcript is spliced to produce the two cDNA precursor molecules: IGF-Ia contains exons 1, 2, 3 and 5, whilst IGF-Ib contains exons 1, 2, 3 and 4 (Rotwein *et al.,* 1987; De Pager-Holthuizen *et al.,* 1990) (Fig. 1.2). Unlike the E domain of IGF-Ia, the E domain of the IGF-Ib precursor is not conserved between species (Tavakkol *et al.,* 1988).

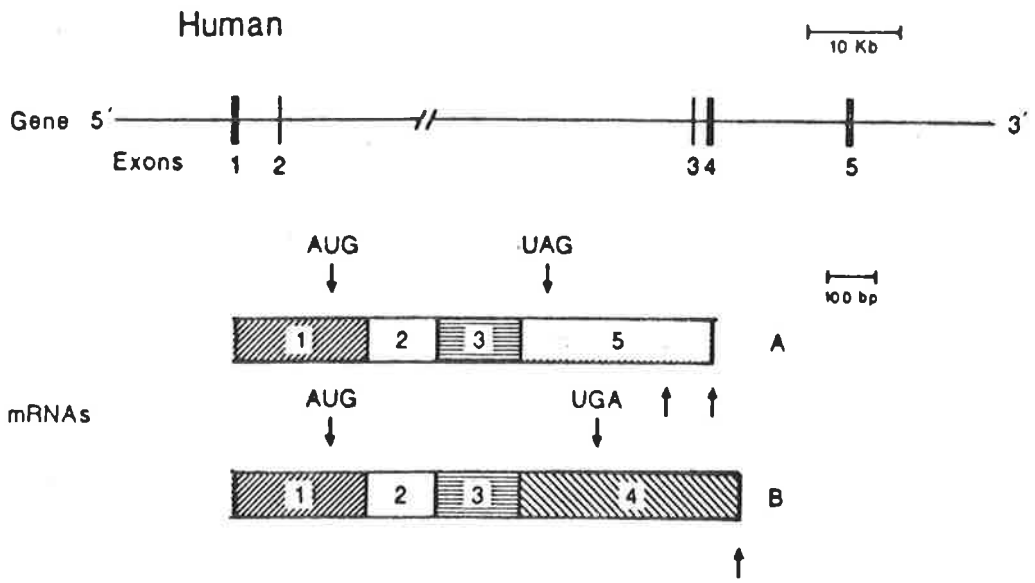
1.3.2 c) mRNA Expression

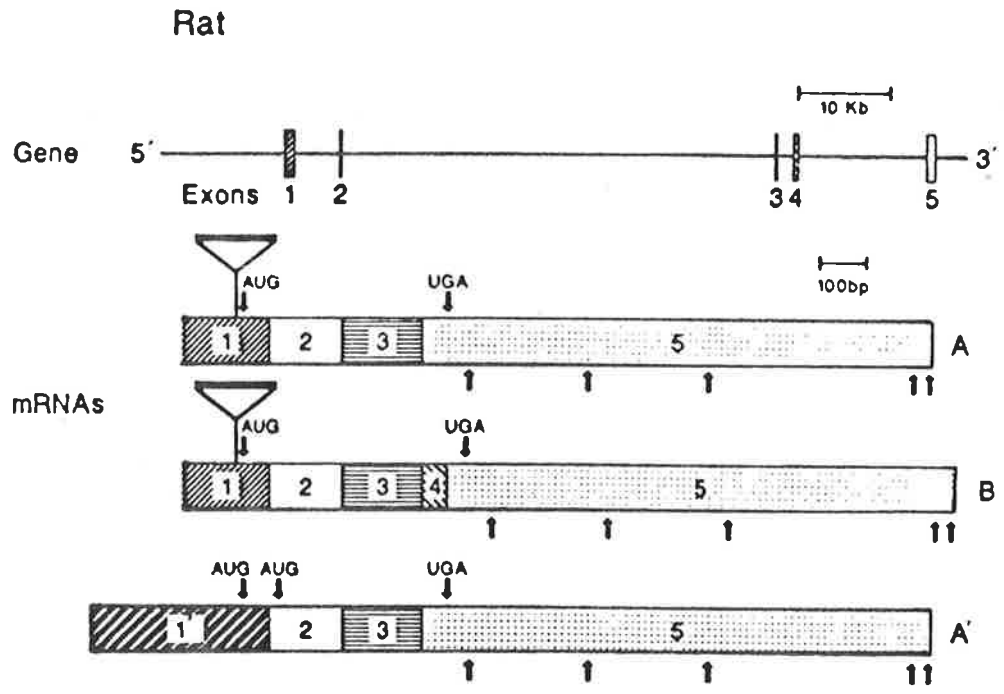
In the rat, processing is further complicated by: alternative splicing that can occur in both the 5' and 3' end of the gene; five polyadenylation sites at the 3' end of exon 5; divergent 3'

Figure 1.2: Structure and expression of the human and rat IGF-I gene and mRNA (Daughaday & Rotwein, 1989).

Exons are numbered; indicated below each gene is the alternative mRNA splicing that generates the different transcripts. IGF-Ia and IGF-Ib are noted. The stripped region in the 5' end of the rat mRNA, marked A' has not been marked on the genomic map. The 5' end of each mRNA is left open since it is not defined. The beginning and end of the coding region for each mRNA is marked by an AUG and UGA/UAG, respectively. Possible alternative polyadenylation sites are indicated by unlabelled arrows.

IGF-I Gene and mRNA Structure





untranslated regions; and a 5' region of variable sequence (Casella *et al.,* 1987; Murphy *et al.,* 1987; Shimatsu *et al.,* 1987; Holthuizen, 1990) (Fig. 1.2). Northern analysis indicates a range of IGF-I mRNA transcripts ranging from 1 to 7.6 kb (Bell *et al.,* 1985a; Han *et al.,* 1988; Rotwein, 1986) (Fig. 1.2). Human IGF-I mRNA can be detected in most adult and fetal tissues (Han *et al.,* 1988). Expression is tissue specific and developmentally regulated.

Rat and mouse IGF-I expression is regulated by differential initiation of transcription and alternative splicing due to the presence of three 5' leader sequences. The expression of the alternative 5' untranslated regions regulates tissue specificity, mRNA stability and mRNA levels developmentally (Lowe *et al.,* 1987; Adamo *et al.,* 1989; Lowe *et al.,* 1989). The half life of rat IGF-I mRNA is affected by the 3' untranslated sequence (Helper *et al.,* 1990).

1.3.3 IGF-II GENE

1.3.3 a) Precursors

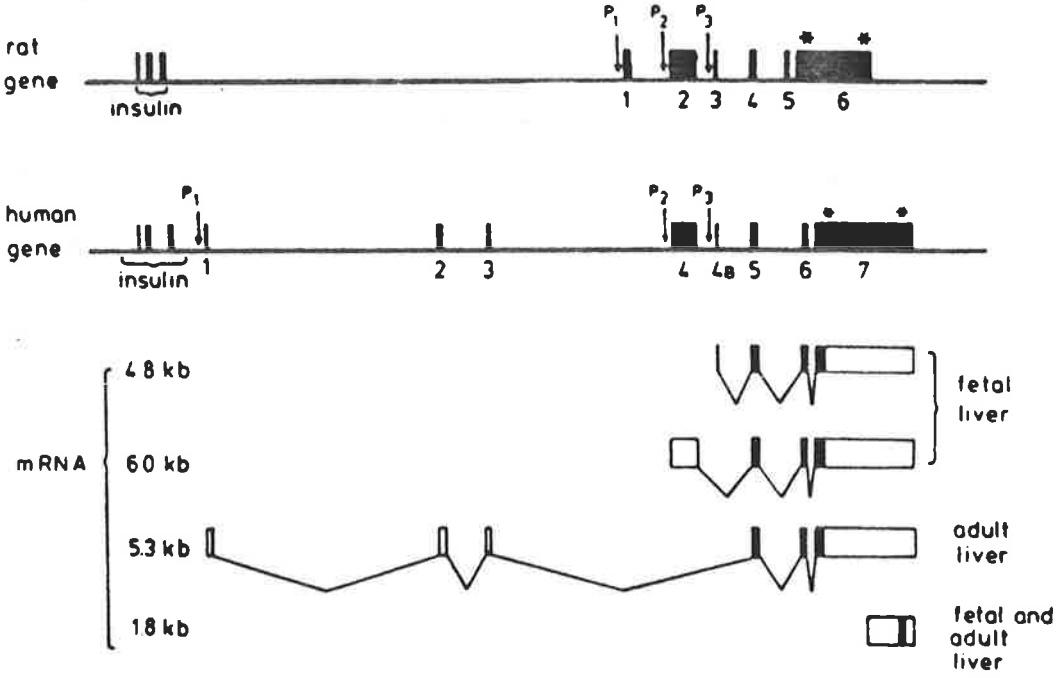
The IGF-II cDNAs for human, rat and mouse encode an 180 amino acid long precursor which contains a 24 amino acid signal peptide and an 89 amino acid carboxy terminus (E domain). In each case this is processed to a 67 amino acid peptide.

1.3.3 b) Gene structure

The human IGF-II gene is located on the short arm of chromosome 11 (Brissenden *et al.,* 1984; Tricoli *et al.,* 1984). The gene spans more than 30 kb of chromosomal DNA, comprising 8 exons. Exons 5, 6, 7 are the coding sequence and 1, 2, 3, 4, 4B are 5' untranslated regions that are differentially expressed. Transcriptional initiation can start at one of five promoters which precede exons 1, 4, 4B (Holthuizen, 1990). In contrast, the rat IGF-II gene has 6 exons. Exons 1, 2, 3 are transcribed from three weak promoters and encode the 5' untranslated regions. Exons 2 and 3 are analogous to human exons 4 and 4B. The leader exons (1, 2, 3) are linked to exons 4, 5 and exon 6 that encode the 3' untranslated region (Sussenbach, 1989).

Figure 1.3: Structure and function of the IGF-II gene and mRNA transcripts in both the human and rat (Sussenbach, 1988).

The exons are numbered and the promoters are indicated by the 'P'. Polyadenylation sites are indicated by asterisks.


rat
gene
insulin
P1
P2
P3
1
2
3
4
5
6
human
gene
insulin
1
2
3
4
4B
5
6
7
mRNA
4.8 kb
6.0 kb
5.3 kb
1.8 kb
fetal
liver
adult
liver
fetal and
adult
liver

1.3.3 c) mRNA Expression

The initiation of transcription is tissue and developmentally specific (Holthuizen, 1990). The size of mRNA (5.3-18 kb) is regulated by: the promoter initiation site; the length of the 5' untranslated region, which may vary between 100 to 1200 bases; and two variable polyadenylation signals at the 3' end contained within exon 7 (Fig. 1.3). Clearly, such mechanisms add to the transcriptional diversity, differential expression and developmental regulation of the IGFs (reviewed, Daughaday and Rotwein, 1989; Kato *et al.*, 1990; Johnson and Ilan, 1990; Rotwein *et al.*, 1990).

1.4 RECEPTORS for IGF

The biological responses to IGF are mediated, in part, via cellular transmembrane receptors. These receptors have been characterised by affinity cross-linking and immunoprecipitation techniques, followed by SDS-polyacrylamide analysis. This section of the review will cover the basic findings. For more comprehensive literature coverage, the reader is referred to reviews of: Rechler and Nissley, (1985); Carpenter, (1987); and Czech, (1989).

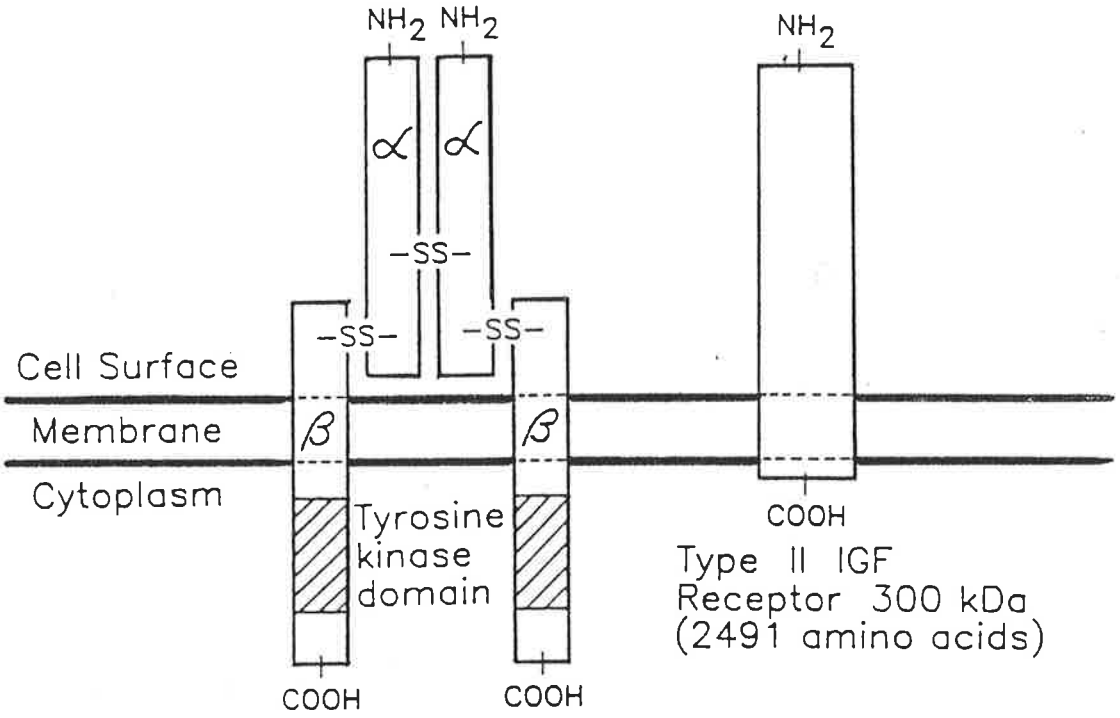
1.4.1 IGF-I RECEPTOR

The insulin and IGF-I receptors are similar in both structure and transmembrane signalling, but display different binding characteristics. The IGF-I receptor binds IGF-I with slightly greater affinity than IGF-II, and insulin with a much lower affinity. The insulin receptor binds insulin with a greater affinity than IGF-I (Massague and Czech, 1982).

The receptor for IGF-I is referred to as the type 1 receptor. It is a glycosylated protein composed of two identical alpha units (Mr 135,000) and two identical beta subunits (Mr 95,000) linked by disulfide bonds into an $alpha_2beta_2$ heterotetrameric complex (Massague and Czech, 1982) (Fig. 1.4). The alpha subunit residing in the extracellular domain is involved in ligand binding and contains an N-terminus rich in cysteine amino acids. The two alpha subunits are joined by class I disulphide bonds and anchored to the plasma membrane by class II disulphide bonds through the extracellular component of the beta subunit. The intracellular

Figure 1.4: Schematic diagram of the type-I and type-II receptors (Morgan *et al.*, 1990).

Proteins are drawn to approximately to scale and orientated in the plasma membrane with the carboxyl terminus inside. The tyrosine kinase domains of the IGF-I receptor is indicated as a hatched boxed.


NH2 NH2
NH2
α α
-SS-
-SS-
-SS-
Cell Surface
Membrane
Cytoplasm
β β
Tyrosine
kinase
domain
COOH
COOH
COOH
Type II IGF
Receptor 300 kDa
(2491 amino acids)
Type I IGF
Receptor 350 kDa
α chain = 125 kDa
(705 amino acids)
β chain = 90 kDa
(627 amino acids)

portion of the beta subunit contains an ATP binding domain (Roth and Cassell, 1983; Ebina, *et al.*, 1987), tyrosine-specific autophosphorylation acceptor sites (Jacobs *et al.*, 1983), protein kinase activity (Zick *et al.*, 1984) and serine and threonine phosphorylation acceptor sites (Yu *et al.*, 1986).

The full amino acid sequence of the receptor was deduced from a cDNA clone isolated by Ullrich (1986). The type I receptor is synthesised as a 1400 amino acid precursor. The precursor is glycosylated and proteolytically processed to the mature alpha and beta subunits, and assembled to produce a mature protein (Ullrich *et al.*, 1986).

The mechanism of ligand binding to a cellular receptor, the internalization of the ligand-receptor complex and the intracellular cascade of events that ensue are not completely understood. Some of the primary events, following internalization are: receptor phosphorylation; inositol phosphate processing; calcium mobilisation; the synthesis of proto-oncogenes; phosphorylation and dephosphorylation by protein kinase of protein K, S6 and other proteins; and pH fluctuations. For reviews the reader is referred to: Krebs, 1984; Stryer, 1986. Berridge, 1985, 1987; Sibley *et al.*, 1987; and Corps, 1990.

The IGF receptors are widely distributed throughout the body and subject to developmental and differential regulation (Alexandrides *et al.*, 1989; Heyner *et al.*, 1989; Werner *et al.*, 1989).

1.4.2 IGF-II RECEPTOR

The receptor for IGF-II termed the type 2 receptor, is a 250,000 kDa glycoprotein. The primary structure was determined from the cloning and sequencing of the receptor cDNA (Morgan *et al.*, 1987) (Fig. 1.4). There are 19 N-linked glycosylation sites in the extracellular domain, which is composed of 15 conserved repeats of 150 amino acids that are only 20 % identical, yet contain a conserved motif of 8 cysteine residues. One major hydrophobic segment represents the transmembrane compartment. The cytoplasmic domain is hydrophillic and contains potential serine, threonine and tyrosine phosphorylation sites. Interestingly, the bovine cation-independent mannose-6-phosphate receptor (CIM6P) is 99.8 % homologous to the

human IGF-II receptor (Oshima *et al.*, 1988). Recent structural, biochemical and immunological evidence has proved that these receptors are identical (Nissley *et al.*, 1990).

The type 2/CIM6P receptor binds IGF-II with greater affinity than IGF-I and does not bind insulin (Rechler and Nissley, 1985). There are distinct binding sites for mannose 6-phosphate and IGF-II on the same receptor molecule (Braulke *et al.*, 1988).

The structural homology and ligand interaction suggests a dual role for the type II/CIM6P receptor. The receptor is involved in the targeting of lysosomal enzymes and may mediate some IGF-II actions via intracellular signals such as G proteins or protein kinase C activation (Nishimoto *et al.*, 1989; Braulke *et al.*, 1990; Nissley *et al.*, 1990). The expression of the IGF-II/CIM6P receptor is developmentally regulated in a manner similar to circulating IGF-II concentrations (Sklar *et al.*, 1989).

The intracellular mechanisms and signals of the IGF receptors and the significance in the regulation of IGF activity are not completely understood.

1.5 BIOLOGICAL ACTIVITIES of IGF

The IGFs were originally investigated and characterised by the measuring $^{35}SO_4$ incorporation into proteoglycans of costal cartilage (Section 1.1.1), insulin-like effects on adipose tissue (Section 1.1.2) and stimulation of thymidine incorporation into chick embryo fibroblasts (Section 1.1.3). Since that time the biological activities of IGF have been extensively researched then both *in vivo* and *in vitro*. This dissertation will briefly discuss the functions of IGF. For more comprehensive reviews the reader is referred to: Hall and Sara, (1983); Froesch *et al.*, (1985); and Froesch and Zapf, (1985).

1.5.1 CELL ASSOCIATED ACTIVITIES

The insulin-like effects mediated by IGF include; enhanced glucose transport, inhibition of glycogen breakdown, epinephrine-stimulated glycerol and free fatty acid release from adipose tissue and lipogenesis (Froesch *et al.*, 1978; Zapf *et al.*, 1981; Baxter, 1986; and

Walton *et al.*, 1989c). Effects of IGF *in vitro* on cell replication and DNA synthesis have been demonstrated in many cell types (Froesch *et al.*, 1985; Baxter, 1986). Other cellular responses include; stimulation of protein synthesis, RNA and DNA synthesis, as well as, inhibition of protein degradation and lipolysis in a variety of cell types (Froesch *et al.*, 1985; Hall and Sara, 1983; Ballard *et al.*, 1986). IGF-I also mediates the mitogenic actions of growth hormone in cartilage tissue (Van Wyk *et al.*, 1974). In addition, IGF can cause cellular differentiation of cell of mesodermal origin, such as osteoblasts, myoblasts, myotubules, and erythroid cell colony formation (Froesch *et al.*, 1985).

1.5.2 *IN VIVO* EFFECTS of IGF

The study of the growth promoting effects of IGF *in vivo* were restricted initially by the shortage of peptide. In 1978, partially purified somatomedins were used to demonstrate an increase in body weight and length in dwarf rats (van Buul and van den Brande, 1978). In 1980, pure peptide was injected into frogs via the dorsal lymph sac causing DNA synthesis and mitogenesis in the lens epithelium (Rothstein *et al.*, 1982). However, it was the availability of recombinant human IGF that has furthered our understanding of the metabolic effects of IGF. Definitive evidence of IGF action as an *in vivo* moderator of GH action came from Shoenle *et al.* (1982). Continuous infusion of IGF-I or IGF-II into hypophysectomised rats resulted in increased body weight, tibeal width and thymidine incorporation into cartilage (Shoenle *et al.*, 1982). Subsequently, IGFs have been infused into various catabolic states and changes in growth parameters such as body weight gain, nitrogen retention, protein turnover and carcass composition have been examined (Tomas *et al.*, 1991 a,b). In addition to growth-promoting activity, IGF has short-lasting effects on insulin target tissues. High concentrations of IGF may induce hypoglycemic effects (Froesch *et al.*, 1985).

1.5.3 REGULATORS OF IGF CONCENTRATIONS

1.5.3 a) Age

Plasma concentrations of IGF-I increase after birth, peak during mid-puberty and then progressively decline with age. The peak of IGF-I during puberty is not obviously related to GH concentrations. Other possible factors influencing the peak in IGF are uncertain (Clemmons and Underwood, 1986). However, the age-associated decline in the IGF-I concentration is related to a reduction in GH secretion (Rudman *et al.*, 1981; Florini *et al.*, 1985).

IGF-II is the dominant peptide in fetal life, declining postnatally in the rat, whereas the IGF-II levels persist in the human and are greater than the IGF-I levels (Daughaday and Rotwein, 1989; Gargosky *et al.*, 1990a).

1.5.3 b) Nutritional status

Nutritional status is important in the regulation of IGF-I (Clemmons and Underwood, 1986), but not IGF-II (Davenport *et al.*, 1988). The fall in human and rat plasma IGF-I correlates with poor nitrogen balance, suggesting a close link between IGF-I and the catabolic condition of fasting (Clemmons *et al.*, 1981, Tomas *et al.*, 1991). Isley *et al.* (1984) showed that calories are a critical determinant of IGF-I concentrations, but if energy intake is not unacceptably low, then protein content of the diet also has significant effects. Extending this observation, Clemmons *et al.* (1985) showed that essential amino acids will augment IGF-I concentrations after refeeding.

1.5.3 c) Hormonal control

The blood concentration of IGF-I is influenced by a variety of hormones. Patients with hypothyroidism have low plasma IGF-I concentrations (Burstein *et al.*, 1979) that are elevated fourfold with thyroxine treatment (Chernausek *et al.*, 1983).

Circulating concentrations of IGF-I but not IGF-II are GH-dependent (Zapf *et al.*, 1981; D'Ercole *et al.*, 1984). Growth hormone deficiency exists with low IGF-I levels while growth hormone (GH) excess coincides with elevated levels of IGF-I. However, there are exceptions,

such as malnutrition, where IGF-I levels decline, but GH concentrations may be high (Froesch and Zapf, 1985). Also, Merimee *et al.* (1982) showed that injecting GH into starved subjects did not increase plasma IGF-I levels.

Prolactin and placental lactogen can stimulate IGF-I synthesis but this effect is variable and dependent upon assay conditions (Froesch and Zapf, 1985).

Estrogen administration will increase circulating IGF-I concentrations in patients with Turners syndrome (Cutter *et al.,* 1985) but will reduce IGF-I levels in ovarectomised, hypophysectomised rats (Murphy and Friesen, 1988). In contrast, uterine expression of IGF-I mRNA is elevated in response to estrogen (For review: Murphy and Friesen, 1990). Testosterone injected into GH-deficient or hypopituitary patients does not alter plasma IGF-I concentration. However, androgens are suggested to cause the pubertal peak in IGF-I (Daughaday and Rotwein, 1988).

There is both direct and indirect evidence for the regulation of hepatic IGF-I expression by insulin (For review: Johnson and Ilan, 1990).

The activity of follicle-stimulating hormone on granulosa cells in culture is markedly potentiated by IGF-I, suggesting a role for the amplification of gonadal function by IGF (Adashi *et al.,* 1990).

IGFBP are major modulators of IGF action (Section 1.6).

1.6 INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBP)

1.6.1 PURIFICATION of IGFBP

Early reports on NSILA indicated two forms of NSILA activity; NSILA-S (IGF-I and IGF-II) and NSILA-P. NSILA-P contained proteins that chromatographed under neutral conditions at 100-150 kDa and 50-70 kDa (Froesch *et al.,* 1963; Burgi *et al.,* 1966; Jakob *et al.,* 1968). In 1977, specific somatomedin binding proteins were detected following neutral chromatography of serum incubated with radiolabelled IGF (Hintz and Liu, 1977; Kaufmann *et al.,* 1977). Since this time, many IGFBP have been isolated and characterized (Table 1.3).

Table 1.3: Isolated IGFBP.

IGFBP	Designated	Reference
AFBP (Amniotic fluid BP)	human IGFBP-1	Drop *et al.*, 1979 Povoa *et al.*, 1984 Busby *et al.*, 1988a
HepG2 BP		Povoa *et al.*, 1985
PP12 placental protein 12		Koistenen *et al.*, 1986
PEG pregnancy associated endometrial alpha globulin		Bell *et al.*, 1985 b
MSA CP	rat IGFBP-2	Knauer *et al.*, 1981
BRL3A IGFBP		Mottola *et al.*, 1986
MDBK BP	bovine IGFBP-2	Szabo *et al.*, 1988
human GHD*-IGFBP	human IGFBP-3	Martin & Baxter, 1986
rat IGFCP	rat IGFBP-3	Zapf *et al.*, 1988
pig IGFBP	porcine IGFBP-3	Walton *et al.*, 1989a
bovine IGFBP	bovine IGFBP-3	Walton *et al.*, 1989b
bone IGFBP	human IGFBP-4	Mohan *et al.*, 1989
24 kDa IGFBP	rat IGFBP-4	Shimonaka *et al.*, 1989
h Bone-derived BP	human IGFBP-5	Bautista *et al.*, 1991
CSF IGFBP	human IGFBP-6	Hossenlopp *et al.*, 1986 Roghani *et al.*, 1989
He(39)L IGFBP		Forbes *et al.*, 1990
AG2804 IGFBP		Martin *et al.*, 1990
U-2 IGFBP		Andress & Birnhaum,1991
vascular IGFBP	?	Bar *et al.*, 1989

* GHD = Growth hormone dependant

In 1989 a systematic nomenclature was adopted to classify the IGFBP based upon biochemical, immunological and sequence information. The acronym for the binding proteins is IGFBP, with a letter prefix to denote the species and an arabic number suffix for classification (Ballard *et al.*, 1989).

1.6.2 ISOLATION of cDNA CLONES for IGFBP

1.6.2 a) IGFBP-1

IGFBP-1 was first cloned from human decidual and HepG2 cell libraries as a preIGFBP-1 of 259 amino acids containing a putative 25 amino acid signal sequence (Julkanen *et al.*, 1988; Lee *et al.*, 1988). Human genomic clones found the IGFBP-1 gene which spans 5.9 kb, to be located on chromosome 7 p12-p13 (Alitalo *et al.*, 1989). Four exons produced a single mRNA of 1.6 kb suggesting no differential splicing (Brinkman *et al.*, 1988a,b; Brewer *et al.*, 1988). The rat IGFBP-1 cDNA displays 66% homology with the human IGFBP-1 sequence (Murphy *et al.*, 1990).

1.6.2 b) IGFBP-2

The full primary structure of human IGFBP-2 was determined from a cDNA clone. The cDNA encoded a 328 amino acid IGFBP precursor with a 39 residue signal sequence. Southern analysis demonstrated that IGFBP-2 was encoded by a single copy gene (Binkert *et al.*, 1989). Brown and coworkers isolate the rat equivalent of IGFBP-2. The cDNA hybridized to a 2 kb mRNA and encoded a precursor protein of 304 amino acids, comprising a 34 residue signal sequence (Brown *et al.*, 1989; Margot *et al.*, 1989). The bovine IGFBP-2 has also been cloned. The IGFBP precursor exists as a signal peptide of 26 amino acids and a mature protein of 284 amino acids. The predicted protein sequences shows 89% homology between the bovine, rat and human IGFBP-2 forms (Upton *et al.*, 1990).

1.6.2 c) IGFBP-3

The human IGFBP-3 cDNA was first isolated by Wood *et al.* (1988). The cDNA predicted contained a 27 amino acid signal sequence and a mature protein of 264 residues. Similarly, the rat IGFBP-3 cDNA clone indicated a protein with a 26 amino acid signal sequence and a 265 amino acid mature protein. The rat IGFBP-3 mRNA is 2.6 kb long and derived from a single gene (Shimasaki *et al.*, 1989; Albiston and Herington, 1990). In addition, the porcine IGFBP-3 cDNA clone has been isolated encoding a mature peptide of 266 amino acids, two more than the human sequence, and has a 2.6 kb mRNA (Shimasaki *et al.*, 1990).

The human IGFBP-3 gene is located on chromosome 3 and spans 8.9 kb of chromosomal DNA. The gene is divided into four exons encoding the IGFBP, whilst exon 5 contains the 3' untranslated region (Cubbage *et al.*, 1990).

1.6.2 d) IGFBP-4

Recently, cDNA clones of IGFBP-4 have been isolated from rat, pig and human. The mature protein consists of 233 amino acids for the rat while the human IGFBP contains four additional residues. The mRNA transcript detected was 2.6 kb (Shimasaki *et al.*, 1990, 1991).

1.6.2 e) IGFBP-5

The rat, pig and human cDNA for IGFBP-5 has been recently isolated using the polymerase chain reaction or cross hybridization techniques (Kiefer *et al.*, 1991; Shimasaki *et al.*, 1991). The mature protein is of 252 amino acids.

1.6.3 TISSUE SPECIFIC EXPRESSION of IGFBP

Northern analysis of human IGFBP-1 showed high levels of expression in the liver, the decidua at term and early pregnancy, and the secretory endometrium. No hybridizable mRNA was detected in the proliferative endometrium, placenta at term, or any other organ (Brinkmann *et al.*, 1988b; Julkanen *et al.*, 1988). In addition, rat IGFBP-1 mRNA was expressed in the kidney and low level expression was detected in the uterus and brain (Murphy

et al., 1990).

Expression of IGFBP-2 was found in the human brain, as well in Jurkat cells, a helper T cell line (Binkert *et al.*, 1989). In the rat, expression was abundant in adult brain, testes, ovaries and kidney. During pregnancy in the rat, IGFBP-2 mRNA is detected in the placenta and uterus after day 12 of gestation with increases up to day 20 (one day prior to delivery). Further, rat IGFBP-2 mRNA appears to be developmentally regulated as levels are high in fetal liver but low in the adult liver (Margot *et al.*, 1989).

The expression levels of human IGFBP-3 are undocumented. However, mRNA of rat IGFBP-3 has been found predominantly in the liver, kidneys and stomach. Heart, adrenal ovary, testis, spleen, lung, large and small intestine all express IGFBP-3 in low amounts (Shimasaki *et al.*, 1989).

The tissue expression of IGFBP-4 and IGFBP-5 mRNA in rat tissues has demonstrated transcription was highest in the liver and kidney, but also evident in the ovary, testis, spleen, adrenal, heart, lung, stomach and brain (Keifer *et al.*, 1991; Shimasaki *et al.*, 1991).

1.6.4 STRUCTURAL CHARACTERISTICS of IGFBP

1.6.4 a) IGFBP-1

IGFBP-1 is a single peptide chain of approximately 25 kDa. IGFBP-1 contains at least five potential O-linked glycosylation sites, but no N-linked glycosylation sites. The N-terminal sequence is cysteine rich and followed by repeated sequences of Pro, Glu, Ser and Thr residues (PEST) that exist in proteins with short half lives (Julkanen *et al.*, 1988). The protein also contains an Arg-Gly-Asp motif (RGD) near the C-terminus that may provide a cellular attachment site (Brewer *et al.*, 1988; Julkanen *et al.*, 1988). IGFBP-1 binds IGF-I with similar or slightly greater affinity than IGF-II (Brewer *et al.*, 1988; Brinkman *et al.*, 1988a,b; Julkanen *et al.*, 1988; Lee *et al.*, 1988).

1.6.4 b) IGFBP-2

IGFBP-2 is a single chain non-glycosylated polypeptide of 31 kDa. It contains cysteine-rich regions at either end of the peptide, like other IGFBP and an RGD sequence near the C-terminus. IGFBP-2 binds IGF-II with a greater affinity than IGF-I (Szabo *et al.*, 1988; Binkert *et al.*, 1989; Brown *et al.*, 1989).

1.6.4 c) IGFBP-3

The molecular mass of IGFBP-3 predicted from the cDNA sequence is 28.5 kDa (Wood *et al.*, 1988), yet purified protein on gel-permeation chromatography and SDS polyacrylamide gel electrophoresis migrates as a series of bands of 40-50 kDa due to N-linked glycosylation (Martin and Baxter, 1986). This protein is the acid-stable component of a 150 kDa complex. The 150 kDa complex consists of: an alpha, acid labile subunit (ALS) that is glycosylated (86 kDa); a beta subunit, IGFBP-3 (40-50 kDa); and a gamma subunit, IGF (7.5 kDa). Formation of the 150 kDa complex requires IGFBP-3 to form a binary complex with IGF before ALS can bind to produce the ternary form. Thus ALS alone cannot bind IGF, and ALS and IGFBP-3 will not complex without IGF. IGFBP-3 binds IGF-I and IGF-II approximately equally (Baxter, 1988; Baxter *et al.*, 1989; Baxter and Martin, 1989a). The kinetics of the ternary complex formation are decreased by salt since the formation of the binary complex is slowed. Glycosaminoglycans can also inhibit ternary complex formation (Baxter, 1990).

1.6.4 d) IGFBP-4

In addition to the 18 cysteine residues conserved in IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-5, IGFBP-4 contains two extra cysteines and binds IGF-II with greater affinity than IGF-I (Mohan *et al.*, 1989; Shimasaki *et al.*, 1989). This IGFBP contains 233 amino acids and demonstrates a molecular mass around 25 kDa on SDS PAGE (Mohan *et al.*, 1989).

1.6.4 e) IGFBP-5

Both rat and human IGFBP-5 consist of 252 amino acids and has a molecular mass of 28577 (Bautista *et al.*, 1991; Keifer *et al.*, 1991). The amino acid sequence indicates no N-linked glycosylation sites and the 18 cysteine residues are conserved (Shimasaki *et al.*, 1991). Similar to IGFBP-4, this binding protein binds IGF-II with a greater affinity than IGF-I (Bautista *et al.*, 1991; Keifer *et al.*, 1991).

1.6.4 f) IGFBP-6

The recent classification for rat and human IGFBP-6 by Shimasaki *et al.* (1991) has shown this protein to share amino acid sequence with IGFBP isolated from human CSF (Hossenlopp *et al.*, 1986; Roghani *et al.*, 1989) and medium from two human fibroblast cell cultures, He(39)L (Forbes *et al.*, 1990) and AG2804 (Martin *et al.*, 1990). This IGFBP also binds IGF-II preferentially, but unlike IGFBP-5, it is glycosylated.

1.6.5 BIOLOGICAL ROLES of IGFBP

The IGFBP may play a passive role, physically transporting IGFs and extending their biological half life in the circulation (Cohen and Nissley, 1975). However, recent studies have provided evidence that IGFBP can both inhibit and enhance the bioactivity and bioavailability of IGF (reviewed: Ooi, 1990).

1.6.5 a) Stimulatory effects

It is important to note that in all reported studies, the acute addition of any IGFBP *in vitro*, inhibits the binding of labelled IGF to receptors. However, some research groups have published evidence for stimulatory effects of complexed IGF. Cornell and coworkers showed that IGF-IGFBP complexes were metabolically active in a fat cell bioassay (Cornell *et al.*, 1987). In hamster kidney fibroblasts, the mitogenic activity of IGF-I was enhanced when combined with IGFBP-3 (Blum *et al.*, 1989). However, DeMellow and Baxter found that depending on whether IGFBP-3 was pre-incubated or co-incubated with IGF, IGFBP-3 could stimulate or

inhibit IGF-induced DNA synthesis in human fibroblasts (DeMellow and Baxter, 1988).

A possible potentiating role for IGFBP-1 has also been investigated. In human fibroblasts, IGFBP-1 has been suggested to bind to the cell surface and aid delivery of IGF to the cellular receptor (DeVroede *et al.,* 1986; Clemmons *et al.,* 1987). The potentiation of IGF action by IGFBP-1 has been shown with human, mouse and chick embryo fibroblasts and porcine smooth muscle cells (Elgin *et al.,* 1987). Busby *et al.* (1989) have suggested a possible mechanism for the delivery of IGF. They have reported multimeric forms of IGFBP-1 that are fully active, and suggest that the binding of this stable complex to the cellular matrix surfaces may promote the presentation of IGF to the cell.

Recently, a role for IGFBP-5 purified from human bone and IGFBP-6 purified from human osteogenic sarcoma culture medium has been shown to potentiate the mitogenic action of IGF-II and IGF-i, respectively. This effect occurred without pre-incubation or the addition of platelet poor plasma to the cell culture medium (Andress and Birnhaum, 1991; Bautista *et al.,* 1991).

1.6.5 b) Inhibitory effects

The inhibitory activities of the IGFBPs have long been recognised. Zapf *et al.* (1978b, 1979) found that the activity of NSILA was inhibited in fat cell assays when complexed to serum carriers (ie. all IGFBP). Human IGFBP-3 has been shown to inhibit IGF action on human fibroblasts (DeMellow and Baxter, 1988). The ability of MSA (rIGF-II) on BRL3A cells to stimulate glucose uptake and DNA synthesis was inhibited in the presence of MSA carrier protein (IGFBP-2) (Knauer and Smith, 1980). More recently, it has been shown that the addition of purified bIGFBP-2 or hIGFBP-1 into chick embryo fibroblasts will inhibit protein accumulation and DNA synthesis (Ross *et al.,* 1989). IGFBP-I has also been found to inhibit IGF actions in adipose tissue and chondrocytes (Drop *et al.,* 1979; Ritvos *et al.,* 1988). In addition, IGFBP-4 was reported to inhibit IGF-stimulated bone cell proliferation (Mohan *et al.,* 1989).

The literature available in this area is immense, so for more detail refer to: Baxter and

Martin, (1989b); Ooi and Herington, (1987); Ooi, (1990). The ability of IGFBP to inhibit or potentiate the biological effects *in vitro* now needs to be extended to *in vivo* studies.

1.6.6 REGULATION of IGFBP

1.6.6 a) Age and Diurnal regulation

In humans, IGFBP-3 concentrations, measured by RIA, increase from early childhood to puberty and then gradually decline with age (Baxter and Martin, 1986). Donahue and co-workers have reported that although neither the number nor the size of the IGFBP changes, the band intensities of all IGFBP were reduced with increasing age as detected by Western-ligand blotting (Donahue et al., 1990). In the pig, the intensities of all IGFBP bands increase with increasing gestational age (McCusker *et al.*, 1985).

On a shorter time scale, IGFBP-1 is diurnally regulated, with concentrations in humans peaking between 2400 and 0600 hours and declining by 1200 hours (Baxter and Cowell, 1987). Interestingly, in patients with Cushing's syndrome, IGFBP-1 concentrations are low and no diurnal regulation is observed (Degerblad *et al.*, 1989). IGFBP-3 is not diurnally regulated, although Jorgensen and coworkers infer that IGFBP-3 displays a circadian pattern (Jorgensen *et al.*, 1990).

1.6.6 b) Nutrition

Nutrition is a major regulator of the IGF. Thus, the close correlation of IGF and IGFBP in the circulation would infer nutrition to be a determinant of IGFBP levels also. In pigs subjected to dietary deprivation, the 43 and 39 kDa IGFBP (IGFBP-3), 34 kDa IGFBP (IGFBP-2) and 24 kDa IGFBP (probably IGFBP-4) bands declined whilst the 29 kDa (probably IGFBP-1) band increased in intensity as measured by Western-ligand blot analysis (McCusker *et al.*, 1989). In agreement, Busby and coworkers have shown that in fasted humans, IGFBP-1 concentrations increase with the length of fasting and decline upon refeeding to control levels (Busby *et al.*, 1988b).

Rats maintained on low protein diets (4%) have low levels of a 40-50 kDa triplet

(IGFBP-3), a 30 kDa and 28 kDa IGFBP, whilst a 24 kDa IGFBP appears unchanged in the serum (Tomas *et al.,* 1991a). In contrast, Straus and Takemoto (1990) have reported increased hepatic expression of rat IGFBP-2 in the liver of rats subjected to 4% protein diets and suggest that IGFBP-2 expression is nutritionally regulated. Fasting rats for 48 hours did not alter IGFBP-3, but reduced the levels a 30 kDa IGFBP and 24 kDa IGFBP, slightly increased IGFBP-2 and increased the expression of IGFBP-2 in the liver (Orlowski *et al.,* 1990). These results indicate a distinct difference in IGFBP levels between protein deprivation and fasting.

1.6.6 c) Growth Hormone (GH)

In 1976, the growth hormone-dependence of the somatomedin carrier protein was reported in hypophysectomised (hypox.) rats treated with GH. The IGF binding patterns were unchanged by the addition of GH, and although the IGF binding affinities were unaltered, the carrying capacity was reduced in hypox. animals (Cohen and Nissley, 1976; Moses *et al.,* 1976, 1979). These findings were extended when growth hormone-deficient patients, injected with GH showed an increase in the 150 kDa IGFBP which declined between 48 and 72 hours after administration (Grant *et al.,* 1986). However it was the RIA for the human growth hormone-dependent binding protein (now called IGFBP-3) that allowed the measurement of IGFBP-3 in various metabolic conditions (Baxter and Martin, 1986). In GH-deficient patients, IGFBP-3 concentrations were 50-80% lower than normal, whilst patients with active acromegaly showed a 2-fold increase in IGFBP-3 concentrations. Western-ligand blotting showed an increase in the IGFBP-3 in conditions such as acromegaly and diminished levels in conditions of genetic or idiopathic GH deficiency (Hardouin *et al.,* 1989). In pig serum, IGFBP-3 concentrations were significantly elevated in chronically GH-infused pigs and reduced in hypox. animals, as measured by RIA (Walton *et al.,* 1989a). A similar increase in IGFBP-3 has been shown in hypox. or protein-deprived rats treated with GH (Clemmons *et al.,* 1989).

Unlike IGFBP-3, IGFBP-1 is inversely regulated by GH. In acromegalic patients the concentration of IGFBP-1 was low and in patients suffering GH deficiency, IGFBP-1 was elevated as measured by RIA (Busby *et al.,* 1988b). Healthy individuals infused with GH

showed a decline in IGFBP-1 concentrations, similar to meal-induced falls in IGFBP-1 (Conover *et al.*, 1990). *In vitro*, IGFBP-1 synthesised and secreted by human fibroblasts was unaffected by the addition of GH to the medium (Conover *et al.*, 1989).

In hypox. rats, IGFBP-2 mRNA levels in the liver were elevated 20-fold and the administration of GH did not restore the mRNA levels to control values. Interestingly, hypophysectomy did not affect mRNA levels of IGFBP-2 in the testes, brain, kidney, skeletal muscle or spleen (Margot *et al.*, 1989).

1.6.6 d) Insulin and Glucose

i) *in vivo*

The early observations relating to streptozotocin (stz) -induced diabetes in rats reported a decline in the 150 kDa IGFBP and an increase in the 40-50 kDa IGFBP. Treatment with insulin, but not GH, induced the 150 kDa peak and reduced the 40-50 kDa form (Zapf *et al.*, 1981). Unterman and coworkers further characterised the changes in IGFBP by Western-ligand blot analysis and found that IGFBP-3 was undetectable while IGFBP-1 was significantly elevated in diabetes. However, treatment with insulin restored the binding profile to control levels (Unterman *et al.*, 1989; Zapf *et al.*, 1989).

