

<http://researchcommons.waikato.ac.nz/>

Research Commons at the University of Waikato

Copyright Statement:

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

The thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from the thesis.

REGULATION OF TISSUE INSULIN-LIKE GROWTH
FACTOR-1 PRODUCTION IN THE POST-NATAL SHEEP
THROUGH THE GROWTH HORMONE AXIS.

A Thesis

Submitted to the University of Waikato
for the degree of Doctor of Philosophy

by

Ke Min Hua

University of Waikato

1993

TABLE OF CONTENTS

	Page Number
Contents	ii
Abstract	ix
Acknowledgements	xii
List of Figures	xiv
List of Tables	xvii
List of Abbreviations	xix
CHAPTER ONE : INTRODUCTION	1
1.1 Growth Hormone (GH)	2
History/Molecular Structure	2
Synthesis Secretion/Regulation	4
Receptors	7
Metabolic Actions	9
1.2 Insulin-Like Growth Factors (IGFs)	12
History/Molecular Structure	12
Variants of IGFs	16
Synthesis/Regulation/Tissue Distribution	19
Receptors	25
Metabolic Actions	28
1.3 IGF Binding Proteins (IGFBPs)	30
IGFBP-1, 2 and 3	30
IGFBP-4, 5 and 6	43
Ovine/Bovine IGFBP	46
Physiological role of the IGFBPs	47
1.4 Theories of GH Action in Stimulating Growth	49

	Somatomedin Hypothesis of GH Action	49
	Dual Effector Theory of GH Action	53
	IGF Action	56
	Role for GH Axis in Lactation in Ruminants	57
1.5	Autocrine/Paracrine and Endocrine Mechanism of IGF	61
1.6	Aims of the Thesis	65
 CHAPTER TWO : MATERIALS AND METHODS		
2.1	Measurement of Metabolites in Plasma	67
	- Measurement of Glucose	67
	<i>Materials</i>	67
	<i>Principle of Assay</i>	67
	<i>Test methodology</i>	70
	- Measurement of Non-Esterified Fatty Acids (NEFA)	70
	<i>Materials</i>	70
	<i>Principle of Assay</i>	72
	<i>Test methodology</i>	72
2.2	Estimation of Haemaglobin (Hb)	73
	Materials	73
	Principle of Assay	73
	Test Procedure	74
2.3	Radioimmunoassay (RIA) for IGF-I	75
	Materials	75
	Radioiodination of IGF-I	76
	<i>Iodogen method</i>	76
	<i>Tracer storage</i>	78
	Antibody Production	80
	<i>IGF-I antiserum</i>	81
	<i>Conjugation procedure</i>	81
	<i>Immunization procedure</i>	81
	<i>Secondary antiserum</i>	81

	IGF-I RIA for Sheep Plasma	82
	<i>Materials</i>	84
	<i>Sample Extraction by AEC Technique</i>	86
	<i>Assay procedure</i>	86
2.4	In Vitro IGF-I Tracer Binding/Size Exclusion Chromatography (SEC)	88
	IGF-I Tracer Binding	88
	IGF-I Binding Activity in Sheep Plasma/SEC at pH7.4	88
2.5	Validation of Sheep Tissue Extraction for IGF-I RIA	89
	Introduction	89
	Tissue Extraction	92
	<i>Tissue sampling</i>	92
	<i>Extracting tissues</i>	92
	<i>Problems with separation of tissue homogenate</i>	93
	Validation	93
	<i>Recovery of IGF-I immunoreactivity from sheep tissue extracts</i>	95
	<i>Examination of IGF-I binding activity in sheep tissue extracts</i>	95
	<i>Conclusion</i>	100
	IGF-I RIA for Tissue Extracts	100
2.6	IGF-I Ligand Blot Analysis	100
	Modified Western Blotting Technique	100
	IGF-I BPs in Adult Sheep Plasma	101
2.7	RIA for Ruminant GH	104
	Materials	104
	GH Iodination	105
	GH Assay	106
2.8	In Situ Hybridisation	106
	Introduction	106

Materials	108
<i>Markers for size determination</i>	108
<i>IGF-I cDNA probes</i>	109
Methods	109
<i>Processing and sectioning tissue</i>	109
<i>Section pre-treatment</i>	112
<i>Hybridisation method</i>	113

CHAPTER THREE : REGULATION OF PLASMA AND TISSUE IGF-1 IMMUNO - REACTIVITY BY NUTRITION AND GH IN POSTNATAL SHEEP.

3.1	Introduction	116
3.2	Materials and Methods	119
	Materials	119
	Animals and Treatment	119
	Blood Sampling	120
	Tissue Processing	121
	Tissue Extraction	121
	Assays	121
	<i>Measurement of glucose, NEFA and GH in plasma</i>	121
	<i>In-situ hybridisation</i>	121
	<i>IGF-1 RIA</i>	122
	<i>Estimation of tissue - extractable IGF-1 derived from entrapment of blood</i>	122
	Validation of AEC Technique for Measuring IGF-I in Extracts of Sheep Tissues	123
	Calculations of Tissue IGF-I concentrations	124
	Biometrics	124
3.3	Results	125
	Nutritional status in the experimental animals	125
	Hormonal status in the experimental animals	125
	Plasma and Tissue IGF-I concentrations	129
	Liver IGF-I mRNA levels monitored by ISH	134
	Correlations between liver IGF-I and IGF-I mRNA levels	134

3.4	Discussion	134
	Nutritional status	137
	Effect of fasting on plasma levels of GH	138
	Nutritional and Hormonal Regulation of IGF-I in plasma and tissues	140
3.5	Summary	145

CHAPTER FOUR : GH STIMULATION OF TISSUE IGF-1 PRODUCTION IN POST-NATAL SHEEP

4.1	Introduction	146
4.2	Material and Methods	149
	Materials	149
	Animals and Treatment	149
	Blood sampling and Tissue processing	150
	Tissue Extraction	150
	Assays	151
	<i>Plasma concentrations of GH</i>	151
	<i>In-situ Hybridisation (ISH) for monitoring tissue IGF-I mRNA</i>	151
	<i>IGF-I RIA</i>	151
	Calculations of Tissue IGF-I concentrations and total content of IGF-I in organ, muscle and plasma	152
	Biometrics	153
4.3	Results	153
	Circulating levels of GH; 24 hr-profile of plasma GH	153
	Circulating levels of glucose; 24 hr-profile of plasma glucose	155
	Circulating levels of IGF-I; 24 hr-profile of plasma IGF-I response to GH	155
	Tissue levels of IGF-I; 24 hr-profile of tissue IGF-I response to GH	155
	Correlations between tissue and plasma IGF-I concentrations	159

	Basal levels and maximal increase in tissue and plasma IGF-I concentrations in response to GH	161
	Basal levels of total IGF-I in organ, muscle, plasma and the increase in total IGF-I in organs after GH administration	161
	IGF-I mRNA levels in liver, kidney and muscle	163
4.4	Discussion	163
	Profiles of plasma GH and glucose in response to exogenous GH	163
	Tissue IGF-I production in response to exogenous GH	166
4.5	Summary	172

CHAPTER FIVE : EFFECT OF GH ADMINISTRATION ON PLASMA LEVELS OF IGFBPs IN POST-NATAL SHEEP

5.1	Introduction	173
5.2	Material and Methods	176
	Material	176
	Animals and Treatment	176
	Tissue extraction	177
	Assays	178
	<i>GH RIA</i>	178
	<i>IGF-1 RIA</i>	178
	<i>IGF-I Ligand blot analysis</i>	179
	Biometrics	180
5.3	Results	180
	Circulating levels of GH	180
	Tissue and plasma IGF-1 response to GH administration	180
	IGFBPs in sheep plasma	180
	Plasma IGFBP response to GH administration	184
5.4	Discussion	191

	Identification of IGFBPs in sheep plasma	191
	IGFBP response to administration of GH	194
5.5	Summary	199
 CHAPTER SIX : GENERAL DISCUSSION		
6.1	Nutritional and GH regulation of tissue IGF-I production	201
6.2	Role for IGF : Paracrine/Autocrine or Endocrine?	207
6.3	Conclusion	212
	REFERENCES	213
	PUBLICATIONS ARISING FROM THIS THESIS	290

ABSTRACT

Somatic growth represents the outcome of interactions between the genetic potential of an individual to grow and environmental factors, prominent amongst these is nutrition. GH, the IGFs, and their respective receptors appear to play important and interrelated roles in co-ordinating these interactions, and as such they are considered one hormonal system 'GH axis' which controls normal post-natal growth.

A large number of external and internal stimuli impinge on the GH axis. Experiments in this thesis were conducted to address whether nutritional status (a powerful external stimulus) or GH (a prominent internal stimulus) treatment differentially alters tissue and plasma IGF-I immunoreactivity, tissue IGF-I mRNA and plasma IGFBPs. GH stimulation of IGF-I in the liver and other tissues and the associated response of circulating IGFBPs was measured with a view to investigating IGF-I production in both hepatic and extra-hepatic tissue and its regulatory control through the GH axis. This would provide an insight into the relative contribution or importance of local versus hepatic production of IGF-I in post-natal sheep.

To achieve the objectives in this study, methods of IGF-I tissue extraction were developed and evaluated in the experiments presented in this thesis to provide an efficient extraction of IGF-I and reliable removal of IGFBP for measurement of IGF-I by RIA in various tissues in sheep that were fed or fasted, with or without administration of exogenous GH. To monitor changes at transcriptional step, levels of IGF-I mRNA in tissues were measured using *in-situ hybridisation*. Responsiveness

of circulating IGFBPs to administration of GH was investigated by IGF-I ligand blot analysis.

Administration of exogenous GH significantly elevated IGF-I levels in liver, kidney, heart, spleen and muscle. The rise in tissue (except muscle) levels of IGF-I preceded that in plasma, suggesting that IGF-I derived from multiple tissues contributes to plasma concentrations of the growth factor. GH stimulated IGF-I mRNA in both hepatic and extra-hepatic tissues. The elevated levels of tissue IGF-I and GH-dependent IGF-I mRNA in both hepatic and extra-hepatic tissues indicate production of IGF-I in the liver and other tissues.

The response of muscle IGF-I to administration of GH followed the rise in plasma IGF-I. This may indicate a late response in local production of IGF-I in the muscle or may reflect the influence of changes in IGF-I plasma concentration, implicating a possible endocrine role for IGF-I in the muscle, although the relative contribution or importance of local versus plasma-origin IGF-I is not certain in this tissue.

Data in this thesis demonstrated that GH not only stimulates IGF-I production but also plays a pivotal role in regulation of circulating levels and tissue availability of IGF-I (ie. tissue distribution of IGF-I) by affecting both IGF-I and its binding proteins either directly or indirectly.

Fasting depressed plasma and tissue levels of IGF-I. The degree of the depression in IGF-I levels varied between tissues. Levels of IGF-I mRNA in liver were

positively correlated with concentrations of liver IGF-I in animals that were well fed with or without GH treatment, but not in those that were fasted or fasted and treated with GH. This indicates that GH regulation of IGF-I production in liver is, to a large extent, dependent on nutritional status. Nutrition therefore plays a critical role in the regulation of IGF-I production in the sheep. The current study has also provided a way to estimate total organ content of IGF-I and the response of this parameter to GH treatment. The GH-induced increase in total liver IGF-I is 4-fold more than the corresponding increase in kidney, 13-fold more than that in heart and 30-fold more than that in spleen. These results support the traditional concept that liver is a major contributor of circulating IGF-I, although the data in this study suggested that IGF-I is synthesised in multiple tissues in the sheep.

In conclusion, the results of the present study are compatible with the concept of both local and classic central production of IGF-I under the influence of GH, and therefore do not exclude either an autocrine/paracrine or an endocrine mechanism of IGF-I action. The relative importance or contribution of each may be related to a tissue-specific fashion of IGF-I gene expression which is differentially regulated by GH, nutrition and numerous other factors.

ACKNOWLEDGEMENTS

I wish to express my thanks to all colleagues and friends, at Growth Physiology and Molecular Biology of Ruakura Agricultural Centre and at the Department of Bioscience of the University of Waikato, who in numerous ways have contributed to this study.

Special thanks to:

Dr J.J. Bass, Dr P.C. Molan and Dr S.C. Hodgkinson, my supervisors in research, for introducing me to the field of protein chemistry and molecular endocrinology. Your enthusiasm, encouragement and critical expertise will never be forgotten.

A.K. Hodges, S. Kirk, A. Molenaar and Dr R. Ord with whom some of the work, in measurement of IGF-I mRNA, presented here was undertaken.

J. Napier for technical advise and reading or correcting my thesis.

B. Edgley, P. Dobbie, D. Petrie and C. Berry for laboratory support. M. Holmes and T. Watson for care of the animals.

Dr S. Spencer, Dr J. Oldham and J. Elliott for valuable and interesting discussions.

Dr R.J. Wilkins for the privilege of working for a period in the Molecular Biology Lab.

Dr N. Cox and J.F. Zhang for biometrics.

G. Patterson-Gray for her major contribution in the typing and presentation of this thesis. M. Bates and P. Fitz-Gerald for some initial typing of this thesis.

Dr P. Allen for unforgettable help.

Ross, Merv, Jim, Steve and John for making me feel at home.

Finally, Quanjin, Wei and my parents for being always 'together' with me.

List of Figures

	Page
1.1 Amino acid sequence of bovine growth hormone.	3
1.2 Primary sequences of human, bovine and ovine IGF-I and II.	14
1.3 Schematic representation of insulin-like growth factors binding proteins 1 to 6.	45
2.1 Purification of radiolabelled IGF-I on a reversed phase Sep-Pak C ₁₈ cartridge.	79
2.2 Superose 12 size exclusion chromatography of normal sheep plasma.	90
2.3a-b Size exclusion chromatography of normal sheep plasma and acid-ethanol-cryo-precipitation extracts of NSP following incubation with ¹²⁵ I-labelled like growth factor-I.	97
2.3c-e Size exclusion chromatography of acid-ethanol-cryo-precipitation extracts of sheep tissues following incubation with ¹²⁵ I-labelled insulin-like growth factor-I.	98
2.3f-h Size exclusion chromatography of acid-ethanol-cryo-precipitation extracts of sheep tissues following incubation with ¹²⁵ I-labelled insulin-like growth factor-I.	99
2.4 IGF-1 ligand blot analysis of adult sheep plasma.	103
2.5 Autoradiography of RNA transcripts (probes for <i>in situ hybridisation</i>) following electrophoresis under denaturing conditions.	110

	Page
3.1 Effect of nutritional manipulation and GH treatment (hGH; 0.15 mg/kg bodyweight per day) on plasma levels of glucose (mmol/l) in 12-month-old ewes during a period of five-day-treatment.	126
3.2 Effect of nutritional and GH treatment (hGH; 0.15 mg/kg bodyweight per day) on plasma levels of NEFA (mmol/l) in 12-month-old ewes during a period of five-day-treatment.	127
3.3 Effect of five-day-fasting on plasma levels on ovine GH (ng/ml) in 12-month-old ewes.	128
3.4 Effect of nutritional manipulation and GH treatment (hGH; 0.15 mg/kg body weight per day) on plasma levels of IGF-I in 12-month-old ewes during a period of five-day-treatment.	130
3.5 Autoradiography of ovine liver sections probed for IGF-I RNA by <i>in-situ hybridization</i> in the four trial groups: Feeding, Fasting, Feeding and daily injection of GH, Fasting and dialy injection of GH.	135
3.6 Correlation between IGF-I concentrations and the relative levels of IGF-I mRNA determined by ISH in 12-month-old ewes after five days of treatment.	136
4.1 Twenty four hour-profile of circulating levels of growth hormone.	154
4.2 Twenty four hour-profile of circulating concentrations of glucose.	156

	Page
4.3 Plasma IGF-I response to bovine growth hormone administration in six-month-old ewes.	157
4.4 Tissue IGF-I response to bovine growth hormone administration in six-month-old ewes.	158
4.5 Muscle IGF-I response to bovine growth hormone administration in six-month-old ewes.	160
4.6 A comparison of the relative increases in total organ IGF-I 13 hours after administration of bovine growth hormone.	164
4.7 IGF-I mRNA levels in liver, kidney and muscle after GH administration.	165
5.1 Plasma levels of growth hormone.	181
5.2 Tissue and plasma IGF-I concentrations in control groups of Dorset x Coopworth ewes at different sampling times.	182
5.3 IGF-I ligand blot analysis of normal sheep plasma, collected from a control ewe 0, 5, 17 and 24 h after one single-dose injection of saline.	185
5.4a IGF-I ligand blot analysis of sheep plasma, collected from a representative 6-month-ewe (lamb #3) 0, 5, 17 and 24 h after GH administration.	186
5.4b IGF-I ligand blot analysis of sheep plasma, collected from a 6-month-ewe (lamb #2) 0, 5, 17 and 24 h after GH administration.	187
5.5 Plasma IGFBP-3 response to GH administration.	188

		Page
5.6	Concomitant increase in plasma/tissue IGF-I and plasma IGFBP-3 in response to GH administration.	189
5.7	Plasma IGFBP response to GH administration (bGH; 0.25 mg/kg body weight).	190
5.8	Basal levels of plasma IGFBPs in control ewe lambs.	192

LIST OF TABLES

		Page
3.1	Effect of nutritional manipulation and GH treatment (hGH; 0.15 mg/kg per day) on plasma IGF-I and tissue IGF-I concentrations in sheep after 5 days of treatment.	131
3.2	Blood contamination of IGF-I in tissues (Haemoglobin assay)	132
3.3	Differences in IGF-I concentration in sheep tissues between two different levels of nutrition after five days of treatment.	133
4.1	The basal levels of IGF-I concentrations in control and the peak concentrations of IGF-I in GH-treated sheep tissues after administration of bovine growth hormone; 0.25 mg/kg body weight.	162
5.1	IGF-I concentrations in sheep tissue and plasma 0, 5, 17 and 24 hours after administration of bovine growth hormone (0.25 mg/kg body weight).	183

LIST OF ABBREVIATIONS

A	Angstrom
ACTH	adrenocorticotrophic hormone
ASP	adult sheep plasma
ALS	acid-labile subunit
BSA	bovine serum albumin
BST hormone)	bovine somatotrophin (recombinant bovine growth hormone)
BP	binding protein
C-terminal	carboxy terminal
Ci	Curie
C/mmol	curies per millimole
cm	centimetre
CPM	counts per minute
CV	coefficient of variation
cDNA	complementary deoxyribonucleic acid
CSF	cerebrospinal fluid
#C	degrees celsius
Da	Dalton
des (1-3) IGF-I	Trauncated variant f IGF-I lacking the amino terminal amino acids
E. coli	Escherichia coli
EDTA	disodium salt of ethylene diaminetetra-acetic acid
FPLC	fast protein liquid chromatography system
NSP	normal sheep plasma
Fc	antibody fragment consisting of the two c-terminal portions of the heavy chains of IgG
<i>g</i>	gravity
GRF	growth hormone releasing factor
GOD	glucose oxidase
GH	growth hormone
g/l	grams per litre
h	hour
h	human when appended to IGF-I
HPLC	high performance liquid chromatography system
HCl	hydrochloric acid
³ H	tritium (tritiated)

¹²⁵ I	iodine-125
¹³¹ I	iodine-131
iv	intra venous
Ile	isoleucine
IgG	immunoglobulin G
IGF	collective term for the insulin-like growth factors
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFBP	IGF binding protein
¹²⁵ I-IGF-I	radioiodinated IGF-I
¹³¹ I-IGF-I	radioiodinated IGF-I
Ka	association (affinity) constant
kb	kilobase
kDa	kilodalton
kd	dissociation constant
LPO	lactoperoxidase
Leu	leucine
Lys	lysine
l	litre
mol	mole
mCi	millicurie
lCi	microcurie
M	molar, mol/l, moles per litre
mM	millimolar, mmol/l
lmol/l	micromolar, lmol/l
lCi/lg	microcurie per microgram
m	metre
mm	millimetre
lm	micrometre
mg	milligram
mg/ml	milligram per millilitre
lg	microgram
min	minute
ml	millilitre
ml/min	millilitre per minute
ll	microlitre
MW	molecular weight
MDBK	fetal bovine kidney cell line
mRNA	messenger ribonucleic acid
nmol	nanomole
nm	nanometre
N-Met IGF-I	amino terminal methionyl recombinant human IGF-I
ng/ml	nanogram per millilitre
NEFA	non esterified fatty acids