In the human, the overall IGFBP binding activity was reduced in patients with insulin-dependent diabetes mellitus (IDDM) and restored by insulin-replacement therapy (Rieu and Binoux, 1985). Subsequently, IGFBP-1 was reported to be regulated by insulin. Concentrations of IGFBP-1 were increased with type 1 and type 2 diabetes and restored with insulin therapy. In addition, patients with either an insulinoma, or euglycemic hyperinsulinemia showed a decline of IGFBP-1 of 40-70% (Suikkari *et al.*, 1988, 1989; Unterman *et al.*, 1990). Administration of oral glucose consistently lowered IGFBP-1 levels in diabetic and non-diabetic pregnant women, men and non-pregnant women. Insulin elicited a 4-fold increase in IGFBP-1 after the nadir of oral glucose. These data suggest a role for IGFBP-1 in the maintenance of glucose homeostasis (Yeoh and Baxter, 1988).

ii) *in vitro*

Additional studies have utilised cell culture systems. Insulin treatment of rat hepatocytes from diabetic and insulin-treated diabetic rats cultured *in vitro* increased IGFBP-1 levels to 70% of normal (Scott and Baxter, 1986). Further, Lewitt and Baxter (1989, 1990) utilised human fetal liver explant cultures to examine the regulation of IGFBP-1. They found that the glucose concentration in the medium was inversely related to the IGFBP-1 production and that production could be inhibited by insulin. Cytochalasin B, a glucose transport inhibitor, as well as two agents that activate the cyclic nucleotide pathway, blocked 2-deoxyglucose uptake and increased IGFBP-1 production.

Other groups have used hepatoma cells and shown an inverse relationship of IGFBP-1 to insulin that are independent of glucose levels. They suggest that the suppressive effects of insulin on IGFBP-1 synthesis is mediated via the insulin receptor (Cotterill *et al.*, 1989; Conover and Lee, 1990; Singh *et al.*, 1990).

Interestingly, insulin addition to bovine fibroblasts causes the induction of IGFBP-3 and an increase in the smaller molecular mass IGFBP (Conover, 1990).

1.6.6 e) Renal failure

In 1986, Baxter and Martin reported an increase in IGFBP-3 in patients with chronic renal failure [CRF] (Baxter and Martin, 1986). In contrast, Liu *et al.* (1990) have reported decreased levels of IGFBP-3 as assessed by Western-ligand blotting. In addition, both Liu *et al.* (1990) and Goldberg *et al.* (1982) found an increase in the smaller molecular mass IGFBP-2 and IGFBP-1, in patients with CRF. The IGF carrying capacity of the IGFBP-3 as part of the 150 kDa complex was also reduced in serum from CRF patients (Liu *et al.*, 1990).

In addition to these factors regulating IGFBP, other hormones and different metabolic conditions (cancer, wound healing) are being examined to determine the physiological significance and role(s) of IGFBP (Clemmons, 1990).

1.7 PREGNANCY

A major portion of my research has been to examine the IGF and IGFBP during pregnancy in the rat and human. In the past two years, interest in this area has grown rapidly. Consequently, to put my research into perspective, this section of the dissertation will be referenced prior to November 1988, the time at which I began my PhD studies on IGFBP.

Decreases in rat IGF during late pregnancy have been reported when IGF was measured as activity on costal cartilage (Bala *et al.*, 1978) and in unextracted sera (Sara *et al.*, 1980). Others have reported no change in circulating rat IGF-I (Daughaday *et al.*, 1982). Sheppard and Bala (1986) measuring rat IGF-I by RIA showed an increase in serum IGF concentrations during early pregnancy and a progressive decline during late pregnancy. In contrast, human IGF-I levels were shown to be elevated during pregnancy (Furlanetto *et al.*, 1978; Bala *et al.*, 1981) as were the IGF-II levels (Wilson, *et al.*, 1982). However, these assays measured serum IGF without the definite removal of interfering IGFBP so that comparison between the studies is difficult.

Meanwhile, Rutanen *et al.* (1982, 1984) and Drop *et al.* (1984) were measured serum concentrations of PP12 and amniotic fluid BP (IGFBP-1), respectively, during human pregnancy and reported a peak of IGFBP-1 at 22-23 weeks which then declined at weeks 32-33. An RIA for human IGFBP-3 was used to show that IGFBP-3 was elevated during the third trimester of pregnancy (Baxter and Martin, 1986).

In summary, IGF are anabolic peptides that are widely distributed throughout the body. They exert their metabolic actions by binding to cellular receptors or circulating binding proteins. Their interaction with a specific receptor, initiates a cascade of intracellular events leading eventually to cellular growth. The other interaction is via the IGFBP, which act as endocrine stores of IGF, as well as modulators of IGF activity, possibly in an autocrine or paracrine way. Additional complexity in the regulation of IGF and IGFBP are due to endocrine and metabolic effectors.

1.8 OBJECTIVES of the STUDY

The insulin-like growth factors (IGF) are growth-promoting peptides whose bioavailability and bioactivity is regulated by IGF binding proteins (IGFBP). The main thrust of my research was to examine the levels and regulation IGF and IGFBP during pregnancy, primarily in the rat, with some extension to pregnant women.

The maternal IGF concentrations were assessed in the absence of potentially interfering IGFBP, IGFBP were measured and characterised immunologically and on the basis of size. The IGF carrying capacity of the circulating IGFBP in sera fractionated on a size-exclusion column was measured. Finally, in rats only, I attempted to alter the maternal nutrient repartitioning by regulating endogenous IGF levels.

The specific aims of this project were:

[1] to characterize the IGFBP in the rat in different biological fluids and in media conditioned by cells;

[2] to measure changes in serum IGF and IGFBP profiles during pregnancy and lactation in the rat;

[3] to modulate the endogenous concentrations of IGF and IGFBP by infusing pregnant rats with IGF-I or GH and examining the effects of treatment on growth; and

[4] to compare the serum IGF and IGFBP profiles during pregnancy in the human with those in the rat.

CHAPTER TWO: CHARACTERIZATION OF INSULIN-LIKE GROWTH FACTOR-BINDING PROTEINS IN SERUM, LYMPH, AMNIOTIC FLUID & CEREBROSPINAL FLUID, AND IN MEDIA CONDITIONED BY BONE-, LIVER- AND MUSCLE-CELLS.

2.1 INTRODUCTION

The insulin-like growth factors (IGF) in the circulation and in media conditioned by cells are complexed to IGF binding proteins (IGFBP). These IGFBP enhance the half-life of IGF in the circulation and may promote or inhibit the biological activity of IGF (Elgin *et al.*, 1987; Szabo *et al.*, 1988; Ballard *et al.*, 1991). The rat IGFBP have been isolated and the sequences of five classes of these are documented (Brown *et al.*, 1989; Murphy *et al.*, 1989; Shimasaki *et al.*, 1989, 1990, 1991). The molecular masses for the rat IGFBP on SDS-polyacrylamide gels are: IGFBP-1, 28 kDa; IGFBP-2, 30 kDa; IGFBP-3, 45-50 kDa; and IGFBP-4.

In rat serum, IGFBP have been characterized using gel permeation chromatography (Moses *et al.*, 1979), affinity cross-linking (D'Ercole and Wilkins, 1984) and Western-ligand blot analysis (Hossenlopp *et al.*, 1987). In media conditioned by bone- muscle- and liver-derived cells, IGFBP have been characterized using Western-ligand blot analysis (Hossenlopp *et al.*, 1987; McCusker *et al.*, 1989; Schmidt *et al.*, 1989).

The aim of this study was to characterize the IGFBP in adult and fetal rat serum, abdominal lymph, amniotic fluid, cerebrospinal fluid (CSF) and in conditioned media from rat bone-, liver- and muscle-derived cells. This study extends previous findings by better defining the IGFBP. In the analysis of IGFBP I have compared the IGFBP in biological fluids with purified IGFBP standards and performed Western-immunoblots with antisera that recognises rat IGFBP-2.

2.2 MATERIALS and METHODOLOGY

2.2.1 Fluid samples

Blood was collected from female Sprague Dawley rats (approximately 200 g; Central animal house, University of Adelaide) by cardiac puncture, and from rat fetuses (17 days gestation) following decapitation. The blood was clotted for 18 hrs at 4°C and the serum was harvested following centrifugation at 1000 g for 30 min. Amniotic fluid was collected from exanguinated pregnant Sprague Dawley rats (approximately 200 g; day 17 of pregnancy). Abdominal lymph was collected from the thoracic duct of anaesthetised female rats by Drs. L. Read and A. Martin. CSF was collected by Mr. P. Rogers from the intracisternal space of eight anaesthetised male rats (approximately 350 g), divided into two pools and centrifuged at 10,000 g for 5 min. All samples were stored at -20^{0}C before analysis.

2.2.2 Cell-conditioned media collection

The following cells were cultured for media collection; their sources are indicated: L6 myoblasts, Dr. M. Gunn, Texas A and M University, College Station, TX, U.S.A.; SD8ArA vascular smooth muscle cells, Dr. J. Funder, Prince Henry's Hospital, Melbourne, Australia; Hep10C, Mr. C. Chandler, CSIRO Division of Human Nutrition, South Australia, Australia; MH_1C_1 hepatoma, American Type Culture Collection, Rockville, M.D., U.S.A; HTC hepatoma, Dr. W.D. Wicks, Department of Pharmacology, University of Colorado, CO, U.S.A.; H35 hepatoma, Dr. Joyce Becker, University of Wisconsin, Madison, WI, U.S.A; osteogenic sarcoma (OS104) and calvarial osteoblasts, Drs. T.J.Martin and N.C. Partridge, Repatriation General Hospital, West Heidelberg, VIC; BRL3A buffalo rat liver cells, Commonwealth Serum Laboratory, Parkville, Victoria, Australia.

The cells were cultured into 75 cm^2 culture flasks in Dulbecco Modified Minimal Essential Medium (DMEM; Flow Laboratories, Stanmore, New South Wales, Australia) containing 5 % (v/v) fetal calf serum, Fungizone (1 µg/ml; E.R. Squibb and Sons, Inc.,

Princeton, N.J., U.S.A.), penicillin G (60 µg/ml; Glaxo, Boronia, Victoria, Australia) and streptomycin (100 µg/ml; Glaxo), incubated in 5 % (v/v) CO_2: 95 % (v/v) air, and a humidified atmosphere at 37°C. Confluent monolayers, obtained after 5 to 7 days in culture, were washed twice for 1.5 hrs in serum-free DMEM (15 ml), after which serum-free DMEM (10 ml) was added to each flask. Following a 48 hr incubation, medium was collected and centrifuged at 1000 g for 15 min. to remove cell debris and stored at -20°C. Prior to analysis, medium samples were concentrated 5- to 20-fold in Centricon PD10 microconcentrators (Amicon, Danvers, MA, U.S.A.).

2.2.3 Peptides and Proteins

Recombinant human (rh) IGF-I was kindly provided by Drs. H.H. Peters and K. Scheibli (CIBA-Geigy, Basel, Switzerland) and rhIGF-II by Lilly Research Laboratories (Indianapolis, IN, U.S.A.). Both peptides were iodinated by Dr. P.C. Owens with carrier-free $Na^{125}I$ (Amersham International plc, Amersham, Bucks, U.K.) to a specific activity between 40 and 70 µCi/µg using the chloramine T method (Van Obberghen-Schilling and Pouyssegur, 1983).

Human IGFBP-1 (hIGFBP-1) was a gift from Dr. R.C. Baxter (Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia). Bovine IGFBP-2 was purified by Ms J. Moss as previously described (Szabo *et al.,* 1988), and porcine as well as bovine IGFBP-3 were purified by Dr. P.E. Walton (Walton *et al.,* 1989a,b).

Antisera for Western-immunoblot analysis were: rabbit anti-bovine (MDBK) IGFBP-2 (Upton *et al.,* 1990); and rabbit anti-human IGFBP-1 kindly provided by Dr. P.D.K. Lee (Texas Childrens Hospital, Houston, TX, U.S.A.).

2.2.4 Preparation of iodinated IGF for *in vitro* labelling of fluids

To ensure the iodinated IGF contained neither aggregates nor breakdown products, the radiolabelled IGFs were separated on a column of TSK fractogel 55(S)HW (Merck, Darmstadt, F.R.G.) at room temperature. Radiolabelled IGF (1 ml) was loaded onto the column (55 cm x 0.5 cm) and eluted at 0.3 ml/min in a mobile phase of sodium phosphate (50 mmol/l, pH 6.5) containing 0.25 % (w/v) bovine serum albumin (BSA; radioimmunoassay grade, Sigma Chemical Co., St. Louis, MO, U.S.A.) that had been filtered and degassed through a 0.22 µ filter prior to chromatography. Fractions (0.6 ml) were collected and the radioactivity measured in a gamma counter. These fractions were assayed for trichloroacetic acid (TCA) precipitability (2.2.4 i) and biological activity (2.2.4 ii).

2.2.4 i) TCA precipitability

A subsample of each fraction (10 µl) was diluted in 1 ml of TSK mobile phase and TCA (100 % w/v) was added to a final concentration of 10 % (v/v). Following an incubation for 30 min on ice, the sample was centrifuged at 2800 rpm at 4°C for 30 min. The supernatant was removed and the radioactivity in both the pellet and supernatant measured in a gamma counter. Fractions eluting in the correct molecular size-region and with 85-100 % precipitability were retained.

2.2.4 ii) Biological activity

Receptor binding of labelled IGFs was assessed by competitive binding studies on L6 myoblasts. Confluent monolayers of L6 myoblasts in 24-place multiwells were washed twice in 1 ml of Hepes buffer (N-2 hydroxyethylpiperazine-N'-2' ethane sulphonic acid (100 mmol/l) containing sodium chloride (150 mmol/l), magnesium sulphate (1.2 mmol/1) and glucose (8 mmol/l) pH 7.4) and BSA (RIA-grade; 0.5 % w/v). Each wash was incubated for 1 hr at 4°C. Subsequently, 400 µl of the Hepes buffer containing BSA (0.5% w/v) with radiolabelled IGF (10,000 c.p.m.) was added to each

well, together with 100 µl of either insulin (20 µg/well; Actrapid, Novo Lab. Pty. Ltd., Copenhagen, Denmark) or rhIGF (100 ng/well), or diluent. After an 18 hr incubation at 4°C, the multiwells were washed twice at 0°C with increasing volumes of Hanks balanced salt solution (Gibco Lab., Grand Island, NY, U.S.A.) containing 0.5 % (w/v) BSA at pH 7.4. Monolayers were dissolved by trituration in 1 ml of sodium hydroxide (0.5 M) containing Triton X-100 (1 % v/v) and the radioactivity was measured in a gamma counter.

Fractions of radiolabelled IGFs with high TCA precipitability and biological activity were pooled, aliquotted and stored at -20°C until needed.

2.2.5 Fractionation of fluids incubated with radiolabelled IGF by fast phase liquid chromatography (FPLC)

Rat fluids were defatted by vortexing with an equal volume of freon (1,1,2 trichloro-1,2,2-trifluoro-ethane), vortexing and then centrifuging at 10,000 g for 5 min. before collecting the aqueous phase. Radiolabelled IGF-I (20 µl; 100,000 c.p.m.) plus 50 µl buffer, or IGF-II (70 µl; 100,000 c.p.m.) was added to each sample (200 µl) in the absence or presence of excess rhIGF-I or rhIGF-II. The peptide amounts are indicated in Table 2.1. The samples (275 µl) were incubated for 18 hrs at 4°C before 200 µl was applied to a Superose 12 FPLC column (10/30; Pharmacia, Piscataway, NJ, U.S.A.). The sample was chromatographed at room temperature by elution at 0.5 ml/min in a buffer containing sodium phosphate (50 mmol/l), sodium chloride (150 mmol/l) and sodium azide (0.2% w/v) at pH 7.2 that had been filtered and degassed through a 0.22µ filter prior to each chromatography. Fractions (0.25 ml) were collected and the radioactivity was measured using a gamma counter.

The Superose 12 column was calibrated using gel permeation chromatography molecular mass standards (Sigma Chemical Co., St. Louis, MO, U.S.A.): thyroglobulin (600 kDa); gamma globulin (150 kDa); bovine serum albumin (69 kDa); ovalbumin (46 kDa); and carbonic anhydrase (30 kDa); as well as iodinated IGF-I or IGF-II (7.5 kDa).

To ensure the chromatographic properties of the column remained unchanged, it was calibrated in this way routinely.

2.2.6 Western-ligand blot analysis

Rats fluids were extracted with Freon as described in Section 2.2.5 and incubated with a (4x concentration) SDS-loading buffer in a ratio of at least 2 loading buffer:1 sample. The sample was heated at 65^{0}C for 15 min. before loading onto a 1.5 mm thick, discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4 % stacking gel and a 10 % separating gel (Laemmli, 1970). The volumes of fluids applied are indicated in the figure legends. Following electrophoresis at a current of 40 mA for 8 hrs, proteins were transferred onto nitrocellulose sheets (0.45 µ; Schleicher and Schuell, Dassel, F.R.G.) at a current of 300 mA for 3 hrs using a Hoeffer Transphor TE42 apparatus (Hoeffer Scientific, San Francisco, CA, U.S.A.). The nitrocellulose was dried and treated in 50 ml of saline buffer (150 mmol/l NaCl and 10 mmol/l Tris at pH 7.4) containing Triton X-100 (1 % v/v) for 30 min, BSA (1 %, w/v) for 120 min, and Tween 20 (0.1 %, v/v) for 15 min. The treated nitrocellulose was incubated in 20 ml of saline buffer containing BSA (1 %, w/v) and Tween 20 (0.1 %, v/v) and ^{125}I-IGF-II (500,000 c.p.m.) for 18 hrs. Subsequently, the sheets of nitrocellulose were washed in saline buffer (50 ml) containing Tween 20 (0.1 %, v/v); twice for 30 min and once for 90 min. All steps were carried out in a shaking water-bath at 4^{0}C. The sheets were dried and exposed to X-ray film (A8323; Konica, Tokyo, Japan) with two intensifying screens (Cronex Hi-plus; Du Pont, Wilmington, MA, U.S.A.). The time of exposure to X-ray film was dependent on sample loading and the specific activity of iodinated IGF. Exposure times are indicated in figure legends.

2.2.6 Western-immunoblots

Samples were subjected to SDS-PAGE and transferred to nitrocellulose as described in Section 2.2.6. The nitrocellulose was blocked with BSA (1 %, w/v) for 18

hrs and washed for three times for 15 min in saline buffer (Section 2.2.5) containing Tween 20 (0.1 %, v/v) before being incubated for 18 hrs in 20 ml of the first antisera at a 1/500 dilution in saline containing BSA (1 %, w/v), Tween (0.1 %, v/v) and sodium azide (0.02 %, w/v) . The nitrocellulose filters were washed again, three times for 15 min in saline buffer containing Tween 20 (0.1 % v/v) before the addition of the second antisera, goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co.) at a 1/1000 dilution in 20 ml of saline containing BSA (1 %, w/v), Tween (0.1 %, v/v) and sodium azide (0.02 %, w/v). After a 4 hr incubation, the nitrocellulose was incubated in 50 ml of buffer comprising Tris (100 mmol/l; pH 9.0), NaCl (100 mmol/l) and $MgCl_2$ (5 mmol/l) to which the substrates, nitro blue tetrazolium (200 µl of 75 mg/ ml in 70 % diethyl formamide) and 5-bromo-4-chloro-3-indoyl phosphate (200 µl of 50 mg/ ml in 100 % diethyl formamide) were sequentially added. The antibody binding was detected by a color reaction that developed within 5-10 min and could be stopped with the addition of EDTA at a final concentration of 100 mmol/l.

2.3 RESULTS

Four regions containing ^{125}I-labelled IGF were observed in the fluids following Superose 12 chromatography; of approximately 200, 150, 40 and 7.5 kDa (Table 2.1 and Figs 2.1-2.3). The regions pooled for different binding areas of serum and lymph were between: 8.75 and 11.25 ml for the 200 kDa region; 11.5 and 14.0 ml for the 150 kDa area; and 14.25 and 16.25 ml for the 40 kDa region. The free radioligand eluted between 16.5 and 20 ml. Fetal serum, CSF and amniotic fluid were pooled between: 8.75 and 11.25 ml for the 200 kDa region; 12.5 and 16 ml for the 40 kDa region; and 16.25 and 19 ml for the free radioligand. These regions were selected to suit best the isolation of regions in each fluid (Figs. 2.1-2.3).

The chromatographic profiles of adult rat serum incubated with ^{125}I-labelled IGF-I and ^{125}I-labelled IGF-II are shown in Fig. 2.1, and the percent radioactivity calculated from each region is listed in Table 2.1. Three ^{125}I-labelled IGF-I regions of

Table 2.1: The percent of ^{125}I-labelled IGF-I and ^{125}I-labelled IGF-II radioactivity in fractions of different rat fluids eluted from Superose 12.

[a] Adult serum was incubated with unlabelled IGF-I (1 µg) and ^{125}I-labelled IGF-I

[b] Adult serum was incubated with unlabelled IGF-I (1.6 µg) and ^{125}I-labelled IGF-II

[c] Adult serum was incubated with unlabelled IGF-II (0.5 µg) and ^{125}I-labelled IGF-I

[d] Adult serum was incubated with unlabelled IGF-II (0.3 µg) and ^{125}I-labelled IGF-II

[e] Fetal serum or lymph or amniotic fluid or cerebrospinal fluid (CSF) were incubated with unlabelled IGF-I at 1 µg and ^{125}I-labelled IGF-I and ^{125}I-labelled IGF-II

[f] Fetal serum or lymph or amniotic fluid or cerebrospinal fluid (CSF) were incubated with unlabelled IGF-II at 0.5 µg and either ^{125}I-labelled IGF-I and ^{125}I-labelled IGF-II.

Each fluid was chromatographed separately; one sample per day with the column being recalibrated between samples.

Table 2.1

RAT FLUID	^{125} I-labelled IGF-I (% of total recovered radioactivity)				^{125} I-labelled IGF-II (% of total recovered radioactivity)			
	200 kDa	150 kDa	40 kDa	7.5 kDa	200 kDa	150 kDa	40 kDa	7.5 kDa
Adult serum	-	24	16	46	4	60	28	7
+ IGF-I [ab]	-	18	16	54	11	53	24	11
+ IGF-II [cd]	-	8	7	72	4	39	25	29
Fetal serum	-	-	55	37	9	-	66	18
+ IGF-I [e]	-	-	21	68	15	-	53	23
+ IGF-II [f]	-	-	12	81	4	-	35	53
Lymph	-	16	24	54	3	52	43	1
+ IGF-I [e]	-	7	24	54	3	52	43	1
+ IGF-II [f]	-	3	4	86	1	13	12	71
Amniotic fluid	-	-	23	70	17	-	60	20
+ IGF-I [e]	-	-	21	68	20	-	49	28
+ IGF-II [f]	-	-	13	77	3	-	22	73
CSF	-	-	19	53	-	-	42	32
+ IGF-I [e]	-	-	5	83	-	-	34	55
+ IGF-II [f]	-	-	3	87	-	-	14	76

Figure 2.1: Superose 12 fast-phase liquid chromatogram of adult rat serum labelled *in vitro*.

Adult rat serum was incubated for 18 hrs at 4^0C with (a)^{125}I-labelled IGF-I or (b)^{125}I-labelled IGF-II in the presence or absence of unlabelled IGF-I (1 µg) or IGF-II (0.5 µg).

To determine the elution volume (Ve) of IGF-binding peaks, fractions (0.25 ml) were collected and the radioactivity recorded. The column was calibrated with molecular mass standards, indicated at the top of the panel: Vo = void volume, G = gamma globulin (150 kDa), B= bovine serum albumin (69 kDa), O= ovalbumin (46 kDa), C = carbonic anhydrase (30 kDa) and I = iodinated IGF-I or IGF-II (7.5 kDa). The radioactivity measured with serum and iodinated IGF is shown as (●). Specificity of the protein peaks was assessed by the addition of unlabelled IGF-I (1 µg (a), 0.3 µg (b); ○) or IGF-II (0.5 µg (a), 0.3 µg (b); □). The regions pooled for Table 2.1 are marked.

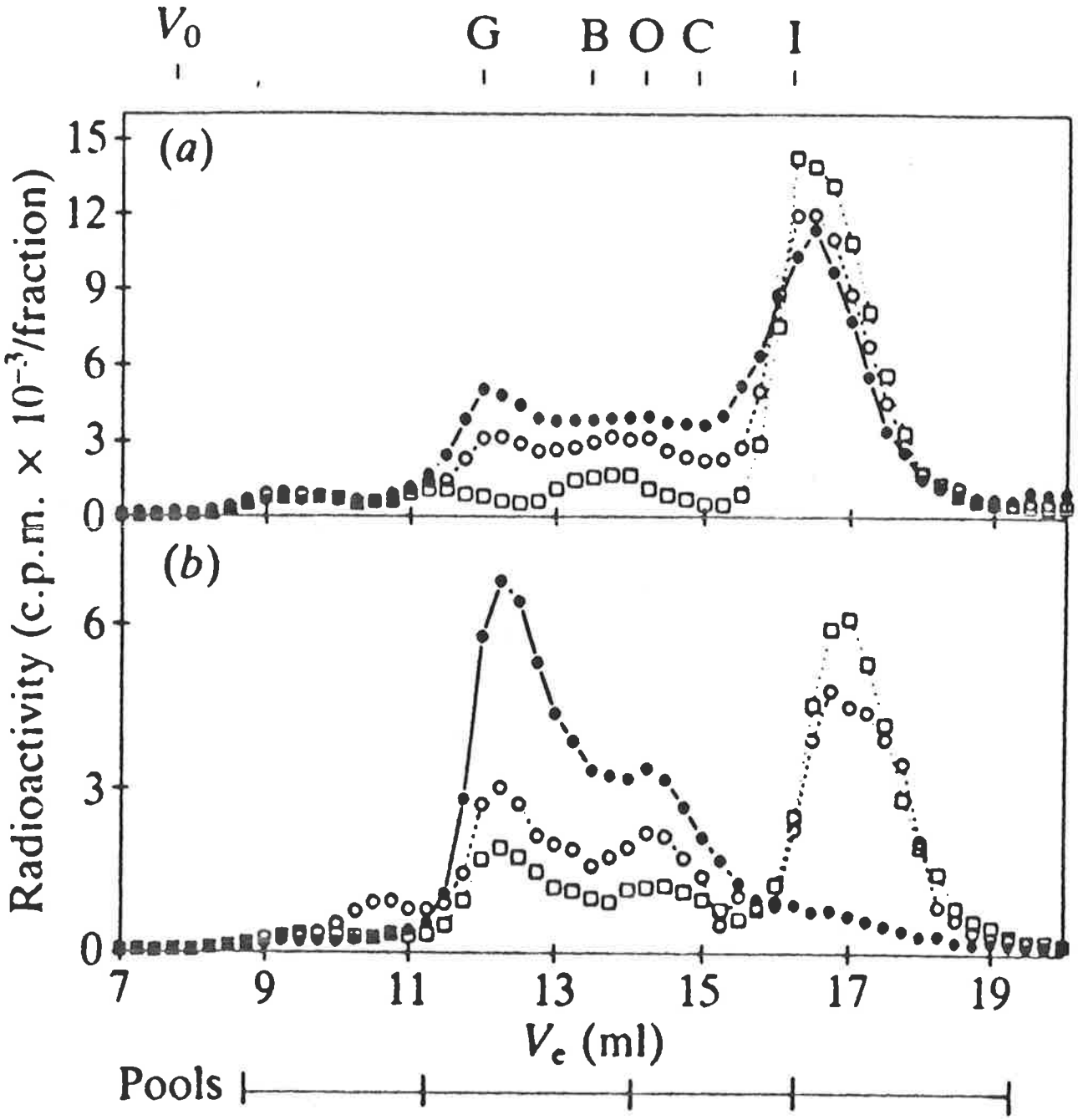

V_0
G
B
O
C
I
(a)
(b)
15
12
9
6
3
0
6
3
0
Radioactivity (c.p.m. × 10^{-3}/fraction)
7
9
11
13
15
17
19
V_e (ml)
Pools

Figure 2.2: Superose 12 fast-phase liquid chromatogram of rat fetal serum labelled *in vitro*.

Fetal serum at 17 days gestation was incubated for 18 hrs at 4^0C with (a)^{125}I-labelled IGF-I or (b)^{125}I-labelled IGF-II in the presence or absence of unlabelled IGF-I (1 µg) or IGF-II (0.5 µg). To determine the elution volume (Ve) of IGF-binding peaks, fractions (0.25 ml) were collected and the radioactivity recorded. The column was calibrated with molecular mass standards, indicated at the top of the panel: Vo = void volume, G = gamma globulin (150 kDa), B= bovine serum albumin (69 kDa), O= ovalbumin (46 kDa), C = carbonic anhydrase (30 kDa) and I = iodinated IGF-I or IGF-II (7.5 kDa). The radioactivity measured with serum and iodinated IGF is shown ●. Specificity of the protein peaks was assessed by the addition of unlabelled IGF-I (1 µg; ○) or IGF-II (0.5 µg ; □). The regions pooled for Table 2.1 are marked. This pooling regime was also applied to amniotic fluid and cerebrospinal fluid chromatographs.

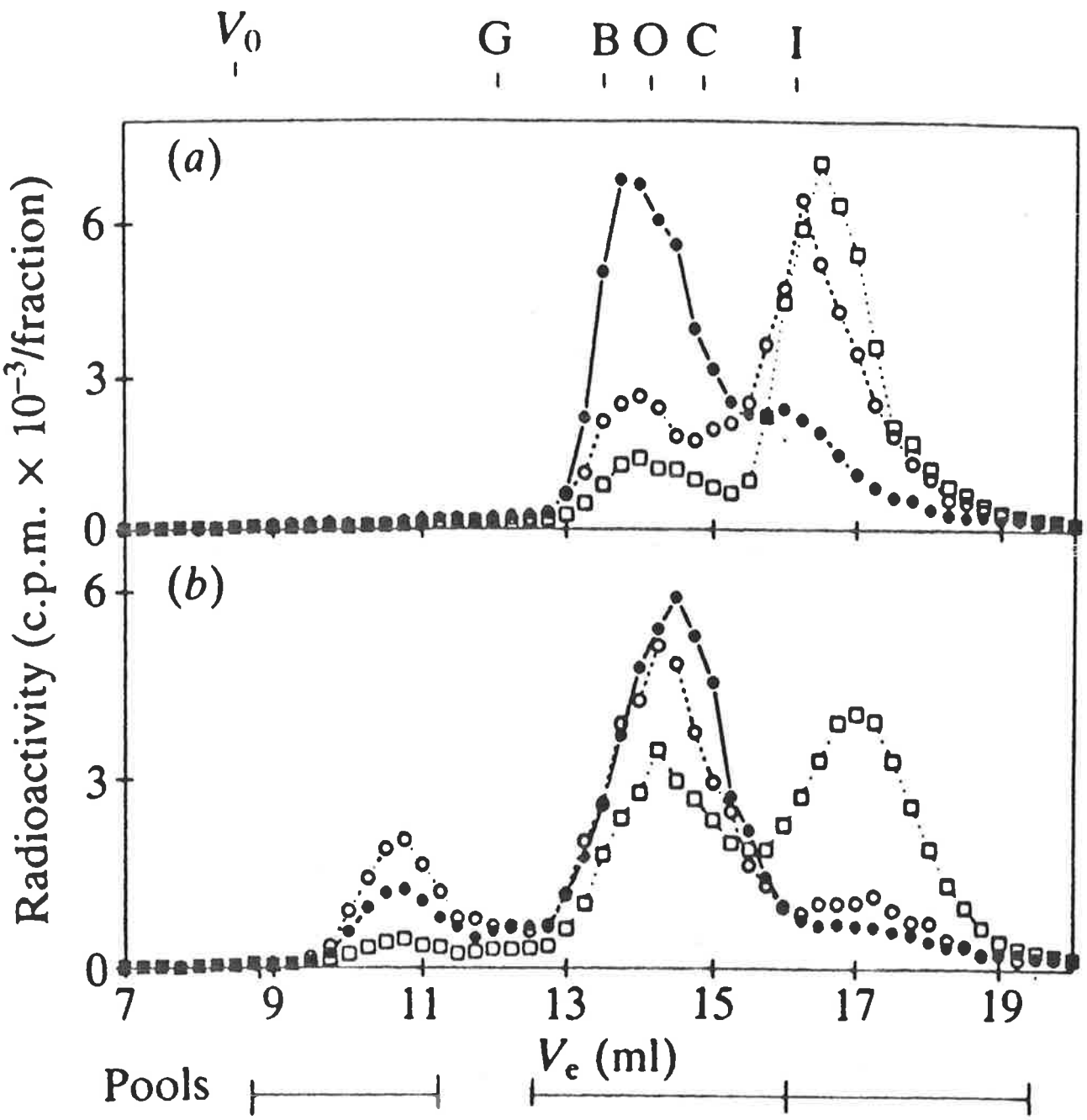
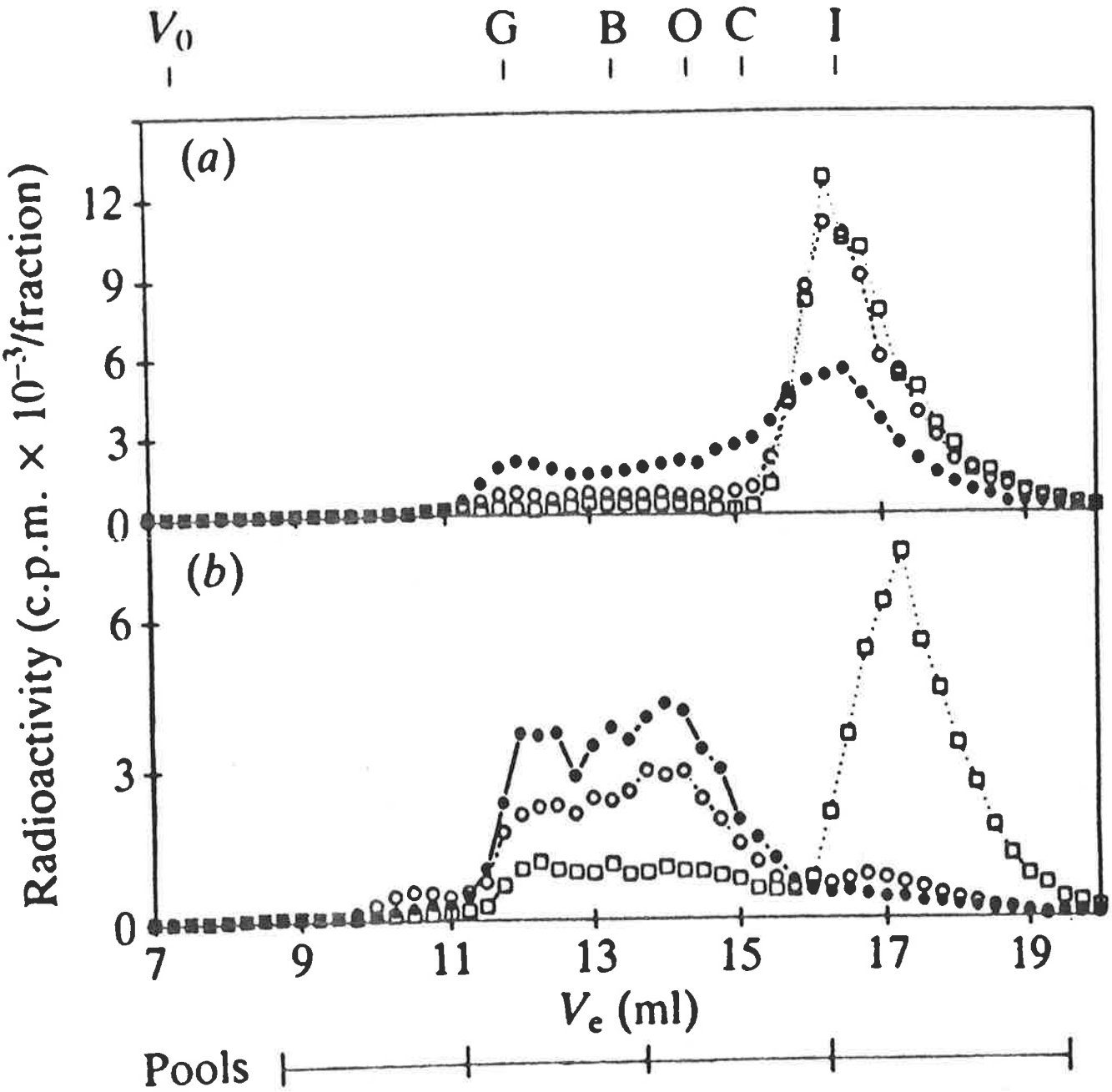

V_0
G
B
O
C
I
(a)
(b)
6
3
0
Radioactivity (c.p.m. × 10^{-3}/fraction)
7
9
11
13
15
17
19
V_e (ml)
Pools

Figure 2.3: Superose 12 fast-phase liquid chromatogram of rat abdominal lymph labelled *in vitro*.

Lymph was incubated for 18 hrs at 4^0C with (a)^{125}I-labelled IGF-I or (b)^{125}I-labelled IGF-II in the presence or absence of unlabelled IGF-I (1 µg) or IGF-II (0.5 µg). To determine the elution volume (Ve) of IGF-binding peaks, fractions (0.25 ml) were collected and the radioactivity recorded. The column was calibrated with molecular mass standards, indicated at the top of the panel: Vo = void volume, G = gamma globulin (150 kDa), B = bovine serum albumin (69 kDa), O = ovalbumin (46 kDa), C = carbonic anhydrase (30 kDa) and I = iodinated IGF-I or IGF-II (7.5 kDa). The radioactivity measured with serum and iodinated IGF is shown as (●). Specificity of the protein peaks was assessed by the addition of unlabelled IGF-I (1 µg (a); ○) or IGF-II (0.5 µg (a); □). The regions pooled for Table 2.1 are marked.


V_0
G
B
O
C
I
(a)
(b)
12
9
6
3
0
6
3
0
7
9
11
13
15
17
19
Radioactivity (c.p.m. × 10^{-3}/fraction)
V_e (ml)
Pools

150 kDa, 40 kDa and 7.5 kDa were detected in adult rat serum. Both IGF-I and IGF-II added in excess reduced the radioactivity in the 150 and 40 kDa regions with a corresponding increase in the 7.5 kDa region; IGF-II appeared more potent than IGF-I in displacing the radioligand (Table 1). Four IGF-II regions of 200 kDa, 150 kDa, 40 kDa and 7.5 kDa were detected in adult serum. Excess IGF-II competed radioligand from all regions except that of free IGF, whereas excess IGF-I decreased radioactivity in the 150 kDa and 40 kDa regions and increased radioactivity in the 200 kDa and free regions (Fig. 2.2, Table 2.1).

Fetal serum exhibited a different pattern of IGF binding from adult serum. Labelled IGF-I chromatographed as the 40 kDa and 7.5 kDa regions (Fig. 2.2 a). Unlabelled IGF-I and IGF-II competed for ^{125}I-labelled IGF-I binding to the 40 kDa region with IGF-II being more potent in this competition (Fig. 2.2, Table 2.1). Radiolabelled IGF-II chromatographed as 200 kDa, 40 kDa and 7.5 kDa regions in fetal rat serum. Unlabelled IGF-II reduced the radioactivity in the 200 kDa and 40 kDa regions, whereas unlabelled IGF-I decreased the radioactivity in the 40 kDa region, but increased the radioactivity in the 200 kDa and 7.5 kDa regions (Fig. 2.2, Table 2.1).

Amniotic fluid displayed a similar chromatographic profile to fetal rat serum when radiolabelled with either ligand (Table 2.1, profiles not shown).

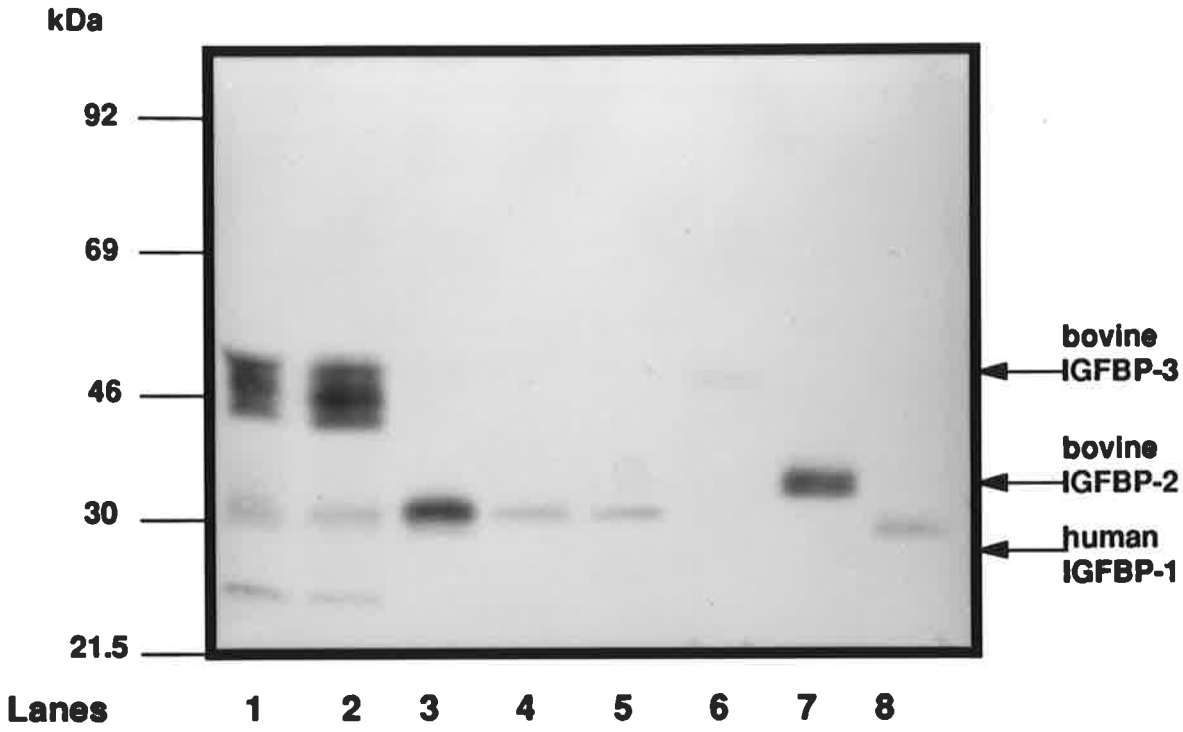
CSF produced a 40 kDa region when incubated with either radioligand, but lacked the 200 kDa region (Table 2.1, profiles not shown).