N-linked	amino linked glycosylation
N-terminal	amino terminal
NSILA	non-suppressible insulin-like activity
O-linked	hydroxy linked glycosylation
PAGE	polyacrylamide gel electrophoresis
PBSA	phosphate buffered bovine serum albumin
pI	isoelectric point
pH	hydrogen ion concentration
Phe	phenylalanine
Pro	proline
P	probability
RIA	radioimmunoassay
RPC	reversed-phase chromatography
rpm	revolutions per minute
rec IGF-I	recombinant human authentic sequence IGF-I
RT	room temperature
RRA	radioreceptor assay
SD	standard deviation
SEM	standard error of the mean
SED	standard error of the difference
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
SRIF	somatostatin
Sm-C	somatomedin-C
TFA	trifluoroacetic acid
TCA	trichloroacetic acid
vol	volume
v/v	vol per volume
w/v	weight per volume
150 kDa	one hundred and fifty kilodalton IGF binding protein
complex	
40-50 kDa	40-50 kDa pool of IGFBP
3D	three dimensional

ABBREVIATIONS IN THE SEQUENCE OF AMINO ACIDS

A	alanine
Ala	alanine
Arg	arginine
Asp	aspartic acid
Asn	asparagine

C	cysteine
Cys	Cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
Met	methionine
N	asparagine
P	proline
R	arginine
Ser	serine
S	serine
Thr	threonine
Tyr	tyrosine
Trp	tryptophan
T	threonine
V	valine
X	an unidentified amino acid

DEDICATION

To my father

This is the way

I have been talking to you

during the past four years

with my love and sorrow

I truly believe that

you did hear

and

you did understand

CHAPTER 1 : INTRODUCTION

Growth occurs by a combination of an increase in the number (hyperplasia) and an increase in the size (hypertrophy) of the component cells; which results in an increase in body size or mass.

From a physiological point of view, growth is a complex process of cell replication, tissue differentiation, matrix formation, cell death and many other interrelated mechanism. It is the product of interactions between the genetic drive to growth and many environmental factors, especially nutrient/substrate supply (1, 2).

The endocrine system can be envisaged as the mechanism by which the genetic and environmental infractions are co-ordinated and cellular replication is modulated. The endocrine control of growth involves the complex interactions of several hormones and growth factors, acting both systemically and locally. Although many of these interactions are poorly understood, much evidence indicates that pituitary growth hormone (GH) is essential for normal post-natal but not necessarily for pre-natal or very early post-natal growth in mammals (3, 4, 5, 361, 362, 363, 352, 364). It has been generally accepted that the actions of GH in regulating post-natal somatic growth are mediated, at least in part, by the insulin-like growth factors (IGFs).

The purpose of this introduction is to review the role of the GH axis in the regulation of post-natal growth in the ruminant.

Growth Hormone (GH)

History/Molecular Structure

GH was first suspected from the work of Evans and Long (477), in 1921, who injected a saline extract of anterior lobes of bovine pituitaries into normal rats with increased growth as a result. Smith in 1926 (375) then showed that the growth of hypophysectomised rats was resumed after administration of a similar anterior pituitary extract. Consequently, the existence of a growth-promoting activity in the anterior lobe of the pituitary was indicated. It was not until 1944, however, that Li *et al.* reported the isolation of GH from bovine pituitaries (6). The primary structure of bovine GH (figure 1.1) consists of some 190 amino acids arranged in a single polypeptide chain containing two disulphide bridges and has an approximate molecular weight of 22 kDa (7). Human GH, first isolated in 1956 (8), has also been extensively studied (9, 10, 365, 366) and is considered to be a complex of hormones (9, 10). The physiological significance of the variants is as yet uncertain.

Evidence suggests that the growth hormones, prolactin and placental lactogens evolved from a common ancestral hormone and share structural and functional characteristics (11, 12). Little species variability between the ovine and bovine GH is evident (3/191 amino acids) but by contrast the human and rat hormones are quite distinct. This is reflected in biological properties of the hormones. For

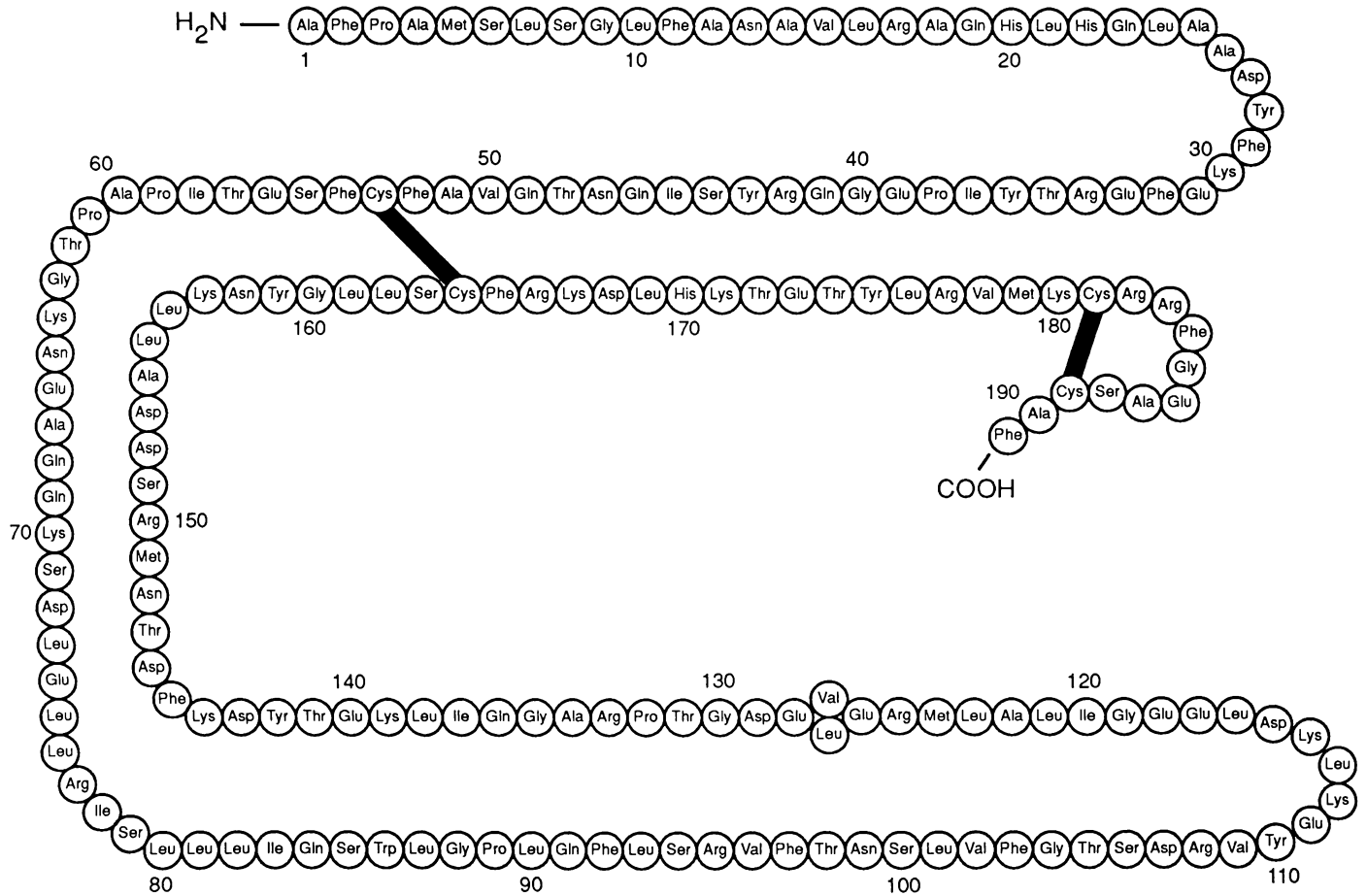


FIGURE 1.1 Amino acid sequence of bGH, data from Wallis (7)

example non-primate GHs have little anabolic activity in the human whereas the human hormone is active in many species (13). Human GH also has marked lactogenic properties compared with other species including sheep and ox (14). GH has a complex three-dimensional structure (15) and it is possible the hormone's broad range of metabolic and anabolic actions may be properties of distinct regions of the GH molecule. Recombinant bovine GH (bGH) and human GH (hGH) have been used in the animal studies reported in this thesis.

Synthesis/Secretion/Control

GH is synthesized by somatotrophic cells in the anterior pituitary (*pars distalis*; 16). Synthesis takes place on membrane-bound polysomes. The primary translation product appears to be a precursor protein of some 25 kDa containing an amino terminal hydrophobic signal sequence of approximately 3 kDa (17, 18).

Maturation of the nascent polypeptide involves proteolytic cleavage of the precursor sequence, disulphide alignment and formation and the hormone is sequestered in secretory granules to await exocytosis. Apart from the pituitary few other sites of GH synthesis are recognised. One such site is the placenta which apparently synthesizes a GH variant in small amounts in addition to placental lactogen and prolactin (19, 20).

The secretion of GH in adult mammals is highly pulsatile demonstrating a marked synchronous ultradian rhythm with peaks of secretory activity occurring at regular intervals. This has been recognised in rats (21), ruminants, (22) and humans.

Pulsatility is apparently necessary for optimal GH activity in some species but not apparent in ruminants (23). Although precise reasons for this are far from certain they may relate to both pulse and receptor dynamics (24).

Secretion of GH is influenced by neural, hormonal and metabolic factors possibly acting through the final common pathway of hypothalamic somatostatin (SRIF) and growth hormone releasing factor (GRF). Somatostatin, a tetradecapeptide, is produced in a wide range of neural and other tissues including pancreas and alimentary tract (25). However evidence suggests that it is SRIF produced in neurones of the periventricular and/or arcuate regions of the hypothalamus that is released at the stalk median eminence into the hypophysial portal circulation and exerts an inhibitory effect on GH secretion(26, 27).

GRF, a 44 amino acid peptide which, by contrast, stimulates GH release, is primarily localised in nuclei of the medial basal hypothalamus (chiefly arcuate and ventromedial) and is also transmitted to the pituitary via the portal circulation (28-31).