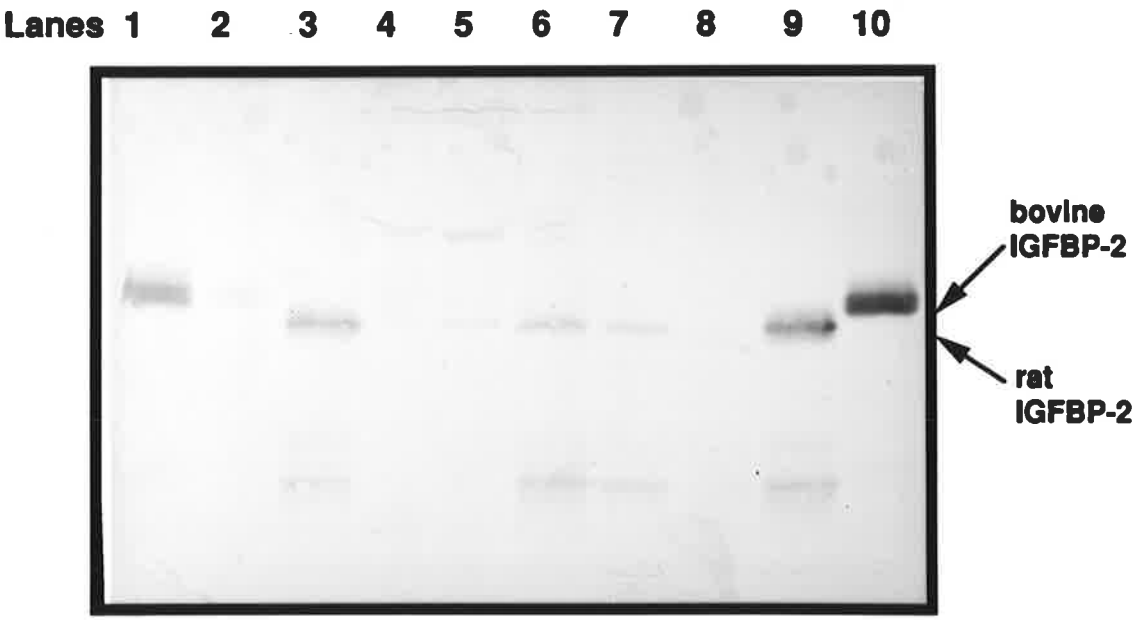
The IGFBP in serum, lymph, amniotic fluid and CSF were further characterised using Western-ligand blot analysis (Fig. 2.4). Treatment of the nitrocellulose with either ^{125}I-labelled IGF-I (data not shown) or ^{125}I-labelled IGF-II (Fig. 2.4) gave identical results, with the exception that fetal and adult serum, as well as lymph, produced a band of approximately 200 kDa with radiolabelled IGF-II only. Adult rat serum contained: a 200 kDa band; a 40-50 kDa triplet that aligned with purified bovine IGFBP-3; a 30 kDa band; a 28 kDa band that aligned with human IGFBP-1 standard; and a 24 kDa band (Fig. 2.4 upper, lane 1). The polyclonal antiserum to bovine IGFBP-

Figure 2.4: Western-ligand blot and Western-immunoblot analysis of rat fluids.

Upper panel: Ligand blot analysis of rat fluids. Samples of biological fluids and pure IGFBP standards were incubated with SDS loading buffer, electrophoresed, transferred to nitrocellulose and probed with ^{125}I-labelled IGF-II. The nitrocellulose was exposed to X-ray film for 7 days. The radiolabelled molecular weight markers are shown. The fluids are: lane 1, adult rat serum (5 µl); lane 2, abdominal lymph (5 µl); lane 3, fetal rat serum at 17 days of gestation (10 µl); lane 4, amniotic fluid at 17 days of gestation (10 µl); and lane 5, cerebrospinal fluid (10 µl). Lanes 6-8 contain 10 ng of purified porcine IGFBP-3, bovine IGFBP-2 and human IGFBP-1, respectively.

Lower panel: Detection of IGFBP in rat fluids that react with bovine IGFBP-2 antiserum. Samples were electrophoresed, transferred and incubated with bovine IGFBP-2 (MDBK-IGFBP) antiserum. The binding was detected by colorimetric reaction as described in 2.2.6. Lane 1 contains 20 ng of purified bovine IGFBP-2 (MDBK-BP) which acts as a positive control. Lane 2 is the negative control containing cell-conditioned media from the H35B hepatoma (25 µl of a four-fold concentration). Lanes 3 and 9 contain 25 µl and 50 µl loading of a 4 times concentrated cell-conditioned media from BRL3A cells (rat IGFBP-2), respectively. Lanes 4-9 contain rat fluids: lane 4, adult serum (10 µl); lane 5, abdominal lymph (10 µl); lane 6, fetal serum at 17 days gestation (20 µl); lane 7, amniotic fluid at 17 days gestation (20 µl); and lane 8, cerebrospinal fluid (20 µl). Lane 9 contains 25 µl of BRL3A-conditioned media which has been concentrated 4 fold (200 µl equivalent); and lane 10, 50 ng of purified bovine IGFBP-2 (MDBK IGFBP).


kDa
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1
Lanes 1 2 3 4 5 6 7 8


Lanes 1 2 3 4 5 6 7 8 9 10
bovine IGFBP-2
rat IGFBP-2

2 revealed a band at 30 kDa implying that rat adult serum contains IGFBP-2 (Fig. 2.4 lower, lane 4).

To determine whether the IGFBP observed in the ligand blots of adult serum were components of the larger complexes detected with neutral size-exclusion chromatography, serum was fractionated on FPLC and fractions were subjected to Western-ligand blot analysis (Fig. 2.5). The 200 kDa region blotted with a faint band at 200 kDa. The 40-50 kDa triplet that aligned with porcine IGFBP-3 was detected in Superose 12 fractions corresponding to the 150 kDa region. This indicates that most of the IGFBP-3 in rat serum exists as the 150 kDa complex (Fig. 2.5). The 28-30 kDa band, which resolved as a doublet, and the 24 kDa band were detected in fractions corresponding to the 40 kDa region observed in Fig. 2.1.

The Western-ligand blot of fetal serum contained two IGFBP of 24 kDa and 30 kDa (Fig. 2.4 upper, lane 3): consistent with the chromatogram in Fig. 2.2. The smaller IGFBP were present in the 40 kDa region, the 150 kDa region was absent. Although the 200 kDa IGFBP detected in Fig. 2.2, it could not be clearly demonstrated by Western-ligand blotting. The 30 kDa band in fetal serum reacted with bovine IGFBP-2 antiserum, as did the smaller molecular mass IGFBP in both fetal serum and purified standard (Fig. 2.4 lower, lane 6). The 28 kDa band observed in adult rat serum was absent in fetal rat serum.

The ligand blot of abdominal lymph (Fig. 2.4 upper, lane 2) indicated the same IGFBP as observed for adult rat serum (Fig. 2.4 upper, lane 1). Further characterization of IGFBP in lymph was assessed following Superose 12 size-exclusion chromatography of adult lymph and subjecting fractions to Western-ligand blot analysis. Fractions corresponding to the 150 kDa region blotted as a 40-50 kDa triplet that aligned with IGFBP-3 standard (Fig. 2.6). Fractions corresponding to the 40 kDa region blotted as a 24 kDa band, a 28 kDa band that aligned with IGFBP-1 and a 30 kDa band that reacted with bIGFBP-2 antisera (Fig. 2.4 lower, lane 5).

The Western-ligand blot of amniotic fluid produced a band of 28-30 kDa (Fig.

Figure 2.5: Western-ligand blot analysis of IGFBP in adult rat serum fractionated on a Superose 12 column under neutral conditions.

Serum was chromatographed and two consecutive fractions (0.25 ml) were pooled (0.5 ml). 50 µl aliquots of these pools and 20 ng of purified IGFBP standards (bovine IGFBP-3, bovine IGFBP-2, human IGFBP-1, respectively) were electrophoresed, transferred to nitrocellulose and incubated with ^{125}I-labelled IGF-II. The X-ray film was exposed for 7 days. The position of the SDS-PAGE molecular mass markers are shown. The indicated Ve (ml) represents the elution volume of the first fraction of the pool. The Superose 12 molecular mass markers corresponding to the fractions subjected to Western-ligand blotting are represented at the top of the panel: gamma globulin, 150 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; and iodinated IGF-II, 7.5 kDa.

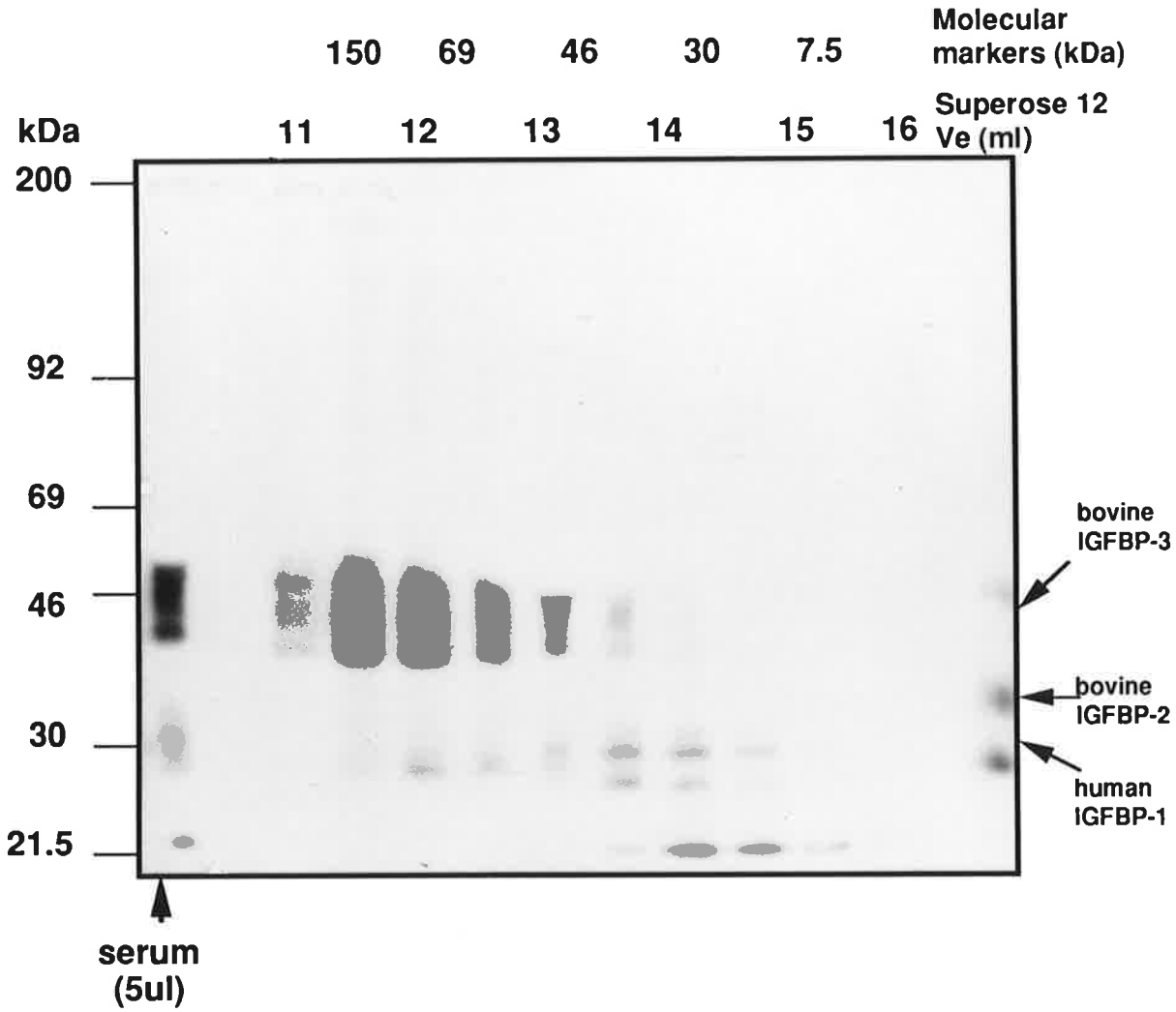
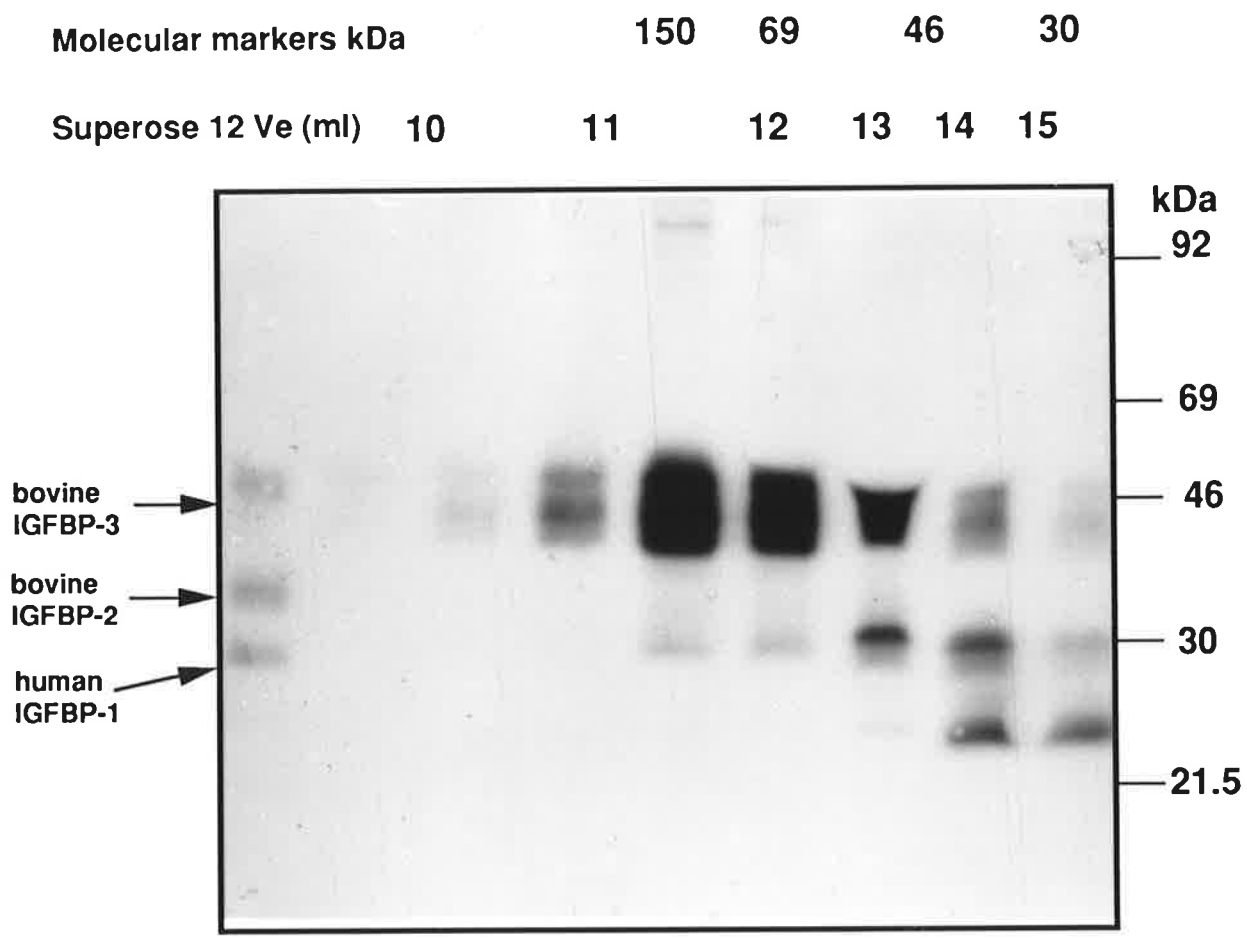

Molecular markers (kDa)
150
69
46
30
7.5
Superose 12 Ve (ml)
kDa
11
12
13
14
15
16
200
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1
serum (5ul)

Figure 2.6: Western-ligand blot analysis of IGFBP in abdominal lymph fractionated on a Superose 12 column under neutral conditions.

Lymph was chromatographed and three consecutive fractions (0.25 ml) were pooled (0.75 ml). An aliquot of 50 µl of these pools and 10 ng of purified IGFBP standards (bovine IGFBP-3, bovine IGFBP-2, human IGFBP-1, respectively) were electrophoresed, transferred to nitrocellulose and incubated with ^{125}I-labelled IGF-II. The X-ray film was exposed for 7 days. The indicated Ve (ml) represents the middle fraction of the pool. The position of the SDS-PAGE molecular mass markers are shown. The Superose 12 molecular mass markers corresponding to the fractions subjected to Western-ligand blotting are represented at the top of the panel: gamma globulin, 150 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa.


Molecular markers kDa
150
69
46
30
Superose 12 Ve (ml)
10
11
12
13
14
15
kDa
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1

2.4 upper, lane 4). This band reacted with bovine IGFBP-2 antisera and aligned with rat IGFBP-2 standard (BRL3A BP) (Fig. 2.4 lower, lane 7). In addition, smaller cross-reactive bands were detected in amniotic fluid (Fig. 2.4 lower, lane 7). CSF contained a single 30 kDa band (Fig. 2.4 upper, lane 5) that did not cross-react with bovine IGFBP-2 antisera (Fig. 2.4 lower, lane 8).

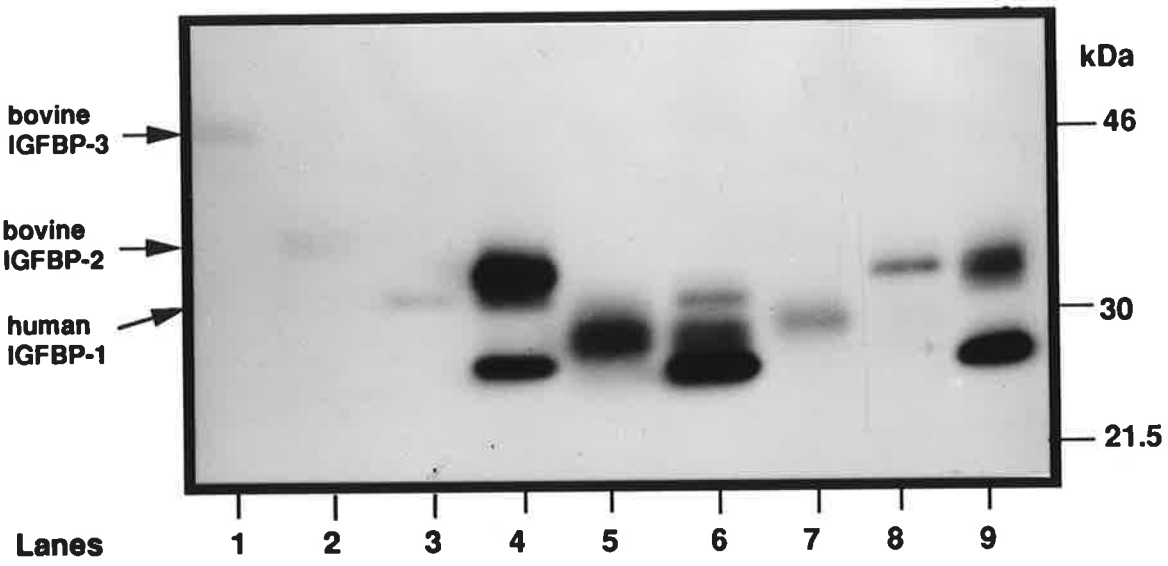
To characterize the IGFBP produced by the cells of different tissues, cell-conditioned media were subjected to Western-ligand blot (Fig. 2.7 upper) and Western-immunoblot analysis (Fig. 2.7 lower).

The liver-derived cell line (BRL3A), the hepatocyte cells (Hep10C) and the hepatomas (MH_1C_1 and H35B) each produced a similar IGFBP pattern: a 30 kDa IGFBP; a 28 kDa IGFBP that aligned with human IGFBP-1 standard; and a 24 kDa IGFBP. Only the pattern for Hep10C is presented (Fig. 2.7 upper, lane 4). Another hepatoma, HTC, produce a 26 kDa IGFBP (Fig. 2.7 upper, lane 5). The two muscle-derived cell-conditioned media contained different combinations of IGFBP. The L6 myoblasts produced a 28 kDa band that aligned with human IGFBP-1 standard, a 26 kDa IGFBP and a 24 kDa IGFBP (Fig. 2.7 upper, lane 6), whereas the vascular smooth muscle cell line (SD8ArA) produced only a 26 kDa IGFBP (Fig. 2.7 upper, lane 7). Calvarial osteoblasts secreted a 30 kDa IGFBP, whilst the osteogenic sarcoma produced an additional 24 kDa IGFBP (Fig. 2.7 upper, lanes 8 & 9). The cross-reactivity of cell-derived IGFBP with bovine IGFBP-2 was examined (Fig. 2.7 lower). The liver-derived cell lines, Hep 10C (not shown) and BRL3A (Fig. 2.7 lower, lanes 3 & 9) produced a band of approximately 30 kDa and faint smaller molecular mass bands. The hepatoma, H35B, did not give a band that cross-reacted with the antisera (Fig. 2.7 lower, lane 2), while the HTC hepatoma produced a cross-reacting triplet of 30-34 kDa (Fig. 2.7 lower, lane 7). Both bone-derived cell lines (calvarial osteoblasts and osteogenic sarcoma) produced a faint band aligning with rat IGFBP-2 standard (Fig. 2.7 lower, lane 4 & 5). The calvarial osteoblasts also showed some cross-reactivity with smaller bands. The muscle-derived cell lines, L6 (Fig. 2.7 lower, lane 8) and SD8ArA (not shown), did not

Figure 2.7: Western-ligand blot and Western-immunoblot analysis of media conditioned by bone-, muscle- and liver-derived cell lines.

Upper panel: Western-ligand blot analysis of rat cell conditioned media. Media were concentrated by diafiltration and then diluted in SDS loading buffer, electrophoresed, transferred to nitrocellulose and incubated with radioligand IGF-II. The position of the SDS-PAGE molecular mass markers are shown. Lanes 1-3 contain 10 ng of purified porcine IGFBP-3, bovine IGFBP-2 and human IGFBP-1, respectively. Lanes 4-9 contain conditioned media from liver-, muscle- and bone-derived cell lines that were concentrated by diafiltration, thus the volumes loaded differ but the protein content of each is equivalent to 200 µl of unconcentrated conditioned media: lane 4, Hep10C liver-derived cell line; lane 5, hepatoma HTC; lane 6, L6 myoblasts; lane 7, vascular smooth muscle; lane 8, calvarial osteoblasts; and lane 9, osteogenic sarcoma.

Lower panel: Immunoblot detection of IGFBP in cell-conditioned media that react with bovine IGFBP-2 antisera. Media were concentrated by diafiltration, electrophoresed, transferred to nitrocellulose and incubated with antiserum. Binding was detected by a colorimetric reaction. Lane 1 contained 50 ng purified bovine IGFBP-2 (MDBK-IGFBP) and lanes 2-8 contained media concentrated by diafiltration but the protein content of each loaded equals 100 µl of unconcentrated conditioned media. Lane 2, hepatoma H35B is a negative control; lane 3, liver-derived BRL3A (rat IGFBP-2) is a positive control; lane 4, calvarial osteoblasts; lane 5, osteogenic sarcoma; lane 6, hepatoma Hep10C; lane 7, hepatoma HTC; lane 8, L6 myoblasts; lane 9, an equivalent to 200 µl of unconcentrated BRL3A conditioned media; and lane 10, 50 ng purified bovine IGFBP-2 (MDBK-IGFBP).


bovine
IGFBP-3
bovine
IGFBP-2
human
IGFBP-1
kDa
46
30
21.5
Lanes
1
2
3
4
5
6
7
8
9

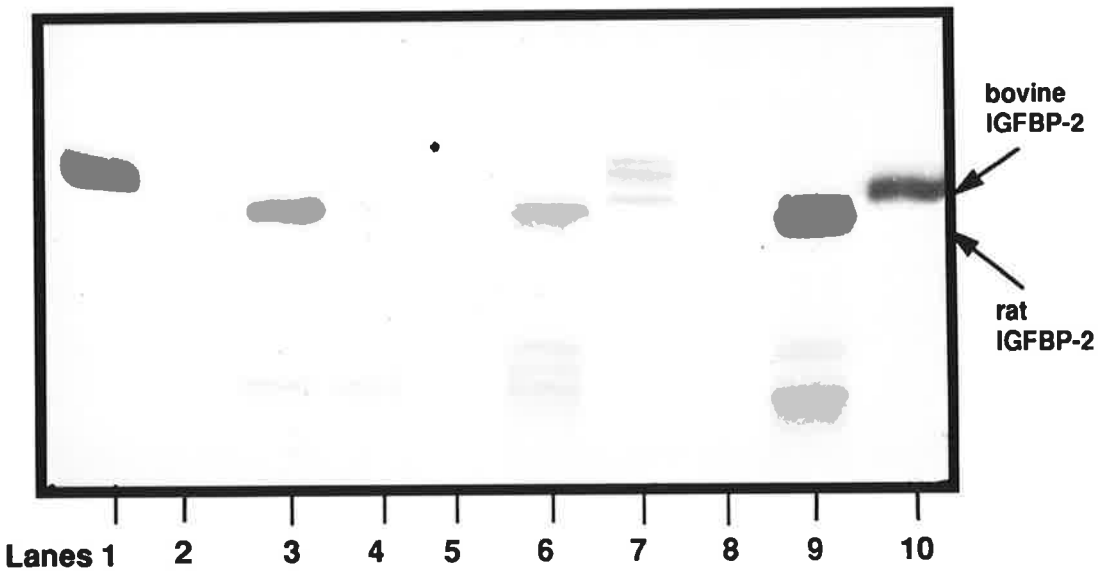

bovine
IGFBP-2
rat
IGFBP-2
Lanes 1
2
3
4
5
6
7
8
9
10

exhibit any cross-reactivity with the IGFBP-2 antiserum. None of the cell types examined produced detectable levels of IGFBP-3.

In neither the rat fluids nor the cell conditioned media did the human IGFBP-1 antisera cross react with any IGFBP, although the human IGFBP-1 standard did work as a positive control. Thus, a binding protein immunologically similar to human IGFBP-1 was not evident in the samples tested.

2.5 DISCUSSION

The five IGFBP identified in the current study include: a 200 kDa IGFBP; IGFBP-3 detected as a 40-50 kDa triplet aligning with IGFBP-3 standard on Western-ligand blot analysis and predominantly arising from the 150 kDa IGF-binding complex; a 30 kDa band which cross-reacted in most fluids with antisera to bovine IGFBP-2; a 28 kDa band aligning with human IGFBP-1, but not cross-reacting with human IGFBP-1 antiserum; and a 24 kDa IGFBP.

The 200 kDa IGFBP was observed in serum, lymph and amniotic fluid during chromatography on a Superose 12 gel-permeation column. The high specificity of this IGFBP for IGF-II, as demonstrated by competition for radiolabelled IGF-II in the presence of IGF-I and IGF-II, suggests this protein is the circulating type-II receptor (Rosenfeld *et al.,* 1987). Keiss and colleagues (1987) had previously detected this circulating receptor in adult rat serum, and Hodgkinson *et al.,* (1989) have recently detected a similar IGFBP in the plasma from fetal and adult sheep. The 200 kDa IGFBP in amniotic fluid may also be the circulating type-II receptor.

Serum and abdominal lymph contained the smaller IGFBP of 30 kDa that cross reacted with bovine IGFBP-2 antiserum, a 28 kDa IGFBP that aligned with human IGFBP-1, and a 24 kDa IGFBP. Similarly, Hossenlopp *et al.* (1987) detected the presence of 24 kDa and 30 kDa bands in adult and fetal rat serum using the Western-ligand blot technique with radiolabelled IGF-I.

Amniotic fluid contained a 30 kDa IGFBP-2. In agreement, Busby *et al.* (1988a)

have also reported a 30 kDa amniotic fluid IGFBP. The presence of IGFBP-1 has been established in a number of species (Ooi and Herington, 1988), but we could not establish the identity of any band as IGFBP-1. CSF contained an IGFBP of 30 kDa. However, this IGFBP was not IGFBP-2 due to its lack of cross reactivity with the bovine IGFBP-2 antisera. An IGF-II preferring IGFBP of a similar molecular mass in CSF was recently reported in sheep (Hodgkinson *et al.*, 1989) and human CSF (Hossenlopp *et al.*, 1987). Romanus *et al.* (1989) detected IGFBP of 34 kDa and 28-30 kDa in human CSF using Western-ligand blotting. These authors determined that the 34 kDa IGFBP cross-reacted with anti-BRL3A IGFBP sera and classified it as IGFBP-2. Lamson *et al.* (1989) also specifically immunoprecipitated the IGFBP from BRL3A cell conditioned media, as well as rat CSF, indicating that the major class of IGFBP in rat CSF is IGFBP-2. Possibly the CSF IGFBP detected in my study is the rat homologue of the 34 kDa IGFBP isolated from human CSF by Roghani *et al.* (1989) which appears to be distinct from other IGFBP.

Only IGFBP of small molecular mass were secreted by the rat cells tested. Liver-derived cells secreted a 30 kDa IGFBP which immunoreacted with bovine IGFBP-2, as well as a 28 kDa and a 24 kDa IGFBP. This result agrees with an earlier report by Hossenlopp *et al.* (1987). These authors found that the major IGFBP secreted by adult rat liver cells, using Western-ligand blot analysis, were a 32 kDa and a 24 kDa IGFBP, but when cell-conditioned media was applied at greater volumes, faint bands of 39 kDa, 41 kDa and 43 kDa IGFBP were detected. However, I was unable to detect the 39 kDa, 41 kDa and 43 kDa IGFBP, even at high concentrations. In addition, the hepatoma, HTC, secreted a 26 kDa IGFBP, but produced a triplet of IGFBP with bovine IGFBP-2 antisera. This result indicates that bovine IGFBP-2 antiserum may recognize more than one species of protein that can not be detected using the Western-ligand blot technique. The muscle-derived L6 cell line produced three IGFBP of 28 kDa, 26 kDa and 24 kDa, consistent with a report by McCusker *et al.* (1989). The vascular smooth muscle cell line secreted only a 26 kDa IGFBP. Calvarial osteoblasts and osteogenic

sarcomas cells each produce a 30 kDa IGFBP that cross-reacted with bovine IGFBP-2 antisera. The sarcoma cell line also produced a 24 kDa IGFBP, suggesting that tumourigenesis can affect IGFBP secretion from cells. Schmidt *et al.* (1989) detected IGFBP characteristic of IGFBP-2, IGFBP-3 and the 24 kDa IGFBP in rat osteoblast conditioned media. However, I did not detect IGFBP-3 production in the osteoblast-derived cells.

Both serum and abdominal lymph contained IGFBP-3 which existed as a 150 kDa complex under neutral, non-dissociating conditions. Binoux and Hossenlopp (1988) detected IGFBP-3 in human lymph by Western-ligand blotting but failed to detect any binding of radiolabelled IGF-II to human lymph IGFBP in the 150 kDa region, and only 5 % of the IGF activity was detected in this range. In addition, Cohen and Nissley (1975) found that lymph from the cysterna chyli of rats contained one-half the somatomedin activity found in serum. Binoux and Hossenlopp (1988) therefore concluded that the 150 kDa complex does not cross the capillary barrier in lymph. I detected IGFBP-3 in fractions of rat abdominal lymph that eluted from a Superose 12 column at 150 kDa. The difference between these results and those of Binoux and Hossenlopp (1988) may be due to relatively high concentrations of endogenous IGF, or the composition of rat and human lymph may be truly different. In support of this later contention, I found that significantly lower volumes of rat lymph were required for detection of IGFBP (10 µl versus 30 µl for the previous human studies).

In conclusion, rat adult serum and abdominal lymph contained a 200 kDa IGFBP (putative type-II receptor extracellular domain) and a 150 kDa IGFBP that contained subunits of 40-50 kDa aligning with IGFBP-3 on Western-ligand blots. Both fluids also contained a 30 kDa IGFBP that was immunoreactive with bovine IGFBP-2, a 28 kDa IGFBP aligning with IGFBP-1 but not cross reacting with human IGFBP-1 antiserum and a 24 kDa IGFBP, possibly IGFBP-4. Fetal serum and amniotic fluid lacked the 150 kDa and 28 kDa IGFBP, but contained IGFBP-2. CSF contained only a 30 kDa IGFBP that was not IGFBP-2. Several smaller molecular mass IGFBP were

detected in media conditioned by liver-, bone- and muscle-derived cells, but none of the cells secreted IGFBP-3. These results allow me to partially classify and to compare the IGFBP in rat fluids with those produced by cultured cells - a wide distribution of IGFBP can be found secreted by different tissues and circulating in various biological fluids. Whether the types of IGFBP secreted by several cultured cell lines are representative of those secreted *in vivo* by the corresponding tissues (from which the cells were derived) remains to be established.

CHAPTER THREE: THE CHARACTERIZATION AND MOLECULAR DISTRIBUTION OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND IGF-BINDING PROTEINS IN RAT SERUM DURING PREGNANCY

3.1 INTRODUCTION

At least five IGFBP types exist in the circulation of rats: a 200 kDa IGFBP, putatively the truncated type-II receptor; a 150 kDa complex containing IGFBP-3 of 40-50 kDa; a 30 kDa IGFBP-2; a 28 kDa IGFBP, possibly IGFBP-1; and a 24 kDa IGFBP that may be IGFBP-4 (Refer to Chapter 2; Gargosky *et al.*, 1990c; Baxter and Martin, 1989a).

The placenta contains IGF receptors (Marshall *et al.*, 1974). Both IGF-I and IGF-II are growth-promoting peptides that are developmentally regulated, suggesting IGF may play a regulatory role during pregnancy and lactation in the rat. Few studies had reported IGF-I concentrations in pregnant animals when this study was commenced. In the rat and mouse, maternal serum IGF-I levels were reported to be depressed in late pregnancy (D'Ercole and Underwood, 1980; Sheppard and Bala, 1986; Chiang *et al.*, 1990). In contrast, Daughaday *et al.* (1982) found little variation in serum IGF-I concentrations but a rise in serum IGF-II with pregnancy in rats. Two longitudinal studies in humans reported a rise in maternal serum IGF-I compared with non-pregnant females (Wilson *et al.*, 1982; Hall *et al.*, 1984). The source of these apparent inconsistencies is not clear, but IGF-binding proteins had been shown to produce artifacts in IGF assays (Mesiano *et al.*, 1988). Thus, it was plausible that some of the precautions to exclude IGFBP interference had not been effective. The IGFBP had not been characterized during the different stages of pregnancy, in any species, although total IGFBP was correlated with IGF-I and IGF-II concentrations in serum (Baxter and Martin, 1986). In addition, the proportions of free and bound IGF had not been established in pregnant rats. Thus, the aim of the present study was to measure serum IGF-I and IGF-II concentrations in the absence of potentially interfering IGFBP, to determine the distribution of IGF amongst the higher molecular mass forms, and to characterize serum IGFBP during pregnancy in the rat.

3.2 MATERIALS and METHODOLOGY

3.2.1 Blood samples

Adult female Sprague-Dawley rats (approximately 250 g), bred in the University of Adelaide animal house, were given free access to standard rat chow and maintained under controlled conditions (26°C, 12 h light: 12 h dark). Females were placed in individual cages with a male rat and copulation was confirmed by visualisation of a vaginal plug. This day was taken as day 1 of pregnancy. Mated rats were subsequently kept in individual cages. Blood samples (350 µl) were collected from the tail vein at 3 to 4 day intervals during pregnancy and throughout lactation, until day 11 postpartum, after which the pups were removed and killed by cervical dislocation. Blood samples were clotted at 4^0C for 18 hrs and sera harvested after centrifugation at 10,000 g for 5 min. Sera were stored at -20^0C.

3.2.2 IGF and IGFBP measurements

Many of these measurements were carried out by Ms. K. Moyse and Ms. M. Pearce under the supervision of Dr. P.C. Owens. Acknowledgments are cited in the figure legends.

Equal volumes of serum from four to six different rats at similar stages of pregnancy or lactation were pooled and diluted to 75 µl/ ml in water plus concentrated mobile phase to dissociate IGF from IGFBP, after which 0.2 ml (containing 15 µl serum) was fractionated through a Waters/ Millipore Protein Pak Column (Waters/Millipore, Lane Cove, New South Wales, Australia) at 1 ml/ min in acetic acid (0.2 mol/l) and triethylamine (0.05 mol/l) at pH 2.8 containing Tween 20 (0.5 % v/v), (mobile phase) as previously described (Owens *et al.*, 1990). Pools of rat sera were chromatographed and 0.25 ml fractions collected to determine the elution position of IGF and IGFBP, as detected by IGF-I radioimmunoassay (RIA) and IGF-II radioreceptor assay (RRA). In subsequent analysis of individual serum samples, four fractions were collected. The first eluted between 6 and 8.5 ml and contained the

IGFBP, the second was an intermediate fraction, the third eluted between 9 and 11.5 ml and contained the IGF, and the fourth was a trailing 1 ml fraction. Four to five samples were collected on different occasions from six rats, and the IGF pools (9.0-11.5 ml) and IGFBP pools (6.0-8.5 ml) were assayed by RIA for IGF-I and RRA for IGF-II. The chromatographic properties of the Protein pak column were checked routinely by following the distribution of radiolabelled IGF after chromatography. The limits of detection in the IGF assays were calculated using the standard curve method described by Baxter (1980); these were 32 pg for IGF-I and 35 pg for IGF-II. For 0.05 ml, assayed from the 2.5 ml pools derived from 0.015 ml serum, the assay sensitivities were 107 µg/l and 117 µg/l for IGF-II.

3.2.3 Molecular size of ^{125}I-labelled IGF-IGFBP complexes determined by size-exclusion chromatography at pH 7.4.

The *in vitro* labelling of rat sera has been previously described (Section 2.2.5). Briefly, serum samples (200 µl) were lipid-extracted and incubated with iodinated IGF-I or IGF-II (5 µl, 100,000 c.p.m.) in the absence or presence of 500 ng IGF-I/ 5 µl or 200 ng IGF-II/ 5 µl. After an 18 hr incubation at 4^{0}C, samples (200 µl) were applied to a Superose 12 FPLC column and chromatographed at 0.5 ml/ min in saline buffer, pH 7.4. Fractions (0.25 ml) were collected and the radioactivity measured in a gamma counter.

3.2.4 Western-ligand blot analysis

Samples were treated in SDS-loading buffer, applied to discontinuous SDS-PAGE (10 % separating gel, 4 % stacking gel), after which the electrophoresed proteins were transferred to nitrocellulose. The sheets of nitrocellulose were treated, probed with iodinated IGF and exposed to X-ray film at -80^{0}C. For details see Section 2.2.6.

3.2.5 The distribution of IGF amongst the higher molecular mass forms.

The molecular sizes of IGFBP-IGF complexes in serum were determined by FPLC size-exclusion chromatography (Section 2.2.5). Briefly, pooled sera (35 µl) was defatted and incubated with 10,000 c.p.m. of radiolabelled IGF-I or IGF-II for 18 hrs at 4°C. Labelled serum (50 µl) was then fractionated though a Superose 12 FPLC column in mobile phase, pH 7.4 at 0.5 ml /min. Fractions (0.25 ml) were collected and the radioactivity in each fraction was determined in a gamma counter. These labelled serum samples were chromatographed before and after chromatography of unlabelled serum at pH 7.4 to ensure the properties of the column were consistent. Fractions (0.5 ml) derived from unlabelled sera were collected in tubes containing Tween 20 (20 µl, 0.5 % v/v) and acidified with 167 µl of a 4-times concentrated mobile phase (acetic acid (800 mmol/l) and triethylamine (200 mmol/l) at pH 2.8 containing Tween 20 (0.5 % v/v)). These fractions were chromatographed on a Protein Pak 125 molecular sieve column at 0.5 ml/ min in 1-times mobile phase using an autoinjector calibrated to apply 600 µl. Upon confirmation that IGF-II could not be measured in sera from rats (data not shown), fractions of 0.25 ml were collected and assayed for IGF-I content. The IGF extracted from fractions across the FPLC profile eluted in the same position as IGF-I and IGF-II standard.

3.2.6 Antisera for IGF assays

Rabbit anti-IGF-I antiserum and goat anti-rabbit serum was provided by Dr. P.C. Owens, CSIRO Division of Human Nutrition, South Australia, Australia. Ovine placental membranes were isolated by Mr. F. Carbone following the method of Baxter and DeMellow (1986). The standards used were rh IGF-I kindly provided by Drs. H.H. Peters and K. Scheibi, Ciba-Geigy, Basle, Switzerland, and recombinant bovine IGF-II by Dr. R Collier, Monsanto Co., St Louis, MO, U.S.A.

3.2.7 IGF-I and IGF-II measurements.

(i) Radioimmunoassay (RIA) for IGF-I

Measurement of IGF-I by RIA involved the addition of 50 µl of the Protein Pak eluate fractions, mobile phase alone or standards in mobile phase to 30 µl Tris (400 mmol/l). This was followed by: 200 µl of RIA assay buffer comprising NaH_2PO_4 (30 mmol/l), protamine sulphate (0.2 % w/v), disodium EDTA (10 mmol/l), NaN_3 (0.2 % w/v) and Tween 20 (0.05 % v/v) at pH 7.5 that had been filtered and degassed through a 0.22 µ filter; 50 µl of radiolabelled IGF-I (20,000 c.p.m.); and 50 µl of anti-IGF-I antiserum in RIA assay buffer at a final dilution of 1/40,000 such that 30-40 % of the added radioactivity would be bound in the absence of competing ligand. The samples (380 µl) were incubated at 4°C for 18 hrs, after which excess goat anti-rabbit serum (50 µl of 1/20 dilution) and rabbit serum (50 µl of 1/250 dilution) were added. Following a further incubation for 1 hr at 4°C, 1 ml of cold polyethylene glycol 6000 (6 % w/v; Sigma) in sodium chloride (150 mmol/l) was added and the tubes were centrifuged in a cold centrifuge at 4000 g for 30 min at 4 °C. After removal of the supernatant by aspiration, the radioactivity in the pellet was measured in a gamma counter. All samples and standards were measured in triplicate. The limits of detection in the IGF-I RIA were calculated using the standard curve method described by Baxter (1980). The assay limit of detection for IGF-I ranged from 20-50 pg.

(ii) Radioreceptor assay (RRA) for IGF-II

Measurement of IGF-II in column fractions involved the addition of 50 µl of column fraction, mobile phase alone, or standard in mobile phase to 30 µl Tris (400 mmol/l) plus 200 µl of RRA buffer 1 comprising Tris (10 mmol/l), BSA (0.5 % w/v) and $CaCl_2$ (10 mmol/l) at pH 7.4, 50 µl radiolabelled IGF-II (20,000 c.p.m.) and 100 µl of ovine placental membranes (final concentration 0.2 mg protein/ml) in RRA buffer 1. This amount of membrane was sufficient to bind 30-40 % of the radiolabelled IGF-II in the absence of competing ligand. The samples were incubated at 4°C for 18 hrs after

which 1 ml of cold RRA buffer 2 containing Tris (10 mmol/l), BSA (0.5 % w/v) and $CaCl_2$ at pH 7.4 was added. The tubes were centrifuged at 4000 rpm for 30 min at 4°C. The supernatants were aspirated and the radioactivity in the pellet was measured in a gamma counter.

The limits of detection in the IGF-II RRA were calculated using the standard curve method described by Baxter (1980). The assay limit of detection for IGF-II ranged from 25-90 pg.

3.2.8 Statistical analysis

Serum IGF-I results were analysed by one-way analysis of variance, and the means compared using the Newman-Keuls test (Montgomery, 1983). Data analysis was performed using Microstat II statistical package (Ecosoft Inc., Indianapolis, IN, U.S.A.).

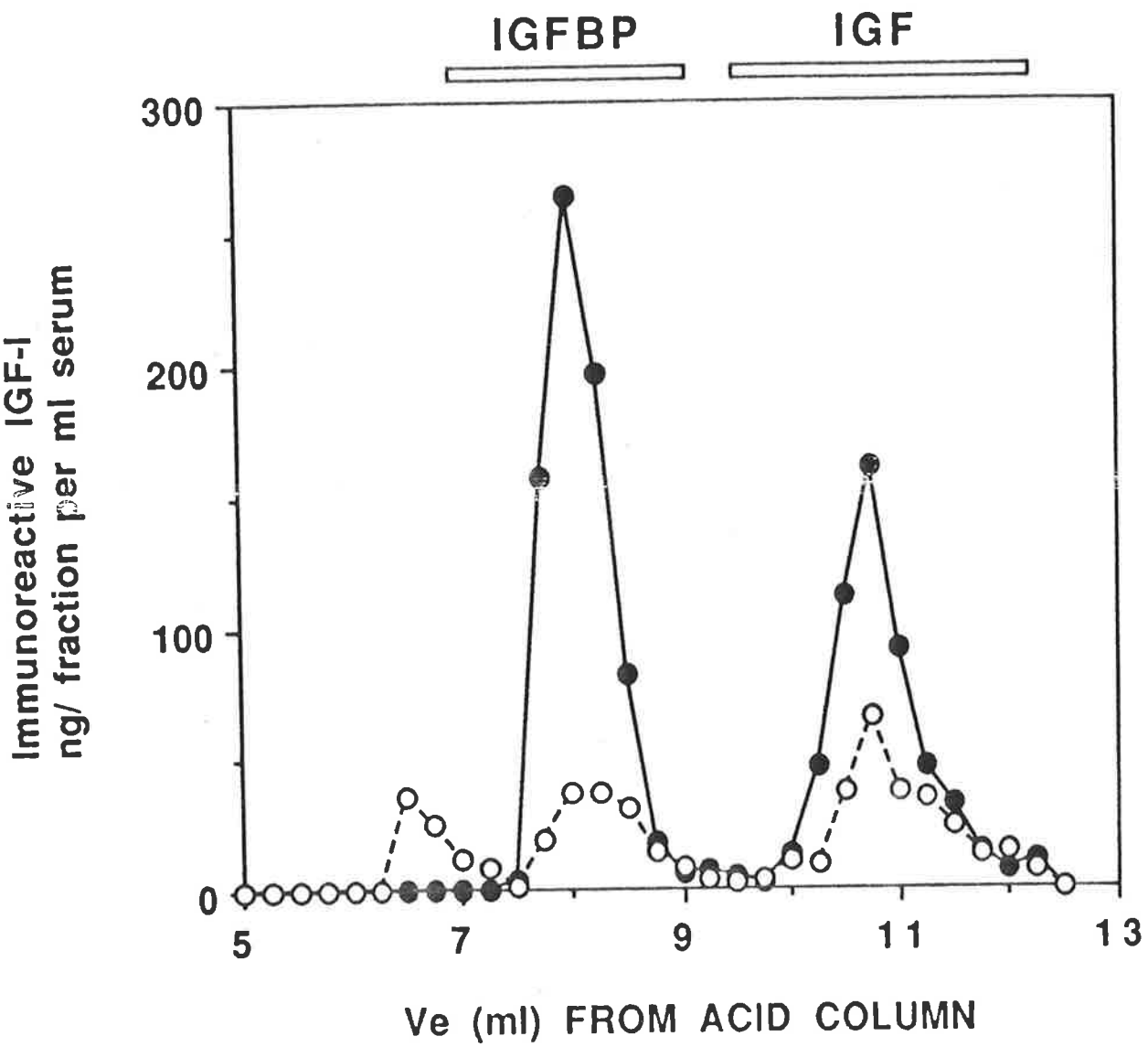
3.3 RESULTS

IGF-I, IGF-II and IGFBP in serum pooled from non-pregnant, pregnant and lactating rats were measured after separating IGF from IGFBP by high performance size-exclusion chromatography under acidic conditions (Fig. 3.1). Two well resolved peaks of activity were detected in sera from non-pregnant rats. The first peak eluting between 7 and 9 ml represented the IGFBP and was detected in identical fractions with both IGF assays. A second peak eluting between 10 and 12 ml was detected in the IGF-I RIA in pooled sera. Human IGF-I standard eluted in the same position as this second peak, as did radiolabelled IGF-I. In sera from rats in late pregnancy, the IGFBP peak was diminished as was the IGF-I peak (Fig. 3.1). In the IGF-II RRA almost all fractions between 10 and 12 ml were below the limit of detection (35 pg). The clear separation of IGF from IGFBP provided quantitation of IGF in the absence of possible IGFBP interference, and a measure of IGFBP-interference in the IGF assay.

To assess the change in the IGF and IGFBP profile during pregnancy, serum samples were pooled from rats at a similar stage of pregnancy, subjected to size-

Figure 3.1: Acid size-exclusion chromatography of pooled sera from non-pregnant and pregnant rats (n = 6) followed by IGF-I RIA.

Sera (200 µl) from non-pregnant rats (●) and sera from rats in late pregnancy (○) was diluted to 15 % (v/v) in acid and subjected to size-exclusion chromatography at pH 2.8. The fractions (0.25 ml) were assayed in an IGF-I RIA (as described in the methods). The limit of detection ranged from 20-42 pg.


IGFBP
IGF
300
200
100
0
5
7
9
11
13
Immunoreactive IGF-I
ng/ fraction per ml serum
Ve (ml) FROM ACID COLUMN

exclusion chromatography at pH 2.8 and the fractions assayed by RIA for IGF-I (Fig. 3.2). For this study a different Protein Pak column was used that displayed slightly altered chromatographic properties; the IGFBP peak eluted between 6 and 8.5 ml and the IGF-I peak between 9.0 and 11.5 ml. Summation of activities in fractions between 9.0 and 11.5 ml showed that sera from days 9-10 and 12-13 of pregnancy contained peaks of IGF-I which were approximately 150% greater than those of serum pooled from rats in late pregnancy (days 16-17 and 19-21). By one day postpartum the serum IGF-I concentration had increased (Fig. 3.1). Summation of IGF-I immunoreactivity between 6 and 8.5 ml showed that IGFBP levels followed a quantitatively similar, but more pronounced pattern in pregnancy and lactation than seen with IGF-I (Fig. 3.2). Thus serum pools from late pregnancy (days 16-17 and days 19-21) had only 10% of the IGFBP activity in the IGF-I RIA compared with those from early pregnancy. During the first three postpartum days the IGFBP levels had recovered to 70% of those seen in early pregnancy.

IGF-I and IGFBP concentrations were also examined in each of six rats individually sampled on three to four occasions in a longitudinal study to determine more precisely the timing of the changes in maternal serum. For this experiment each serum sample which was subjected to size-exclusion chromatographed at pH 2.8, resolved to produce an IGF region (9-11.5 ml) and an IGFBP region (6-8.5 ml) pool. IGF-I concentrations in all sera collected between days 9-13 of pregnancy were greater than 500 ng/ ml. By day 16 IGF-I had fallen to an average of 45 % ($p < 0.05$) of the early pregnancy means and did not significantly recover by 1-3 days postpartum (Fig. 3.3). The amounts of IGFBP detected followed a similar trend to IGF-I (data not shown). However, IGFBP activity was undetectable (< 107 ng/ ml serum) in all 9 rat samples collected between days 16 and 21 of pregnancy, whereas significant activity was detected in 10 of the 12 samples collected at other occasions.

Measurement of the IGFBP by IGF-I RIA or IGF-II RRA gave an indirect measure of total IGFBP based on the ability of IGFBP to inhibit the binding of

Figure 3.2: Acid size-exclusion chromatography of pooled sera from rats at different stages of pregnancy followed by IGF-I RIA.

200 µl of serum was diluted to 15 % (v/v) and subjected to size-exclusion chromatography at pH 2.8. The fractions (0.25 ml) were assayed in an IGF-I RIA. Each panel represents a pool of serum from 4-6 rats at similar stages of pregnancy. The limit of the assay sensitivity was 0.16 ng/ 0.25 ml fraction.

I wish to acknowledge Kirsty Moyse's technical assistance in these assays.

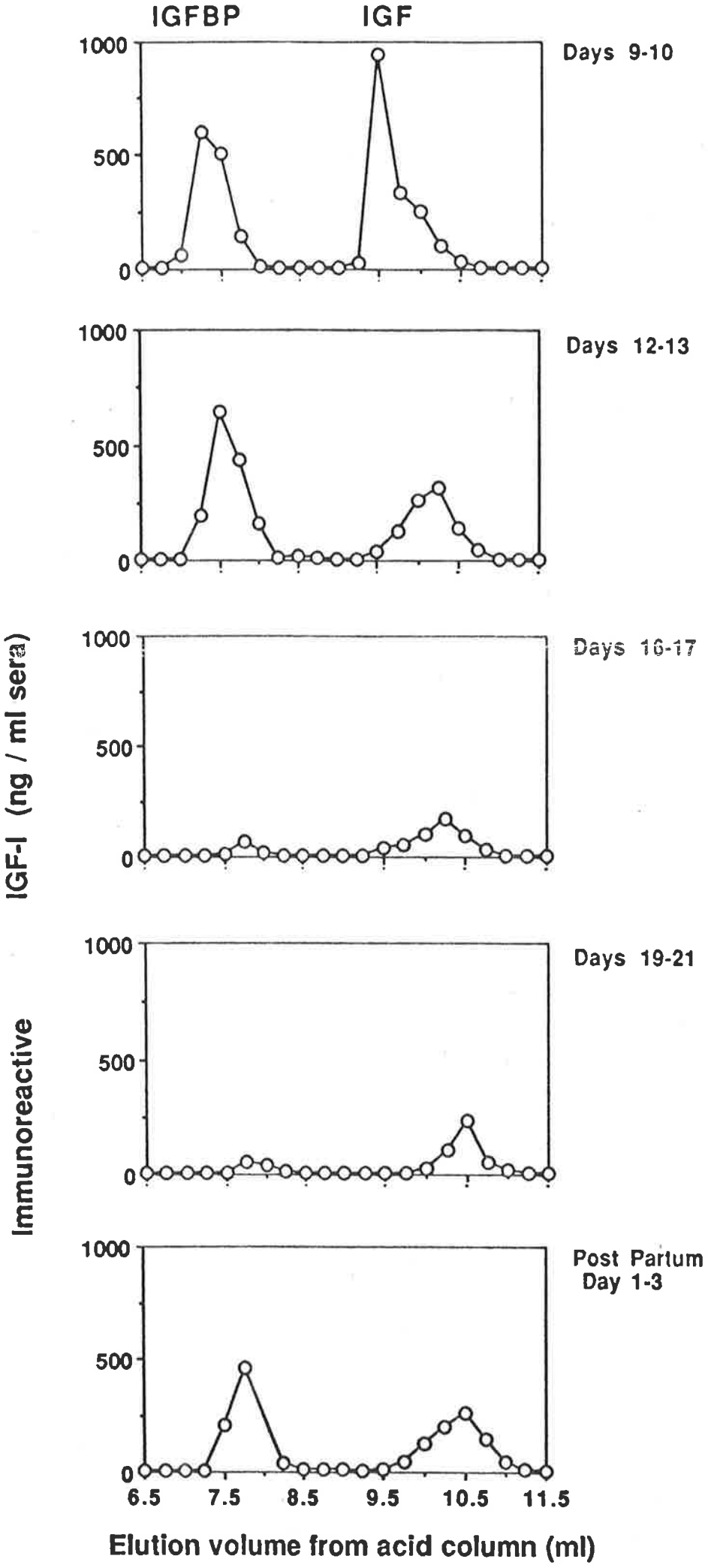
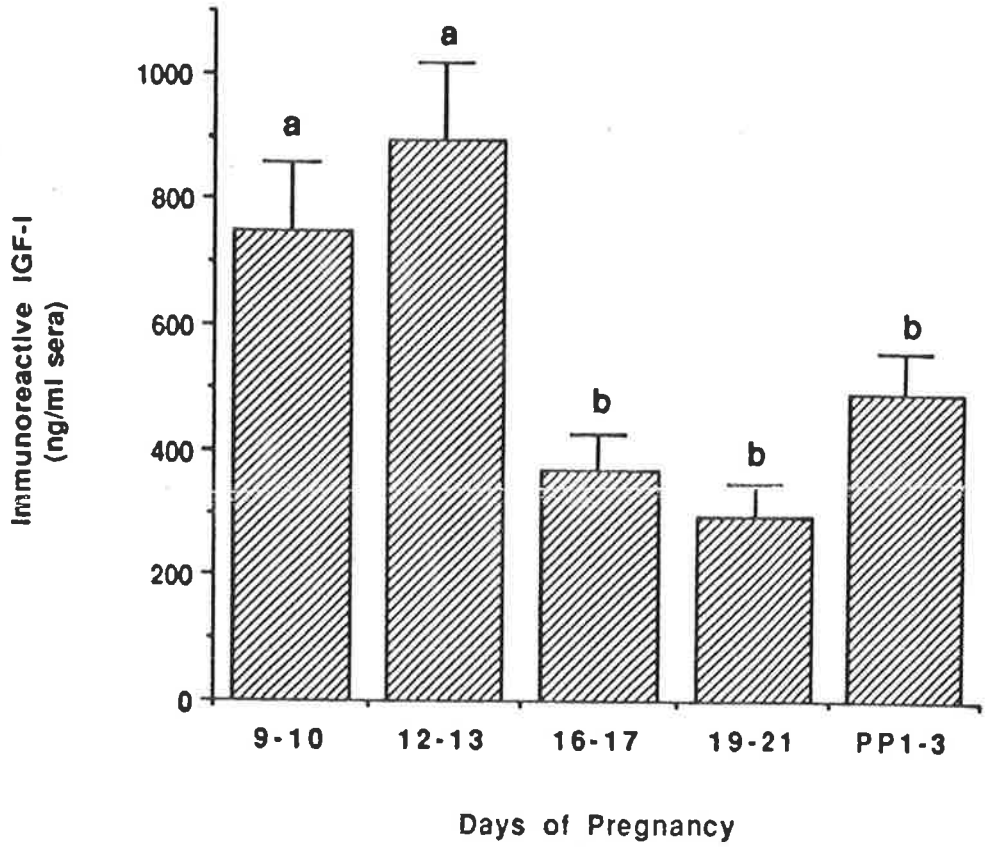

IGFBP
IGF
1000
500
0
Days 9-10
Days 12-13
Days 16-17
Days 19-21
Post Partum
Day 1-3
IGF-I (ng / ml sera)
Immunoreactive
6.5
7.5
8.5
9.5
10.5
11.5
Elution volume from acid column (ml)

Figure 3.3: Immunoreactive IGF-I in acid-chromatographed sera from rats throughout pregnancy.

Serum from individual animals was subjected to acid-chromatography, the IGF-I pool collected (2.5 ml fraction) and all samples measured in one assay. Values are means ± S.E.M. for between 4 and 6 animals at each stage. The lowest detectable concentrations were 107 ng per ml of serum. To determine whether the IGF-I levels differed during pregnancy, data were analyzed using ANOVA and the Newman-Keuls test. Histogram labelled with different letters are significantly different ($P < 0.05$).

I wish to acknowledge Kirsty Moyse's technical assistance in these assays.

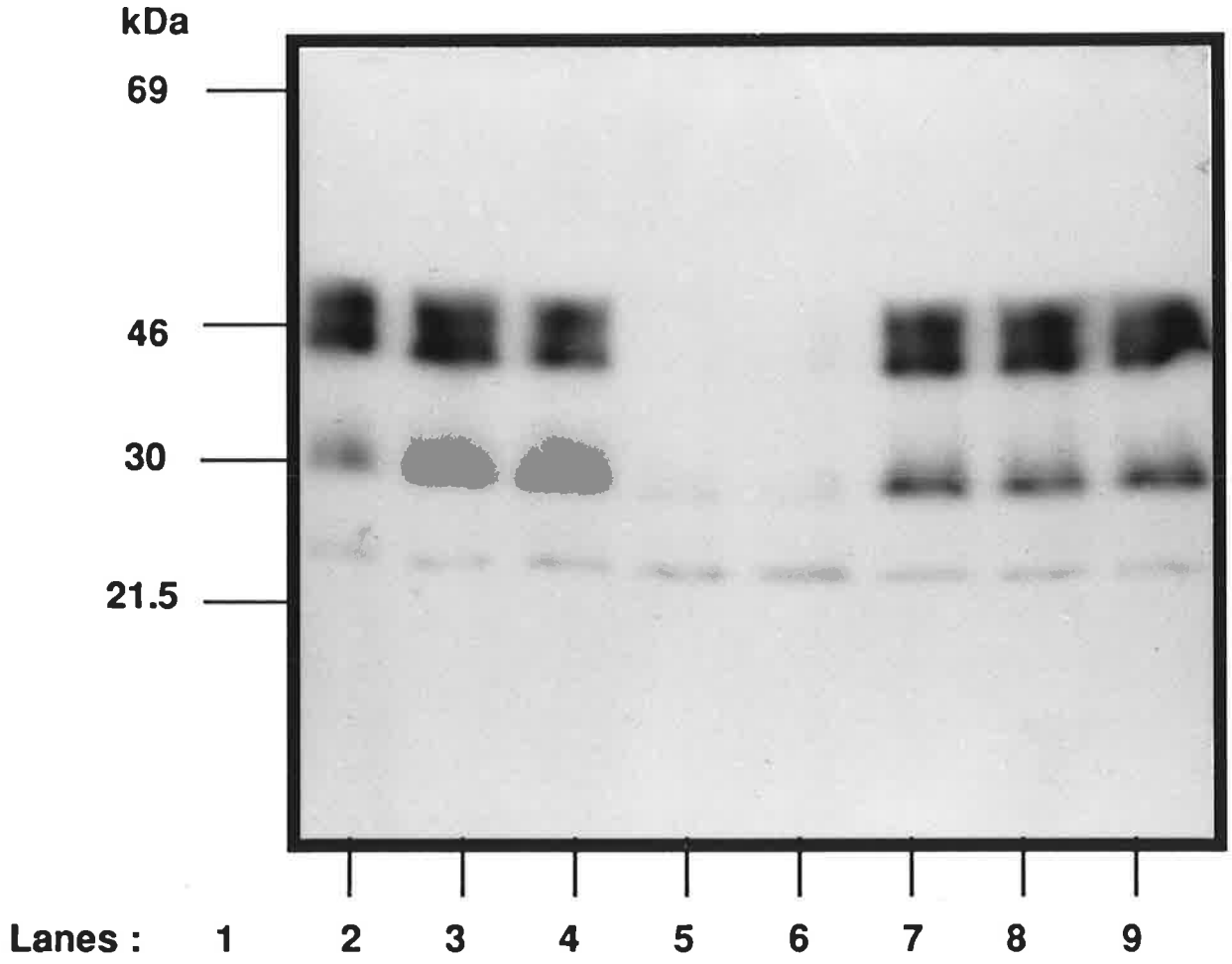

Immunoreactive IGF-I
(ng/ml sera)
1000
800
600
400
200
0
a
a
b
b
b
9-10
12-13
16-17
19-21
PP1-3
Days of Pregnancy

^{125}I-labelled IGF to the antibody or receptor, respectively. Thus, to characterize the changes in the classes of IGFBP, blood samples from individual animals before, during and after pregnancy were collected and the sera subjected to Western-ligand blot analysis. An autoradiograph profile of sequential samples from one rat is shown in Fig. 3.4. Early pregnancy serum contained a 40-50 kDa triplet, a 28-30 kDa doublet and a 24 kDa band. A similar pattern was observed in virgin female or male rats (data not shown). This pattern was maintained up to day 13, although only the results from days 3 to 9 are shown in the figure. By day 16 a dramatic change in the IGFBP pattern had occurred; the 40-50 kDa triplet became undetectable and the intensity of the 28-30 kDa doublet was markedly diminished. The 24 kDa band appeared slightly elevated during days 15-21 of pregnancy. This change in the IGFBP pattern was maintained until delivery. One day postpartum, the signal intensity of IGFBP-3, the 28-30 kDa IGFBP and the 24 kDa IGFBP had returned to early pregnancy levels and were maintained at least until day 11 of lactation. This temporal profile of IGFBP levels was observed in four other pregnant rats studied (data not shown).

To determine the molecular mass of binding proteins under neutral, non-dissociating conditions, samples of serum were labelled *in vitro* with ^{125}I-labelled IGF-II and subjected to Superose 12 gel filtration (Fig. 3.5 and 3.6, upper panel). The specificity of binding *in vitro* was demonstrated by the addition of unlabelled IGF-I or IGF-II. Serum from non-pregnant rats chromatographed as two distinct areas of radioactivity of 150 kDa and 40-50 kDa (Fig. 3.5, upper panel). The addition of unlabelled IGF-I decreased radioactivity in the 150 kDa and 40-50 kDa peaks, but increased radioactivity in the 200 kDa and free IGF regions of the chromatograph. Unlabelled IGF-II reduced the radioactivity in all peaks with a corresponding increase in the free IGF peak. Serum from rats at 17-19 days pregnancy incorporated less radioactivity into the 150 kDa peak, whilst more radioactivity was measured in the 40-50 kDa peak (Fig. 3.6, upper panel). Again unlabelled IGF-I "chased" radioactivity into both the 200 kDa and free IGF region, reducing the radioactivity measured in the 150

Figure 3.4: Western-ligand blot analysis of serum samples collected throughout pregnancy and postpartum.

Serum from an individual rat throughout pregnancy was subjected to Western-ligand blot analysis. The autoradiograph shown is from a Western-ligand blot probed with ^{125} I-labelled IGF-II. Lanes 1 shows the positions of labelled molecular mass markers. Lanes 2-9 contain 10 µl of serum from the same animal. Lane 2, day 3 pregnancy; lane 3, day 5; lane 4, day 9; lane 5, day 16; lane 6, day 19; lane 7, day 1 postpartum; lane 8, day 3 postpartum and lane 9, day 11 postpartum. The nitrocellulose was exposed to X-ray film for 7 days at -20^{0}C.


kDa
69
46
30
21.5
Lanes :
1
2
3
4
5
6
7
8
9

kDa and 40-50 kDa regions (Fig. 3.6, upper panel). Unlabelled IGF-II reduced the radioactivity measured in all IGFBP regions and increased the radioactivity in the free IGF region. The profiles were similar with ^{125}I-labelled IGF-I, although no radioactivity in the 200 kDa region was detected (data not shown).

The IGF-labelled binding protein complexes were further characterized by subjecting the neutral Superose 12 fractions to Western-ligand blot analysis and probing them with iodinated IGF-II (Fig. 3.5 and 3.6, lower panel). Sera from non-pregnant rats, shown in figure 3.5 (lower panel), demonstrated a faint 200 kDa band aligning with the 200 kDa region from neutral chromatography. A 40-50 kDa triplet on the Western-ligand blot that aligned with the porcine IGFBP-3 standard was found in the 150 kDa peak column fractions. In addition, the smaller 40-50 kDa peak blotted as 30 kDa, 28 kDa and 24 kDa bands. In sera from rats in late pregnancy, the 200 kDa IGFBP region Western-ligand blotted as a faint 200 kDa band and the 30 kDa, 28 kDa and 24 kDa bands were detected in the 40-50 kDa region of the Superose 12 chromatograph. In contrast, the 40-50 kDa triplet aligning with IGFBP-3 was undetectable in sera from late pregnancy (days 17-19) (Fig. 3.6, lower panel).

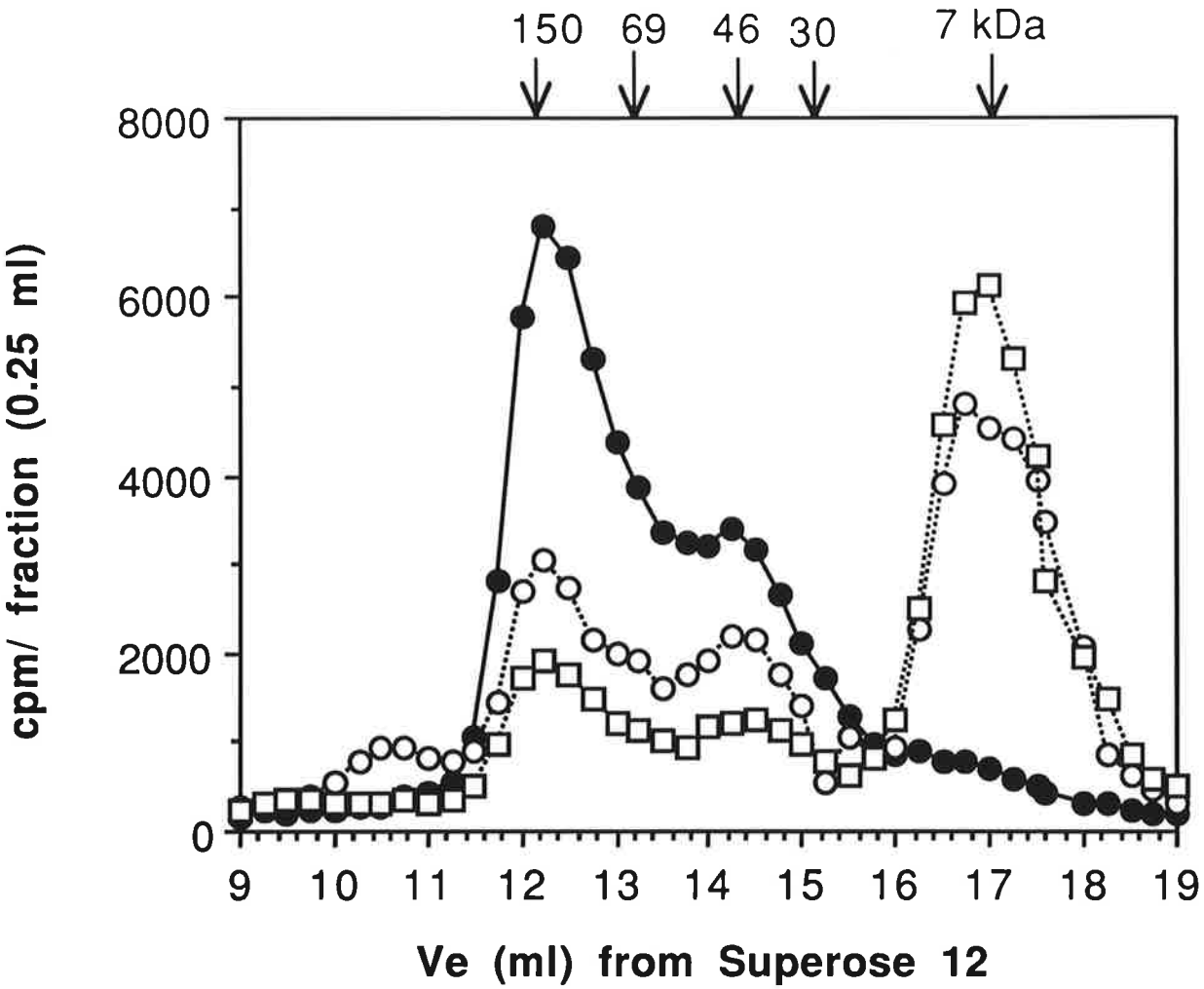
These results showed a decline in detectable IGFBP-3 during late pregnancy. They also suggest a decline in IGF-I concentrations and accordingly less of the 150 kDa complex. To assess the distribution of IGF-I amongst the IGFBP, sera from non-pregnant and pregnant rats were fractionated on a size-exclusion column at pH 7.4 to resolve the different IGFBP complexes from unbound IGF. Each fraction was further chromatographed on a size-exclusion column under acid conditions to dissociate IGF-IGFBP complexes of IGF and IGFBP. The separation of IGF was essential to measure the IGF concentration because IGFBP causes artifacts in IGF radioligand assays. The IGF-containing fractions from the acid column were assayed by RIA for IGF-I.

Labelled sera incorporated radioactivity into three distinct size-classes as assessed by Superose 12 chromatography, similar to Fig 3.5; a 150 kDa region eluting between 10-13 mls, a 40-50 kDa region eluting between 13-15 mls, and free IGF eluting

Figure 3.5: Western-ligand blot of IGF-binding proteins in serum from non-pregnant rat after fractionation on a Superose 12 size-exclusion column at neutral pH.

Upper panel : serum was pooled from 20 non-pregnant rats, incubated *in vitro* with ^{125}I-labelled IGF-II in the presence or absence of IGF-I (500 ng) or IGF-II (200 ng) and applied to a Superose 12 column at pH 7.4. The profile of ^{125}I-IGF-II binding is plotted (●). The chromatograph of ^{125} I-IGF-II + IGF-I are represented (○) and of ^{125} I-IGF-II + IGF-II (□). The column standards represented at the top of the figure are: 150 kDa, gamma globulin; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase; and 7.5 kDa, ^{125} I-IGF-II.

Lower panel: 50 µl of column fractions (each two fractions pooled), undiluted serum (5 µl) and 10 ng of purified IGFBP standards were diluted in SDS-loading buffer, electrophoresed, transferred to nitrocellulose sheets and probed with IGF-II radioligand. The positions of molecular mass standards and the IGFBP standards are shown. The nitrocellulose was autoradiographed for 7 days.


150
69
46
30
7 kDa
8000
6000
4000
2000
0
cpm/ fraction (0.25 ml)
9
10
11
12
13
14
15
16
17
18
19
Ve (ml) from Superose 12

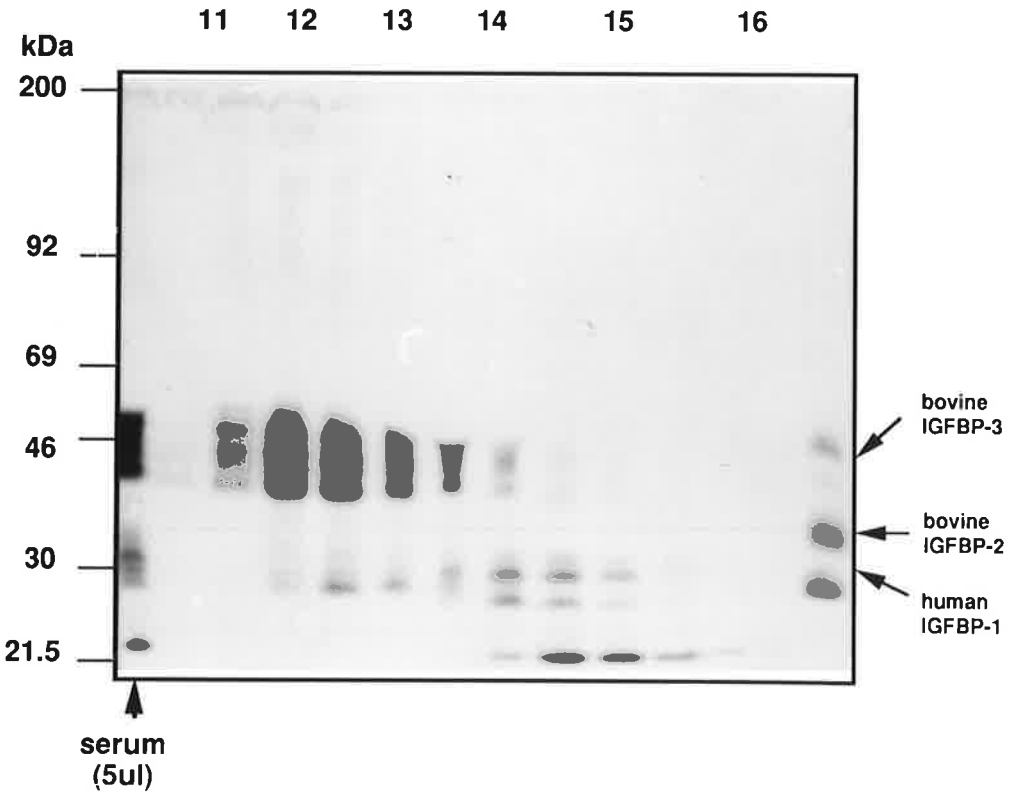
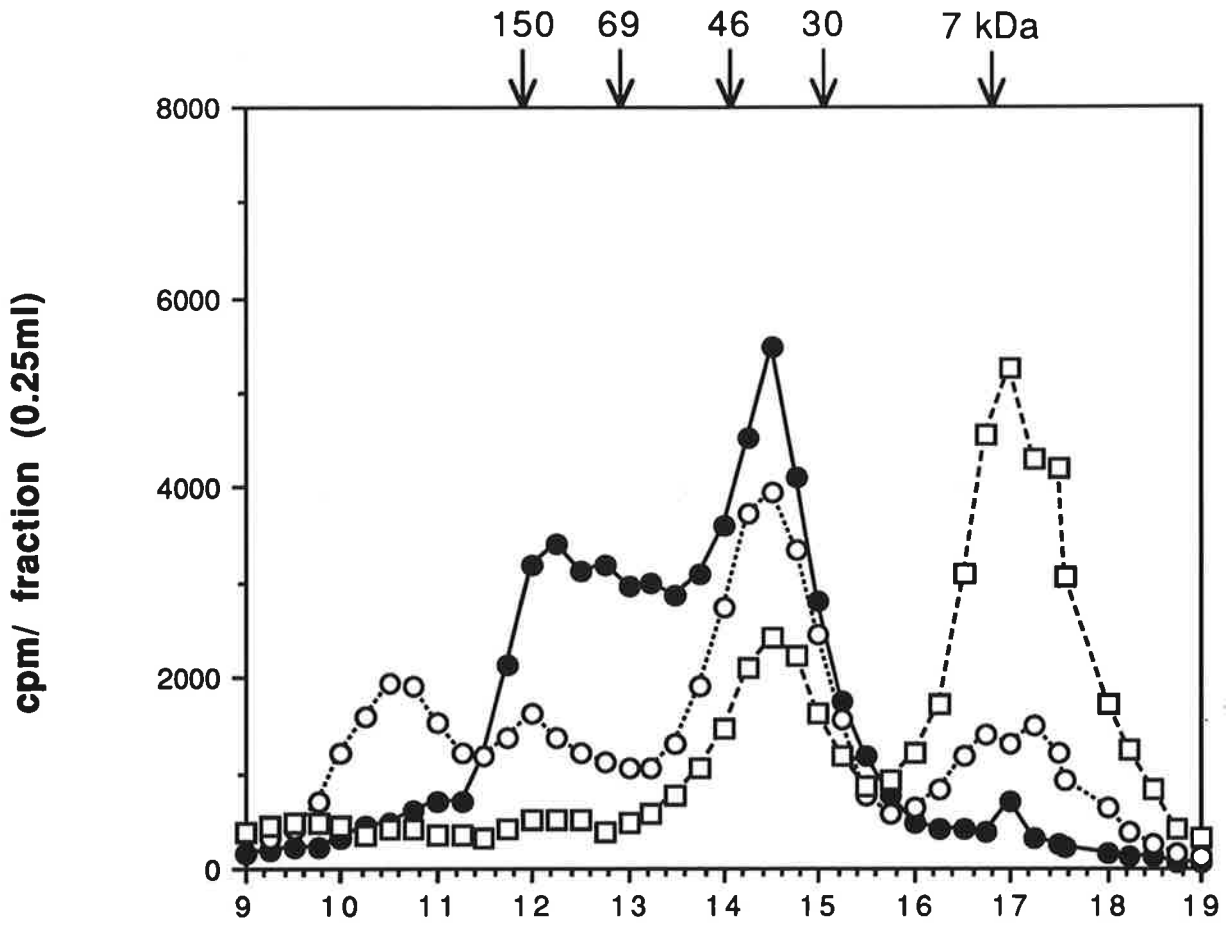
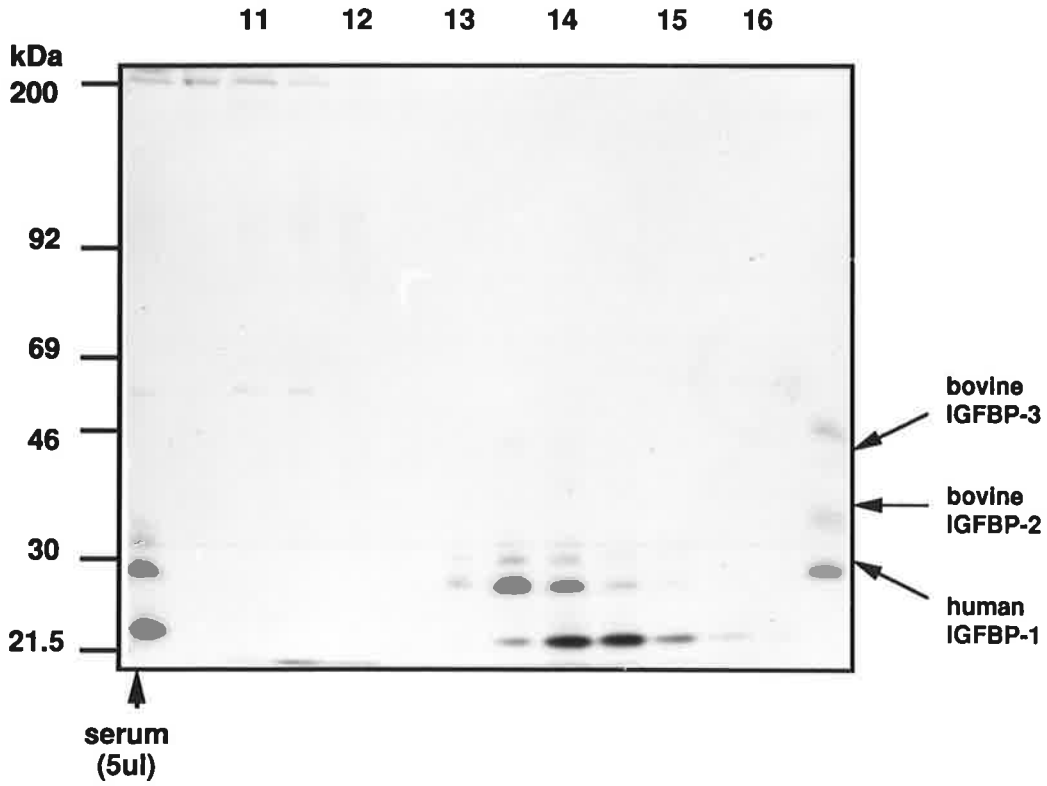

11
12
13
14
15
16
kDa
200
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1
serum (5ul)

Figure 3.6: Western-ligand blot analysis of IGF-binding proteins in sera from rats in late pregnancy (day 19) after fractionation on a Superose 12 size-exclusion column at neutral pH.

Upper panel : Sera from 2 pregnant rats were pooled, incubated *in vitro* with ^{125}I-labelled IGF-II with and without unlabelled IGF-I or IGF-II (as described in Figure 3.5) and applied to a Superose 12 column at pH 7.4. The profiles of ^{125}I-IGF-II binding (●), ^{125} I-IGF-II + IGF-I (○) and ^{125} I-IGF-II + IGF-II (□) are plotted. The column standards represented at the top of the figure are: 150 kDa, gamma globulin; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase; and 7.5 kDa, ^{125} I-IGF-II.

Lower panel: 50 µl of column fractions (each two fractions pooled), serum (5 µl) and 10 ng of purified IGFBP were diluted in SDS-loading buffer, electrophoresed, transferred on to nitrocellulose sheets and probed with IGF-II radioligand. The positions of molecular mass standards are indicated. The nitrocellulose was autoradiographed for 7 days.


150
69
46
30
7 kDa
8000
6000
4000
2000
0
cpm/ fraction (0.25ml)
9
10
11
12
13
14
15
16
17
18
19
Ve (ml) from Superose 12


11
12
13
14
15
16
kDa
200
92
69
46
30
21.5
serum
(5ul)
bovine
IGFBP-3
bovine
IGFBP-2
human
IGFBP-1

between 15-17 mls (Fig. 3.7, upper panel). Fractions collected when unlabelled sera from the same regions were chromatographed on Superose 12 at pH 7.4 were assessed for their IGF-I content. In sera from non-pregnant rats, IGF-I (Fig. 3.7, lower panel) was predominantly carried by the 150 kDa complex and the remainder was carried by the 40-50 kDa region of the Superose 12 fractions. Negligible quantities of unbound IGF were detected (Fig. 3.7, lower panel). In contrast, serum pooled from rats during late pregnancy had little IGF-I in the 150 kDa complex. Most of the IGF-I was carried by the smaller 40-50 kDa region (Fig. 3.6, lower panel). To determine the recovery of IGF and the validity of our measurements, the IGF-I content in same serum pools calculated after a single chromatographic step (Fig. 3.1) was compared against the sum of IGF-I recovered following dual chromatography (Fig. 3.7). The recoveries ranged from 70%-85%.

3.4 DISCUSSION

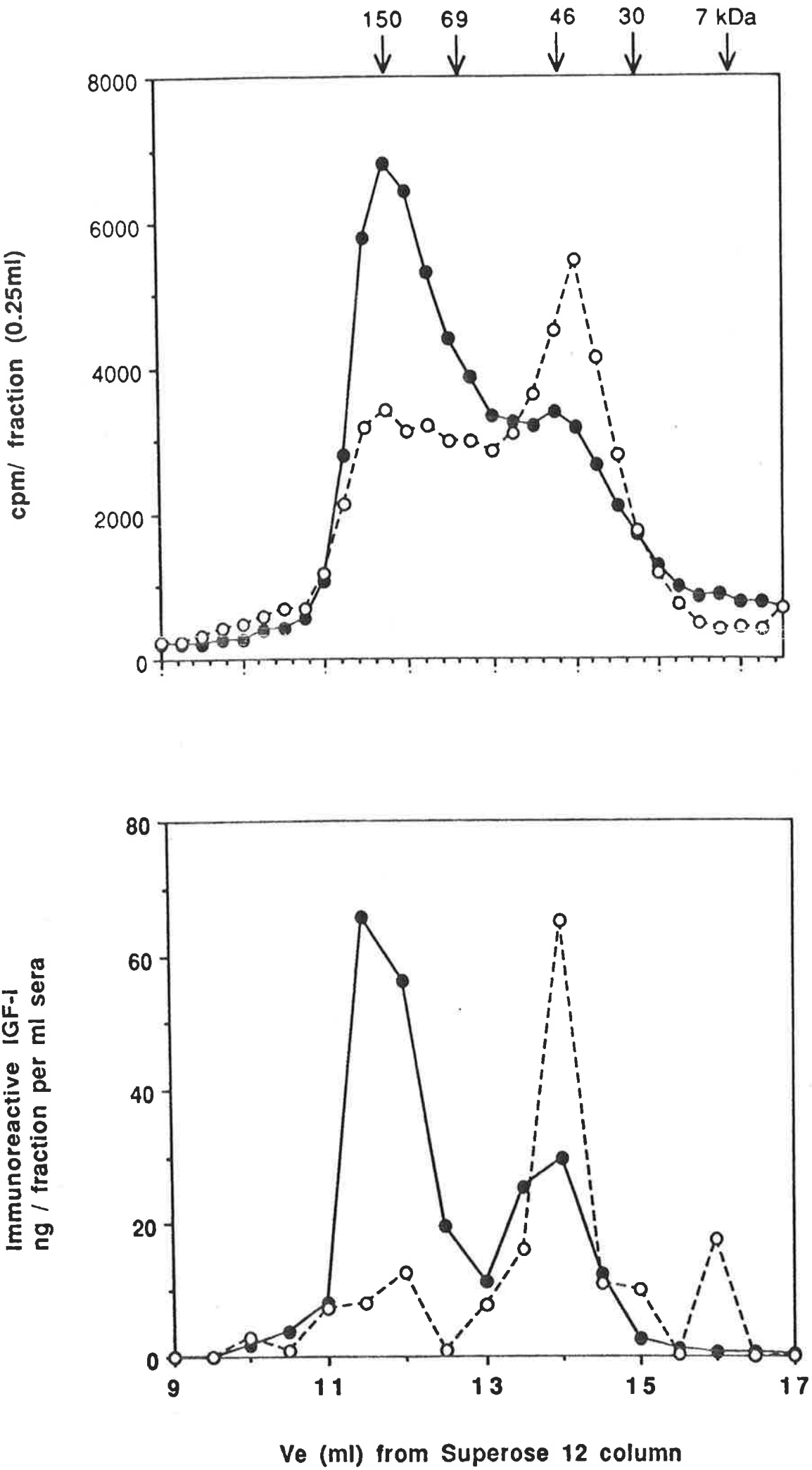
In the current study we have measured maternal IGF and IGFBP concentrations and characterized the changes in the IGFBP profile during pregnancy and lactation in rats. Maternal serum IGF-I levels were highest in early to mid-pregnancy and lowest in late pregnancy. This result is consistent with previous and more-recently reported IGF-I concentrations measured in the pregnant rat and mouse (D'Ercole and Underwood, 1980; Sheppard and Bala, 1986; Chiang *et al.*, 1990; Davenport *et al.*, 1990). I could not detect IGF-II in the sera from non pregnant or pregnant rats thus, confirming the results of Moses and colleagues (1980). In contrast, Donovan *et al.*, (1991) have reported that serum IGF-II concentrations measured by RIA are very low but unaltered during pregnancy in the rat.

IGFBP levels measured by interference in the RIA or by Western-ligand blotting mimicked IGF-I concentrations during pregnancy in that levels declined after day 13. Baxter and Martin (1986) have reported a parallelism between IGF-I and IGFBP concentration in serum. Further, Hardouin *et al.* (1989) suggested that IGFBP synthesis

Figure 3.7: The distribution of IGF amongst higher molecular mass IGFBP in sera from non-pregnant and pregnant rats.