Increasing evidence suggests a close inter-relationship between nutritional status and the somatotrophic axis and this occurs at several levels. For example the arcuate hypothalamus which contains GRF-secreting neurones also contains neurones which regulate feeding. Circulating concentrations of GH are closely related to nutrition (444, 445, 450-456). Thus levels are higher and the GH response to GRF challenge greater in undernourished sheep compared with adequately fed counterparts as in

cattle (55, 42, 32-35). Some evidence suggests GH secretion is related to the protein rather than caloric content of intake (36, 37). In a major study Breier and colleagues examined the effect of nutritional status on pulsatile GH release in growing steers (42). Increased GH secretion associated with under-nutrition was observed in that study without an obvious change in pulse frequency. Cattle in peak lactation also demonstrate higher concentrations of GH which may result from inadequate nutritional intake (53, 38). It is considered that increased GH secretion in under-nutrition may serve to mobilise energy from adipose tissue to meet metabolic requirements (65, 53).

Further studies have indicated that the hepatic GH receptor is an important second level for interaction between nutrition and the somatotrophic axis. This is discussed more fully below. In addition to nutritional influences, GH secretion in ruminants appears to be sexually dimorphic (40, 41) related to developmental stage (43, 44, 41), and demonstrates some diurnal and seasonal variability (22, 39). Factors affecting GH secretion via feedback mechanisms at the hypothalamic and/or pituitary level include plasma substrates, insulin, GH itself, sex steroids (reviewed in 45) and importantly insulin-like growth factor-I (IGF-I; 46). Recent reports in 1992 from Ninth International Congress of Endocrinology (383) revealed that neither IGF-I nor IGF-II alone plays a physiologically important role in feedback regulation of pulsatile GH secretion at a central nerve system level, and that central administration of IGF-I and IGF-II, in combination, inhibited GH bursts 2-3 h after injection. Both GH pulse amplitude and mean plasma GH levels were markedly suppressed compared with vehicle-injected controls, suggesting a synergistic

interaction between these two growth factors in central control of GH secretion (383).

Receptors

The wide range of GH actions are believed to result from the initial interaction of GH with its membrane-bound receptor. GH receptors are present in many cell types including hepatocytes, adipocytes, skeletal muscle cells, chondrocytes, osteoblasts and β islets from a number of species (47, 49). The receptor generally appears as a glycoprotein consisting of dimers and trimers of 70-100 kDa subunits. Recently the rabbit and human liver GH receptor has been cloned (47, 48) and shown to consist of an N-terminal extra-cellular domain of some 250 amino acids with a 350 residue intracellular region.

The cloned proteins consist of single polypeptide chains which are capable of binding GH. However evidence from electrophoresis with immunological and affinity cross-linking suggests heterogeneity of the GH receptor (49). The GH receptor also has tyrosine kinase activity and becomes phosphorylated on ligand binding (50). The extra-cellular domain of the GH receptor has recently been shown to be very similar to a GH-binding protein originally identified in rabbit plasma, and evidence suggests that the GH receptor is also present in the cytosol and possibly the nucleus of target cells (51).

In the ruminant the GH receptor is developmentally regulated and sensitive to endocrine and metabolic control. In sheep, the binding of ovine GH (oGH) to the

liver is minimal before birth (52) which is consistent with the view that pre-natal somatic growth is not significantly regulated by GH, but rises to adult values within a few days of parturition (307). This is paralleled by an increase in plasma IGF-I (310). Hepatic GH binding also increases markedly at puberty and during pregnancy, and roles for insulin, oestrogens and GH itself in up-regulating the GH receptor have also been proposed (53). Nutrition has a marked effect on GH receptors in the steer (54), increasing both hepatic GH receptor number and affinity and similar results having been demonstrated in lambs under nutrition (55). Emergence of the high affinity binding site in animals on a high plane of nutrition has been carefully described, and is associated with increased plasma IGF-I concentrations and liveweight gain, carcass leanness and milk production (53). Evidence suggests that GH can induce its own receptor. Hypophysectomy in sheep reduces GH binding which can be partially restored by exogenous GH. Treatment of lambs with high doses of bGH for relatively long periods (0.55 mg/kg/day, for eight weeks) has been found to induce a 2.5 fold increase in oGH binding, this being mostly due to a marked increase in the high-affinity binding site (55). Nutritional dependence of GH receptors in extra-hepatic tissues is less well described.

The major changes in GH receptors described suggest that this may be an important locus for regulatory control in the somatotrophic axis (57). Oestrogens, which are established growth promotants in ruminants, are also believed to act at least partially at the level of the GH receptor. While oestradiol 17 β affects circulating GH only slightly, the steroid has been found to increase high affinity hepatic binding several-

fold on higher planes of nutrition (54).

It is tempting to speculate that amongst the GH receptor subtypes there are variants which mediate anabolic and catabolic actions. Accordingly these would be regulated differently to meet the demands of a wide variety of physiological conditions, for example high and low nutritional intakes. However, evidence that conditions which activate GH receptors in adipocytes (catabolic processes) are similar to those in liver (anabolic actions) would suggest that this is not the case (47).

Metabolic Actions

Growth hormone shows great diversity of biological actions which can broadly be classified as either anabolic (*i.e.* stimulate cell proliferation) or metabolic. These are chiefly concerned with nutrient partition. For example, actions of GH in adipose tissue are catabolic whereas those in muscle, cartilage and bone are anabolic. That GH can have such a wide variety of apparently opposing effects is perhaps the major reason for its central role in the regulation of post-natal growth and development, and explains why it is the subject of on-going inquiry and research with respect to both animal production and human health.

Growth hormone causes a marked reduction in adiposity (56, 58) which is directly related to the relative rates of triglyceride synthesis and degradation. GH markedly decreases lipid synthesis in many species. Thus glucose utilisation by adipocytes is reduced; this is paralleled by a decline in key lipogenic enzymes (glucose-6-

phosphate dehydrogenase, fatty acid synthetase). Stimulatory effects of insulin on fat cell metabolism including lipogenesis and glucose transport are also blunted by GH (reviewed in 59). By contrast lipolysis is increased. This effect is quite marked but may be indirect since GH appears to increase sensitivity to lipolytic stimuli.

Long-term treatment of growing lambs with GH also markedly increases circulating non-esterified fatty acids (NEFA), this being associated with a significant decrease in carcass fat (328). Circulating concentrations of GH are elevated on fasting in ruminants and in humans but not in rats. Elevated GH during fasting may serve to mobilise nutrients from adipose tissue to maintain homeostasis (11, 65, 62).

GH has a marked stimulatory effect on milk production in lactating ruminants (61). Although increases in milk yield of 20-30% can be achieved by daily injection (commonly as recombinant bGH), the mechanism by which this occurs is poorly understood. However, much evidence (270, 271, 331, 332, 336) suggests that this is not a direct effect of GH on the mammary gland but indirect. One important effect relates to substrate supply. Thus GH treatment appears to chronically increase lipolytic activity in lactating ruminants as illustrated by a progressive rise in NEFA, the effect of which is to divert fat reserves to milk production. This effect appears to be greater in animals in negative energy balance (62, 63) with fat reserves to mobilise.

GH also has direct effects on carbohydrate metabolism. Following bolus administration, acute insulin-like and prolonged (anti-insulin-like) diabetogenic

effects are observed in several species as revealed by transient hypoglycemia followed by elevation of plasma glucose. This hyperglycemia is often associated with increased insulin concentrations, the classical manifestation of an insulin-resistant state (60, 65). Increased plasma concentrations of glucose may result in part from reduced insulin sensitivity and reduced glucose uptake by peripheral tissues, particularly muscle, and also to increased hepatic glucose output in response to GH. Insulin is elevated in GH-induced insulin resistance, possibly as a result of the stimulatory effect of glucose on insulin release or alternatively via a direct effect of GH on the β islet. GH is elevated in fasting ruminants, which in addition to mobilising lipid may have the advantage of sparing glucose for homeostatic processes (53).

When GH is administered to an animal it causes general nitrogen retention. Accordingly levels of blood urea and urinary nitrogen fall. Hepatic degradation of amino acids is reduced as is amino acid delivery from peripheral tissues to liver (65). These effects are due largely to stimulation of protein synthesis in many tissues including muscle and liver. The effect is rapid, so although in the longer-term GH actions on protein synthesis may be mediated by other factors, particularly the IGFs, the short-term effect appears to be direct (11).

Thus GH appears to enhance the activity of the protein synthetic apparatus and to be involved in the induction of a wide range of proteins, having structural, storage and regulatory functions. Amongst the latter the IGFs and their binding proteins are of major importance in the control of growth and development. They are topics

of the investigative work of this thesis and are discussed in following sections (1.2 and 1.3). Further examples of specific proteins induced by GH are enzymes involved in amino acid metabolism, such as tyrosine aminotransferase (64).

Growth hormone also has physiological effects concerned with facilitating substrate supply. A good example of this is the increased mammary blood flow observed in dairy cattle treated with bGH (61).

Insulin-Like Growth Factors (IGFs)

History/Molecular Structure

The insulin-like growth factors (IGF-I and -II) are mitogenic polypeptides which have been implicated in the regulation of pre and post-natal growth in many species.

Evidence for the IGFs emerged from three different lines of investigation. First was the concept that GH action on skeletal tissues occurs via an intermediary factor originally termed sulphation factor (72) and latter somatomedin (76). Secondly was the observation that many cells in culture need serum to proliferate. In 1972 serum factors were isolated (77) which, when added to culture medium, stimulated replication. These were collectively termed multiplication stimulation activity (MSA) and subsequently it was realised that hepatocytes in culture synthesize and secrete MSA (78). The third line of investigation relates to the insulin-like actions of IGF.

Serum has insulin-like activity on muscle and adipose tissue far in excess of its insulin content. The insulin-like effects of serum were only slightly suppressible with anti-insulin serum, and were determined to be due to a factor termed non-suppressible insulin-like activity (NSILA) (79). Full structural analysis and characterisation has subsequently revealed that these factors belong to the closely related family now known as the insulin-like growth factors (IGF-I and II).

Variants of somatomedin termed A, C and one form of NSILA (-I) have subsequently been demonstrated to be the same peptide (now termed IGF-I), and MSA and NSILA-2 are the rat and human homologues of a second peptide (IGF-II) (80).