Upper panel: Sera (35 µl) pooled from 20 non-pregnant rats (●) and 2 rats in late pregnancy (○) were labelled *in vitro* with either iodinated IGF-I or IGF-II, fractionated by Superose 12 chromatography and the radioactivity incorporated was measured and plotted. The column standards represented at the top of the figure are: 150 kDa, gamma globulin; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase; and 7.5 kDa, ^{125} I-IGF-II.

Lower panel: Unlabelled sera (200 µl) was fractionated by Superose 12 chromatography and then individual fractions were acidified, applied to size-exclusion chromatography at pH 2.8 and the IGF-containing fractions analysed by RIA. The same serum pools described above were used: non-pregnant sera (●) and pregnant sera (○).


150
69
46
30
7 kDa
8000
6000
4000
2000
0
cpm/ fraction (0.25ml)
80
60
40
20
0
9
11
13
15
17
Immunoreactive IGF-I
ng / fraction per ml sera
Ve (ml) from Superose 12 column

was coordinated with IGF-I synthesis. The results of our examination of IGF binding proteins and IGF-I concentrations during pregnancy supported this concept because IGF-I and IGFBP changed in a concerted manner.

Serial sampling of serum from individual animals during pregnancy and immediately postpartum enabled further assessment of the changes in IGFBP classes. The decline in IGFBP-3 and in the 28-30 kDa IGFBP was observed in late pregnancy. Low levels of both continued until delivery, after which they increased and remained unchanged throughout early lactation (postpartum days 3-11). The 24 kDa IGFBP was only slightly elevated during late pregnancy. The decline in IGFBP-3 has been very recently confirmed by other researchers in both the rat and human (Davenport *et al.*, 1990; Gargosky *et al.*, 1990b; Guidice *et al.*, 1990). My studies on human sera will be discussed in Chapter 5 of this dissertation.

Using size-exclusion chromatography, I showed that serum from pregnant rats contained a 200 kDa peak, a diminished 150 kDa peak and an elevated 40-50 kDa peak compared with serum from non-pregnant rats. Ronge and Blum (1989) reported comparable results in cattle. Using a similar technique, they found that serum from dairy cows towards the end of pregnancy contained a reduced 150 kDa IGFBP, whereas the 45-65 kDa peak was unaltered. I extended my characterization of the binding proteins by employing Western-ligand blot analysis. The 200 kDa region blotted as a 200 kDa band that was observed only with IGF-II radioligand. The molecular mass of this protein and the relative affinity for IGF-II rather than IGF-I suggests this IGFBP is the putative soluble type II receptor (Keiss *et al.*, 1987). The 150 kDa peak appeared as a 40-50 kDa triplet that aligned with the porcine IGFBP-3 standard, in agreement with the characterization of human IGFBP-3 by Baxter and Martin (1989b). In addition, the 24 kDa, 28 kDa and 30 kDa IGFBP observed with Western-ligand blot analysis may represent IGFBP-4, IGFBP-1 and IGFBP-2, respectively, on the basis of the molecular masses of these rat proteins (Brown *et al.* 1989; Murphy *et al.* 1989; Shimasaki *et al.*, 1990). These results agree with my observations with unfractionated

serum and with those of Yang *et al.* 1989.

The IGF-I content of the IGFBP complexes was also measured in both non-pregnant and pregnant rats. In sera from non-pregnant rats, IGF-I was predominantly carried by the 150 kDa IGFBP-IGF complex and the remainder was detected in the 40-50 kDa IGFBP-IGF complex. This study confirmed that in sera from non-pregnant rats, IGFBP-3 was present as a 150 kDa complex that carried the majority of IGF in the circulation. The converse was observed in sera from rats in late pregnancy; most of the IGF-I was carried in the 40-50 kDa IGFBP-IGF complex. I believe this is the first time that IGF had been measured in rat sera fractionated by size-exclusion chromatography in the absence of IGFBP that would otherwise interfere in the IGF assay. The 150 kDa complex has been reported to be composed of three parts: an acid-labile subunit (90 kDa); IGFBP-3 (50 kDa); and IGF (7.5 kDa). The formation of the ternary 150 kDa complex requires IGFBP-3 to bind IGF and form a binary complex to which the acid-labile component can bind (Baxter, 1988). Thus I speculate that either the decline of detectable IGFBP-3 or of IGF-I in sera from rats in late pregnancy is associated with a decline in the 150 kDa complex formation.

The decline of both IGF-I and IGFBP-3 in late pregnancy may result from changes in growth hormone (GH) status since IGF-I and IGFBP-3 are GH dependent (Baxter and Martin, 1989a; Daughaday and Rotwein, 1989). However Carlsson et al. (1990) recently reported that basal levels of circulating GH and GH pulse amplitude increased during pregnancy. This increase in GH levels occurred at the same stage of pregnancy that IGF-I and IGFBP declined, perhaps indicating a state of GH resistance. Chiang *et al.* (1990) have also suggested that GH resistance occurs during late pregnancy in the rat. These authors observed a decrease in circulating IGF-I levels, reduced growth-promoting properties of the internal milieu (as judged by growth and development of fetal transplants placed under the kidney capsule) and decreased maternal skeletal growth during late pregnancy. Furthermore, very high doses of GH (10 mg/ kg/ day) were required to restore growth promotion of the internal milieu to

non-pregnant levels (Chiang *et al.*, 1990). Alternatively, it was recently reported that the decline of IGFBP-3, detected by Western-ligand blotting, was due actually an artifact due to a pregnancy-associated protease that degraded the IGFBP during the procedure (Davenport *et al.*, 1990; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990). The protease activity was observed when serum from pregnant rats was mixed with serum from non-pregnant rats or purified IGFBP-3 standard, incubated and subjected to Western-ligand blotted. The IGFBP profile of non-pregnant rat serum was destroyed by sera from pregnant rats (Davenport *et al.*, 1990; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990).

Since unbound IGF-I is rapidly cleared from the circulation (Ballard *et al.*, 1991), the lower levels of IGFBP-3, the major serum carrier, would result in reduced serum concentrations of circulating IGF-I, consistent with our observed results. In agreement, Davenport *et al.*, (1990) have reported an increased rate of clearance of IGF-I from the maternal circulation during late pregnancy. These authors have also indicated a reduced rate of transcription of IGF-I mRNA in the maternal liver, which would compound the decline in circulating IGF-I levels.

I suggest that the fall in IGF-I during late pregnancy may benefit the rapidly growing fetus. At this stage the fetus requires a greater nutrient supply in order to sustain rapid growth (Metcalfe *et al.*, 1988). If the pregnant rat could not meet these demands through dietary intake, catabolism of maternal proteins could supplement the substrate requirements of the fetus. Increased rates of muscle protein degradation in the mother have been reported in late pregnancy, perhaps as a means to increase the supply of amino acids and other substrates to the fetus (Morton and Goldspink, 1986; Ling *et al.*, 1987; Metcalfe *et al.*, 1988). This state of catabolism is consistent with our observation of low IGF-I levels, because IGF-I has been shown to suppress net muscle protein catabolism both via a stimulation of the synthetic pathway and an inhibition of the degradative pathway (Ballard *et al.*, 1986). Moreover, infusion of IGF-I into rats has been shown to lower circulating amino acid by reducing protein degradation

(Jacob *et al.*, 1989).

In conclusion, during pregnancy in the rat, IGF-I is the major IGF present in serum, IGF-I concentrations are diminished after day 13 and remain low for the duration of pregnancy. IGFBP-3 levels appear to be lower during late pregnancy compared against non-pregnancy levels when measured by Western-ligand blot analysis and indirectly by IGF-I carrying capacity. The levels of IGFBP-3 or IGF-I may be low due to: a state of GH resistance; or a pregnancy-associated IGFBP protease that degrades IGFBP-3. However, whether the protease results are artifacts of the *in vitro* system or actual physiological changes can not be directly assessed without appropriate techniques to measure the IGFBP in rat sera. Nonetheless, I suggest that low IGF-I may promote muscle protein catabolism in the mother, to provide supplementary nutrients to the fetus.

CHAPTER FOUR : ADMINISTRATION OF INSULIN-LIKE GROWTH FACTOR-I, BUT NOT GROWTH HORMONE, INCREASES MATERNAL WEIGHT GAIN IN LATE PREGNANCY WITHOUT AFFECTING FETAL OR PLACENTAL GROWTH.

4.1 INTRODUCTION

The anabolic effects of both growth hormone (GH) and insulin-like growth factor-I (IGF-I) have been demonstrated in non-pregnant rats (Evans and Simpson, 1931; Hizuka *et al.*, 1986; Phillips *et al.*, 1988; Sillence and Etherton, 1989). GH may promote growth both directly and also indirectly via its stimulation of IGF-I synthesis. The IGFs, mitogenic for many different tissues (Froesch *et al.*, 1985) are modulated by at least four IGF binding proteins (IGFBP) recently categorized as IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 (Ballard *et al.*, 1990; Shimasaki *et al.*, 1990).

The pregnancy-associated changes in rat IGFBP and IGF have been discussed in Chapter 3 of this dissertation. During early pregnancy, rat serum IGF-I levels are elevated (Sheppard and Bala, 1986; Davenport *et al.*, 1990; Gargosky *et al.*, 1990b), consistent with the anabolic phase of early pregnancy (Metcalfe *et al.*, 1988). The decline in circulating IGF-I coincided with the late-pregnancy catabolic state where maternal blood glucose is decreased and maternal protein reserves are catabolized to supplement dietary amino acids (Metcalfe *et al.*, 1988). This catabolism is selective as hepatic protein accumulation continued throughout pregnancy (Harris and Kretchmer, 1988).

Rat IGFBP-3 levels also decline in late pregnancy and recover postpartum (Davenport *et al.*, 1990; Gargosky *et al.*, 1990b; Guidice *et al.*, 1990) perhaps due to a pregnancy-specific protease (Davenport *et al.*, 1990; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990).

This study aims to reverse the depressed serum IGF-I and IGFBP concentrations during late pregnancy and to determine the impact of these changes on maternal, fetal and placental growth. I chose to infuse recombinant human GH (rhGH) that has been shown to regulate the synthesis of IGF-I and IGFBP-3 (Baxter and Martin, 1986; Daughaday and Rotwein, 1989), and recombinant human (rh) IGF-I that will increase circulating IGF-I concentrations and initiate IGFBP synthesis in certain states of low serum IGF and IGFBP (Tomas *et al.*, 1991).

4.2 MATERIALS and METHODOLOGY

4.2.1 Peptides

Recombinant human IGF-I and GH were provided by Genentech Inc., South San Francisco, CA, U.S.A. Purified IGFBP standards used for Western-ligand blot analysis are described in Section 2.2.6.

4.2.2 Experimental Design

The study was a composite of three trials, each conducted with three to five animals per treatment group. The responses between the trials were not statistically significant ($P > 0.05$) as determined from one-way analysis of variance. Thus, the three studies were combined to provide a suitable number of animals for each treatment group.

Virgin Sprague Dawley rats (approximately 250 g) were fed *ad libitum* standard rat chow and maintained under controlled conditions (26°C, 12 hrs light and dark). Females were placed in individual cages with a male rat. Every six hours, animals were checked for vaginal plugs. The day of plug detection was termed day 1 of pregnancy and the male was removed. Dams were weighed daily and blood samples (350 µl) were taken from the tail at days 0, 3, 6, 9, 14, 17 and 20 of pregnancy. Serum was harvested following an 18 hr, 4°C incubation and centrifugation at 10,000 g for 5 min. The serum was stored at -20°C. On day 11 of pregnancy, the rats were lightly anaesthetized and an Alzet model 2001 Osmotic pump (Alza, Palo Alto, CA, U.S.A.) was implanted subcutaneously in the back. Pumps were filled with 0.1 mol acetic acid/ l as excipient for vehicle-treated animals, or rhGH diluted in sterile water to deliver 2.4 mg/kg per day, or rhIGF-I diluted in 0.1 mol acetic acid/l to deliver 1.4 mg/kg per day. The peptide doses were based on results of previous studies (Tomas *et al.*, 1991). On day 21, one day prior to the expected delivery date, the rats were killed using CO_2 asphyxiation. Litter number and individual fetal and placental weights were recorded.

4.2.3 Analysis of serum IGF and IGFBP

Maternal serum samples (20 µl) were analysed for their IGF content by Ms. M. Pearce under the supervision of Dr. P. Owens. Acid-ethanol extraction and radioimmunoassay for IGF-I was used for individual rat serum samples as previously described (Owens *et al.,* 1990). Serum IGFBP were analysed by Western-ligand blot analysis (Section 2.2.6).

4.2.4 Statistical Analysis

Treatment effects on single parameters in each group were analysed by one-way analysis of variance (ANOVA) and the means compared using a Newman-Keuls test (Montgomery, 1983). Data analysis were performed using the Microstat II Software Package (Ecosoft Inc., Indianapolis, IN, U.S.A.). Where parameters and co-variates were measured repeatedly over time, the effects of treatment (grouping factor), time (repeated measures factor), their interaction and the influence of co-variates were analysed by repeated measures ANOVA (program 5V, BMDP Statistical Software, University of California Press). This program was designed for analysis of unbalanced models due to incomplete data sets and hence, was the most appropriate for examining the effect of treatment on fetal and placental weights. The magnitude and significance of a specific treatment on parameters was determined from the estimated regression coefficients, their standard errors and co-variance matrix (BMDP Manual, Vol 2, pp. 1102-1103).

I wish to thank Dr. Julie Owens for her assistance with these statistical calculations.

4.3 RESULTS

Serum from each rat, at each collection time, was subjected to acid-ethanol extraction after which the IGF-I concentration was determined by radioimmunoassay (Fig. 4.1). During the first nine days of pregnancy, serum IGF-I concentrations increased from 340 ng/ ml to 500 ng/ ml. Mini osmotic pumps were implanted on day 11 of pregnancy. In the vehicle-treated group, IGF-I levels declined. Rats infused with GH showed an apparent increase in IGF-I early in the treatment period, although the increase was not statistically significant. Otherwise, the IGF-I pattern for GH-treated rats was similar to the vehicle-treated rats. Administration of IGF-I caused a 1.9 fold increase in IGF-I three days after pump implantation, with the concentrations significantly elevated ($P < 0.01$) throughout the treatment period when compared with either vehicle- or GH-treated groups.

To examine circulating IGFBP, sera from each treatment group were pooled and subjected to Western-ligand blot analysis. Sera from non-pregnant rats (Fig. 4.2, lane 2) blotted as a 40-50 kDa triplet that aligned with purified bovine IGFBP-3, a 32 kDa band, a 28 kDa band that aligned with human IGFBP-1 and a 24 kDa band. Note that this pattern was obtained with 1 µl serum while 5 µl was electrophoresed from the pregnant rats (Fig. 4.2, lanes 3-8). This pattern was maintained until day 14 of pregnancy, after which the 40-50 kDa triplet became undetectable and the 28 and 32 kDa bands diminished in intensity (data not shown). To examine the period of change in the IGFBP profile, serum pools were compared at days 17 and 20 of pregnancy (Fig. 4.2). Vehicle-treated animals essentially lacked the 40-50 kDa triplet at days 17 and 20 of pregnancy (Fig. 4.2, lanes 3 & 4). The intensities of the 24, 28 and 32 kDa bands were reduced, but detectable at both days. Infusion of GH produced a small increase in the 40-50 kDa IGFBP triplet at day 17 only (Fig. 4.2, lane 5). IGF-I treatment induced a small increase in 40-50 kDa IGFBP, principally at day 17 (Fig. 4.2, lane 7), although these bands were also detectable at day 20 (Fig. 4.2, lane 8). The intensity of the 32 kDa band was increased with IGF-I treatment for both days 17 and 20 of pregnancy.

Figure 4.1: Immunoreactive insulin-like growth factor-I measured in acid-ethanol extracted serum from individual rats in each treatment group.

The groups represented are those treated with vehicle (●), growth hormone (○) and IGF-I (■). Values are the means ± S.E.M. for nine to ten animals at each time point. The lowest detectable concentration was 64 ng /ml. The asterisk indicates a significant difference ($P<0.01$) between the IGF-I treated rats compared with both vehicle- and growth hormone- treated rats (ANOVA and the Newman Keuls test).

I would like to acknowledge Madeline Pearce for her technical support with the IGF-I RIAs.

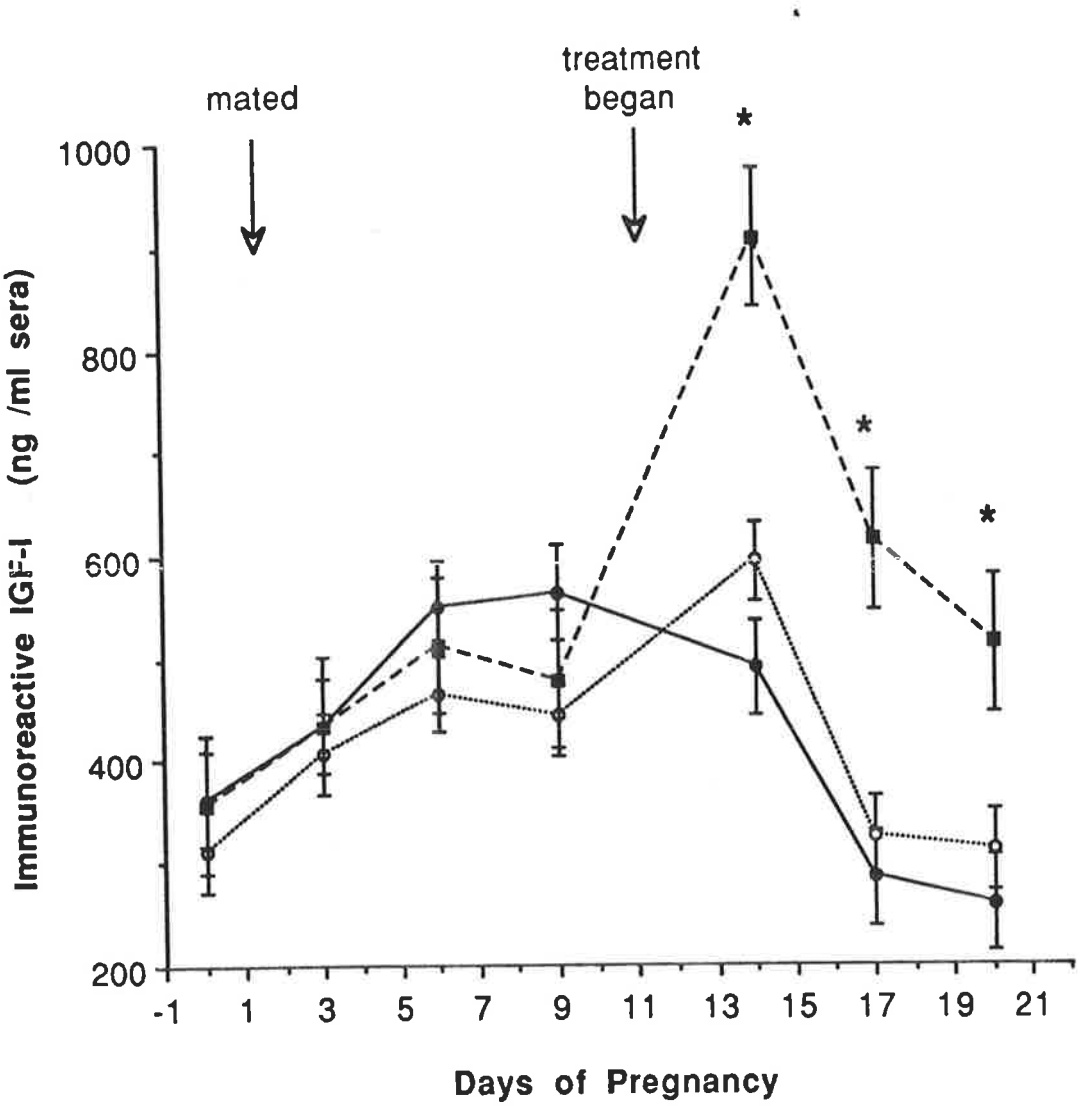
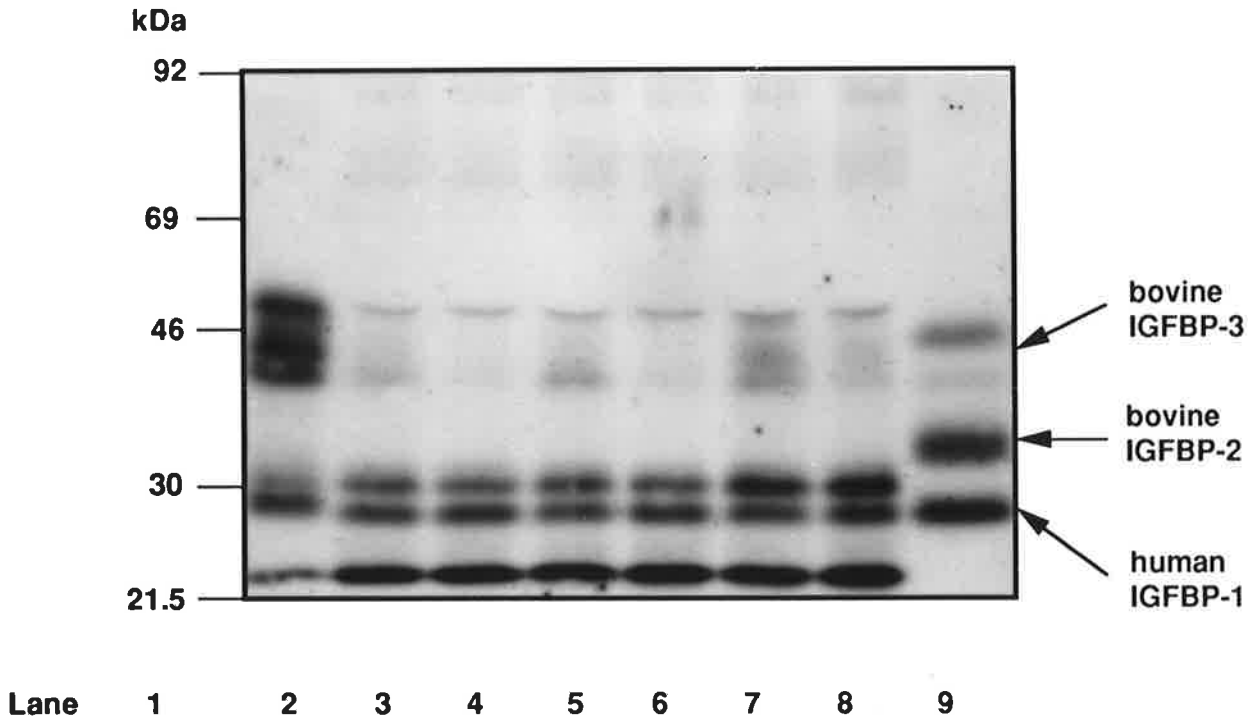

mated
treatment began
*
*
*
1000
800
600
400
200
Immunoreactive IGF-I (ng /ml sera)
-1
1
3
5
7
9
11
13
15
17
19
21
Days of Pregnancy

Figure 4.2: Western-ligand blot analysis of pooled serum at days 17 and 20 of pregnancy.

Serum pooled from rats (n= 9-10) at days 17 and 20 were incubated in SDS-loading buffer, electrophoresed, transferred to nitrocellulose and probed with iodinated IGF. The autoradiograph shown is the result of probing the nitrocellulose with ^{125}I-labelled insulin-like growth factor-II (IGF-II). Lane 1, radiolabelled molecular mass markers; lane 2, pool of non-pregnant rat sera (1 µl); lanes 3 & 4, sera (5 µl) from vehicle-infused pregnant rats at days 17 and 20, respectively; lanes 5 & 6, sera (5 µl) from growth hormone-infused pregnant rats at days 17 and 20, respectively; lanes 7 & 8, sera (5 µl) from IGF-I-infused pregnant rats; and lane 9, 15 ng each of bovine IGFBP-3, bovine IGFBP-2 and human IGFBP-1. The autoradiograph was exposed for 38 days.


kDa
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1
Lane 1 2 3 4 5 6 7 8 9

Clearly, neither GH nor IGF-I administration restored the IGFBP pattern to non-pregnant levels.

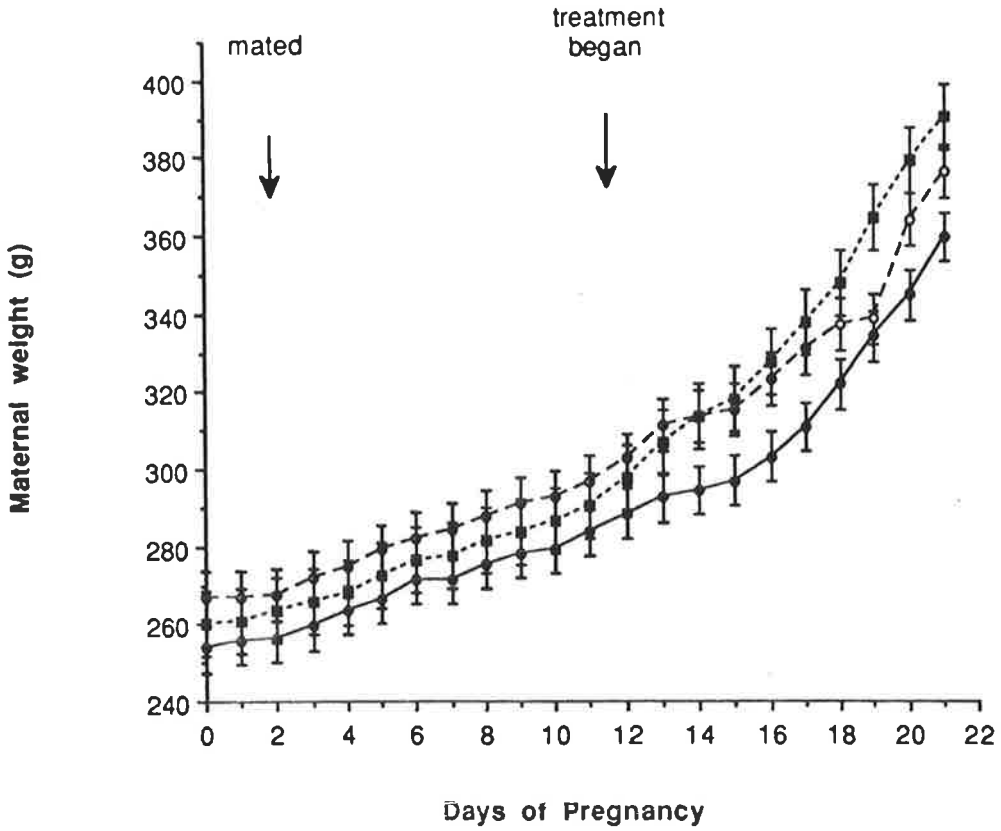
Administration of IGF-I accelerated the rate of increase in maternal weight during pregnancy (treatment x time, P=0.015) (Fig 4.3. upper panel). The cumulative change in maternal weight from the day of pump implantation was similar for the vehicle-infused (72 g) and GH-infused rats (77 g) (Fig. 4.3, lower panel). IGF-I treated rats increased their weight by 99 g, a 27 % increase above the control (P = 0.0003) (Fig. 4.3, lower panel). "Empty maternal weights", calculated from the total weight at day 21 less fetal and placental weights, were increased significantly (P = 0.048) by day 21 as a result of IGF-I treatment (Table 4.1).

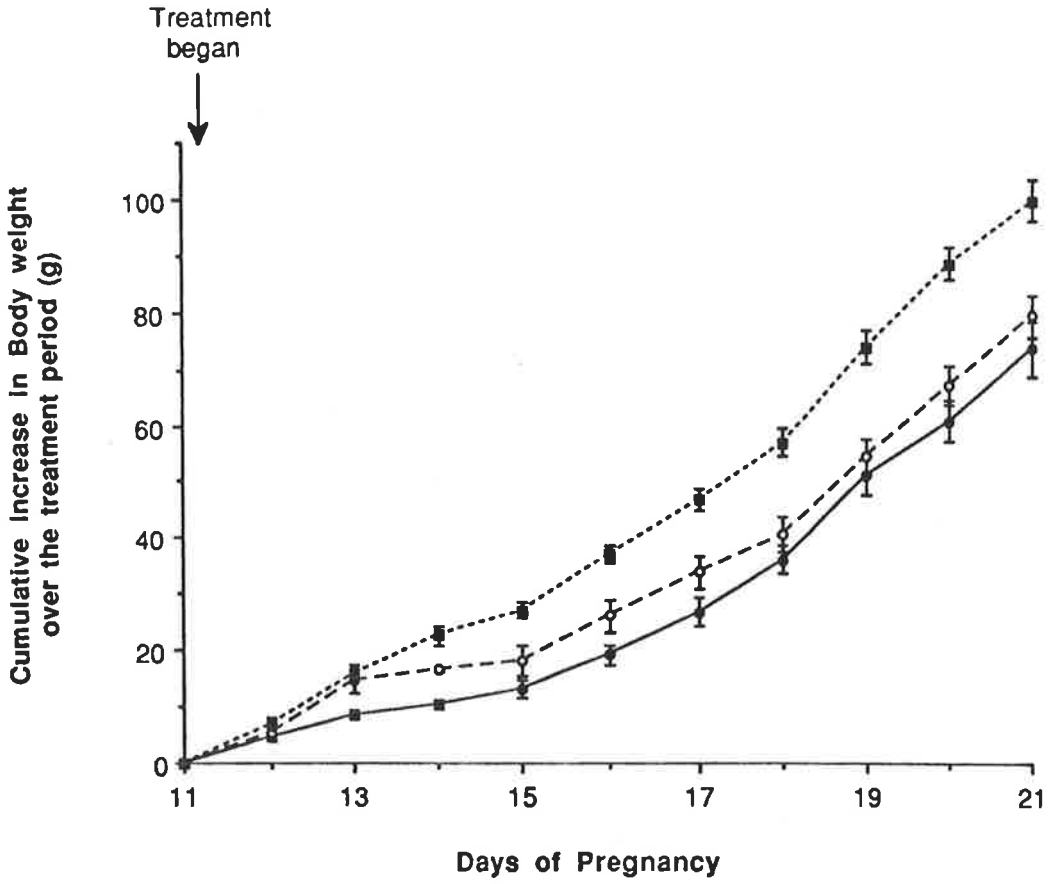
Average fetal weights and average placental weights, as well as the fetal : placental ratio were not significantly different between treatment groups as assessed by one way analysis of variance (Table 4.1). Summation of fetal weights and placental weights for each dam to compensate for differing litter number also showed no significant effect of treatment (Table 4.1). However, this statistical analysis had limitations because it was based on averaged fetal weights and did not account for any differences in litter number or maternal weight. Pups from larger litters tend to be smaller than those from smaller litters (Dempster *et al.*, 1984) and preterm maternal size is highly correlated with infant birth weight and placental weight (Metcalfe *et al.*, 1988). Accordingly, analysis of the effects of treatment was extended using an unbalanced repeated measures model with litter number measured postpartum and maternal weight measured throughout gestation as co-variates. Fetal weights were not significantly effected by treatment (P=0.41) while litter number (P=0.33) and maternal weights (P=0.50) as co-variates were not significant with this analysis. Placental weights were not significantly changed by treatment (P=0.13), and litter number was not a significant influence (P=0.25), but maternal weight did influence placental weight gain (P=0.006). The ratio of fetal to placental weight was not significantly different between treatment groups (P=0.31) or affected by litter number (P=0.53) or maternal

Figure 4.3: Maternal weight gain following growth hormone or insulin-like growth factor-I administration during late pregnancy.

Upper panel: Maternal weight values (g) represent the means of nine to ten rats ± S.E.M. The groups are, pregnant rats infused with vehicle (●), growth hormone (○) and insulin-like growth factor-I (■).

Lower panel: Cumulative maternal weight change during the treatment period. The groups are depicted as above. The values represent the means of nine to ten animals ± S.E.M.


mated
treatment began
Maternal weight (g)
400
380
360
340
320
300
280
260
240
0
2
4
6
8
10
12
14
16
18
20
22
Days of Pregnancy


Treatment began
Cumulative Increase in Body weight over the treatment period (g)
100
80
60
40
20
0
11
13
15
17
19
21
Days of Pregnancy

size (P=0.76). Thus, neither fetal weight, placental weight nor the fetal : placental ratio showed statistical significance using co-variate analysis.

4.4 DISCUSSION

During late pregnancy there is a net degradation of maternal muscle protein, accompanied by a reduction in glucose uptake in non-uterine tissues (Metcalfe *et al.*, 1988). IGF-I suppresses net protein catabolism (Ballard *et al.*, 1986) and enhances glucose uptake (Jacob *et al.*, 1989). Thus, the coordinate decline of serum IGF-I during late pregnancy in the rat was consistent with a maternal catabolic state. The aims of my experiment were to replenish the diminished serum IGF-I concentration observed during late pregnancy in the rat, to assess the changes in serum IGFBP and to examine the effects on maternal, fetal and placental weights.

I examined the possible role of GH in regulating serum IGF-I and IGFBP-3 concentrations in late pregnancy because both IGF-I and IGFBP-3 are GH dependent (Baxter and Martin, 1986). In addition, GH administration to hypophysectomised or protein-deprived rats restores depressed IGFBP-3 concentrations (Clemmons *et al.*, 1989). During pregnancy in the human, pituitary-derived GH concentrations decline and concentrations of the placental GH variant are increased (Eriksson, 1989). However, reported changes in GH concentrations during late pregnancy in the rat are less clear. There are reports of inhibited pituitary GH gene expression (Kumar and Biswar, 1988), unaltered immunoreactive GH concentrations (Madon *et al.*, 1989), increased GH levels (Carlsson *et al.*, 1990) and GH resistance in late pregnancy (Chiang *et al.*, 1990).

I found that infusion of GH at 2.4 mg/ kg per day did not significantly elevate IGF-I levels during late pregnancy. Maternal, fetal and placental weights were not significantly different from the control animals, and GH infusion did not significantly alter the IGFBP profile. Similar to these results, Chiang *et al.* (1990) found that injections of porcine GH at 2.5 mg/ kg per day into pregnant rats over a similar time

Table 4.1: Maternal, fetal and placental weights of rats on day 21 of pregnancy following 10 days of growth hormone (GH) or insulin-like growth factor-I (IGF-I) infusion.

Values are means ± S.E.M. for ten rats in the vehicle and IGF-I groups and for nine rats in the GH group.

TREATMENT GROUPS			
Parameter	**Vehicle**	**GH**	**IGF-I**
	Measurement		
Empty Maternal Weight	322 ± 4.6	335.6 ± 8.5	348 ± 7.8*
Maternal Weight Change (days 11-21)	71.7 ± 4.40	76.8 ± 4.27	98.9 ± 4.10**
§Mean Litter Number	12 ± 0.51	11 ± 0.48	12 ± 0.79
§Mean Fetal Weight	2.52 ± 0.18	2.72 ± 0.25	2.92 ± 0.28
§Mean Placental Weight	0.50 ± 0.01	0.47 ± 0.02	0.50 ± 0.02
Total Fetal Weight	30.2 ± 3.5	29.9 ± 2.50	34.8 ± 2.61
Total Placental Weight	5.85 ± 0.32	5.23 ± 0.21	6.13 ± 0.40
Fetal: Placental Ratio	5.05 ± 0.28	5.72 ± 0.40	5.75 ± 0.36

§ The litter number, fetal and placental weights were averaged for each dam, and then the mean calculated for each treatment group.

* $P < 0.05$, ** $P < 0.0005$ compared with control (ANOVA and Newman-Keuls)

period had no affect on the reduced growth-promoting properties of the "internal milieu", as determined by transplantation of fetal paws under kidney capsules of pregnant rats. They found that concentrations of 10 mg/ kg per day were required to initiate growth. This result led to a concept of GH resistance in late pregnancy. Such a state of GH resistance during late pregnancy may explain my results. Alternatively, the lack of IGF-I or IGFBP induction by rhGH may be because due to the prolactin-like effect of human GH in the rat. However, this was not quantitated. Alternatively, the GH binding protein (GHBP) may have sequestered the exogenous GH, but the literature on GHBP during pregnancy is scant. In the human, low affinity GHBP levels are increased during pregnancy whilst levels of the high affinity GHBP, the major GH carrier, are unchanged (Baumann *et al.*, 1989). The physiological relevance in the human is unknown and extension to the rat is not possible. Nonetheless, Chiang *et al.*, (1990) did not observe any anabolic effect of GH even though the porcine GH used displays only somatogenic properties in the rat, strengthening the concept of GH resistance in late pregnancy in this species.

Infusion of IGF-I into pregnant rats during late pregnancy when IGF-I concentrations were otherwise depressed, significantly increased circulating IGF-I levels. The most notable result of IGF-I infusion was an increase in the maternal weight gain during the treatment period. Neither fetal nor placental weights were influenced by IGF-I treatment, possibly consistent with a diversion of nutrients to maternal tissues. The average litter numbers for each treatment group were virtually identical, suggesting that treatment did not result in fetal death.

The fetus is a major site of amino acid utilization in late pregnancy (Metcalfe *et al.*, 1988). Infusion of IGF-I into normally growing rats lowers circulating amino acids by reducing protein degradation (Jacob *et al.*, 1989). Thus, the presence of increased IGF-I concentrations in the maternal circulation may reduce the availability of amino acids to the fetus. However, Domenech *et al.* (1986) have shown that despite a marked decrease in maternal total amino acid concentrations when the mother was subjected to

dietary deprivation, fetal plasma total amino acid concentrations were unaffected. They suggested that the preservation of fetal amino acid concentrations was due to enhanced placental transport of amino acids. This may explain our observation of increased maternal weight without altering the outcome for fetal or placental development.

Following the administration of IGF-I, the 40-50 kDa IGFBP (IGFBP-3) became detectable, and the 32 kDa IGFBP was significantly increased in late pregnancy. The IGF-I-induced increase of IGFBP in the sera of pregnant rats was less pronounced than that observed in nutritionally-deprived rats (Tomas *et al.*, 1991). One reason for the limited IGFBP induction could be proteolytic degradation of IGFBP. Recent reports have demonstrated the presence of a pregnancy-specific protease in the sera of rats and humans that degrades IGFBP-3 to smaller fragments of 30-14 kDa (Davenport *et al.*, 1990; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990). This protease may degrade the IGFBP-3 synthesised following IGF-I administration. The increase in the smaller molecular mass IGFBP supports the notion that the protease is specific for IGFBP-3 (Davenport *et al.*, 1990; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990).

The rate of IGF-I clearance is reported to be increased five-fold in pregnant rats (Davenport *et al.*, 1990). However, the decline of IGF-I levels in IGF-I administered rats paralleled the vehicle group, suggesting that the dynamics of IGF-I clearance was unaltered. It seems plausible that the elevation of the 32 kDa IGFBP by IGF-I may have increased the serum carrying capacity for IGF-I, a situation that could increase the half life and bioactivity of IGF.

The increased maternal weight gain following IGF administration, without affecting fetal and placental weights, suggests a modification in the mode of maternal nutrient repartitioning during late pregnancy.

CHAPTER FIVE: CIRCULATING IGF-I, IGF-II AND IGFBP-3 INCREASE DURING HUMAN PREGNANCY

5.1 INTRODUCTION

During pregnancy the maternal metabolism adapts to the demands of a rapidly growing conceptus. The rat maternal carcass muscle is catabolized to supply amino acids to the fetus and glucose uptake by non-uterine tissues is reduced during the second half of pregnancy (Metcalfe *et al.,* 1988). These metabolic changes coincide with a decline in circulating IGF-I concentrations (Davenport *et al.*, 1990; Gargosky *et al.*, 1990b; Guidice *et al.*, 1990). IGF-I enhances net protein anabolism and stimulates glucose uptake (Froesch *et al.,* 1985; Ballard *et al.* 1986), suggesting that IGF may regulate, in part, the metabolism of pregnant rats. The rat is commonly used as an experimental model for the human yet the two species are distinct in energy metabolism and hormonal adaptation during pregnancy. In contrast to humans, during pregnancy in the rat the fetal growth rate is rapid over a short gestation period and food intake for the rat is increased (Metcalfe *et al.,* 1988). Furthermore, in the rat skeletal growth is reduced during pregnancy, possibly due to the demand of many rapidly growing conceptuses (Chiang *et al.,* 1990). This situation is not observed in the human. As a result of the species differences, I was interested in the circulating IGF and IGFBP levels during pregnancy in the human.

In an early study, somatomedin (IGF) bioactivity was found to be low during early and late pregnancy in humans but similar to non-pregnant controls near week 28 and at term (Bala *et al.,* 1978). Furlanetto *et al.* (1978) reported a progressive rise in serum immunoreactive somatomedin-C during gestation, which declined after delivery. In agreement, Bala *et al.,* (1981) reported higher levels of immunoreactive somatomedin in serum of women near term were reported and two longitudinal studies reported a rise in maternal serum IGF-I compared with non-pregnant females (Wilson *et al.,* 1982; Hall *et al.,* 1984). Wilson *et al.* (1982) also reported high levels of IGF-II in late pregnancy. In these earlier measurements of IGF in the blood of pregnant woman, samples were not rigorously treated to remove the IGFBP which have been shown to interfere in the IGF assays (Mesiano *et al.,* 1988). In addition, the individual IGFBP

forms had not been examined. The aims of this study were to measure IGF-I and IGF-II and to characterise the IGFBP in the circulation of pregnant women.

5.2 MATERIALS and METHODOLOGY

5.2.1 Sample collection

Following ethical approval and the consent of the donors, Ms. Ross Green at the Queen Victoria Hospital collected 10 ml blood samples by venipuncture from healthy women aged 18-35 years. The blood clotted after 18 hrs at 4^0C and serum was harvested following centrifugation at 10,000 g for 5 min. Serum was pooled from non-pregnant women (n=7) and from women with uncomplicated pregnancies (n = 10) for analysis. Samples were stored at -20^0C until needed.

5.2.2 Measurement of IGF-I and IGF-II

The IGF-I and IGF-II measurements were done by Ms. K. Moyse under the supervision of Dr. P. Owens. Acknowledgments are cited in the figure legends.

Differences between the documented protocol in Section 3.2.2 are that a 40 µl equivalent of pooled serum, rather than 15 µl of pooled rat serum, was fractionated through the Waters/ Millipore Protein Pak Column at 0.5 ml/ min and 0.25 ml fractions were collected. The pooled serum was chromatographed three times to give 0.75 ml fractions. IGF-I was detected by RIA and IGF-II by RRA as described in Section 3.2.7 and IGFBP characterized using Western-ligand blot analysis (Section 2.2.6).

Ms. K. Moyse also measured IGF-I in individual serum samples by acid-ethanol extraction and RIA as described previously (Owens *et al.*, 1990).

5.2.3 Analysis of IGF binding proteins

IGFBP were analysed by Western-ligand blot analysis (Section 2.2.6), Western-immunoblot analysis (Section 2.2.7) and size-exclusion chromatography of serum labelled *in vitro* with radiolabelled IGF (Section 2.2.5).

5.2.4 The distribution of IGF amongst the higher molecular mass forms.

The molecular sizes of IGFBP-IGF complexes in serum were determined by FPLC size-exclusion chromatography (Section 3.2.5) and the IGF content of these IGFBP fractions measured by RIA for IGF-I and RRA for IGF-II (Sections 3.2.6 and 3.2.7).

Analysis of human serum required 0.4 ml acid column fractions rather than 0.25 ml to permit measurement of the IGF-I and IGF-II content in triplicate.

5.2.5 Statistical analysis

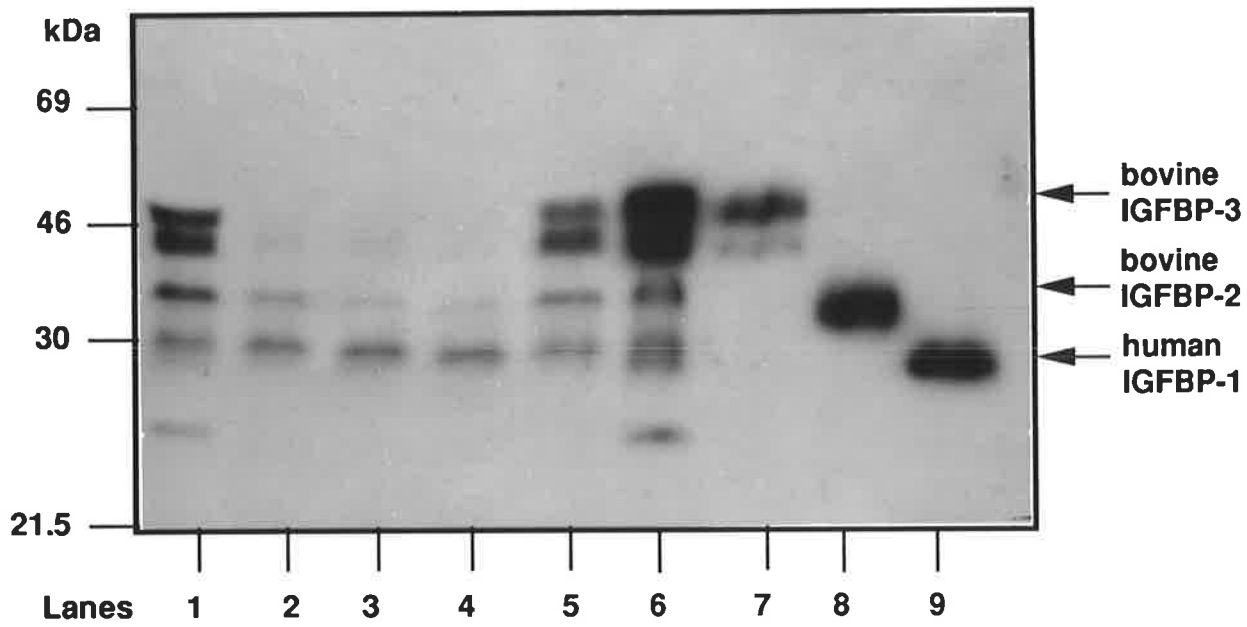
Serum IGF-I results were analysed by one-way analysis of variance, and the means compared using the Newman-Keuls test (Montgomery, 1983). Data analysis was performed using Microstat II statistical packages (Ecosoft Inc., Indianapolis, IN, U.S.A.).

5.3 RESULTS

To characterize the different classes of circulating IGFBP, sera from women at a similar stage of pregnancy were pooled and subjected to Western-ligand blot analysis. An autoradiographic profile of sequential samples is shown (Fig. 5.1). Sera from non-pregnant women produced a 40-50 kDa triplet that aligned with bovine IGFBP-3 standard, a 36 kDa IGFBP, a 28 kDa IGFBP aligning with human IGFBP-1 standard and a 24 kDa IGFBP (Fig. 5.1, lane 6). Serum collected during early pregnancy and 3-4 days postpartum contained similar bands, but at lower labelling intensities (Fig. 5.1, lanes 1 & 5, respectively). After weeks 7-8 of pregnancy, the 40-50 kDa triplet and the

Figure 5.1: Western-ligand blot of serum samples from non-pregnant, pregnant and lactating women.

Serum were pooled from 7-10 women at similar stages of pregnancy and subjected to Western-ligand blot analysis. The nitrocellulose was probed with ^{125}I-labelled IGF-II and exposed to X-ray film for 7 days. The ^{14}C molecular mass markers are shown. Lanes 1-6 contain 5 µl of serum at different stages of pregnancy. Lane 1, weeks 7-8; lane 2, the first trimester, weeks 16-21; lane 3, the second trimester, weeks 27-30; lane 4, the third trimester, weeks 33-38; lane 5, postpartum, 3-4 days; and lane 6, non-pregnant. Lane 7-9 contain 10 ng of purified bovine IGFBP-3, bovine IGFBP-2 and human IGFBP-1, respectively.


kDa
69
46
30
21.5
Lanes 1 2 3 4 5 6 7 8 9
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1

36 kDa IGFBP became undetectable. Only the 28 kDa IGFBP was evident and the intensity of labelling of this band increased until delivery (Fig. 5.2, lanes 2-4). Analysis of these bands by Western-immunoblotting with either human IGFBP-3 or IGFBP-1 was unsuccessful due to high non-specific binding of the antisera and lack of sensitivity even though different incubation conditions and several different second antibody detection systems were used (data not shown).

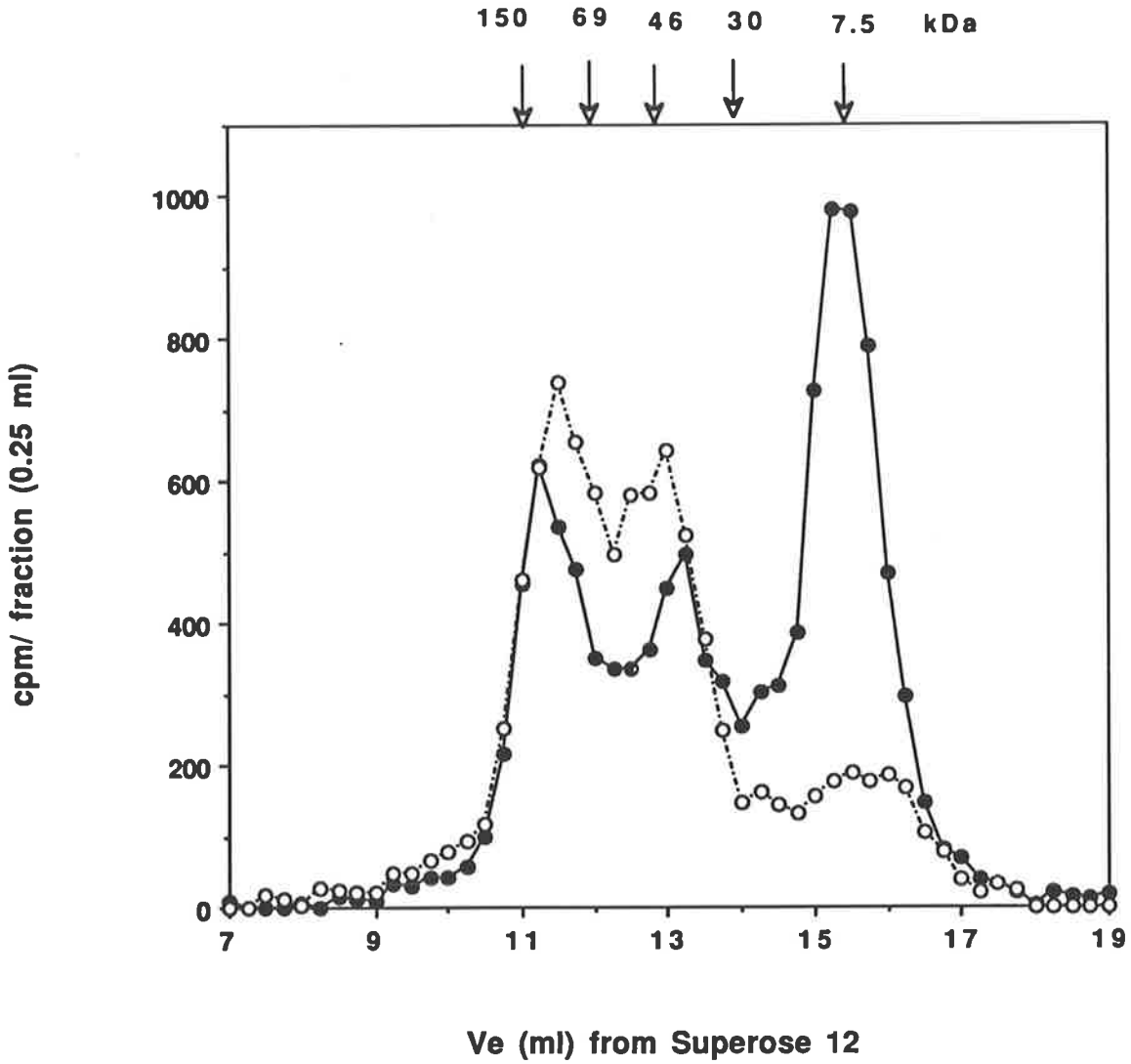
To determine the molecular mass of binding proteins under neutral, non-dissociating conditions, the same serum pools were labelled *in vitro* with either ^{125}I-labelled IGF-I or ^{125}I-labelled IGF-II and subjected to size-exclusion chromatography at pH 7.4 (Fig 5.2 & 5.3, upper panel). Sera from non-pregnant women chromatographed as three ^{125}I-IGF-containing regions: a 150 kDa IGFBP complex eluting between 11 and 12.75 ml; a 40-50 kDa IGFBP complex eluting between 13 and 14.75 ml; and a free IGF region eluting between 15 and 17.5 ml (Fig. 5.2, upper panel). Sera from women in the third trimester chromatographed predominantly as a 150 kDa IGFBP complex and a reduced 40-50 kDa IGFBP complex (Fig. 5.3, upper panel). The specificity of binding *in vitro* was demonstrated by the addition of unlabelled IGF-I or IGF-II. In preliminary experiments, excess unlabelled IGF-I and IGF-II displaced the radioactivity from both the 150 kDa and 40-50 kDa IGFBP complexes and increased the radioactivity in the free IGF region (data not shown).

The IGF-labelled IGFBP complexes were further characterized by subjecting the neutral Superose 12 fractions to Western-ligand blot analysis (Fig. 5.2 & 5.3, lower panel). In sera from non-pregnant women, the 150 kDa IGFBP complex Western-ligand blotted as a 40-50 kDa doublet that aligned with bovine IGFBP-3 standard. The 40-50 kDa IGFBP complex of the Superose 12, Western-ligand blotted as fainter IGFBP bands of 36 kDa, 28 kDa and 24 kDa (Fig. 5.2, lower panel). In contrast, sera from women in late pregnancy lacked the 40-50 kDa IGFBP-3 doublet arising from the 150 kDa IGFBP complex and lacked the 36 kDa and 24 kDa IGFBP bands shown to be derived from the 40-50 kDa IGFBP complex in serum of non-pregnant women. Only the 28 kDa band

Figure 5.2: Western-ligand blot analysis of IGF-binding proteins in sera from non-pregnant women after fractionation on a Superose 12 size-exclusion column at neutral pH.

Upper panel : Sera from 7 non-pregnant women were pooled, incubated *in vitro* with ^{125}I-labelled IGF-I or IGF-II and applied to a Superose 12 column at pH 7.4. The distribution of radioactivity for ^{125}I-labelled IGF-I (●) or IGF-II (○) in serum from non-pregnant women. The Superose 12 standards represented at the top of the figure are: 150 kDa, gamma globulin; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase; and 7.5 kDa, ^{125} I-IGF-II.

Lower panel: Sera were pooled from seven non-pregnant women and chromatographed through a size-exclusion column at pH 7.4. Aliquots (25 µl) of 0.5 ml fractions from the Superose 12 column were subjected to Western-ligand blot analysis. Lane 1 contains the radiolabelled (^{14}C) molecular mass markers; lane 2, a subsample of the fractionated sera (2 µl); lanes 3-14, a 25 µl aliqout of fractions from the Superose 12 size-exclusion column eluting between 11.5 ml-16 ml; and lane 15, 10 ng of purified bovine IGFBP-3, bovine IGFBP-2 and human IGFBP-1. The autoradiograph was exposed to X-ray film for 7 days.


150
69
46
30
7.5
kDa
1000
800
600
400
200
0
cpm/ fraction (0.25 ml)
7
9
11
13
15
17
19
Ve (ml) from Superose 12

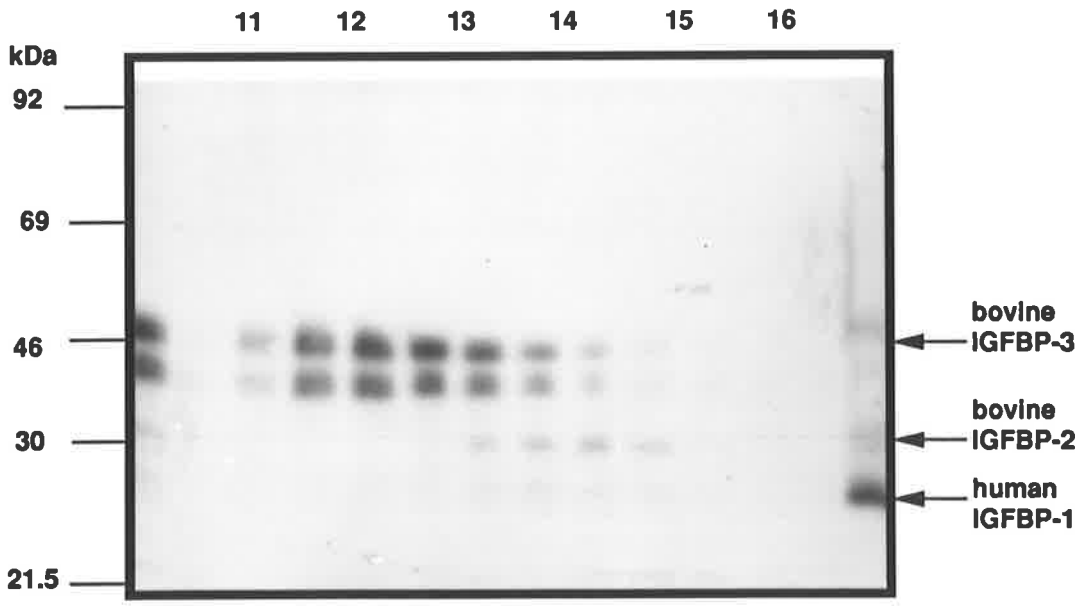
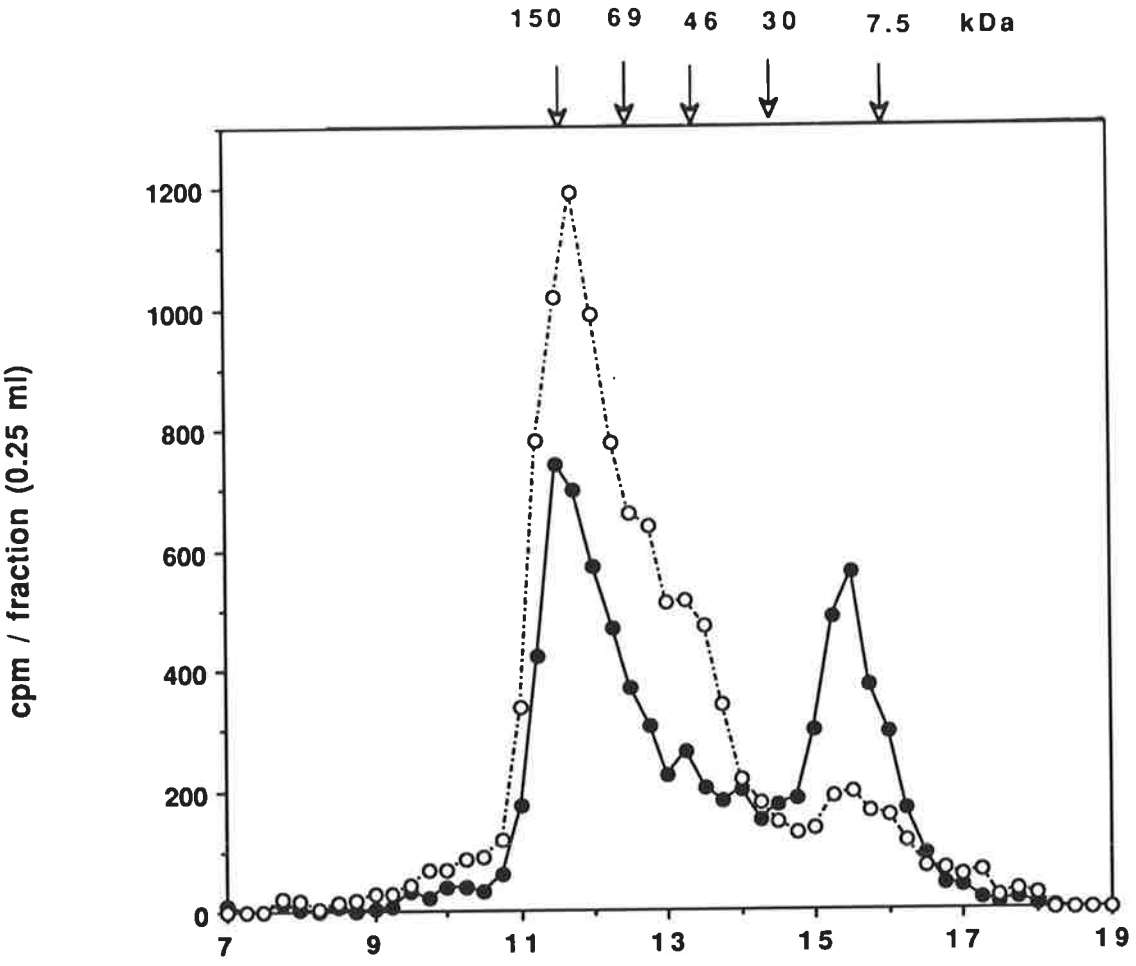
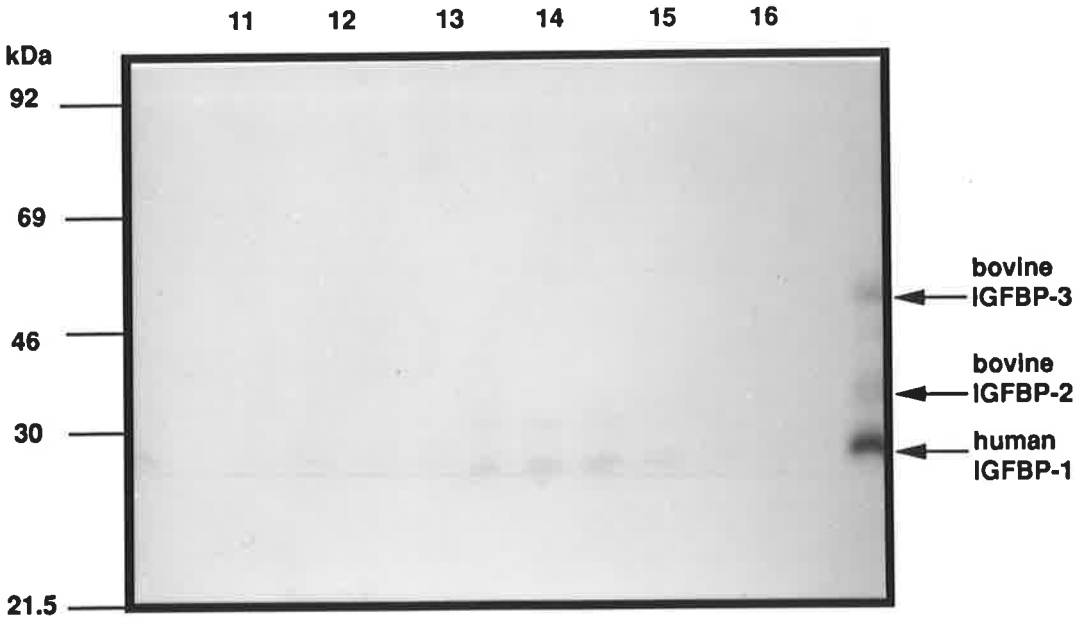

11
12
13
14
15
16
kDa
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1

Figure 5.3: Western-ligand blot analysis of IGF-binding proteins in sera from women in late pregnancy after fractionation on a Superose 12 size-exclusion column at neutral pH.

Upper panel : Sera from 10 women between weeks 33-38 of pregnancy were pooled, incubated *in vitro* with ^{125}I-labelled IGF-I or IGF-II and applied to a Superose 12 column at pH 7.4. The distribution of radioactivity for ^{125}I-labelled IGF-I serum (●) or IGF-II (○) is shown. The size-exclusion column standards represented at the top of the figure are: Vo, void volume; 150 kDa, gamma globulin; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase; and 7.5 kDa, ^{125} I-IGF-II.

Lower panel: serum from women in late pregnancy were pooled and chromatographed through a size-exclusion column at pH 7.4. Aliquots (25 µl) of 0.5 ml fractions from the Superose 12 column were subjected to Western-ligand blot analysis. Lane 1 contains the radiolabelled (^{14}C) molecular mass markers; lane 2, a subsample of the fractionated sera (2 µl); lanes 3-14, a 25 µl aliqout of fractions from the Superose 12 size-exclusion column eluting between 11.5 ml-16 ml; and lane 15, 10 ng of purified bovine IGFBP-3, bovine IGFBP-2 and human IGFBP-1. The autoradiograph was exposed to X-ray film for 7 days.


150
69
46
30
7.5
kDa
1200
1000
800
600
400
200
0
cpm / fraction (0.25 ml)
7
9
11
13
15
17
19
Ve (ml) from Superose 12


11
12
13
14
15
16
kDa
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1

derived from the 40-50 kDa IGFBP complex was evident (Fig. 5.3, lower panel).

IGFBP and IGF are closely associated in the circulation, yet most of the IGFBP could not be detected by Western-ligand blot analysis during late pregnancy. To address this point, IGF concentrations were measured in serum fractionated by size-exclusion chromatography at pH 2.8 (Fig. 5.4). In sera from non-pregnant women, a high molecular mass activity eluted from the column between 7 and 8.25 ml, which represented the IGFBP. The IGFBP are detected because their association with IGF radioligands reduces the radioactivity bound to antibodies or receptors in the IGF assays. In pregnant and lactating women, the serum IGFBP activity was low or undetectable in either assay. To further characterise the IGFBP, fractions corresponding to the 7-8.5 ml region were subjected to Western-ligand blot analysis (Fig 5.5). In sera of non-pregnant women this IGFBP region contained: a 40-50 kDa IGFBP-3 doublet; a 36 kDa IGFBP; a 28 kDa IGFBP; and a 24 kDa IGFBP (Fig. 5.5, upper panel). In contrast, the 40-50 kDa IGFBP was undetectable and the 36 kDa IGFBP very faint in the same fractions from women in late pregnancy (Fig. 5.5, lower panel).

A third peak, intermediate peak eluting between 8-9 ml was detected only in sera from pregnant and lactating women by IGF-II RRA (Fig. 5.1). In sera from women in late pregnancy, fractions corresponding to the intermediate peak Western-ligand blotted as extremely faint bands of 30 kDa, 24 kDa and 21 kDa. These smaller IGFBP were not seen in sera from non-pregnant women (Fig. 5.5, lower panel).

The IGF-I and IGF-II activity eluted between 9.25 to 11 ml, the same position as IGF standards. Summation of the activity in this region showed that the concentration of IGF-II was consistently higher than IGF-I (Table 5.1). The IGF-II levels increased in early pregnancy and peaked in the second trimester, after which they declined to non-pregnant levels at 3-4 days postpartum. The sum of IGF-I activity also showed a progressive increase that peaked in the third trimester and declined 3-4 days postpartum (Table 5.1). Measurement of IGF-I concentrations by acid column chromatography versus acid-ethanol extraction were compared. IGF-I concentrations

Figure 5.4: Size-exclusion chromatography at pH 2.8 of sera pooled from non-pregnant, pregnant and lactating women followed by IGF-I RIA and IGF-II RRA.

200 µl of serum was diluted to 40 % (v/v) in mobile phase pH 2.8 and chromatographed. Fractions (0.4 ml) were assessed for IGF-I (●) and IGF-II (○) levels. The assay limit of detection for IGF-I ranged from 20-42 ng/ fraction and for IGF-II ranged from 0.1-0.2 ng/ fraction.

I wish to acknowledge Ms. K. Moyse's technical assistance in these assays.

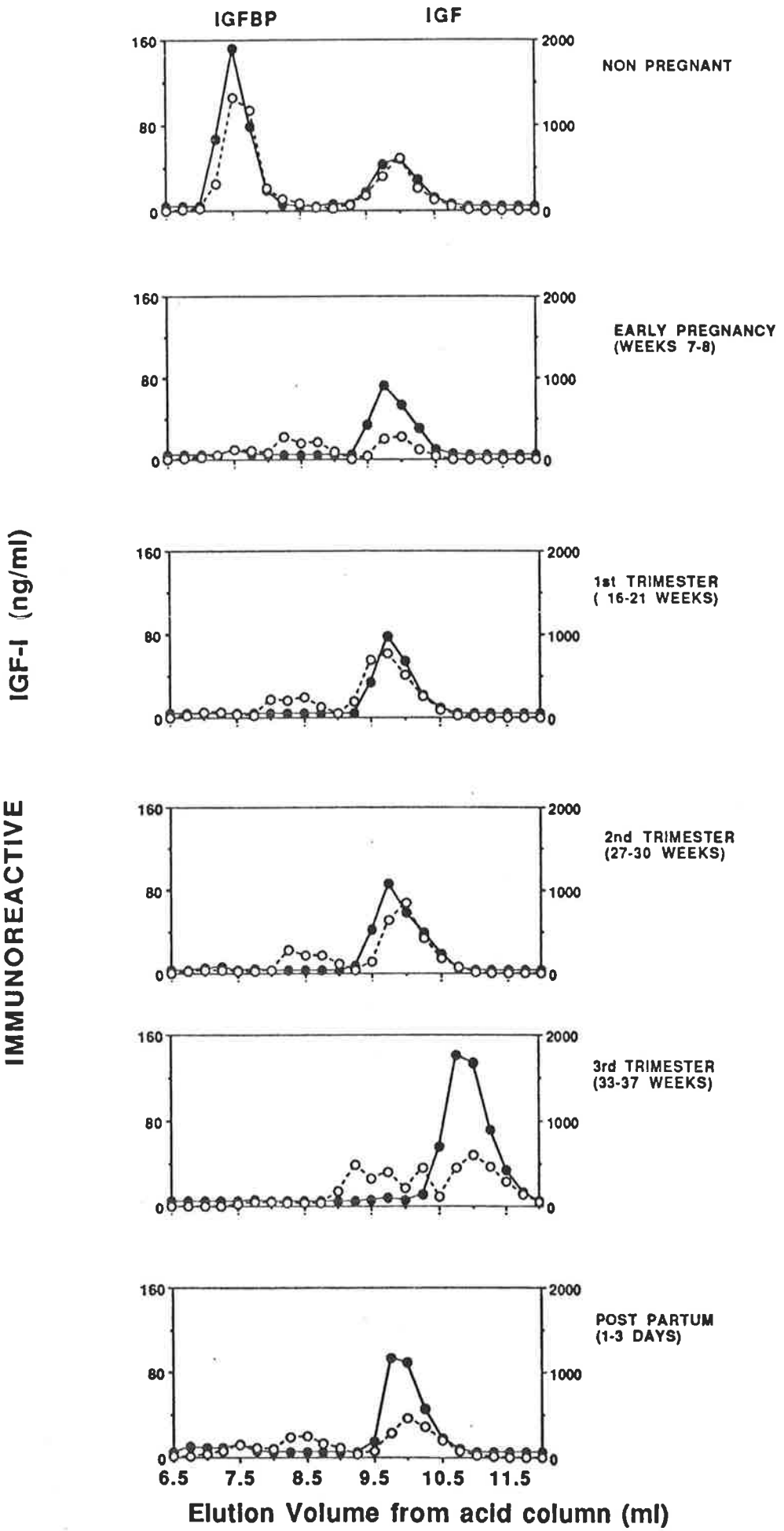
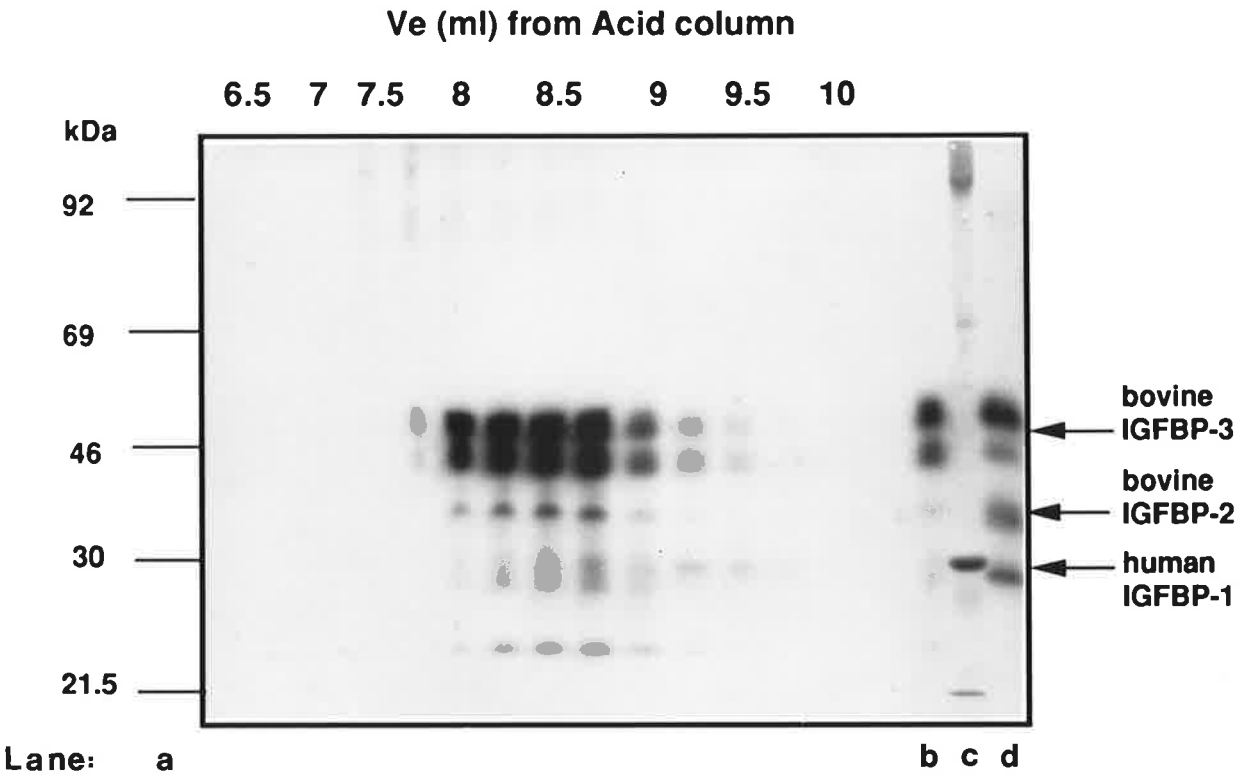

IGFBP
IGF
NON PREGNANT
EARLY PREGNANCY
(WEEKS 7-8)
1st TRIMESTER
(16-21 WEEKS)
2nd TRIMESTER
(27-30 WEEKS)
3rd TRIMESTER
(33-37 WEEKS)
POST PARTUM
(1-3 DAYS)
160
80
0
2000
1000
0
IMMUNOREACTIVE
IGF-I (ng/ml)
RADIORECEPTOR ACTIVE
IGF-II (ng/ml)
6.5
7.5
8.5
9.5
10.5
11.5
Elution Volume from acid column (ml)

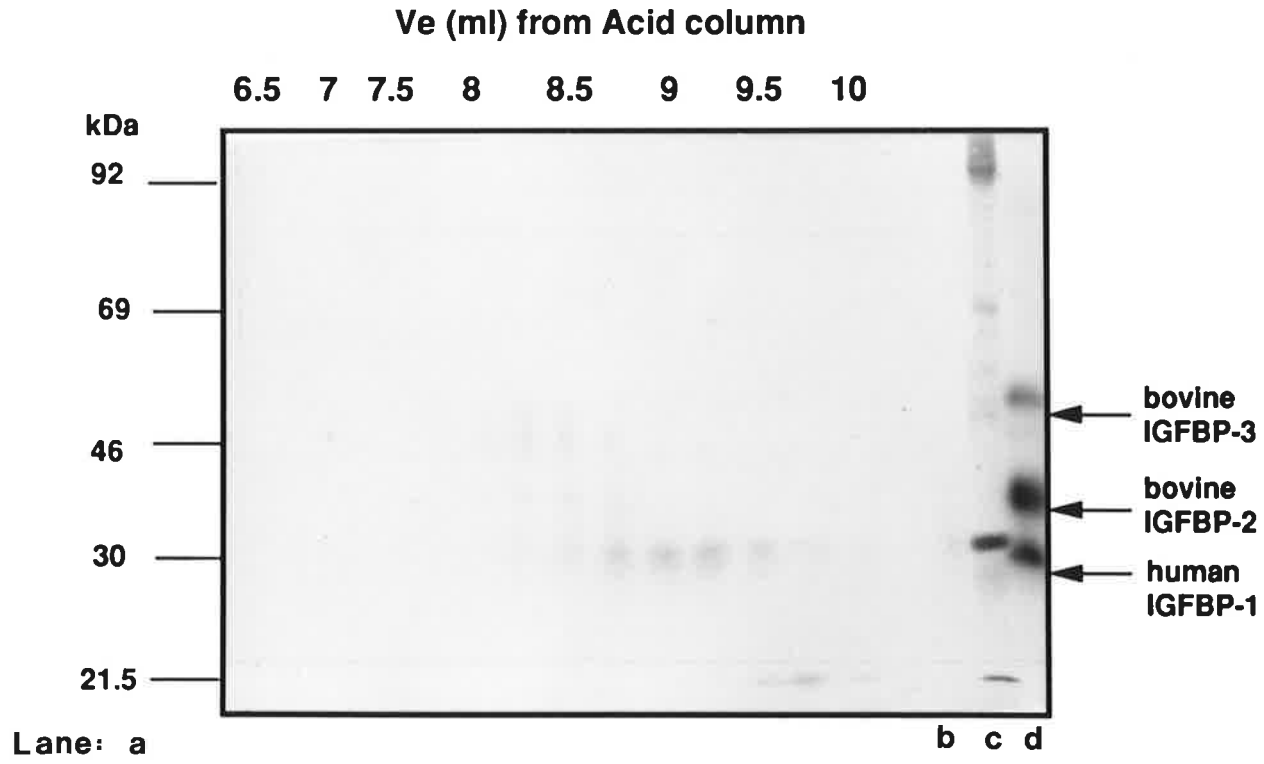
Figure 5.5: Western-ligand blot analysis of sera fractionated by size-exclusion chromatography at pH 2.8.

Upper panel: Serum pooled from non-pregnant women (n = 7).

Serum (200 µl) was diluted to 40 % (v/v) and chromatographed. 0.25 ml fractions of three consecutive chromatographs were collected and pooled. 50 µl of each 0.75 ml fraction was neutralised with 10 µl Tris (1.2 M) and incubated with 15 µl SDS loading buffer. 30 µl (6 µl serum equivalent) was subjected to Western-ligand blot analysis. Lanes marked "a" and "c" contain ^{14} C molecular mass markers, lane "b" contains serum (3 µl) and lane "d" contains bovine IGFBP-3, bovine IGFBP-2 and human IGFBP-1 (10 ng each). The remaining lanes contain fractions between 6.5-10 ml. The autoradiograph was probed with radiolabelled IGF-II and exposed to X-ray film for 30 days.

Lower panel: Serum pooled from women in the third trimester of pregnancy (n = 10). Serum was treated as described above.


Ve (ml) from Acid column
6.5 7 7.5 8 8.5 9 9.5 10
kDa
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1
Lane: a b c d


Ve (ml) from Acid column
6.5 7 7.5 8 8.5 9 9.5 10
kDa
92
69
46
30
21.5
bovine IGFBP-3
bovine IGFBP-2
human IGFBP-1
Lane: a b c d

determined by acid column analysis were consistently higher than those found by acid-ethanol extraction of individual sera, but a similar pattern of change with pregnancy and lactation were observed with both techniques (Table 5.1).

Western-ligand blot analysis and chromatography at pH 2.8 showed very low or undetectable levels of the IGFBP, especially IGFBP-3. If IGFBP levels are really low while the IGF-I and IGF-II concentrations are increased, then less IGF-I and IGF-II would circulate as part of the IGFBP complexes and more would circulate free. Consequently, the amounts of IGF-I and IGF-II in the two major IGFBP size-classes and in the free pool were measured in the same serum pools of non-pregnant and pregnant women. Serum was labelled *in vitro* with ^{125}I-IGF-I or ^{125}I-IGF-II and subjected to Superose 12 size-exclusion chromatography at pH 7.4 (Fig. 5.6 a). The distribution of radiolabelled IGF was consistent with previous results. Unlabelled serum was subjected to size-exclusion chromatography at pH 7.4. The fractions were collected, acidified and applied to a size-exclusion column at pH 2.8. The IGF-I and IGF-II content of each fraction was then measured. In sera from non-pregnant women, both IGF-I (Fig. 5.6 b) and IGF-II (Fig. 5.6 c) were present predominantly in the 150 kDa complex. Most of the remaining IGF-I and IGF-II were found in the 40-50 kDa region of the Superose 12 fractions and relatively little IGF was "free" (Fig. 5.6). Similarly, in sera from women in the third trimester of pregnancy, both IGF-I (Fig. 5.6 b) and IGF-II (Fig. 5.6 c) were also carried predominantly by the 150 kDa region, whilst the remaining IGF was carried by the 40-50 kDa region. Some "free" IGF-I and IGF-II were also detected (Fig. 5.6). Overall there was an *increase* during pregnancy in the quantity of IGF-I and IGF-II present in the 150 kDa complex. The sum of IGF-I and IGF-II concentrations in the 150 kDa IGFBP complex in sera from women in late pregnancy was greater than in sera from non-pregnant women (Fig. 5.6 d).

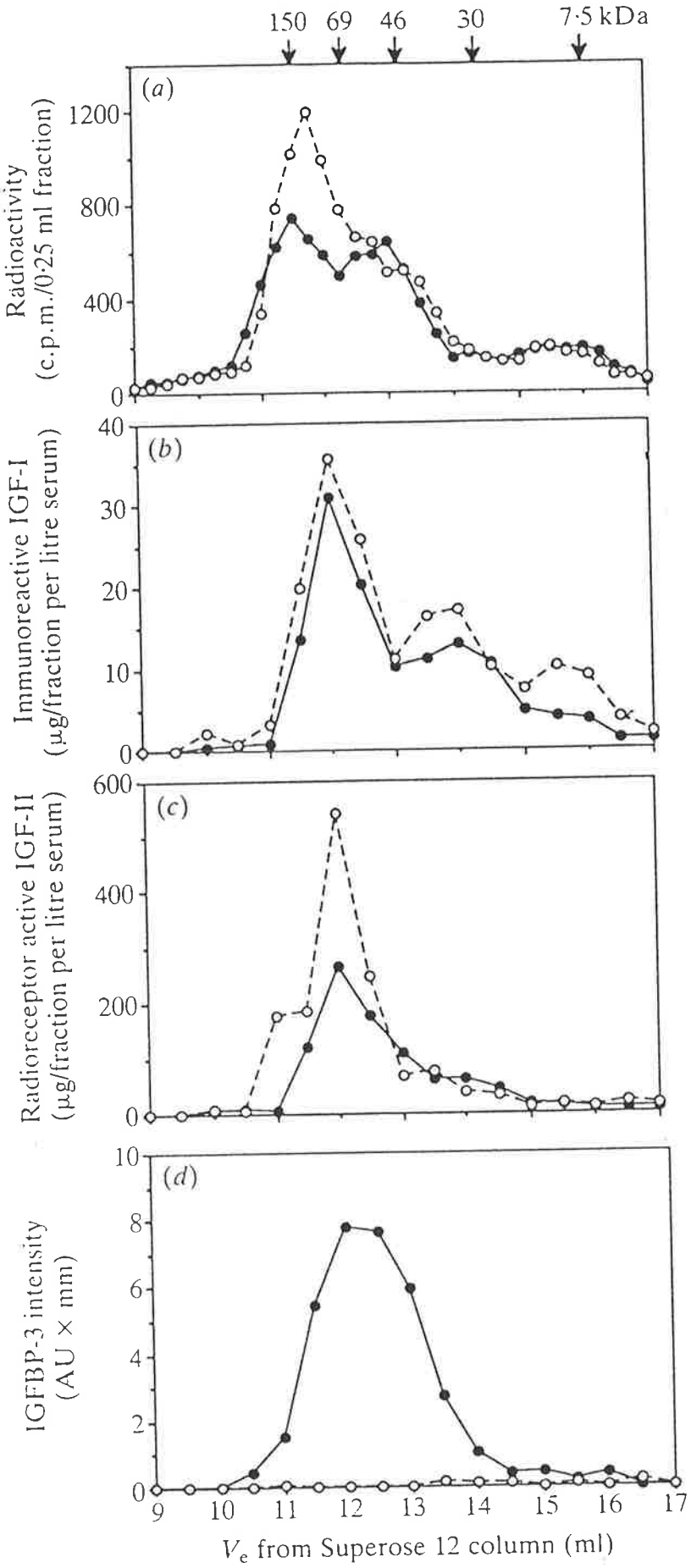
Figure 5.6: IGF-I, IGF-II and IGFBP in sera pooled from non-pregnant (n=10) and pregnant (n=7, 33-38 weeks gestation) women.

Panel A: Size distribution of radioactivity in serum incubated overnight with iodinated IGF-II and then fractionated on a Superose 12 size-exclusion at pH 7.4.

Panel B: Unlabelled sera were subjected to size-exclusion chromatography at pH 7.4 and then individual fractions were further chromatographed at pH 2.8. The resulting IGF-containing fractions from the acid chromatograph were assayed for IGF-I.

Panel C: IGF-II assay of the same fractions shown in panel B.

Panel D: Sera that had been fractionated by Superose 12 (panel A) was subjected to Western-ligand blot analysis and the intensity of the 46 kDa doublet on the autoradiograph was measured by densitometric scanning. Both gels were prepared and treated identically, and the IGFBP-3 standard loaded on each gel gave similar intensity values.



	non-preg (μg/l sera)	preg (μg/l sera)
11-13 ml:	71	92
13-15 ml:	47	73
15-17 ml:	21	31

	non-preg (μg/l sera)	preg (μg/l sera)
11-13 ml:	548	924
13-15 ml:	232	225
15-17 ml:	57	35

Table 5.1: Serum IGF-I and IGF-II concentrations during pregnancy and lactation in women.

	ng/ ml		
Group (number of subjects)	**Acid-ethanol extraction (means ± SEM)**	**Acid column chromatography of serum pools**	
	IGF-I	**IGF-I**	**IGF-II**
Non-pregnant (n=7)	122 ± 23	160	1637
Early pregnancy weeks 7-8 (n=10)	201 ± 30	210	741
First Trimester weeks 16-21 (n=9)	183 ± 21	204	2205
Second Trimester weeks 27-30 (n=10)	222 ± 41	253	2316
Third Trimester weeks 33-38 (n=10)	349 ± 56	458	2134
Non-pregnant, lactating (n=10) 3-4 days postpartum	203 ± 34	274	1496

Serum IGF-I measured by acid-ethanol extraction of single serum samples from each of 56 women were analysed by one-way analysis of varience. IGF-I was higher in pregnant than non-pregnant women ($P < 0.005$) and greatest in the third trimester as determined by t-test after Bonferroni adjustment of critical values for multiple comparisons ($P < 0.01$).

Serum IGF-I and IGF-II were calculated by summing the area in the low molecular mass fractions (9.25-11 ml) after size-exclusion chromatography at pH 2.8 of serum pooled from each group (Fig. 5.1).

These analysis were made by Ms. K. Moyse under the supervision of Dr. P. Owens.

5.4 DISCUSSION

In the present study, IGF-I, IGF-II and IGFBP were measured in the sera of women before, during and after pregnancy. In addition, the molecular mass distributions of immunoreactive IGF-I and receptor-active IGF-II, as well as the types of IGFBP detected by Western-ligand blotting in different size classes of IGF-IGFBP complexes were assessed in sera of non-pregnant and pregnant women.

Maternal serum IGF-I and IGF-II levels were measured by both acid-ethanol extraction and the more rigorous removal of IGFBP by size-exclusion chromatography at pH 2.8 showed a similar pattern: maternal serum IGF-I and IGF-II concentrations were elevated during pregnancy and declined postpartum. This was consistent with previous reports of elevated serum IGF-I and IGF-II (Bala *et al.*, 1981; Wilson *et al.*, 1982; Hall *et al.*, 1984).

Analysis of IGFBP in sera from non-pregnant women by size-exclusion chromatography and Western-ligand blot analysis showed that the 150 kDa complex contained IGFBP-3 (40-50 kDa doublet on SDS gel electrophoresis) and that the 40-50 kDa region contained IGFBP of the 34 kDa, 28 kDa and 24 kDa classes. Identically treated rat sera showed similar results (Gargosky *et al.*, 1990b). Also, Hardouin *et al.* (1987) have examined human sera by Western-ligand blot and found that the 150 kDa complex contained a 40-50 kDa IGFBP-3 while the 40-50 kDa complexes contained 34, 28 and 24 kDa IGFBP.