IGF-I and II are single chain polypeptides, the primary structures of which were first reported by Rinderknecht and Humbel in 1978 (81, 82). They have approximately 70% identity and are also highly homologous with the insulin precursor (pro-insulin). Thus they have regions corresponding to the B and A chains of insulin, and an inter-connecting peptide relating to the C-peptide of pro-insulin which is present in the mature peptide. In addition the IGFs have short carboxyl-terminal extensions on the A chain termed the D-peptide (Figure 1.2). IGF-I is a basic peptide (pI 8.2) of 70 amino acids with molecular weight of approximately 7650 Da while IGF-II is acidic with 67 amino acids (MW 7470 Da).

In addition to the human, IGF-I has been isolated from rat serum (83) as well and the complete sequence has been deduced from the nucleotide sequence of the rat

	1				5					10			
hIGF-I				Gly	Pro	Glu	Thr	Leu	Cys	Gly	Ala	Glu	Leu
bIGF-I				Gly	Pro	Glu	Thr	Leu	Cys	Gly	Ala	Glu	Leu
oIGF-I				Gly	Pro	Glu	Thr	Leu	Cys	Gly	Ala	Glu	Leu
hIGF-II	Ala	Tyr	Arg	Pro	Ser	Glu	Thr	Leu	Cys	Gly	Gly	Glu	Leu
bIGF-II	Ala	Tyr	Arg	Pro	Ser	Glu	Thr	Leu	Cys	Gly	Gly	Glu	Leu
oIGF-II	<i>Ala</i>	Tyr	Arg	Pro	Ser	Glu	Thr	Leu	Cys	Gly	Gly	Glu	Leu
		15					20					25	
hIGF-I	Val	Asp	Ala	Leu	Gln	Phe	Val	Cys	Gly	Asp	Arg	Gly	Phe
bIGF-I	Val	Asp	Ala	Leu	Gln	Phe	Val	Cys	Gly	Asp	Arg	Gly	Phe
oIGF-I	Val	Asp	Ala	Leu	Gln	Phe	Val	Cys	Gly	Asp	Arg	Gly	Phe
hIGF-II	Val	Asp	Thr	Leu	Gln	Phe	Val	Cys	Gly	Asp	Arg	Gly	Phe
bIGF-II	Val	Asp	Thr	Leu	Gln	Phe	Val	Cys	Gly	Asp	Arg	Gly	Phe
oIGF-II	<i>Val</i>	<i>Asp</i>	<i>Thr</i>	<i>Leu</i>	<i>Gln</i>	<i>Phe</i>	<i>Val</i>	<i>Cys</i>	<i>Gly</i>	<i>Asp</i>	<i>Arg</i>	<i>Gly</i>	<i>Phe</i>
							B						
				30					35				
hIGF-I	Tyr	Phe	Asn	Lys	Pro	Thr	Gly	Tyr	Gly	Ser	Ser	Ser	Arg
bIGF-I	Tyr	Phe	Asn	Lys	Pro	Thr	Gly	Tyr	Gly	Ser	Ser	Ser	Arg
oIGF-I	Tyr	Phe	Asn	Lys	Pro	Thr	Gly	Tyr	Gly	Ser	Ser	Ser	Arg
hIGF-II	Tyr	Phe	Ser	Arg	Pro	Ala	-	-	Ser	Arg	Val	Ser	Arg
bIGF-II	Tyr	Phe	Ser	Arg	Pro	Ser	-	-	Ser	Arg	Ser	Asn	Arg
oIGF-II	<i>Tyr</i>	<i>Phe</i>	<i>Ser</i>	<i>Arg</i>	<i>Pro</i>	<i>Ser</i>	<i>-</i>	<i>-</i>	<i>Ser</i>	<i>Arg</i>	<i>Ser</i>	<i>Asn</i>	<i>Arg</i>
							C						
		40				45				50			
hIGF-I	Arg	Ala	Pro	Gln	Thr	Gly	Ile	Val	Asp	Glu	Cys	Cys	Phe
bIGF-I	Arg	Ala	Pro	Gln	Thr	Gly	Ile	Val	Asp	Glu	Cys	Cys	Phe
oIGF-I	Arg	Ala	Pro	Gln	Thr	Gly	Ile	Val	Asp	Glu	Cys	Cys	Phe
hIGF-II	Arg	Ser	Arg	-	-	Gly	Ile	Val	Glu	Glu	Cys	Cys	Phe
bIGF-II	Arg	Ser	Arg	-	-	Gly	Ile	Val	Glu	Glu	Cys	Cys	Phe
oIGF-II	<i>Arg</i>	<i>Ser</i>	<i>Arg</i>	<i>-</i>	<i>-</i>	<i>Gly</i>	<i>Ile</i>	<i>Val</i>	<i>Glu</i>	<i>Glu</i>	<i>Cys</i>	<i>Cys</i>	<i>Phe</i>
			55				60						65
hIGF-I	Arg	Ser	Cys	Asp	Leu	Arg	Arg	Leu	Glu	Met	Tyr	Cys	Ala
bIGF-I	Arg	Ser	Cys	Asp	Leu	Arg	Arg	Leu	Glu	Met	Tyr	Cys	Ala
oIGF-I	Arg	Ser	Cys	Asp	Leu	Arg	Arg	Leu	Glu	Met	Tyr	Cys	Ala
hIGF-II	Arg	Ser	Cys	Asp	Leu	Ala	Leu	Leu	Glu	Thr	Tyr	Cys	Ala
bIGF-II	Arg	Ser	Cys	Asp	Leu	Ala	Leu	Leu	Glu	<i>Thr</i>	Tyr	Cys	Ala
oIGF-II	<i>Arg</i>	<i>Ser</i>	<i>Cys</i>	<i>Asp</i>	<i>Leu</i>	<i>Ala</i>	<i>Leu</i>	<i>Leu</i>	<i>Glu</i>	<i>Ala</i>	<i>Tyr</i>	<i>Cys</i>	<i>Ala</i>
							A						
					70								
hIGF-I	Pro	Leu	Lys	Pro	Ala	Lys	Ser	Ala					
bIGF-I	<i>Pro</i>	Leu	Lys	Pro	Ala	Lys	Ser	Ala					
oIGF-I	<i>Ala</i>	Leu	Lys	Pro	Ala	Lys	Ser	Ala					
hIGF-II	Thr	-	-	Pro	Ala	Lys	Ser	Glu					
bIGF-II	Thr	-	-	Pro	Ala	Lys	Ser	Glu					
oIGF-II	<i>Thr</i>	<i>-</i>	<i>-</i>	<i>Pro</i>	<i>Ala</i>	<i>Lys</i>	<i>Ser</i>	<i>Glu</i>					
							D						

Disulphides - IGF-I 6 - 48, 18 - 61, 47 - 52

Figure 1.2 Primary Sequences of human (h), bovine (b) and ovine (o) IGF-I and II. The italic areas indicate residues where the ovine peptides diverge from their bovine homologues.

gene (84). Only three substitutions were identified between rat and human IGF-I, one each in the B, C and A regions with one further in the D region of mouse IGF-I which has also been sequenced (85). Bovine IGF-I has been purified and sequenced from adult serum and found to be identical to human IGF-I (86). Recently IGF-I has been purified from ovine serum (87). The sheep hormone differs from the human and bovine peptides by a single amino acid substitution at position 66 (Ala in ovine, Pro in the human, 88). Partial sequence data has also been reported for the chicken IGF-I (89).

The amino acid sequence of the major forms of rat IGF-II (MSA) has been determined using peptide isolated from conditioned medium of Buffalo rat hepatocytes in culture. The sequence has five substitutions compared with human IGF-II (90). The sequence of bovine IGF-II differs from the human hormone at three of the same four positions as the rat hormone (Ser for Ala at position 32; Ala for Val at 35 and Asp for Ser at 36) (86). Ovine IGF-II purified from adult plasma differs from the bovine peptide at a single residue (62 being Ala in ovine IGF-II and Thr in bovine IGF-II; 87). Characterisation of mouse IGF-II has revealed that it differs from the human hormone at six positions (91; Figure 1.2).

Two different IGF-I precursor peptides have been predicted in the human using cDNA cloning techniques (termed IGF-IA and IGF-IB; 92, 93). The primary translation product of IGF-IA contains 153 amino acids while the IGF-IB precursor has 195 residues. The sequences for these two variants of pre-pro-IGF-I are identical at the amino terminal but diverge towards the carboxyl-terminal (E Domain). The functional significance of these variant forms remains to be

established but they may differ in their GH dependence. The primary translation product of IGF-II (pre-pro- IGF-II) appears to be similarly complicated (94).

Post-translational processing of the precursor proteins for IGF-I and II involves proteolytic cleavage of the hydrophobic signal sequence, disulphide alignment and removal of the extended carboxyl-terminal E domains. The IGFs appear unusual amongst polypeptide hormones in that they are not stored in secretory vacuoles awaiting a trophic stimulus, but rather they are secreted as produced (95).

IGF-I and II genes have been characterised in the human and rat. In the human the IGF-I gene is located on chromosome 12 whereas the gene for IGF-II is found on the short arm of chromosome 11 in a cluster that also includes the genes encoding insulin and tyrosine hydroxylase. The human and rat IGF-I genes are large complex structures containing at least 5 exons of 1.9-50 kb, each of which contain some coding information (96, 97).

Processing of the primary IGF-I gene transcript appears to be extremely complex with a wide range of translation products (0.7-7 kb) revealed by Northern analysis which appear to vary in their pattern of tissue distribution and GH-dependence (99).

The human IGF-II gene is also extremely complex consisting of some eight exons ranging over 30 kb of genome (reviewed in 98).

Variants of IGFs

Reports of several structural variants of IGF-I and II have appeared in the

literature. Large forms ranging in size from 10-20 kDa have been reported which are believed to result from alternative post-translational processing of the IGF precursor proteins. For example, there are variants with the E peptide attached (reviewed in 95). Rinderknecht and Humbel (82) have reported that up to 25% of plasma IGF-II lacks the N-terminal alanine, and an IGF-II peptide, with an internal extension of four amino acids at position 29 has also been identified (100).

Blum and colleagues have isolated several somatomedin variants with charges, which bind IGF antisera. The forms did not appear to vary in molecular size or to be due solely to deamidation since some were more basic than the major variant. Chromatofocusing revealed only minor development changes in distribution of these forms and they were apparently not due to allelic variation in samples (101, 102).

By far the most significant IGF-I variant reported to date is a form lacking the amino-terminal Gly-Pro-Glu sequence (des(1-3)IGF-I). Purified from bovine colostrum and human brain, des (1-3) IGF-I may arise from alternative splicing of IGF-I mRNA or, more likely, the action of a highly specific amino-peptidase. Evidence suggests that des (1-3) IGF-I has high bioactivity (103, 104, 327) and it is discussed more fully in following sections.

Recently IGF-I produced by recombinant DNA technology has become widely available. Several types of IGF peptide have been produced by this approach. The first, expressed in *Escherichia coli* (*E.coli*), has the 70 amino acid sequence of authentic IGF-I and an additional methionyl residue at the amino terminal (105). This may be chemically removed by cyanogen bromide treatment but substitution

of a second methionine (position 59) is then required to avoid further to avoid further cleavage at the second site. A second IGF peptide produced in this fashion has the natural glycine residue at the amino terminal but a conservative methionine to threonine substitution at position 59.

Following direct expression in *E. coli* recombinant peptides are not usually secreted but found in an insoluble form in cytoplasmic inclusion bodies (106). These may be recovered by centrifugation and the peptide resolubilised under reducing and denaturing conditions (107). At this stage cleavage of the N-terminal methionine may be performed and refolding achieved by removal of chaotropic conditions. More recently it has been possible to genetically engineer IGF-I with the authentic amino acid sequence in the yeast *Saccharomyces cerevisiae* (108).

Native IGF-I has three disulphide bonds connecting cysteine residues 6-48, 47-52 and 18-61. A problem with *in vitro* refolding is that disulphide mis-matching may occur. Indeed refolding of IGF-I in aqueous buffer following expression in *E. coli* has been shown to result in a disulphide mismatched variant (Cys 6-47, 48-52 and 18-61) of approximately 30% abundance (300). Other variants are also possible and one (Cys 6-52, 18-61, 47-48) may also be present in low concentrations following refolding of *E. coli*-derived IGF peptides.

Post-translational processing in yeast is much more similar to that in higher eukaryotes. Recombinant peptides are exported from the site of synthesis on the endoplasmic reticulum via the Golgi apparatus to secretory vessels and may be recovered from culture media. Signal sequence removal and disulphide alignment

occur during the secretory process and disulphide mis-matching appears to be much less of a problem (569).

Synthesis/Regulation/Tissue Distribution

GH is the major hormonal influence on plasma IGF-I at least in post-natal animals. This has been recognised since early observations by Salmon and Daughaday (72) on sulphation activity in normal rats and its reduction in serum from GH-deficient hypophysectomised animals. Disorders of GH-deficiency such as hypopituitarism are often associated with suppressed IGF-I (109, 110) and conversely the hormone is often increased in states of GH excess such as acromegaly and pituitary gigantism (111). Administration of GH to normal humans results in a 2-3 fold increase in plasma IGF-I over a long time frame (6-24 h) suggesting that the pituitary hormone is stimulating synthesis and not simply release of IGF-I (112). Exogenous GH also increases plasma IGF-I in sheep (55) and cattle (54) but again only after a delay of several hours (113).

In normal physiological states other factors also have important effects on the regulation of IGF-I. These include developmental stage (114, 115) nutrition (117, 118) and to some extent other endocrine factors, particularly sex steroids (116) and insulin (119).

In the human, IGF-I is low in the neonatal period, increases with pre-pubertal age to a maximum at puberty and declines thereafter with increasing age (115). Concentrations are relatively constant with no diurnal rhythm apparent. The pre-pubertal rise in IGF-I is apparently linked to the increase in sex steroids which occur

at this time (116). In steers steroids have also been found to increase plasma IGF-I, but only in animals on a high plane of nutrition (54 -56).

In sheep, plasma IGF-I concentrations are relatively low before birth, increasing with the development of GH dependence in the post-natal period to a maximum at ~40 days and declining to adult levels thereafter (150-300 ng/ml). The decline is coincident with weaning and may be nutritionally determined reflecting the transition to dependence on the rumen (310) rather than a developmental change.

Nutritional status is a major factor in the regulation of IGF-I. A broad range of experiments have demonstrated that plasma concentrations are reduced by fasting and restored by re-feeding in several species. Controlled nutritional studies in ruminants including steers (42), lambs (120, 55) and dairy cattle have also revealed the suppressive effect of reduced nutritional intake on plasma IGF-I. Evidence suggests that in this state the ruminant is relatively GH-resistant. Thus at low levels of nutrition plasma GH concentrations are actually increased, demonstrating marked pulsatility, compared with animals on normal nutrition (42). Despite this, IGF-I levels remain low. Thus a paradoxical divergence of GH/IGF levels is observed in such animals. Evidence suggests that the high affinity hepatic GH receptor is itself nutritionally regulated (54). The capacity of the high affinity growth hormone receptor decreases with malnutrition and correlates positively with growth rate and IGF-I. So it has been postulated that the high affinity growth hormone receptor may mediate the growth hormone control of IGF-I, and explain the paradoxical negative correlation between growth hormone and IGF-I found in many animals, when nutritional status is altered.

Some evidence suggests that nutritional regulation of IGF-I may involve insulin and be exerted at the level of the somatotrophic receptor (121). It is possible that the influence of insulin on IGF-I production involves increasing GH sensitivity (GH-dependence) although more direct actions are also possible. For example, IGF-I output from cultured rat hepatocytes and perfused livers from diabetic rats is very low and increased by insulin replacement (122, 123).

There are complex interactions between insulin, the IGFs and their receptors. In addition, insulin may stimulate the release of IGFs (422). This is likely to be particularly important in the perinatal period where growth is independent of GH and dependent on substrate availability (423). In the pancreatectomized sheep foetus, IGF-I is reduced (424). Insulin infusion increases IGF-I in the pig foetus (425). It seems likely that in the postnatal ruminant, where substrate availability remains an important determinant of growth, substrate and possibly insulin remain important determinants of IGF release. In the adult rat, insulin stimulates IGF-I release (426, 427). Whether this effect is direct or indirect via insulin's actions on the somatotrophic receptor (428) has not been elucidated. The answer to this question may offer important insights into methods of manipulating the somatotrophic axis.

Modulation of IGF bioactivity by nutritional factors may also involve somatomedin inhibitors. These factors were characterised as a result of disparity between estimates of IGF by biological and immunological methods in different nutritional states (124, 125). Several somatomedin inhibitors have been partially characterised including proteinaceous factors of 16 kDa. Relationship of the

inhibitors to the IGF binding proteins remains to be established (126, 127).

A wide range of data from rat liver perfusion studies and organ culture indicate that liver is a major source of IGF peptides. Important studies reported by Baxter and colleagues (123) and by Schwander (339) have examined the production of IGF-I by adult rat hepatocytes in culture and by intact perfused liver, and concluded that hepatic production could fully account for circulating concentrations of IGF-I (123, 339).

Nonetheless it is now widely accepted that IGF-I is synthesized in multiple peripheral tissues in addition to the liver (338), although the contribution of such material to total circulating IGF is uncertain. Initial studies using immunocytochemistry (129, 131) and/or tissue-extraction and immunoassay (341) demonstrated the presence of IGF-I in several extra-hepatic tissues including muscle, cartilage, kidney, fat and brain. Tissue IGF-I immunoreactivity was found to vary markedly, with concentrations being relatively low in muscle, cartilage and fat, 3-4 fold higher in kidney and very low in brain (130). A wide variation of GH-dependence of tissue IGF-I was also observed. Thus hypophysectomy was found to have relatively little effect on tissue IGF-I in cartilage and brain but, by contrast, muscle IGF-I demonstrated a high degree of GH-dependence being reduced >5-fold by pituitary removal (5). Tissue concentrations of IGF-I were generally found to be relatively low compared with blood (serum). The studies that the presence of IGF-I in several extra-hepatic tissues could not however confirm that the IGF-I peptide was produced locally and not derived from blood.

More recently several RNA hybridisation studies, performed mostly on the rat, have confirmed that IGF-I is synthesised in a wide range of extra-hepatic tissues including musculoskeletal tissues (5, 133, 128, 99). IGF-I mRNA is expressed in developing tissues and organs particularly those of mesodermal and neuroectodermal origin (132). Expression of IGF-I mRNA has been examined in chondrocytes in relation to epiphyseal cartilage growth and endochondral bone formation and also in skeletal muscle. Significantly, IGF-I mRNA demonstrates a high degree of GH-dependence in such tissues (5, 134). This evidence suggests a central role for locally-produced IGF-I in somatic growth (21).

Evidence suggests that gene transcription may represent a further level for regulatory control of IGF-I by nutrition (135, 136). Thus fastsing of rats was found to suppress total hepatic IGF-I mRNA by approximately 80%. The phenomena was not restricted to liver however and IGF-I mRNA was reduced ~60% in kidney and muscle, and ~30-40% in stomach, brain and testis. Levels of mRNA splicing variants were found to be essentially co-ordinately regulated within a tissue (5).

Studies of IGF-I tissue expression in ruminants have not been as extensive as in the rat (128). In lactating dairy cattle, however, intense IGF-I immunofluorescence has been observed around individual alveoli of lactating mammary tissue suggesting association of IGF-I with the mammary epithelium (331). Although this does not indicate whether the growth factor was produced locally or 'blood-derived' it did appear to be GH dependent.

Besides plasma, IGF-I has been identified in several extra-vascular fluids. These include lymph (137), milk (138), colostrum (570), cerebrospinal fluid (139), seminal plasma (140) follicular fluid (141), urine (142), and amniotic fluid (145). Concentrations of IGF-I in lymph have been reported to be 30-40% of those in plasma. IGF-I has been identified in mammary secretions from several species. Although not the primary growth factor of human and mouse milk (EGF is, and IGF appears to be present only at very low concentrations), in ruminants it appears that IGF-I is predominant (299). In colostrum IGF-I is present at concentrations 2-3 times those of plasma, declining to 50% by day 4 post-partum. Bovine colostrum also contains high concentrations of the des (1-3) IGF-I variant proving a convenient source for purification. IGF-I is also present in cerebrospinal fluid (CSF) but at relatively low concentrations compared with plasma (139).