The IGF content of the IGFBP and free regions was determined in sera from non-pregnant women. I found that both IGF-I and IGF-II were predominantly found in the 150 kDa region while the remainder was carried by the 40-50 kDa and very little was free. Thus, in sera from non-pregnant women, IGFBP-3 is present as a 150 kDa complex that carries the majority of the circulating IGF.

In contrast to the situation in non-pregnancy serum, when serum from women in late pregnancy was examined by techniques involving denaturing conditions, I observed severely diminished levels of IGFBP-3 in the 150 kDa complex as well as

smaller IGFBP. These results agree with our previous observations in both the human and rat (Chapter 3), as well as those of Hossenlopp *et al.* (1990) and Guidice *et al.* (1990) with unfractionated serum from pregnant women. One explanation is based on the recent discovery of a pregnancy-associated IGFBP-protease found in the sera of pregnant rats and humans. The protease has been found to degrade IGFBP-3 into smaller fragments of 34 kDa and 26-20 kDa (Hossenlopp *et al.*, 1990; Guidice *et al.*, 1990), which display a reduced affinity for IGF-II and will not bind IGF-I (Binoux *et al.*, 1991). I have consistently found that these IGFBP fragments of 30, 26 and 24 kDa show a preferential binding to IGF-II, measured by interference in the RRA and Western-ligand blots. Thus, the intermediate peak seen following size-exclusion chromatography at pH 2.8 combined with either an IGF-II RRA or Western-ligand blot analysis may be proteolysed fragments of IGFBP-3. However, these smaller bands were not seen in Western-ligand blots of sera fractionated at pH 7.4 or unfractionated. It is possible that the volume of serum loaded and the resolution was inadequate to detect the small bands with low affinity for IGF-II. However, the very low levels of IGFBP-3 during late pregnancy are in conflict with measurement of free versus bound IGF in sera that showed more IGF-I and IGF-II in the 150 kDa complex in pregnancy. The 150 kDa complex is composed of three parts: an acid-labile subunit (90 kDa); IGFBP-3 (50 kDa); and IGF-I or IGF-II (7.5 kDa). The formation of the ternary 150 kDa complex occurs in two steps. First, IGFBP-3 binds IGF-I or IGF-II to form a binary complex. Only then can the acid-labile component bind and the 150 kDa complex form (Baxter, 1988). Thus, very low levels of IGFBP-3 in sera from women in late pregnancy, as indicated by Western-ligand blot analysis and undetectable levels in the IGF assays, should be associated with a decline in the formation of the 150 kDa complex and be accompanied by low IGF-I and IGF-II levels. However, the present study clearly shows that there is more IGF-I and IGF-II in the 150 kDa complex in sera from pregnant women. If IGFBP-3 is an absolute requirement in the formation of the 150 kDa complex in pregnancy then the increase in IGF-I and IGF-II during late pregnancy must be

accompanied by an overall increase in IGFBP-3 levels. Interestingly, this pregnancy-associated increase in IGFBP-3 is not detectable by Western-ligand blot analysis or by size-exclusion chromatography under acidic conditions. Baxter and Martin (1986) have reported concentrations of immunoreactive IGFBP-3 of approximately 0.1 µmol/l IGFBP-3 in sera from non-pregnant women and 0.2 µmol/l in sera from pregnant women. From the total concentrations of IGF-I and IGF-II measured in the 150 kDa complex in repeated analysis of these pooled sera in the present study, and assuming equimolar amounts of IGFBP-3 in this complex, I calculated there should be between 0.09-0.1 µmol/l of IGFBP-3 in sera from non-pregnant women and 0.17-0.19 µmol/l IGFBP-3 in sera from pregnant women.

The apparent inconsistencies in the literature and our contradictory observations of high levels of IGF-I and IGF-II, and high levels of IGFBP-3 as part of the 150 kDa complex, but low levels of IGFBP as detected by Western-ligand blot analysis, can be explained as altered affinity or stability of IGFBP-3 during pregnancy. A pregnancy-associated form of IGFBP-3, or a post translationally modified IGFBP-3 may be more susceptible to IGFBP-protease(s). Alternatively, normal IGFBP-3 may be modified by proteases during human pregnancy such that its ability to renature after treatment with acid or SDS is reduced. The present study shows clearly that the structural integrity of IGFBP-3 as part of the 150 kDa complex is maintained even in pregnancy.

In conclusion, IGF-I, IGF-II and IGFBP-3 (as judged by IGF content of the 150 kDa complex) are elevated in the serum of women in late pregnancy. However, the IGFBP-3 form found in the circulation during pregnancy may have reduced affinity for IGF or reduced stability rendering it virtually undetectable by assays that are dependent on the ability of IGFBP-3 to bind IGF.

The physiological relevance of increased concentrations of IGF-I, IGF-II, and IGFBP-3 of reduced stability or affinity is unclear, but these changes may combine to increase the bioavailability of the IGF.

CHAPTER SIX: SUMMARY AND PROSPECTS

6.1 SUMMARY

In this chapter of my dissertation, I have summarised my PhD research on the IGF in the field of pregnancy and speculated on the physiological significance of this work. Suggestions for the future direction of research in this area are presented in section 6.2.

In the past decades the insulin-like growth factors (IGF) have been of scientific and monetary interest due to their anabolic potential and have been thought to play a role in the regulation of mammalian cellular growth. The ability of IGF to promote growth has been recently confirmed by *in vivo* studies with increased skeletal growth, body weight gain and nitrogen retention (Hizuka *et al.,* 1986; Phillips *et al.,* 1988; Lemmy *et al.,* 1991; Tomas *et al.,* 1991a,b). *In vitro* studies have shown that IGF can increase net protein accumulation, glucose storage and promote cellular proliferation and differentiation.

In the past five years, six IGF binding proteins (IGFBP) have been characterized and shown to modulate IGF activity. They are classified as IGFBP-1 to IGFBP-6. The IGFBP can modulate the biological activity of IGF by either sequestering the IGF and inhibiting its action, or promoting the presentation of IGF to the cell. The IGFBP added a level of complexity to the regulation of IGF-induced growth.

Much of my PhD research involved investigations of the roles of IGF and IGFBP during pregnancy. Requirements for energy and nutrients are increased during pregnancy such that the maternal metabolism and nutrient distribution are modified. In the early phase of pregnancy the demands of the conceptus are relatively small. This period, referred to as the anabolic phase, is characterized by weight gain and deposition of fats and protein (Metcalfe *et al.,* 1988). The middle to late phase of pregnancy is a period of increased placental hormone production (placental lactogens, chorionic gonadotropins, hypothalamic peptides) as well as rapid fetal growth. This period is referred to as the catabolic phase of pregnancy. Thus, in the rat the maternal carcass, which has increased during early pregnancy is partially catabolized (Metcalfe *et al.,*

1988). Hence, during pregnancy the maternal metabolism is shifted to first deposit nutrients and energy stores and then to transfer these stores to the fetus. This alteration may be regulated, in part, by IGF and IGFBP which have been shown to regulate and promote growth.

The IGF and IGFBP concentrations in the maternal circulation may play a role in fetal growth and development (Davenport *et al.*, 1990; Guidice *et al.*, 1990).

The research presented in this dissertation investigated the role of IGF and IGFBP during pregnancy in the rat and human.

1. RAT

The rat was examined initially because: mating can be easily detected and timed; the gestation period is relatively short (21 days); handling and routine blood sampled could be achieved; and invasive techniques could be used.

In chapter two the IGFBP found in biological fluids and in media conditioned by cells were characterised following the establishment and modification of techniques such as: Western-ligand blot analysis; size-exclusion chromatography; and immunological analysis. The IGFBP analysed were diverse in both molecular mass as well as affinities for IGF.

In chapter three, I reported that IGF-I, measured by RIA, was the predominant circulating IGF in the maternal circulation. I could not detect IGF-II by RRA. Donovan *et al.* (1991) have reported extremely low levels of IGF-II, measured by RIA, which did not change with pregnancy. The IGF-I concentration increased during pregnancy until day 13, after which IGF levels declined. One to three days postpartum the IGF-I concentrations returned to non-pregnant levels. The decline in IGF-I concentrations may be due to an increased rate of clearance or a reduced rate of IGF-I mRNA transcription in the liver (Davenport *et al.*, 1990). In parallel with the fall in IGF-I levels, IGFBP concentrations declined at day 13 and recovered 1-3 days postpartum. The decline of IGFBP and their carrying capacity for IGF-I involved principally IGFBP-

3, which normally carries up to 80 % of the circulating IGF-I. Nonetheless, the hepatic mRNA levels of IGFBP-3 in the rat are unchanged (Donovan *et al.*, 1991).

The decline of serum IGFBP-3 has been reported to be due to a very recently-discovered pregnancy-specific IGFBP-protease that degrades the IGFBP-3 into small fragments. These fragments are difficult to detect by Western-ligand blotting due to their reduced affinity for IGF (Davenport *et al.*, 1990; Binoux *et al.*, 1991). Since uncomplexed IGF is rapidly cleared from the circulation (Ballard *et al.*, 1991), the decline in IGFBP would potentiate the increased clearance of IGF-I.

The physiological consequence of IGFBP degradation and reduced circulating levels of IGF-I may be to modulate the maternal metabolism: high IGF-I concentrations coincided with the anabolic period; and low IGF-I concentrations and low IGFBP with the catabolic phase. Thus, the interplay between IGF-I and IGFBP concentrations may regulate the maternal metabolism and affect nutrient repartitioning to the fetus.

To examine the hypothesis that during pregnancy in the rat, maternal IGF-I levels modulate the nutrient distribution, rats were infused during the second half of pregnancy (day 11-21) with either IGF-I or growth hormone (GH). This work was reported in chapter four.

Administration of rhGH did not elevate IGF-I or IGFBP-3 levels and did not change maternal, fetal or placental weights. Infusion of rhIGF-I significantly increased circulating IGF-I levels, weakly induced IGFBP-3 and a 32 kDa IGFBP, and most notably increased the rate of maternal weight gain by 27 %.

This was the first reported study of IGF-I or GH infusion into pregnant rats. The absence of a GH effect could be due to a species effect of GH as human GH displays lactogenic properties in the rat and may have been sequestered by mammary tissues, or may be due to GH resistance during late pregnancy. This has been discussed in chapter four. The growth effects of IGF-I suggest that infusion of the growth factor during the late, catabolic stage of pregnancy might redirect nutrients to the dam without altering conceptus growth. This protection may have been conferred by one or more of the

following parameters: an increase in food intake; an enhanced efficiency of food conversion as seen in IGF-I-administered rats in other catabolic states (Tomas *et al.*, 1991 a,b); and enhanced or altered placental function. Nonetheless, increased circulating concentrations of IGF in the dam did not influence fetal or placental growth.

2. HUMAN

In chapter 5, the circulating concentrations of IGF and IGFBP were measured during human pregnancy.

IGF-I and IGF-II concentrations did not increase until the third or second trimester, respectively, and then declined to reach non-pregnant levels 1-3 days postpartum. This increase in IGF contrasts with the situation in the rat.

An additional complexity was the change in the circulating IGFBP concentrations. Analysis of IGFBP by techniques that relied on the IGFBP binding IGF and involved either acidification or denaturing conditions showed an apparent absence of IGFBP-3 and the smaller IGFBP after week 7 of pregnancy. Techniques that measured IGFBP by either RIA or indirectly by measuring the IGF content of IGFBP under neutral, non-dissociating conditions showed an increase of IGFBP-3 and the smaller IGFBP during late pregnancy.

During late pregnancy, a protease has been reported to degrade the IGFBP-3 to smaller fragments which display a reduced affinity for the IGFs. My results suggest that the stability and affinity of IGFBP-3 is altered during pregnancy possibly by the IGFBP-protease.

The physiological significance of elevated IGF and IGFBP levels during pregnancy in the human is uncertain. Possibly studies on the IGFBP-protease (6.2) can provide some insight into the mechanism of IGF regulation during pregnancy.

These studies raise questions that need to be addressed:

1. What is the physiological significance of the IGFBP-protease?

The susceptibility of the IGFBP to proteolytic degradation implies a role for IGFBP-proteases in the regulation of the biological activity of IGF.

Recently, Campbell and Novak (1991) showed that plasmin, a serine protease, could dissociate IGF from IGFBP and that the presence of plasmin in the media from bone cells could protect IGF from the inhibitory effects of the IGFBP. Plasminogen, which may be converted to plasmin by plasmin activators, is an example of a protease precursor that is elevated during pregnancy (Metcalfe *et al.,* 1988).

Proteolysis of an IGFBP which causes reduced affinity or stability of the IGFBP for IGF would increase the bioavailability of IGF to target tissues. This may lead to the promotion of cellular growth and differentiation. The IGFBP-proteases may provide a mechanism for the regulation of IGF-induced cellular growth. Thus, in catabolic conditions such as states of muscle wasting and nitrogen loss, the interaction between the IGFBP and the protease may act to alleviate the condition by releasing IGF.

2. Why are the IGF and IGFBP concentrations in pregnancy different between the rat and human?

One possibility is that the pregnancy-associated IGFBP-protease is synthesised by a pregnancy-derived tissue ie., the placenta, which has been shown to contain many membrane, lysosomal and golgi-linked proteases (Gossrau *et al.,* 1987). If the placenta is responsible for the concentration of IGFBP-protease in the maternal circulation then a dam with 9 to 16 conceptus may have a greater circulating concentration of protease activity than the human with a single conceptus. This concentration effect in the rat, may explain the extremely low levels of IGFBP-3 in the presence of unaltered hepatic mRNA transcription levels (Donovan *et al.,* 1991).

Alternatively, the energy metabolism and hormonal adaptation during

pregnancy may be quite different in the rat and human. In contrast to humans, the fetal growth rate in the rat is rapid over a short gestation period and food intake is increased in the rat by 40-60 % (Metcalfe *et al.,* 1988). The pregnant rat maintains a relatively large fetal mass when compared to maternal weight (Battaglia & Meschia, 1986). Furthermore, in the rat skeletal growth is reduced during pregnancy, possibly due to the demand of many rapidly growing conceptuses (Chiang *et al.,* 1990). These situations either do not occur or are less pronounced in the human possibly because of the relatively low fetal: maternal weight ratio (Battaglia & Meschia, 1986). The very low levels of IGF-I and IGFBP-3 may be required in the rat to induce a catabolic state such that adequate nutrients and energy can be directed to the many conceptuses. However, the human with a smaller metabolic demand due to a generally single conceptus may have evolved a more subtle mechanism to regulate IGF bioavailability and bioactivity.

6.2 FUTURE WORK

The IGFBP-protease may be a novel modulator of IGF action and subsequently, growth. A project to investigate the IGFBP-protease would further our understanding of the role and regulation of IGF and IGFBP.

1. The development of a quantitative assay to measure IGFBP-protease activity.

The aim would be to develop a sensitive, quantitative assay to measure IGFBP-proteases.

Current reports detect the IGFBP-protease by "mixing" experiments (Davenport *et al.,* 1990; Guidice *et al.,* 1990; Hossenlopp *et al.,* 1990). The assay involves incubation of non-pregnancy serum or a purified IGFBP with pregnancy serum containing the protease activity. The resulting loss of the IGFBP is detected by Western-ligand blot analysis. The studies are not quantitative but do allow an assessment of IGFBP-protease activity. A radioligand assay to measure the IGFBP proteolytic activity in any

sample would be very useful. Such an assay for IGFBP-protease activity could be based on solid-phase radioimmunoassay principles: the sample of interest would be incubated with iodinated IGFBP and then treated with IGF covalently linked to an appropriate carrier. Proteolysed IGFBP has a reduced affinity for IGF and would not precipitate. The difference between bound versus free levels of radioactivity would provide a measure of proteolytic activity. This assay would require some development and optimisation of influences such as temperature, time of incubation, substrate concentration and buffer pH. The linearity and reproducibility of the assay would also need to be further investigated.

The development of this assay would permit a large number of fractions or samples to be routinely assayed. The assay could be faster and simpler than current techniques and very beneficial for quantitative measurement of proteolytic activity.

2. Localisation of the IGFBP-protease in the circulation.

The IGFBP-protease activity has been detected in plasma and serum of pregnant women and rats (Davenport *et al.,* 1990; Gargosky *et al.,* 1990 a,b; Guidice *et al.,* 1990; Hossenlopp *et al.,* 1990). The location and concentration of IGFBP-protease in the circulation is not known.

The aim of this study would be to localise the protease which has been reported in the blood to determine:

1. whether the IGFBP-protease is constitutively expressed in blood cells of non-pregnant and pregnant women, but may not be detectable during non-pregnancy due to low intracellular concentrations or restricted release into the plasma;

2. if the IGFBP-protease is a pregnancy-associated phenomenon, with concentrations being elevated by increased synthesis during pregnancy.

3. in which cells the protease is produced, ie. blood cells, placenta, liver.

To determine the occurrence of IGFBP-protease in the blood cells, mixing

experiment and the specific protease assay which will be developed (Section 1) could be utilized. Blood collected from non-pregnant and pregnant women would be assessed for IGFBP-protease activity. Alternatively, tissues such as placentae collected at term, or liver and kidney specimens at autopsy, could be either cultured *in vitro* or tissue extracts examined for protease activity using the same techniques described above.

3. The biochemical characterisation of the IGFBP-protease.

The IGFBP-protease activity, as detected by mixing experiments is inhibited at pH 4 and limited in the presence of high concentrations of protease inhibitors such as phenylmethane sulphonylfluoride (PMSF) and EDTA, but not leupeptin (Hossenlopp *et al.*, 1990). However little else is known or at least not published.

Further characterization of the IGFBP-protease utilizing size-exclusion and specific affinity columns, substrate specificity, the pH optima and further inhibition experiments would provide biochemical knowledge on the IGFBP-protease.

4. Comparative studies.

The objective of this study would be to compare the IGF and IGFBP concentrations in the blood of pregnant animals with and without protease activity. Sheep have been reported to not express any IGFBP-protease (Guidice & Dsupin, 1991) unlike the human and rat.

I would examine the circulating concentrations of IGF, IGFBP and IGFBP-protease, the occurrence of proteolytic activity, as well as other possible metabolic factors such as different metabolic adaptations, conceptus development and fetal number. This may provide some insight into the physiological significance of the IGFBP-protease and correlate activity to important growth parameters in pregnancy. This study could extend our understanding of the role of IGF in fetal and maternal growth.

I speculate that the analysis of the IGFBP-protease may help our understanding of IGF-induced growth. IGFBP-proteases have been reported in severe illness when a patient is cachexic (Holly *et al.*, 1991) as well as pregnancy. Fundamental research on the IGFBP-protease could in the long term lead to: a greater understanding of the regulation of growth and subsequently permit treatment of a states of reduced growth (ie., intrauterine growth retardation) or states of excessive growth (ie., diabetes); the treatment of catabolic conditions such as burns, cancer and cachexic patients to promote convalescence; or may provide a detection system to detect the loss of cellular anabolism.

REFERENCES

Adamo,M., Lowe,W.L., LeRoith,D. & Roberts,C. (1989). Insulin-like growth factor messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed during development in the rat. Endocrinology **124,** 2737-2744.

Adashi,E.Y., Resnick,C.E., Hernandez,E.R., Svoboda,M.E. & Van Wyk,J.J. (1990). Ovarian IGF-I: Basic concepts leading to pharmaceutical application. Molecular & Cellular Biology of insulin-like growth factors and their receptors. Editors, Le Roth,D. & Raizadsa,M.K. pg.141-152.

Albiston,A. & Herington,A.C. (1990). Cloning and characterisation of the growth hormone-dependent insulin-like growth factor binding protein (IGFBP-3) in the rat. Biochemical and Biophysical Research Communications **166,** 892-897.

Alexandrides,T., Moses,A.C. & Smith,R.J. (1989). Developmental expression of receptors for insulin, insulin-like growth factor I (IGF-I), and IGF-II in rat skeletal muscle. Endocrinology **124,** 1064-1076.

Alitalo,T., Kontula,K., Koistenen,R., Aalto-Setala,K., Julkanen,M., Janne,O.A., Seppala,M. & de la Chapelle,A. (1989). The gene encoding human low molecular weight insulin-like growth factor binding protein (IGFBP-25): regional localisation to 7p12-p13 and description of a DNA polymorphism. Human Genetics **83,** 335-338.

Andress,D.L. & Birnhaum,R.S. (1991). A novel human insulin-like growth factor binding protein secreted by osteoblast-like cells. Biochemical and Biophysical Research Communications **176,** 213-218.

Bala,R.M., Wright,C., Bardai,A. & Smith,G.R. (1978). Somatomedin bioactivity in serum and amniotic fluid during pregnancy. Journal of Clinical Endocrinology and Metabolism **46,** 649-652.

Bala,R.M., Lopatka,J., Leung,A., McCoy,E. & McArthur,R.G. (1981). Serum immunoreactive somatomedin levels in normal adults, pregnant women at term, children at various ages, and children with constitutionally delayed growth. Journal of Clinical Endocrinology and Metabolism **52,** 508-512.

Ballard,F.J., Read,L.C., Francis,G.L., Bagley,C.J. & Wallace,J.C. (1986). Binding properties and biological potencies of insulin-like growth factors in L6 myoblasts. Biochemical Journal **223,** 223-230.

Ballard,J., Baxter,R., Binoux,M., Clemmons,D., Drop,S., Hall,K., Hintz,R., Rechler,M., Rutanen,E. & Schwander,J. (1989). On the nomenclature of the IGF binding proteins. Acta Endocrinologica **121,** 751-752.

Ballard,F.J., Knowles,S.E., Walton,P.E., Edson,K., Owens,P.C., Mohler,M.A. & Ferraiolo,B.L. (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.

Bar,R.S., Booth,B.A., Boes,M. & Dake,B.L. (1989). Insulin-like growth factor-binding proteins from vascular endothelial cells: purification, characterisation, and intrinsic biological activities. Endocrinology **125,** 1910-1920.

Battaglia,F.C. & Meschia,G. (1986). Fetal and placental growth. An Introduction to Fetal Physiology Academic Press,Inc. Harcourt Brace Jovanovich Publishers.

Baumann,G., Shaw,M.A. & Amburn,K. (1989). Regulation of plasma growth hormone-binding proteins in health and disease. Metabolism **38,** 683-689.

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

Baxter,R.C. (1980). Simplified approach to confidence limits in radioimmunoassay. Clinical Chemistry **26,** 763-765.

Baxter,R.C. (1986). The somatomedins: insulin-like growth factors. Advances in Clinical Chemistry **25,** 49-115.

Baxter,R.C. (1988). Characterisation of the acid labile subunit of the growth hormone-dependent insulin-like growth factor binding protein complex. Journal of Clinical Endocrinology and Metabolism **67,** 265-272.

Baxter,R.C. (1990). Glycosaminoglycans inhibit formation of the 140 kDa insulin-like growth factor binding complex. Biochemical Journal **271,** 773-777.

Baxter,R.C. & Cowell,C.T. (1987). Diurnal regulation of growth hormone-independent binding protein for insulin-like growth factors in human plasma. Journal of Clinical Endocrinology and Metabolism **65,** 432-440.

Baxter,R.C. & DeMellow,J.S.M. (1986). Measurement of insulin-like growth factor-II by radioreceptor assay using ovine placental membranes Clinical Endocrinology **24,** 267-278.

Baxter,R.C. & Martin,J.L. (1986). Radioimmunoassay of growth hormone-dependent insulin-like growth factor binding protein in human plasma. Journal of Clinical Investigation **78,** 1504-1512.

Baxter,R.C. & Martin,J.L. (1989a). Structure of the Mr 140,000 growth hormone dependent insulin-like growth factor binding protein complex:determination by reconstitution and affinity labelling. Proceedings of the National Academy of Science of the U.S.A. **86,** 6898-6902.

Baxter,R.C. & Martin,J.L. (1989b). Binding proteins for the insulin-like growth factors : Structure, regulation and function. Progress in Growth Factor Research **1,** 49-68.

Baxter,R.C., Martin,J.L. & Beniac,V.A. (1989). High molecular weight insulin-like growth factor binding protein complex: purification and properties of the acid labile subunit from human serum. Journal of Biological Chemistry **254,** 11843-11848.

Becks,H., Asling,W., Simpson,M.E., Li,C.H. & Evans,H. (1949). The growth of hypophysectomized female rats following chronic treatment with pure pituitary growth hormone. Growth **13,** 175-189.

Bell,G.I., Merryweather,J.P., Sanchez-Pescador,R., Stempien, M.M., Priestley,L., Scott,J. & Rall,L.B. (1984). Sequence of a cDNA clone encoding human preproinsulin-like growth factor-II. Nature **310,** 775-777.

Bell,G.I., Gerhard,D.S., Fong,N.M., Sanchez-Pescador,R. & Rall,L.B. (1985a). Isolation of the insulin-like growth factor genes: insulin-like growth factor genes are contiguous. Proceedings of the National Academy of Science of the U.S.A. **82,** 6450-6454.

Bell,S.C., Patel,S., Hales,M., Kirwan,P.H. & Drife,J.O. (1985b). Immunochemical identification and characterisation of pregnancy-associated endometrial alpha1 and alpha2 globulins secreted by the human endometrium and decidua. Journal of Reproduction and Fertility **74,** 1202-1204.

Bell,G.I., Stempien,M.M., Fong,N.M. & Rall,L.B. (1986). Sequences of liver cDNAs encoding two different mouse insulin-like growth factor I precursors. Nucleic Acid Research **14,** 7873-7882.

Berridge, M. (1985). The molecular basis of communication within the cell. Scientific American **253,** 142-152.

Berrridge,M. (1987). Inositol lipids and proliferation. Biochimica et Biophysica Acta **907,** 33-45.

Binkert,C., Landwehr,J., Mary,J-L., Schwander,J. & Heindrich,G. (1989). Cloning, sequence analysis and expression of a cDNA encoding a novel insulin-like growth factor binding protein (IGFBP-2). The EMBO Journal **8,** 2497-2502.

Binoux,M. & Hossenlopp,P. (1988). Insulin-like growth factor (IGF) and IGF binding proteins: comparison of human serum and lymph. Journal of Clinical Endocrinology and Metabolism **67,** 507-514.

Binoux, M., Hossenlopp, P., Lassare, C. and Sergovia, B. (1991). Degradation of IGF binding proteins by proteases: physiological implications. 2nd International IGF symposium, San Francisco, Keynote Address.

Blum,W.F., Ranke,M.B. & Bierich,J.R. (1986). Isolation and partial characterisation of six somatomedin-like peptides from human plasma Cohn fraction IV. Acta Endocrinologica **111,** 271-284.

Blum,W.F., Jenne,E.W., Reppin,F., Kietzmann,K., Ranke,M.B. & Bierich,J.R. (1989). Insulin-like growth factor-I (IGF-I)-binding protein complex is a better mitogen than free IGF-I. Endocrinology **125,** 766-772.

Blundell,T.L., Bedarkar,S., Rinderknecht,E. & Humbel,R.E. (1978). Insulin-like growth factor: A model for tertiary structure accounting for immunoreactivity and receptor binding. Proceedings of the National Academy of Science of the U.S.A. **75,** 180-184.

Braulke,T., Causin,C., Waheed,A., Junghans,U., Hasilik,A., Maly,P., Humbel,R.E. & von Figura,K. (1988). Mannose 6-phosphate/ insulin-like growth factor II receptor: distinct binding sites for mannose 6-phosphate and insulin-like growth factor II. Biochemical and Biophysical Research Communications **150,** 1287-1293.

Braulke,T., Tippmer,S., Chao,H. & 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.

Brewer,M.T., Stetler,G.L., Squires,C.H., Thompson,R.C., Busby,W.H. & Clemmons,D.R. (1988). Cloning, characterisation, and expression of a human insulin-like growth factor binding protein. Biochemical and Biophysical Research Communications **152,** 1289-1297.

Brinkman,A., Groffen,C.A.H., Kortleve,D.J. & Drop,S.L.S. (1988a). Organisation of the gene encoding insulin-like growth factor binding protein IBP-1. Biochemical and Biophysical Research Communications **157,** 898-907.

Brinkman,A., Groffen,C.A.H., Kortleve,D.J., Geurts van Kessel,A. & Drop,S.L.S. (1988b). Isolation and characterisation of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IBP-1). The EMBO Journal **7,** 2417-2423.

Brissenden,J.E., Ullrich,A. & Franke,U. (1984). Human chromosomal mapping of genes for insulin-like growth factor-I and II and epidermal growth factor. Nature **310,** 781-784.

Brown,A.L., Chiariotti,L., Orlowski,C.C., Mehlman,T., Burgess,W.H., Ackerman,E.J., Bruni,C.B. & Rechler,M.M. (1989). Nucleotide sequence and expression of a cDNA encoding a fetal rat binding protein for insulin-like growth factors. Journal of Biological Chemistry **264,** 5148-5154.

Brown,W.M., Dziegielewska,K.M., Foreman,R.C. & Saunders,N.R. (1990). The nucleotide and deduced amino acid sequences of insulin-like growth factor II cDNAs from adult bovine and fetal sheep liver. Nucleic Acid Research **18,** 4614.

Burgi,H., Muller,W.A., Humbel,R.E., Labhart,A. & Froesch,E.R. (1966). Non-suppressible insulin-like activity of human serum. I. Physiochemical properties, extraction and partial purification. Biochemica et Biophysica Acta **121,**349-359.

Burstein,P.J., Draznin,B., Johnson,C.J. & Schalch,D.S. (1979). The effect of hypothyroidism on growth, serum growth hormone and growth hormone-dependent somatomedin, insulin-like growth factor, and its carrier protein in rats. Endocrinology **104,** 1107-1111.

Busby,W.H., Klapper,D.G. & Clemmons,D.R. (1988a). Purification of a 31,000-dalton insulin-like growth factor binding protein from human amniotic fluid: isolation of two different forms. Journal of Biological Chemistry **263,** 14203-14210.

Busby,W.H., Synder,D.K. & Clemmons,D.R. (1988b). Radioimmunoassay of a 26,000-dalton plasma insulin-like growth factor-binding protein: control by nutritional variables. Journal of Clinical Endocrinology and Metabolism **67,** 1225-1230.

Busby,W.H., Hossenlopp,P., Binoux,M. & Clemmons,D.R. (1989). Purified preparations of the amniotic fluid-derived insulin-like growth factor-binding protein contain multimeric forms that are biologically active. Endocrinology **125,** 773-777.

Campbell,P.C. & Novak,J.F. (1991). The availability of insulin-like growth factor (IGFs) in bone may involve interaction with IGF binding proteins (IGFBPs) and proteases. 2nd International IGF Symposium San Francisco, California, January 12-16. Abstract C50.

Cao,Q., Duguay,S.J., Plisetskaya,E., Steiner,D.F. & Chan,S.J. (1989). Nucleotide sequence and growth hormone-regulated expression of salmon insulin-like growth factor I mRNA. Molecular Endocrinology **3,** 2005-2010.

Carlsson,L., Eden,S. & Jansson,J.-O. (1990). The plasma growth hormone in conscious rats during late pregnancy. Journal of Endocrinology **124,** 191-198.

Carlsson-Skwirut,C., Jornvall,H., Holmgren,A., Andersson,C., Vergman,T., Lundquist,G., Sjogren,B. & Sara,V.R. (1986). Isolation and characterisation of variant IGF-I as well as IGF-2 from adult human brain. FEBS letters **201,** 46-50.

Carlsson-Skwirut,C., Andersson,C. & Sara,V.R. (1987). Circulating forms of human foetal somatomedin. Acta Endocrinologica **114,** 37-40.

Carpenter,G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. Annual Review of Biochemistry **56,** 88-914.

Cassella,S.J., Smith,E.P., Van Wyk,J.J., Joseph,D.R., Hynes,M.A., Hoyt,E.C. & Lund,P.K. (1987). Isolation of rat testis cDNAs encoding an insulin-like growth factor I precursor. DNA **6,** 325-330.

Chernausek,S.D., Underwood,L.E., Utiger,R.D. & Van Wyk,J.J. (1983). Growth hormone secretion and plasma somatomedin-C in primary hypothyroidism. Clinical Endocrinology **19,** 337-334

Chiang, M., Russell, S., M. & Nicoll, C.,S. (1990). Growth-promoting properties of the internal milieu of pregnant and lactating rats. American Journal of Physiology **258,** E98-E102.

Clemmons,D.R. (1990). Insulin-like growth factor binding proteins. Trends in Endocrinology and Metabolism Nov/Dec, 412-417.

Clemmons,D.R., Klibanski,A., Underwood,L.E., McArthur,J.W., Ridgway,E.C, Beitins,I.Z. & Van Wyk,J.J. (1981). Reduction in immunoreactive somatomedin-C during fasting humans. Journal of Clinical Endocrinology and Metabolism **53,** 1247-1250.

Clemmons,D.R., Seeks,M.M. & Underwood,L.E. (1985). Supplemental amino acids augment the somatomedin-C response to refeeding after fasting. Metabolism **34,** 391-395.

Clemmons,D.R. & Underwood,L.E. (1986). Somatomedin-C/Insulin-like growth factor I in acromegaly. Clinics in Endocrinology and Metabolism **15,** 629-653.

Clemmons,D.R., Elgrin,R.G., Hans,V.K.M., Cassella,S.J., D'Ercole,A.J. & Van Wyk,J.J. (1987). Cultured fibroblast monolayers secrete a protein that alters the cellular binding of somatomedin-C/ insulin-like growth factor I. Journal of Clinical Investigation **77,** 1548-1556.

Clemmons,D.R., Thissen,J.P., Maes,M., Ketelsledgers,J.M. & Underwood,L.E. (1989). Insulin-like growth factor-I (IGF-I) infusion into hypophysectomised or protein-deprived rats induces specific IGF-binding proteins in serum. Endocrinology **125,** 2967-2972.

Cohen,K.L. & Nissley,S.P. (1975). Comparison of somatomedin activity in rat serum and lymph. Endocrinology **97,** 654-658.

Conover,C.A. (1990). Regulation of insulin-like growth factor (IGF)- binding protein synthesis by insulin and IGF-I in cultured bovine fibroblasts. Endocrinology **126,** 3139-3145.

Conover,C.A., Liu,F., Powell,D., Rosenfeld,R.G. & Hintz,R.L. (1989). Insulin-like growth factor binding proteins from cultured human fibroblasts: characterisation and hormonal regulation. Journal of Clinical Investigation **83,** 852-859.

Conover,C.A., Butler,P.C., Wang,M., Rizza,R.A. & Lee,P.D.K. (1990). Lack of growth hormone effect on insulin-associated suppression of insulinlike growth factor binding protein 1 in humans. Diabetes **39,** 1251-1256.

Conover,C.A. & Lee,P.D.K. (1990). Insulin regulation of insulin-like growth factor binding protein production in cultured HepG2 cells. Journal of Clinical Endocrinology and Metabolism **70,** 1062-1067.

Cornell,H.J., Enberg,G. & Herington,A.C. (1987). Preferential association of the insulin-like growth factors I and II with metabolically inactive and active carrier-bound complexes in serum. Biochemical Journal **241,** 745-750.

Corps,A.N. (1990). Signalling at the insulin and insulin-like growth factor receptors: transduction probed by transfection. Journal of Endocrinology **124,** 349-351.

Cotterill,A.M., Cowell,C.T. & Silink,M. (1989). Insulin and variation in glucose levels modify the secretion rates of the growth hormone-independent insulin-like growth factor binding protein-1 in the human hepatoblastoma cell line HepG2. Journal of Endocrinology **123,** R17-R20.

Cubbage,M.L., Suwanichkul,A. & Powell,D.R. (1990). Insulin-like growth factor binding protein-3: Organisation of the human chromosomal gene and demonstration of promoter activity. Journal of Biological Chemistry **265,** 12642-12649.

Cutter,L., Van Vliet,G., Conte,F.A., Kaplan,S.L. & Grumbach,M.M. (1985). Somatomedin-C levels in children and adolescents with gonadal dysgenesis: differences from age-matched normal females and effect of chronic estrogen replacement therapy. Journal of Clinical Endocrinology and Metabolism **60,** 1087-1092.

Czech, A. (1989). Signal transmission by the insulin-like growth factors. Cell **59,** 235-238.

Daughaday,W.H., Hall,K., Raben,M.S., Salmon,W.D., Van den Brande,J.L. and Van Wyk,J.J. (1972). Somatomedin: Proposed designation for sulphation factor. Nature **235,** 107.

Daughaday,W.H., Parker,K.A., Borowski,S., Trivedi,B. & Kapadia,M. (1982). Measurement of Somatomedin-related peptides in fetal, neonatal, and maternal rat serum by insulin-like growth factor (IGF) I radioimmunoassay, IGF-II radioreceptor assay (RRA), and multiplication-stimulating activity RRA after acid-ethanol extraction. Endocrinology **110,** 575-581.

Daughaday,W.H., Hall,K., Raben,M.S., Salmon,W.D., Van den Brande,J.L. & Van Wyk,J.J. (1987). Letter to the Editor: On the nomenclature of the somatomedins and insulin-like growth factors. Journal of Clinical Endocrinology and Metabolism **65,** 1075-1076.

Daughaday,W.H. & 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.

Davenport,M.L., Svoboda,M.E., Koerber,K.L., Van Wyk, J.J., Clemmons,D.R. & Underwood,L.E. (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.

Dawe,S.R., Francis,G.L., McNamara,P.J., Wallace,J.C. & Ballard,F.J. (1988). Purification, partial sequences and properties of chicken insulin-like growth factors. Journal of Endocrinology **117,** 173-181.

D'Ercole,J.A. & Underwood,L.E. (1980). Ontogeny of somatomedin during development in the mouse. Developmental Biology **79,** 33-45.

D'Ercole,A.J. & Wilkins,J.R. (1984). Affinity labelled somatomedin-C-binding proteins in rat sera. Endocrinology **114,** 1141-1144.

Degerblad,M., Povoa,G., Thoren,M., Wivall,I-L. & Hall,K. (1989). Lack of diurnal rhythm of low molecular weight insulin-like growth factor binding protein in patients with Cushing's syndrome. Acta Endocrinologica **120,** 195-200.

DeMellow,J.S. & Baxter,R.C. (1988). Growth hormone-dependent insulin-like growth factor (IGF) binding protein inhibits and potentiates IGF-I-stimulated DNA synthesis in human skin fibroblasts. Biochemical and Biophysical Research Communications **156,** 199-204.

Dempster,A.P., Patel,C.M., Selwyn,M.R. & Roth,A.J. (1984). Statistical and computational aspects of mixed model analysis. Applied Statistics **33,** 203-214.

Denko,C.W. & Bergenstal,D.M. (1955). The effect of hypophysectomy and growth hormone on S^{35} fixation in cartilage. Endocrinology **57,** 76-86.

De Pagter-Holthuizen,P., Jansen,M., Van Schaik, F.M.A., van der Kammen,R.A., Oosterwijk,C., Ven den Brande,J.L. & Sussenbach,J.S. (1987). The human insulin-like growth factor II contains two development-specific promotors. FEBS Letters **214,** 259-264.

De Pagter-Holthuizen,P., Jansen,M., van der Kammen,R.A., Van Schaik,F.M.A. & Sussenbach,J.S. (1988). Differential expression of the human insulin-like growth factor II gene. Characterisation of the mRNAs and an mRNA encoding a putative IGF-II-associated protein. Biochimica Biophysica Acta **950,** 282-295.

DeVroede,M.A., Tseng,L.Y-H., Katsoyannis,P.G., Nissley,S.P. & Rechler,M.M. (1986). Modulation of insulin-like growth factor I binding to human fibroblast monolayer cultures by insulin-like growth factor carrier proteins released to the incubation media. Journal of Clinical Investigation **77,** 602-613.

Domenech,M., Gruppuso,P.A., Nishino,V.T., Susa,J.B. & Schwartz,R. (1986). Preserved fetal plasma amino acid concentrations in the presence of maternal hypoaminoacidemia. Pediatric Research **20,** 1071-1076.

Donahue,L.R., Hunter,S.J., Sherblom,A.P. & Rosen,C. (1990). Age-related changes in serum insulin-like growth factor-binding proteins in women. Journal of Clinical Endocrinology and Metabolism **71,** 575-579.

Donovan,S.M., Lamson,G., Hintz,R.L. & Rosenfeld,R.G. (1991). Hepatic insulin-like growth factor binding protein (IGFBP) mRNA expression during pregnancy in the rat. 2nd International IGF Symposium San Francisco, California, January 12-16. Abstract C49.

Drop,S.L.S., Valiquette,G., Guyda,H.J., Corvol,M.T. & Posner,B.I. (1979). Partial purification and characterisation of a binding protein for insulin-like activity (ILAs) in human in amniotic fluid: A possible inhibitor of insulin-like activity. Acta Endocrinologica **90,** 505-518.

Drop,S.L.S., Kortleve,D.J., Guyda,H.J. & Posner,B.I. (1984). Immunoassay of a somatomedin-binding protein from human amniotic fluid: Levels in fetal, neonatal and adult sera. Journal of Clinical Endocrinology and Metabolism **59,** 908-915.

Dulak,N.L. & Temin,H.M. (1973a). A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts. Journal of Cellular Physiology **81,** 153-160.

Dulak,N.L. & Temin,H.M. (1973b). Multiplication stimulating activity for chick embryo fibroblasts from rat liver cell conditioned medium: a family of small polypeptides. Journal of Cellular Physiology **81,** 161-170.

Ebina,Y., Araki,E., Taira,M., Shimada,F., Mori,M., Craik,C.S., Siddle,K., Pierce,S.B., Roth,R.A. & Rutter,W.J. (1987). Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. Proceedings of the National Academy of Science of the U.S.A. **84,** 704-708.

Elgrin,R.G., Busby,W.H. & Clemmons,D.R. (1987). An insulin-like growth factor (IGF) binding protein enhances the biological response to IGF-I. Proceedings of the National Academy of Science of the U.S.A. **84,** 3254-3258.

Enberg,G., Carlquist,M., Jornvall,H. & Hall,K. (1984). The characterisation of somatomedin-A, isolation by micro computer-controlled chromatography, reveals an apparent identity to insulin-like growth factor I. European Journal of Biochemistry **143,** 117-124.

Eriksson,L. (1989). Growth hormone in human pregnancy. Acta Obstetricia et Gynecologica Scandinavica **Suppl 147,** 3-38.

Evans,H.M. & Simpson,M.E. (1931). Hormones of the anterior pituitary. American Journal of Physiology **98,** 511-546.

Fawcett,D.H. & Bulfield,G. (1990). Molecular cloning, sequence analysis and expression of putative chicken insulin-like growth factor-I cDNAs. Journal of Molecular Endocrinology **4,** 201-211.

Florini,J.R., Priny,P.N., Yitello,M.V. & Hintz,R.L. (1985). Somatomedin-C levels in healthy young and older men: relationship to peak and 24 hr levels of growth hormone. Journal of Gerontology **40,** 2-7.

Forbes,B.E., Ballard,F.J. & Wallace,J.C. (1990). An insulin-like growth factor-binding protein purified from medium conditioned by a human lung fibroblast cell line (He[39]L) has a novel N-terminal sequence. Journal of Endocrinology **126,** 497-506.

Francis,G.L., Read,L.C., Ballard,F.J., Bagley,C.J., Upton,F.M., Gravestock,P.M. & Wallace,J.C. (1986). Purification and partial sequence analysis of insulin-like growth factor-I from bovine colostrum. Biochemical Journal **233,** 207-213.

Francis,G.L., Upton,F.M., Ballard,F.J., McNeil, K.A. & Wallace,J.C. (1988). Insulin-like growth factors 1 and 2 in bovine colostrum. Biochemical Journal **251,** 95-103.

Francis,G.L., McNeil,K.A., Wallace,J.C., Ballard,F.J. & Owens,P.C. (1989a). Sheep insulin-like growth factors I and II: Sequences, activities and assays. Endocrinology **124,** 1173-1183.