Regulatory control of IGF-II, as with its biological actions, does not appear to be as clearly defined as IGF-I. However the peptide shows some degree of GH and nutritional dependence and demonstrates marked species-specific developmental regulation. GH-dependence appears less than for IGF-I. Although lower concentrations are seen in hypopituitary states, IGF-II is not elevated in conditions of GH excess (419, 420). Continuous treatment with GH did not modify IGF-II concentrations in 24 to 72 h cultures of 21-day-old foetal rat calvariae (421). Indeed the apparent GH-dependence of IGF-II may be more a reflection of the GH-dependence of the IGF binding proteins than IGF-II itself. Nonetheless GH is known to increase IGF-II in some circumstances, for example hypohysectomised rats (reviewed in 5), and limited data suggests that

this is also the case in the dairy cow (143, 144).

Marked differences in developmental control of IGF-II are observed amongst species. In plasma from late gestation foetal rats IGF-II, concentrations are 20-100 times those in maternal plasma. A rapid fall is observed in the first 3 days of post-natal life after which plasma IGF-II is considered undetectable (146). By contrast in the human fetus a progressive increase in serum IGF-II is observed in the later half of pregnancy, rising to peak levels at term of ~80% adult levels (~600 ng/ml). In foetal sheep serum IGF-II is about 3-fold higher during the last 100 days of gestation than in adult animals (~800 ng/ml). Circulating IGF-II declines rapidly during the perinatal period in sheep (310, 147).

Receptors

Biological functions of the insulin-like growth factors occur via the initial interaction of hormone with membrane-bound receptors. Three such proteins have been implicated in IGF signal transmission; the insulin receptor, and the type-I and type-II IGF receptors. The insulin receptor and the type-I receptor (which has primary specificity for IGF-I) share many structural features. Thus they have heterotetrameric structures consisting of two extra-cellular α chains of approximately 130 kDa coupled by disulphide bonds to two β subunits of 90-95 kDa which traverse the membrane and have large cytoplasmic domains. The subunits are linked in a β - α - α - β fashion. The type-I receptor has an affinity for IGF-II that is only 2-3 times lower than for IGF-I itself ($K_d = 1.5 \times 10^{-9} \text{M}$), but has a low affinity ($K_d = 10^{-7} \text{M}$) for insulin (148, 149).

By contrast the insulin receptor has highest affinity for insulin with approximately 100-fold lower affinity for IGF-I and evidence suggests that IGF-II actually has higher affinity for the insulin receptor than does IGF-I (149).

The insulin and type 1 receptors have intra-cellular tyrosine kinase domains (150), are autophosphorylated as early events in ligand signal transduction and stimulate the phosphorylation of other cytoplasmic proteins. Insulin, IGF-I and II each activate the signalling pathways of the insulin and type I IGF receptors at concentrations proportional to their binding affinities (151).

High homology between the insulin and type 1 receptors together with observations that insulin and IGF-I can each stimulate metabolic and/or anabolic actions through either receptor raise important questions about how specific biological functions of insulin and IGF are segregated. Very different patterns of distribution of the insulin and type 1 receptors amongst tissue types may be one mechanism by which this may be achieved (149). Thus the rapid metabolic effects of insulin are largely accounted for by liver, muscle and fat which express high concentrations of insulin receptor but by contrast growing tissues express relatively greater numbers of IGF than insulin receptors. Thus cells appear to respond selectively to either ligand depending on which receptor is displayed (152).

Soos and Siddle (153) have reported that the insulin and type 1 IGF receptors can form disulphide-linked heteroreceptor complexes. This adds another level of complexity to understanding the mechanisms by which the ligands may

mediate separate biological functions and suggests that biological functions of the hormones are intimately linked.

In contrast to the type-I IGF receptor, the type-II receptor with primary affinity for IGF-II consists of a single polypeptide chain of approximately 220 kDa (151). The protein has a relatively large extra cellular domain containing several repeat sequences with high cysteine content, a single transmembrane segment and a short cytoplasmic domain. Studies with high quality recombinant ligands reveal that IGF-I has <1% affinity for this receptor while insulin has no discernible affinity at all. The type 2 receptor does not appear to have tyrosine kinase activity but may itself be phosphorylated (148) and may signal via a G-protein pathway (154).

The type-II receptor is present in most cell types and has high homology with the cation-independent mannose-6-phosphate (Man-6-P) receptor (155). This protein has the apparently unrelated function of cycling amongst sub-cellular compartments including Golgi, lysosomes and plasma membrane transporting proteins containing Man-6-P functional groups (156). These include lysosomal enzymes and the TGF β precursor. A modified form of the type 2/Man-6-P receptor is present at high concentrations in the circulation of foetal rats (313) and in sheep serum (312). Like IGF-II itself physiological functions are difficult to assign to this receptor and it is possible IGF-II binding occurs not for cellular signalling but for some as yet unresolved reason such as processing, degradation or perhaps competitive inhibition of IGF-II binding to type 1 or insulin receptors (151).

Metabolic Actions

The IGFs are acutely hypoglycaemic, demonstrating insulin-like actions on the classical target tissues of insulin (157). Thus the IGFs stimulate glucose transport (158) and metabolism and synthesis of lipid, protein and glycogen (159,160, 161). Additionally the IGFs inhibit glycogen breakdown and IGF-I at least inhibits catecholamine-stimulated lipolysis in adipocytes.

The soleus muscle of mice has been used to examine the insulin-like actions of IGF *in vivo*. In this tissue IGF stimulates glucose transport, as demonstrated by 2-deoxyglucose influx, glycolysis as revealed by increased lactate formation, and glycogen and protein synthesis with ~10% the potency of insulin. This is in general agreement with estimates of the relative potency of insulin and IGF-I in the perfused rat heart (~25%; 161, 162).

Intravenous bolus injection of IGF-I and II induces a rapid fall in blood sugar to a minimum between 15 and 30 minutes. Studies with ¹⁴C-glucose have demonstrated that the radioactivity is largely incorporated into the classical insulin-target tissues though the adipose tissue sensitivity of IGF-I appears to be only 1-2% that of insulin compared with ~10% in soleus muscle and 20-30% in perfused rat heart (161, 162). These are acute, short term effects which are observed in spite of long circulating half-lives of the IGFs (Section 4.0).

The metabolic effects of the IGFs are demonstrable only on administration of relatively large amounts of peptide which exceed available capacity of the plasma IGF binding proteins. In this sense the experiments may be non-physiologic and their metabolic significance is questionable (163).

Cascieri and colleagues have examined bioactivity of an IGF-I variant with selected mutations in the N-terminal B-Chain (B-chain mutant; 164). This variant bound very poorly to circulating IGF binding proteins and was found to be at least four-fold more potent than native IGF-I at stimulating ^{14}C -glucose incorporation into rat diaphragm glycogen *in vivo*. This was interpreted as supporting the hypothesis that the plasma IGFBP inhibits IGF-I bioactivity (165, 166, 167).

Acute metabolic properties of IGF-II are not well reported because of a shortage of peptide for experimentation. However several reports have recently emerged. IGF-II, as IGF-I, appears to be a potent hypoglycaemic agent in humans and hypotensive effects were also noted (168). Lewis and colleagues have reported potent lipolytic effects of IGF-II in sheep adipocytes. Others however have failed to repeat these observations (169). More recent work showed that the blood glucose levels were suppressed by IGF-II injection. The hypoglycemic effect of IGF-II was greater than that of IGF-I (578).

IGF Binding Proteins (IGFBPs)

It has been known for over 18 years that IGF-I and II exist in blood complexed to binding proteins (BPs) (170). Presence of IGFBP was first demonstrated by *in vitro* IGF tracer binding and size exclusion chromatographic techniques at neutral pH which revealed BPs of apparent molecular weights of 150 kDa and 30-60 kDa. More recently a plethora of proteins with IGF binding qualities have been characterised from several body fluids and species including sheep and cattle and from cells in culture. However naming of these variants has been unsystematic and increasingly complicated by, for example, different analytical techniques being used.

Consequently it was the consensus of an international workshop on the IGF binding proteins to institute a system for naming fully characterised (sequenced) BP (171). Three distinct groups of BP were recognised at that meeting (IGFBP-1, 2 and 3). At the 2nd International IGF Symposium, sequence data on three further BPs were reported and general agreement reached to designate these IGFBP-4, 5 and 6 (172). The IGFBPs are reviewed in the following section.

IGFBP-1, 2 and 3

IGFBP-1

Physicochemistry

IGFBP-1 was first characterised by its specific interference in an IGF-I radio-

receptor assay (173). Members of this group have been named IBP-1 (174), placental protein 12 (175), BP-28 (176), IGFBP-25 (177), and pregnancy-associated endometrial α 1-globulin (178). These proteins have been purified from human endometrium, placenta, amniotic fluid and the Hep G2 hepatoma cell line by conventional and IGF-I affinity chromatographic techniques. IGFBP-1 has a pI of 4.8-4.9 and an apparent molecular weight on SDS PAGE of ~28kDa (non-reduced, 179). On gel filtration chromatography IGFBP-1 elutes with apparent molecular weights of 30-60 kDa (180, 345, 181).

The primary structure of IGFBP-1 has been inferred by cDNA sequencing. The mature protein consists of 234 amino acids and has a predicted molecular weight of 25.3 kDa, less than predictions by other analytical techniques (177). The difference may be due to glycosylation (up to 5% carbohydrate content has been reported) at one or more putative O-linked glycosylation sites (174). IGFBP-1 also has an abundance of cysteine residues (18 in total) located towards the amino and carboxy-terminals, a structural feature in common with most other characterised BPs (182). In addition, IGFBP-1 possesses an Arg-Gly-Asp ubiquitin recognition sequence towards the carboxyl-terminal which may be important for cell surface attachment (182). One report suggests that IGF binding occurs towards the amino-terminal region since this is highly conserved between BP and a 21 kDa N-terminal fragment reportedly retains binding activity (183).

Evidence suggests that IGFBP-1 has similar affinity (~ 6 /nmol) for IGF-I and II

(176). In competitive protein binding assays using either radio-labelled IGF-I or IGF-II as tracer, IGF-I and II appear equally effective at inhibiting tracer binding but by contrast the des (1-3) analog of IGF-I was approximately 100-fold less potent (185). Although this suggests a role for the N-terminal tripeptide of IGF-I in interaction with IGFBP-1, it is interesting that IGF-II, which also has high affinity for IGFBP-1, has a distinct amino-terminal region (86). Evidence suggests that it is the region from Glu 3 to Cys 6, and possibly beyond. This region is identical in IGF-I and II, and is essential for binding to BP (186). The region on IGFBP-1 involved in binding IGF-I is also contentious.