Francis,G.L., Owens,P.C., McNeil, K.A., Wallace,J.C. & Ballard,F.J. (1989b). Purification, amino acid sequences and assay cross-reactivities of porcine insulin-like growth factor-I and II. Journal of Endocrinology **122,** 681-687.

Froesch,E.R. & Zapf,J. (1985). Insulin-like growth factors and insulin: comparative aspects. Diabetologica **28,** 485-493.

Froesch,E.R., Burgi,H., Ramseier,E.B., 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.

Froesch,E.R., Schmidt,Chr., Schwander,J. & Zapf,J. (1985). Actions of insulin-like growth factors. Annual Review of Physiology **47,** 443-467.

Furlanetto,R.W., Underwood,L.E., Van Wyk,J.J. & Handwerger,S. (1978). Serum immunoreactive somatomedin-C is elevated late in pregnancy. Journal of Clinical Endocrinology and Metabolism **47,** 695-698.

Gargosky,S.E., Owens,P.C., Walton,P.E., Robinson,J.C., Wallace,J.C. and Ballard,F.J. (1990a). Circulating levels of insulin-like growth factors increase and molecular forms of their serum binding proteins change with human pregnancy. Biochemical Biophysical Research Communication **170,** 1157-1163.

Gargosky,S.E., Walton,P.E., Owens,P.C., Wallace,J.C. and Ballard,F.J. (1990b). Insulin-like growth factor-I (IGF-I) and IGF binding proteins both decline in the rat during late pregnancy. Journal of Endocrinology **127,** 183-290.

Gargosky,S.E., Walton,P.E., Wallace,J.C. and Ballard,F.J. (1990c). Insulin-like growth factor binding proteins (IGFBP) in the rat: characterization of IGFBP in serum, lymph, cerebrospinal and amniotic fluids, and in media conditioned by liver, bone and muscle cells. Journal of Endocrinology **127,** 391-400.

Goldberg,A.C., Trivedi,B., Delmez,J.A., Harter,H.R. & Daughaday,W.H. (1982). Uremia reduces serum insulin-like growth factor I, increases insulin-like growth factor II and modifies their serum protein binding. Journal of Clinical Endocrinology and Metabolism **55,** 1040-1045.

Gossrau,R., Graf,R., Ruhnke,M. & Hanski,C. (1987). Proteases in the human full-term placenta. Histochemistry **86,** 405-413.

Gown,L.K., Hampton,B., Hill,D.J., Schlueter,R.J. & Perdue,J.F. (1987). Purification and characterisation of a unique high molecular weight form of insulin-like growth factor II. Endocrinology **121,** 449-458.

Grant,M.B., Schmetz,I., Russell,B., Harwood,H.J. Silverstein,J. & Merimee,T.J. (1986). Changes in insulin-like growth factors I and II and their binding protein after a single intramuscular injection of growth hormone. Journal of Clinical Endocrinology and Metabolism **63,** 981-984.

Guidice,L.C., Farrell,E.M., Pham.H., Lamson,G. & Rosenfeld,R.G. (1990). Insulin-like growth factor binding protein in maternal serum throughout gestation and in the puerperium: affects of a pregnancy-associated protease activity. Journal of Clinical Endocrinology and Metabolism **71,** 806-816.

Guidice,L.C. & Dsupin,B.A. (1991). Species specificity of the pregnancy-specific insulin-like growth factor binding protein protease. 2nd International IGF Symposium San Francisco, California, January 12-16. Abstract C46.

Hall,K. & Sara,V.R. (1983). Growth and somatomedins. Vitamins and Hormones **40,** 175-233.

Hall,K., Enberg,G., Hellem,E., Luidin,G., Ottosson-Seeberger,A., Sara,V., Trystad,O. & Ofverholm,U. (1984). Somatomedin levels in pregnancy: longitudinal study in healthy subjects and patients with growth hormone deficiency. Journal of Clinical Endocrinology and Metabolism **59,** 587-594.

Hampton,B., Burgess, W.H., Marshak,D.R., Cullen,K.J. & Perdue, J.F. (1989). Purification and characterisation of an insulin-like growth factor II variant from human plasma. Journal of Biological Chemistry **264,** 19155-19160.

Han,V.K.M., Lund,P.K., Lee,D.C. & D'Ercole,A.J. (1988). Expression of somatomedin/ insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterisation and tissue distribution. Journal of Clinical Endocrinology and Metabolism **66,** 422-429.

Hardouin,S., Hossenlopp,P., Segovia,B., Seurin,D., Portolan,G., Lassare,C. & Binoux,M. (1987). Heterogeneity of insulin-like growth factor binding proteins and relationships between structure and affinity. European Journal of Physiology **170,** 121-132

Hardouin,S., Gourmelen,M., Noguiez,P., Seurin,D., Roghani,M., Le Bouc,Y., Povoa,G., Merimee,T.J., Hossenlopp,P. & Binoux,M. (1989). Molecular forms of serum insulin-like growth factor-binding proteins in man: relationships with growth hormone and IGFs and physiological significance. Journal of Clinical Endocrinology and Metabolism **69,** 1291-1301.

Harris,J.E. & Kretchmer,N. (1988). Synthesis of hepatic protein during pregnancy in the rat. Journal of Nutrition **118,** 1319-1324.

Hasselbacher,G.K., Schwab,M.E., Pasi,A. & Humbel,R.E. (1985). Insulin-like growth factor II (IGF-II) in human brain: regional distribution of IGF-II and of higher molecular mass forms. Proceedings of the National Academy of Science of the U.S.A. **82,** 2153-2157.

Helper,J.E., Van Wyk,J.J. & Lund,P.K. (1990). Different half lives of insulin-like growth factor I mRNAs that differ in length of 3' untranslated sequence. Endocrinology **127,** 1550-1552.

Hey,A.W., Browne,C.A., Simpson,R.J. & Thorburn,G.D. (1989). Simultaneous isolation of insulin-like growth factors I and II from adult sheep serum. Biochimica et Biophysica Acta **997,** 27-35.

Heyner,S., Smith,R.M. & Schultz,G.A. (1989). Temporal regulated expression of insulin and insulin-like growth factors and their receptors in early mammalian development. BioEssays **11,** 171-186.

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

Hintz,R.L., Liu,F. & Rinderknecht,E. (1980). Somatomedin-C shares the carboxy-terminal antigenic determinants with insulin-like growth factor-I. Journal of Clinical Endocrinology & Metabolism **51,** 672-673.

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,S.C., Moore,L., Napier,J.R., Davis, S.R., Bass,J.J. & Gluckman,P.D. (1989). Characterisation of insulin-like growth factor binding proteins in ovine fluids. Journal of Endocrinology **120,** 429-438.

Holly,J.M.P., Davies,S.C., Cotterill,A.M., Coulson,V.J., Ross,R.J.M., Miell,J.P., Abdulla,A.F. & Wass,A.J.M. (1991). Severe illness induces an IGFBP-3 specific protease in the circulation; the activity of which is nutritionally dependant. 2nd International IGF Symposium San Francisco, California, January 12-16. Abstract C48.

Holthuizen,P. (1990). The structure and expression of the human insulin-like growth factor genes. Molecular & Cellular Biology of insulin-like growth factors and their receptors. Editors, Le Roith,D. & Raizadsa,M.K. pp. 97-105.

Honegger,A. & Humbel,R.E. (1986). Insulin-like growth factors I and II in fetal and adult bovine serum. Journal of Biological Chemistry **261,** 569-575.

Hossenlopp,P., Seurin,D., Segovia-Quinson,B. & Binoux,M. (1986). Identification of an insulin-like growth factor-binding protein in human cerebrospinal fluid with a selective affinity for IGF-II. FEBS Letters **208,** 439-444.

Hossenlopp,P., Seurin,D., Segovia-Quinson,B., Portolan,G. & Binoux,M. (1987). Heterogeneity of insulin-like growth factor binding proteins and relationships between structure and affinity. 2. Forms released by human and rat liver in culture. European Journal of Biochemistry **170,** 133-142.

Hossenlopp,P., Segovia,B., Lassarre,C., Roghani,M., Bredon,M. & Binoux,M. (1990). Evidence for enzymatic degradation of insulin-like growth factor-binding proteins in the 150 kDa complex during pregnancy. Journal of Clinical Endocrinology and Metabolism **71,** 797-805.

Humbel,R.E. & Rinderknecht,E. (1978). Chemistry of NSILA (=IGF): Structural homology to proinsulin. Proceedings FEBS meetings **48,** 55-58.

Isley,W.L., Underwood,L.E. & Clemmons,D.R. (1984). Change in plasma somatomedin-C response to ingestion of diets with variable energy and protein content. Journal of Parenteral and Enteral Nutrition **8,** 407-411.

Jacob,R., Barrett,E., Plewe,G., Fagin,K.D. & Sherwin,R.S. (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.

Jacobs,S., Kull,F.C., Earp,H.S., Svodoba,M.S., Van Wyk,J.J. & Cuatrecasas,P. (1983). Somatomedin-C stimulates the phosphorylation of the B subunit of its own receptor. Journal of Biological Chemistry **258,** 9581-9584.

Jakob,A., Hauri,Ch. & Froesch,E.R. (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,F.M.A., Ricker,A.T., Bullock,B., Woods,D.E., Gabbay,K.H., Nussbaum,A.l., Sussenbach,J.S. & Van de Brande,J.L. (1983). Sequence of cDNA encoding human insulin-like growth factor I precursor. Nature **306,** 609-611.

Jansen,M., van Schaik,F.M.A., van Tol,H., Van de Brandt,J.L. & Sussenbach,J.S. (1985). Nucleotide sequences of cDNAs encoding precursors of human insulin-like growth factor II (IGF-II) and an IGF-II variant. FEBS Letters **179,** 243-246.

Johnson,T.R. & Ilan,J. (1990). Expression of IGF-I in liver. Molecular & Cellular Biology of insulin-like growth factors and their receptors. Editors, Le Roith,D. & Raizadsa,M.K. pp. 129-140.

Jorgensen,J.O.L., Blum,W.F., Moller,N., Ranke,M.B. & Christiansen,J.S. (1990). Circadian patterns of serum insulin-like growth factor (IGF) II and IGF binding protein 3 in growth hormone-deficient patients and age-and sex-matched normal subjects. Acta Endocrinologica **123,** 257-262.

Julkanen,M., Koistenen,R., Aalto-Setala,K., Seppala,M., Janne,O.A. & Kontula,K. (1988). Primary structure of human insulin-like growth factor-binding protein/ placental protein 12 and tissue-specific expression of its mRNA. FEBS Letters **236,** 295-302.

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.

Kato,H., Miura,Y., Okoshi,A., Umezewa,T., Takahashi,S. & Noguchi,T. (1990). Dietary and hormonal factors affecting the mRNA level of IGF-I in rat liver in vivo and in primary cultures of rat hepatocytes. Molecular & Cellular Biology of insulin-like growth factors and their receptors. Editors, Le Roith,D. & Raizadsa,M.K. pp. 125-129.

Kallincos,N.C., Wallace,J.C., Francis, G.L. & Ballard, F.J. (1990). Chemical and biological characterisation of chicken insulin-like growth factor-II. Journal of Endocrinology **124,** 89-97.

Kaufmann,U., Zapf,J., Torretti,B. & Froesch,E.R. (1977). Demonstration of a specific serum carrier protein of non-suppressible insulin-like activity *in vitro*. Journal of Clinical Endocrinology and Metabolism **44,** 160-166.

Keifer,M.C., Ioh,R.S., Bauer,D.M. & Zapf,J. (1991). Molecular cloning of a new human insulin-like growth factor binding protein. Biochemical and Biophysical Research Communications **176,** 219-225.

Keiss,W., Greenstein,L.A., White,R.M., Lee,L. & Rechler,M.M. (1987). Type II insulin-like growth factor receptor is present in rat serum. Proceedings of the National Academy of Science of the U.S.A. **84,** 7720-7724.

Klapper,D.G., Svoboda,M.E. & Van Wyk,J.J. (1983). Sequence analysis of Somatomedin-C: confirmation of identity with insulin-like growth factor I. Endocrinology **112,** 2215-2217.

Knauer,D.J. & Smith,G.L. (1980). Inhibition of biological activity of multiplication-stimulating activity by binding to its carrier protein. Proceedings of the National Academy of Science of the U.S.A. **77,** 7252-7256.

Knauer,D.J., Wagner,F.W. & Smith,G.L. (1981). Purification and characterisation of multiplication-stimulating activity (MSA) carrier protein. Journal of Supramolecular Structure and Cellular Biochemistry **15,** 177-191.

Koistenen,R., Kalkkinen,N., Huhtala,M-L., Seppala,M., Bohn,H. & Rutanen,E-M. (1986). Placental protein 12 is a decidual protein that binds somatomedin and has an identical N-terminal amino acid sequence with somatomedin-binding protein from human amniotic fluid. Endocrinology **118,** 1375-1378.

Krebs,E.G. (1984). The phosphorylation of proteins: a major mechanism for biological regulation. Biochemical Society Transactions **19,** 813-820.

Kumar,V. & Biswas, D.K. (1988). Dynamic state of site specific DNA methylation concurrent to altered prolactin and growth hormone gene expression in the pituitary gland of pregnant rats. Journal of Biological Chemistry **263,** 12645-12652.

Laemmli,U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature **227,** 680-685.

Lamson,G., Pham,H., Oh,Y., Ocrant,I., Schwander,J. & Rosenfeld,R.G. (1989). expression of the BRL3A insulin-like growth factor binding protein (rBP-30) in the rat central nervous system. Endocrinology **123,** 1100-1102.

Le Bouc,Y., Dreyer,D., Jaeger,F., Binoux,M. & Sondermeyer,P. (1986). Complete characterisation of the human IGF-I nucleotide sequence isolated from newly constructed adult liver cDNA library. FEBS Letters **196,** 108-112.

Le Bouc,Y., Noguiez,P., Sondermeyer,P., Dreyer,D., Girard,F. & Binoux,M. (1987). A new 5' non-coding region for human placental insulin-like growth factor II mRNA expression. FEBS Letters **222,** 181-185.

Lee, Y-L., Hintz,R.L., James,P.M., Lee,P.D.K., Shively,J.E. & Powell,D.R. (1988). Insulin-like growth factor (IGF) binding protein complementary deoxyribonucleic acid from human HepG2 hepatoma cells: predicted protein sequence suggests an IGF binding domain different from those of the IGF-I and IGF-II receptors. Molecular Endocrinology **2,** 404-411.

Lemmy,A.B., Martin,A.A., Read,L.C., Tomas,F.M., Owens,P.C. & Ballard,F.J. (1991). IGF-I and the truncated analogue des-(1-3)-IGF-I enhances growth in rats after gut resection. American Journal of Physiology **260,** E213-E219.

Lewitt,M.S. & Baxter,R.C. (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,M.S. & Baxter,R.C. (1990). Inhibitors of glucose uptake stimulate the production of insulin-like growth factor binding protein (IGFBP-1) by human fetal liver Endocrinology **126,** 1533-1533.

Ling,P.R., Bistrian,B.R., Blackburn,G.L. & Istfan,N. (1987). Effect of fetal growth on maternal protein metabolism in postabsorptive rat. American Journal of Physiology **252,** E380-E390.

Liu,F., Powell,D.R. & Hintz,R.L. (1990). Characterisation of insulin-like growth factor binding proteins in human serum from patients with chronic renal failure. Journal of Clinical Endocrinology and Metabolism **70,** 620-628.

Lowe,W.L., Roberts,C.T., Lasky,S.R. & Le Roith,D. (1987). differential expression of alternative 5' untranslated regions in mRNAs encoding rat insulin-like growth factor I. Proceedings of the National Academy of Sciences of the U.S.A. **84,** 8946-8950.

Lowe,W.L., Adamo,M., Le Roith,D. & Roberts,C.T. (1989). Expression and stability of insulin-like growth factor-I (IGF-I) mRNA splicing variants in the GH3 rat pituitary cell line. Biochemical and Biophysical Research Communications **162,** 11764-1179.

Madon,R.J., Ensor,D.M. & Flint,D.J. (1989). Growth hormone concentrations do not vary over the reproductive cycle of the female rat. Medical Science Research **17,** 1001-1002.

Margot,J.B., Binkert,C., Mary,J-L., Landwehr,J., Heinrich,G. & Schwander,J. (1989). A low molecular weight insulin-like growth factor binding protein from rat: cDNA cloning and tissue distribution of its messenger RNA. Molecular Endocrinology **3,** 1053-1060.

Marquardt,H. & Todaro,G.J. (1981). Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures. Journal of Biological Chemistry **256,** 6859-6865.

Marshall,R.N., Underwood,L.E., Voina,S.J., Foushee,D.B. & Van Wyk,J.J. (1974). Characterization of the insulin and somatonedin-C receptors in human placental membranes. Journal of Clinical Endocrinology and Metabolism **39,** 283-288.

Maruyama,K., Nagasawa,H., Isogai,A., Tamura,S., Ishizaki,H. & Suzuki,A. (1990). Synthesis of bombyxin-IV, an insulin-like heterodimeric peptide from the silkworm, *Bombyx mori*. Peptides **11,** 169-171.

Massague,J. & Czech,M.P. (1982). The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. Journal of Biological Chemistry **257,** 5038-5045.

Martin,J.L. & Baxter,R.C. (1986). Insulin-like growth factor-binding protein from human plasma: purification and isolation. Journal of Biological Chemistry **261,** 8754-8760.

Martin,J.L., Willetts,K.E. & Baxter,R.C. (1990). Purification of a novel insulin-like growth factor-II binding protein from transformed human fibroblasts. Journal of Biological Chemistry **265,** 4124-4130.

McCusker,R.H., Campion,D.R., Jones,W.K. & Clemmons,D.R. (1985). The insulin-like growth factor-binding proteins of porcine serum: Endocrine and nutritional regulation. Endocrinology **125,** 501-509.

McCusker,R.H., Camacho-Hubner,C. & Clemmons, D.R. (1989). Identification of the types of insulin-like growth factor binding proteins that are produced by muscle cell *in vitro*. Journal of Biological Chemistry **246,** 7795-7800.

Merimee,T.J., Zapf,J. & Froesch,E.R. (1982). Insulin-like growth factors in fed and fasted states. Journal of Clinical Endocrinology and Metabolism **55,** 999-1002.

Mesiano,S., Young,I.R., Browne,C.A. & Thorburn,G.D. (1988). Failure of acid-ethanol treatment to prevent interference by binding proteins in radioligand assays for the insulin-like growth factors. Journal of Endocrinology **119,** 453-460.

Metcalfe,J., Stock,M.K. & Barron,D.H. (1988). Chapter 53: Maternal physiology during gestation. The Physiology of Reproduction 2145- 2176. Edited by E. Knobil, J. Neill, Ewing, L. L., Greenwald, G. S., Markert, C. L. & Pfaff, D. W. Raven Press, Ltd., New York.

Mohan,S., Bautista,C.M., Wergedal,J. & Baylink,D.J. (1989). Isolation of an inhibitory insulin-like growth factor (IGF) binding protein from bone cell-conditioned medium: A potential local regulator of IGF action. Proceedings of the National Academy of Science of the U.S.A. **86,** 8338-8342.

Montgomery,D.C. (1983). Design and analysis of experiments (second edition). Printed by John Wiley & Sons, USA.

Morgan,D.O., Edman,J.C., Standring,D.N., Fried,V.A., Roth,R.A. & Rutter,W.J. (1987). Insulin-like growth factor II receptor as a multifunctional binding protein. Nature **329,** 301-307.

Morton,A.J. & Goldspink,D.F. (1986). Changes in protein turnover in rat uterus during pregnancy. American Journal of Physiology **250,** E114-E120.

Moses,A.C., Nissley,S.P. & Cohen,K.L. (1976). Specific binding of a somatomedin-like polypeptide in rat serum depends on growth hormone. Nature **263,** 137-141.

Moses,A.C., Nissley,S.P., Passamani,J. & White,R.M. (1979). Further characterisation of growth hormone dependent somatomedin-binding proteins in rat serum and demonstration of somatomedin-binding proteins produced by rat liver cells in culture. Endocrinology **104,** 534-546.

Moses,A.C., Nissley,S.P., Short,P.A., Rechler,M.M. & Podskalny,J.M. (1980). Purification and characterisation of multiplication-stimulating activity: Insulin-like growth factors purified from rat-liver-cell-conditioned medium. European Journal of Biochemistry **103,** 387-400.

Mottola,C., MacDonald,R.G., Brackett,J.L., Mole,J.E., Anderson,J.K. & Czech,M.P. (1986). Purification and amino terminal sequence of an insulin-like growth factor-binding protein secreted by rat BRL-3A cells. Journal of Biological Chemistry **261,** 11180-11188.

Murphy,L.J., Bell,G.I., Duckworth,M.L. & Freisen,H.G. (1987). Identification, characterisation and regulation of a complementary deoxyribonucleic acid which encodes insulin-like growth factor I. Endocrinology **121,** 684-691.

Murphy,L.J. & Friesen,H.G. (1988). Differential aspects of estrogen and growth hormone on uterine and hepatic insulin-like growth factor I gene expression in the ovarectomised hypophysectomised rat. Endocrinology **122,** 325-332.

Murphy, L. J., Ballejo, G., Croze, F., Luo, J. and Kennedy, T. (1989). Identification and characterisation of a rat decidual insulin-like growth factor binding protein cDNA. Proceedings of the Endocrine Society 71 st Annual Meeting, abstract no. 259.

Murphy,L.J., Seneviratne,C., Ballejo,G., Croze,F. & Kennedy,T.G. (1990). Identification and characterisation of a rat decidual insulin-like growth factor-binding protein complementary DNA. Molecular Endocrinology **4,** 329-336.

Murphy,L.J. & Friesen,H.G. (1990). Regulation of the insulin-like growth factor gene by estrogen. Molecular & Cellular Biology of insulin-like growth factors and their receptors. Editors, Le Roith,D. & Raizadsa,M.K. pp. 153-168.

Nishimoto,I., Murayama,Y., Katada,T., Ui,T. & Ogata,E. (1989). Possible direct linkage of insulin-like growth factor-II receptor with guanine nucleotide-binding protein. Journal of Biological Chemistry **264,** 14029-14038.

Nissley,P., Keiss,W. & Sklar,M. (1990) The insulin-like growth factor II/ Mannose 6-phosphate receptor. Molecular & Cellular Biology of insulin-like growth factors and their receptors. Editors, Le Roith,D. & Raizadsa,M.K. pp. 359-362.

Ooi,G.T. (1990). At the cutting edge. Insulin-like growth factor binding proteins (IGFBPs): more than just 1,2,3. Molecular and Cellular Endocrinology **71,** C39-43.

Ooi,G.T. & Herington,A.C. (1987). Review. The biological and structural characterisation of specific serum binding proteins for the insulin-like growth factors. Journal of Endocrinology **118,** 7-18.

Orlowski,C.C., Brown,A.L., Ooi,G.T., Yang,Y,W-H., Tseng,L.Y-H. & Rechler,M.M. (1990). Tissue, developmental and metabolic regulation of messenger ribonucleic acid encoding a rat insulin-like growth factor-binding protein. Endocrinology **126,** 644-652.

Oshima,A., Nolan,C.M., Kyla,J.W., Grubb,J.H. & Sly,W.S. (1988). The human cation-independent mannose-6-phosphate receptor: cloning and sequence of the full length cDNAs and expression of functional receptor in COS cells. Journal of Biological Chemistry **263,** 2553-2562.

Owens,P.C., Johnson,R.J., Campbell,R.G. & Ballard,F.J. (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.

Pancak-Roessler,M.K. & Lee, P.D.K. (1990). Insulin-like growth factor-I and insulin-like growth factor-binding protein in the toad, *Bufo woodhousi.* General and Comparative Endocrinology **78,** 263-272.

Parker,M.W., Johanson,A.J., Rogol,A.D., Kaiser,D.L. & Blizzard,R.M. (1984). Effect of testosterone on somatomedin-C concentrations in pre-pubertal boys. Journal of Clinical Endocrinology and Metabolism **58,** 87-90.

Phillips,A.F., Persson,B., Hall,K., Lake,M., Skottner,A., Sanengen,T. & Sara,V.R. (1988). The effects of biosynthetic insulin-like growth factor-I supplementation on somatic growth, maturation and erythropoiesis in the neonatal rat. Pediatric Research **23,** 298-305.

Povoa,G., Enberg,G., Jornvall,H. & Hall,K. (1984). Isolation and characterisation of a somatomedin-binding protein from mid-term human amniotic fluid. European Journal of Biochemistry **144,** 199-204.

Povoa,G., Isaksson,M., Jornvall,H. & Hall,K. (1985). The somatomedin-binding protein isolated from human hepatoma cell line is identical to the human amniotic fluid somatomedin-binding protein. Biochemical and Biophysical Research Communications **128,** 1071-1078.

Rechler,M.M. & Nissley,S.P. (1985). The nature and regulation of the receptors for insulin-like growth factors. Annual Review of Physiology **47,** 425-445.

Rieu,M. & Binoux,M. (1985). Serum levels of insulin-like growth factor (IGF) binding protein in insulin-dependent diabetes during an episode of severe metabolic decompensation and the recovery phase. Journal of Clinical Endocrinology and Metabolism **60,** 781-785.

Rinderknecht,E. & Humbel,R.E. (1976). Polypeptides with non-suppressible insulin-like and cell growth promoting activities in human sera: Isolation, chemical characterisation, and some biological properties of forms I and II. Proceedings of the National Academy of Science of the U.S.A. **73,** 2365-2369.

Rinderknecht,E. & Humbel,R.E. (1978a). The amino acid sequence of human insulin-like growth factor-I and its structural homology with proinsulin. Journal of Biological Chemistry **253,**2769-2776.

Rinderknecht,E. & Humbel,E.R. (1978b). Primary structure of human insulin-like growth factor-II. Febs Letters **89,** 283-286.

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.

Roberts,C.T., Lasky,S.R., Lowe,W.L., Seaman,W.T. & LeRoith,D. (1987a). Molecular cloning of rat insulin-like growth factor I complementary deoxyribonucleic acid: differential messenger ribonucleic acid processing and regulation by growth hormone in extrahepatic tissues. Molecular Endocrinology **1,** 243-248.

Roberts,C.T., Lasky,S.R., Lowe,W.L. & LeRoith,D. (1987b). Rat IGF-I cDNAs contain multiple 5' untranslated regions. Biochemical and Biophysical Research Communication **146,** 1154-1158.

Roghani,M., Hossenlopp,P., Lepage,P., Balland,A. Binoux,M. (1989). Isolation from human cerebrospinal fluid of a new insulin-like growth factor-binding protein with a selective affinity for IGF-II. FEBS Letters **255,** 253-258.

Romanus,J.A., Tseng,Y.Y-H., Yang,Y.H-. & Rechler,M.M. (1989). The 34 kilodalton insulin-like growth factor binidng protien in human cebrospinal fluid and the A673 Rhabdomysarcoma cell line are human homologues of the rat BRL3A binding proteins. Biochemical and Biophysical Research Communications **163,** 875-881.

Ronge,H. & Blum,J.W. (1989). Insulin-like growth factor I binding proteins in dairy cows, calves and bulls. Acta Endocrinologica **121,** 153-160.

Rosenfeld,R.G., Conover,C.A., Hodges,D., Lee,P.D.K., Misra,P., Hintz,R.L. & Li,C.H. (1987). Heterogeneity of insulin-like growth factor-I affinity for the insulin-like growth factor II receptor: comparison of natural, synthetic and recombinant DNA-derived insulin-like growth factor-I. Biochemical and Biophysical Research Communications **143,** 199-205.

Ross,M., Francis,G.L., Szabo,L., Wallace,J.C. & Ballard,F.J. (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.

Roth,R.A. & Cassell,D.J. (1983). Insulin receptor: Evidence that it is a protein kinase. Science **219,** 299-301.

Rothstein,H. (1982). Regulation of the cell cycle by somatomedin. International Review of Cytology **78,** 127-232.

Rotwein,P. (1986). Two insulin-like growth factor-I messenger RNAs are expressed in human liver. Proceedings fo the National Academy of Science of the U.S.A. **83,** 77-81.

Rotwein,P., Folz,R.L. & Gordon,J.I. (1987). Biosynthesis of human insulin-like growth factor I. the primary translation product of IGF-I mRNA contains an unusual 48 amino acid signal peptide. Journal of Biological Chemistry **262,** 11807-11812.

Rotwein,P., DeVol,D., Lajara,P., Betchel,P. & Hammerman,M. (1990). Physiological regulation of insulin-like growth factor expression. Molecular & Cellular Biology of insulin-like growth factors and their receptors. Editors, Le Roith,D. & Raizadsa,M.K. pp. 125-129.

Rubin,J.S., Mariz,I., Jacobs,J.W., Daughaday,W.H. & Bradshaw,R.A. (1982). Isolation and partial sequence analysis of rat basic somatomedin. Proceedings of the National Academy of Science of the U.S.A. **83,** 4904-4907.

Rudman,.D.G., Moffitt,S.D., Fernhoff,P.M., Mc Kensie,W.J., Kenny,J.M. & Bain,R.P. (1981). The relationship between growth velocity and serum somatomedin-C concentrations. Journal of Clinical Endocrinology and Metabolism **52,** 622-627.

Rutanen,E-M., Bohn,H. & Seppala,M. (1982). Radioimmunoassay of placental protein 12: Levels in amniotic fluid, cord blood, and sera of healthy adults, pregnant women, and patients with trophoblastic disease. American Jóurnal of Obstetrics and Gynecology **144,** 460-463.

Rutanen,E-M., Seppala,M., Pietila,R. & Bohn,H. (1984). Placental protein 12 (PP12): Affecting levels in late pregnancy. Placenta **5,** 243-248.

Salmon,W.D. & Daughaday,W.H. (1957). A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage *in vitro*. The Journal of Laboratory and Clinical Medicine **49,** 825-836.

Sara,V.R., Hall,K., Lins,P-E. & Fryklund,L. (1980). Serum levels of immunoreactive somatomedin A in the rat: some developmental aspects. Endocrinology **107,** 622-625.

Sara,V.R., Carlsson-Skwirut,C., Andersson,C., Hall,E., Sjogren,B., Holmgren,A. & Jornvall,H. (1986). Characterisation of somatomedins from human fetal brain: Identification of a variant form of insulin-like growth factor I. Proceedings of the National Academy of Science of the U.S.A. **83,** 4904-4907.

Schmidt,C., Zapf,J. & Froesch,E.R. (1989). Production of carrier proteins for the insulin-like growth factors (IGFs) by rta osteoblasts. FEBS letters **244,** 328-332.

Scott,C.D. & Baxter,R.C. (1986). Production of insulin-like growth factor I and its binding protein in rat hepatocytes from diabetic and insulin-treated diabetic rats. Endocrinology **119,** 2346-2352.

Sheppard,M.S. & Bala,R.M. (1986). Profile of immunoreactive insulin-like growth factor-I during gestation in Wistar rats. Canadian Journal of Physiological Pharmacology **64,** 521-524.

Shen,S.J., Daimon,M., Wang,C.Y., Jansen,M. & Ilan,J. (1986). Isolation of an insulin-like growth factor II cDNA with a unique 5' untranslated region form the placenta. Proceedings of the National Academy of Science of the U.S.A. **85,** 1947-1951.

Shimasaki,S., Koba,A., Mercado,M., Shimonaka,M. & Ling,N. (1989). Complementary DNA structure of the high molecular weight rat insulin-like growth factor binding protein (IGFBP-3) and tissue distribution of its mRNA. Biochemical and Biophysical Research Communications **165,** 907-912.

Shimasaki,S., Uchiyama,F., Shimonaka,M. & Ling,N. (1990). Molecular cloning of the cDNAs encoding a novel insulin-like growth factor-binding protein from rat and human. Molecular Endocrinology **4,** 1451-1458.

Shimasaki,S., Shimonaka,M., Bicsak,T. & Ling,N. (1991). Isolation and molecular characterization of three novel IGFBPs. 2nd International IGF Symposium San Francisco, California, January 12-16. Abstract C54.

Shimatsu,A. & Rotwein,P. (1987). Mosaic evolution of the insulin-like growth factors: organisation, sequence, and expression of the rat insulin-like growth factor I gene. Journal of Biological Chemistry **262,** 7894-7900.

Shimonaka,M., Schroeder,R., Shimasaki,S. & Ling,N. (1989). Identification of a novel binding protein for insulin-like growth factors in adult rat serum. Biochemical and Biophysical Research Communications **165,** 189-195.

Shoenle,E., Zapf,J., Humbel,R.E. & Froesch,E.R. (1982). Insulin-like growth factor I stimulates growth in hypophysectomised rats. Nature **296,** 252-253.

Shuldiner,A.R., Nirula,A., Scott,L.A. & Roth,J. (1990). Evidence that *Xenopus laevis* contains two different non-allelic insulin-like growth factor genes. Biochemical and Biophysical Research Communications **166,** 223-230.

Sibley,D.R., Benovic,J.L., Caron,M.G. & Lefkowitz,R.J. (1987). Regulation of transmembrane signaling by receptor phosphorylation. Cell **48,** 913-922.

Sillence, M.N. & Etherton, T.D. (1989). Changes in serum concentrations of ACTH, corticosterone and growth following morning or evening injection of female rats with porcine growth hormone. Journal of Endocrinology **123,** 113-119.

Singh,A., Hamilton-Fairley,D., Koistenen,R., Seppala,M., James,V.H.T., Franks,S. & Reed,M.J. (1990). Effect of insulin-like growth factor-I (IGF-I) and insulin on the secretion of sex hormone binding globulin and IGF-I binding protein (IBP-1) by human hepatoma cells. Journal of Endocrinology **124,** R1-R3.

Sklar,M.M., Keiss,W., Thomas,C.L. & Nissley,S.P. (1989). Developmental expression of the tissue insulin-like growth factor II/ mannose 6-phosphate receptor in the rat. Journal of Biological Chemistry **264,** 16733-16738.

Slatter,J.D.H., Samaan,N., Fraser,R. & Stillman,D. (1961). Immunological studies with circulating insulin. British Medical Journal **1,** 1712-1715.

Stempien,M.M., Fong,N.M., Rall,L.B. & Bell,G.I. (1986). Sequence of a placental cDNA encoding the mouse insulin-like growth factor II precursor. DNA **5,** 357-360.

Straus,D.S. & Takemoto,C.D. (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.

Stryer,L. (1986). G proteins: A family of signal transducers. Annual Review of Cell Biology **2,** 319-419.

Suikkari,A-M., Koivisto,V.A., Rutanen,E-M., Yki-Jarvinen,H., Karonen,S-L. & Seppala,M. (1988). Insulin regulates the serum levels of low molecular weight insulin-like growth factor-binding protein.Journal of Clinical Endocrinology and Metabolism **66,** 266-272.

Suikkari,A-M., Koivisto,V.A., Koistenen,R., Seppala,M. & Yki-Jarvinen (1989). Dose-response characteristics for suppression of low molecular weight plasma insulin-like growth factor-binding protein by insulin. Journal of Clinical Endocrinology and Metabolism **68,** 135-140.

Sussenbach,J.S. (1989). The gene structure of the insulin-like growth factor family. Progress in Growth Factor Research **1,** 33-48.

Szabo,L., Mottershead,D.G., Ballard,F.J. & Wallace,J.C. (1988). The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires N-terminal tripeptide in IGF-I for binding. Biochemical and Biophysical Research Communications **151,** 718-726.

Tavakkol,A., Simmen,F.A. & Simmen,R.C.M. (1988). Porcine insulin-like growth factor I (IGF-I): complementary deoxyribonucleic acid cloning and uterine expression of messenger ribonucleic acid encoding evolutionary conserved IGF-I peptides. Molecular Endocrinology **2,** 264-681.

Temin,H.M. (1971). Stimulation by serum of multiplication of stationary chicken cells. Journal of Cellular Physiology **78,** 161-170.

Tricoli,J.V., Rall,L.B., Scott,J., Bell,G.I. & Shows, T.B. (1984). Localisation of insulin-like growth factor genes to human chromosomes 11 and 12. Nature **310,** 284-286.

Thorndyke,M.C., Purvis,D. & Plisetskaya,E.M. (1989). Insulin-like immunoreactivity in the brain of two Hagfishes, *Eptatretus stouti* and *Myxine glutinosa*. General and Comparative Endocrinology **76,** 371-381.

Tomas,F.M., Knowles,S.E., Owens,P.C., Read,L.C., Chandler,C.S., Gargosky,S.E. & Ballard,F.J. (1991a). 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,F.M., Knowles,S.E., Owens,P.C., Read,L.C., Chandler,C.S., Gargosky,S.E. & Ballard,F.J. (1991b). Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with IGF-I and especially des(1-3) IGF-I. Biochemistry Journal (in press).

Ullrich,A., Berman,C.H., Dull,T.J., Gray,A. & Lee,J.M. (1984). Isolation of the human insulin-like growth factor-I gene using a synthetic DNA probe. EMBO Journal, **3,** 361-364.

Ullrich,A., Gray,A., Tam,A.W., Yang-fang,T., Tsubokawa,C., Collins,W., Henzel,T.R., LeBon,S., Kathuria,C., Chen,S., Jacobs,U., Franke,J., Ramachrandran,J. & Fugita-Yamaguchi,Y. (1986). Insulin-like growth factor-I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO Journal **5,** 2503-2572.

Unterman,T.G., Oehler,D.T. & Becher,R.E. (1989). Identification of a type 1 insulin-like growth factor binding protein (IGFBP) in serum from rats with diabetes mellitus. Biochemical and Biophysical Research Communications **163,** 882-887.

Unterman,T.G., Patel,K., Mahathre,K.V., Rajamohan,G., Oehler,D.T. & Becker,R.E. (1990). Regulation of low molecular weight insulin-like growth factor binding proteins in experimental diabetes mellitus. Endocrinology **126,** 2614-2624.

Upton,Z., Szabo,L., Wallace,J.C, & Ballard,F.J. (1990). Characterisation and cloning of a bovine insulin-like growth factor-binding protein. Journal of Molecular Endocrinology **5,** 77-84.

van Buul,S. & van den Brande,J.L. (1982). Cellular growth in organs of Snell-dwarf mice during treatment with growth hormone, thyroxine and plasma fractions containing somatomedin activity. Acta Endocrinologica **99,** 150-160.

Van Obbergen-Schilling,E. & Pouyssegur,J. (1983). Mitogen potentiating action and binding of insulin-like growth factor in Chinese hamster fibroblasts. Experimental Cell Research **151,** 207-214.

Van Wyk,J.J., Underwood,L.E., Hintz,R.L., Clemmons,D.R., Voina,S.J. & Weaver, R.P. (1974). The somatomedins: a family of insulin-like hormones under growth hormone control. Recent Progress in Hormone Research **30,** 259-318.

Van Wyk,JJ., Svoboda,M.E. & Underwood,L.E. (1980) Evidence from radioligand assays that somatomedin-C and insulin-like growth factor-I are similar to each other and different from other somatomedins. Journal of Clinical Endocrinology and Metabolism **50,** 206-208.

Walton,P.E. & Etherton,T.D. (1989a). Effect of porcine growth hormone and insulin-like growth factor-I (IGF-I) on immunoreactive IGF-binding protein concentration in pigs. Journal of Endocrinology **119,** 153-160.

Walton,P.E., Baxter,R.C., Burleigh,B.D. & Etherton,T.D. (1989b). Purification of the serum acid-stable insulin-like growth factor binding protein from the pig (*Sus scrofa*). Comparative Biochemical Physiology **92B,** 561-567.

Walton,P.E., Gopinath,R. & Etherton,T.D. (1989c). Porcine insulin-like growth factor (IGF) binding protein blocks IGF-I action on porcine adipose tissue. Proceedings of the Society for Experimental Biology and Medicine **190,** 315-319.

Werner,H., Woloschak,M., Adamo,M., Shen-Orr,Z., Roberts,C.T. & LeRoith,D. (1989). Developmental regulation of the rat insulin-like growth factor I receptor gene. Proceedings of the National Academy of Science of the U.S.A. **86,** 7451-7455.

Whitfield,H.J., Bruni,C.B., Frunzio,R.E., Terell,J.E., Nissley,S.P. & Rechler,M.M. (1984). Isolation of a clone encoding a rat insulin-like growth factor-II precursor. Nature **312,** 277-280.

Wilson,D.M., Bennett,A., Adamson,D., Nagashima,R.J., Liu,R., DeNatale,M., Hintz,R.L. & Rosenfeld,R.G. (1982). Somatomedins in pregnancy: A cross-sectional study of insulin-like growth factors I and II and somatomedin peptide content in normal human pregnancies. Journal of Clinical Endocrinology and Metabolism **55,** 858-861.

Wong,E.A., Ohlsen,S.M., Godfredson,J.A., Dean,D.M. & Wheaton,J.E. (1989). Cloning of ovine insulin-like growth factor-I cDNAs: heterogeneity in the mRNA population. DNA **8,** 649-657.

Wood,W.I., Cachianes,G., Henzel,W.J., Winslow,G.A., Spencer,S.A., Hellmiss,R., Martin,J.L. & Baxter,R.C. (1988). Cloning and expression of the growth hormone-dependent insulin-like growth factor-binding protein. Molecular Endocrinology **2,** 1176-1185.

Yang,Y.W-H., Wang,J-F., Orlowski,C.C., Nissley,P. & Rechler,M.M. (1989). Structure, specificity, and regulation of the insulin-like growth factor-binding proteins in adult rat serum. Endocrinology **125,** 1540-1555.

Yeoh,S.I. & Baxter,R.C. (1988). Metabolic regulation of the growth hormone independent insulin-like growth factor binding protein in human plasma. Acta Endocrinologica **119,** 465-473.

Yu,K.T., Peters,M.A. & Czech,M.P. (1986). Similar control mechanisms regulate the insulin and Type I insulin-like growth factor receptor kinases. Journal of Biological Chemistry **261,** 11341-11349.

Zapf,J., Schoenle,E. & Froesch,E.R. (1978a). Insulin-like growth factors I and II: The 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., Rinderknecht,E., Humbel,R.E. & Froesch,E.R. (1978b). Non-suppressible insulin-like activity (NSILA) from human serum: recent accomplishments and their physiological implications. Metabolism **27,** 1803-1828.

Zapf,J., Schoenle,E., Jagars,G., Sand,I., Grunwald,J. & Froesch,E.R. (1979). Inhibition of the action of nonsuppressible insulin-like activity on isolated rat fat cells by binding to its carrier protein. Journal of Clinical Investigation **63,** 1077-1084.

Zapf,J., Froesch,E.R. & Humbel,R.E. (1981). The insulin-like growth factor of human serum: chemical and biological characterisation and aspects of their possible physiological role. Current topics in Cellular Regulation Horecker,B.L. & Stadtman,E.R. (eds). Academic Press, New York.

Zapf,J., Pescatore,P., Scheiwiller,E. & Froesch,E.R. (1981). Growth hormone (GH), insulin and insulin-like growth factor (IGF): interdependent regulation and functional co-operativity. Journal of Clinical Investigation **67,** abstract 10.

Zapf,J., Born,W., Chang,J-Y., James,P., Froesch,E.R. & Fischer,J.A. (1988). Isolation and NH_2-terminal sequences of rat serum carrier proteins for insulin-like growth factors. Biochemical and Biophysical research Communications **156,** 1187-1194.

Zapf,J., Hauri,C., Waldvogel,M., Futo,E., Hasler,H., Binz,K., Guler,H.P., Schmidt,C. & Froesch,E.R. (1989). Recombinant human insulin-like growth factor I induces its own specific carrier protein in hypophysectomised and diabetic rats. Proceedings of the National Academy of Science of the U.S.A. **86,** 3813-3817.

Zick,Y., Sasaki,N., Rees-Jones,R.W., Grunberg,G., Nissley,S.P. & Rechler,M.M. (1984). Insulin-like growth factor-I (IGF-I) stimulates tyrosine kinase activity in purified receptors from rat liver. Biochemical and Biophysical Research Communication **119,** 6-13.

Zumstein,P.P., Luthi,C. & Humbel,R.E. (1985). Amino acid sequence of a variant pro-form of insulin-like growth factor-II. Proceedings of the National Academy of Science of the U.S.A. **82,** 3169-3172.