Brinkman and colleagues have produced evidence that the C-terminal region of IGFBP-1 is essential for IGF-I binding (184), in contrast to earlier reports (183). Recently results from Schuller et al in 1992 suggested that the most likely part of the epitope to which monoclonal antibody A (against IGFBP-I) is directed contributes to the IGF binding domain of IGF-I (367).

IGFBP-1 is encoded by a single gene (187). Transcription of the gene results in mature mRNA of ~1.5 kb containing four exons (174). The first encodes the highly conserved cysteine rich region while the fourth exon contains the Arg-Gly-Asp consensus sequence. IGFBP-1 is expressed in a tissue-specific and developmentally specific manner. Although widely distributed in foetal tissues (188, 190) it appears to be expressed in relatively few tissues in the adult, such as liver, decidua and endometrium (189). Additionally levels of expression are much greater in foetal than adult liver.

Regulation

A rapidly accumulating pool of evidence suggests that IGFBP-1 plays a very active role in metabolism (191, 337). In the human circulating levels demonstrated marked diurnal rhythmicity, apparently regulated by metabolic status (192, 324). Thus IGFBP-1 rises steadily with fasting and rapidly declines on refeeding. Oral glucose causes a marked suppression and insulin-induced hypoglycaemia a marked elevation of IGFBP-1 (571, 193). Fasting (194), prolonged exercise (193), and diabetes mellitus (325) have also been found to elevate plasma IGFBP-1. Furthermore insulin has been found to suppress IGFBP-1 in conditions where blood glucose is maintained by clamping techniques (196). Evidence suggests a close inverse relationship between plasma insulin and IGFBP-1 (325, 330).

Insulin appears to suppress IGFBP-1, at least, at two levels. Firstly it has been found to inhibit IGFBP-1 production by cultured hepatocytes. Secondly it appears to increase transcapillary movement and distribution of IGFBP-1 into tissues (195). Thus in a significant series of studies Bar and colleagues (335) demonstrated, using isolated perfused rat hearts, that the capillary endothelium represents a selective boundary to the passage of IGFBP into the extra-vascular space. Furthermore insulin was found to differentially regulate transcapillary movement, increasing transfer of IGFBP-1, decreasing transfer of endothelial cell BP and apparently having no effect on movement of IGFBP-2 (335). Thus the endothelium may be an important level for metabolic regulation of IGFBP

distribution into the tissues. What the experiment did not reveal however is whether the IGFBPs are translocated bound to IGF or in a free form.

Another situation in which IGFBP-1 is increased, consistent with suppressed insulin is anorexia nervosa (194), and IGFBP-1 is inhibited in acromegaly and by glucocorticoid (dexamethasone) administration (197).

IGFBP-2

Physicochemistry

Buffalo rat hepatocytes (BRL-3A) in culture secrete a second binding protein of 31-33 kDa which has been extensively characterised (198, 199). The protein has been purified and amino-terminal sequence data reported to position 34 (200). Brown and colleagues isolated a clone encoding this protein from a BRL-3A cDNA library (201). It encodes a BP precursor consisting of a 34 residue signal sequence and a 270 residue mature protein containing 18 cysteine residues, an Arg-Gly-Asp cell surface recognition sequence and no N-glycosylation sites, sharing many of these features with IGFBP-1. About 70% of the deduced sequence of this protein was confirmed by direct amino acid sequencing. As with IGFBP-1 the gene for the BRL-3A protein contains 4 exons and is transcribed into mRNA of ~2kb (182).

The deduced amino acid sequence has only moderate homology with either IGFBP-1 or IGFBP-3 (40-50% at the amino and carboxyl-terminals) but has

much more (82 and 96%) in common with a further protein purified from a foetal bovine kidney cell line (MDBK) (301). For this reason the BP from BRL-3A/MDBK is considered to belong to a group of distinct IGF carriers now termed IGFBP-2 (171).

Competitive binding studies have revealed an approximately 10-fold higher affinity for IGF-II than IGF-I for the MDBK protein, especially when IGF-II was the tracer ligand (185). This characteristic is shared with a slightly longer (34 kDa) BP identified and purified from human CSF (206). Binkert and colleagues have searched a foetal human cDNA library and identified a protein with high (>85%) sequence homology to IGFBP-2 (202). Additionally the human CSFBP was recognised by a panel of antisera to IGFBP-2. Thus, at least one BP in CSF would appear to be a human equivalent of rat IGFBP-2 (182, 309).

Regulation

Expression of IGFBP-2 is developmentally and metabolically regulated (182, 203). IGFBP-2 mRNA is expressed at high levels in multiple tissues of the foetal rat but declines post-natally except in brain (204, 205).

Metabolic regulation of IGFBP-2 mRNA occurs in rat liver (182). Thus expression is increased, by fasting, in rats made diabetic by streptozotocin and by hypophysectomy. Changes in expression are reflected in changes in serum levels. Thus increased serum levels have been observed by SDS electrophoresis

with ligand blotting and western blotting in fasted and hypophysectomised rats. IGFBP-2 also appears to be regulated by fasting, insulin and growth hormone in human serum (207, 208).

Developmental expression of IGFBP-2 appears synchronous with IGF-II in the rat. Levels are high in the fetus and decline post-natally (182, 209). This situation may be completely different in ruminants. Although IGF-II is higher in foetal than neo-natal serum of sheep, concentrations do not decline as completely as in the rat, and the adult sheep has IGF-II in excess of IGF-I. Thus IGFBP-2 expression in the adult sheep may be expected to be greater than in the rat (210).

Recent reports on IGFBP-2 regulation in domestic animals suggest that the BP is suppressed by GH administration and increased in negative energy balance, in line with observations from the rat and human (326, 11, 368, 369).

IGFBP-3

Physicochemistry

IGFBP-3 was first characterised from human plasma by Martin and Baxter in 1986 (298) using IGF-II affinity and reversed-phase chromatographic techniques. The protein migrates as a characteristic doublet on SDS/PAGE of apparent molecular weight 53 and 47 kDa (nonreduced; 212, 213). The functional

significance of these forms has not been resolved. They are each able to bind IGF-I and II, IGF-II demonstrating slightly higher affinity than IGF-I. They apparently have the same amino-terminal sequence, but are thought to represent glycosylation variants (321).

IGFBP-3 isolated from porcine and rat sera are also glycosylated, exist as doublets and have greater than 72% N-terminal sequence homology with the human protein (304, 213). Full primary sequence data for the human and rat proteins have been deduced by cDNA sequencing (214, 215). A mature protein of 264 amino acids (molecular mass 28.7 kDa) is predicted, sharing many structural features with IGFBP-1 and IGFBP-2. Thus the 18 cysteine residues located at the amino and carboxy-terminals and encoded in exons 1 and 4 are conserved, and there is greater than 30% overall homology with human IGFBP-1, and IGFBP-2. Additionally several N- and O-glycosylation sites are observed. IGFBP-3 is heavily glycosylated, which is believed to account for the large difference in observed molecular weight for IGFBP-3 (47 and 53 kDa) from that predicted by cDNA sequencing (28.7 kDa) (215, 216). IGFBP-3 immunoreactivity in plasma is also identified at ~20 KDa. This species may represent a degradation fragment of the intact BP (217).

Size exclusion chromatography of human plasma at neutral pH reveals the bulk of IGF immunoreactivity complexed at 120-160 kDa. By contrast following acidification the IGF is identified at the molecular weight of the free peptide while the IGF binding activity elutes at 30-60 kDa. Two hypotheses have been

proposed to explain this phenomena. Firstly, that multimeric complexes of several IGF molecules and binding subunits constitute the high molecular weight complex (218), and secondly, that in addition to IGF and the 50 kDa binding protein a second non-IGF binding subunit exists which converts the IGF-BP complex to the observed 150 kDa ternary complex. The second theory, originally proposed by Furlanetto (219) and Hintz (220) has gained ascendancy, largely as a result of the investigative work of Baxter and colleagues (303), who reported the purification of a factor from plasma which is able to convert IGF/IGFBP-3 complexes from ~60kDa to 140 kDa. The factor is not an IGF binding protein and has negligible affinity for 'free' IGFBP-3 but binds with moderate affinity ($\sim 5 \times 10^8 \text{ M}^{-1}$) to IGF/IGFBP-3 complexes. The factor migrates as a glycoprotein doublet of 84 and 86 kDa on SDS-PAGE and is irreversibly denatured by mild acidification (pH 4.5). For this reason it has been termed the acid-labile-subunit (ALS) (302). Steric considerations relating to the IGF-I affinity chromatography applied to the purification of ALS suggest that it interacts with IGFBP-3 at a site remote from the IGF binding site. Furthermore, ALS does not bind to IGFBP-1 or 2 so it is tempting to speculate that it binds to the divergent central region of IGFBP-3 encoded by exons 2 and 3. However both IGFBP-3 and ALS are heavily glycosylated so it is possible that this is an important structural feature for complex formation/stabilisation. ALS is present at high concentrations in plasma ($\sim 20 \text{ } \mu\text{g/ml}$), well in excess of total IGF and IGFBP-3 (221).

According to the proposed model the 150 kDa IGFBP complex is by definition saturated since binding of IGF to IGFBP-3 (53 kDa) is required before complex

formation with ALS (85 kDa). However the possibility of free IGFBP-3 in the circulation is consistent with this model. Two lines of evidence, however, suggest that, in human plasma at least, IGFBP-3 is saturated with IGF. Firstly the concentration of IGFBP-3 in plasma is approximately equal to the sum of IGF-I and II concentrations. Secondly almost all IGFBP-3 immunoreactivity is found at ~140kDa on neutral pH/SEC and not at 50-60 kDa, suggesting that it is complexed to ALS (322). Nevertheless the possibility that under particular physiological conditions or in other species, IGFBP-3 exists in plasma in a 'free' form cannot be excluded.

Distribution

Shimasaki has examined the tissue distribution of rat IGFBP-3 mRNA by northern analysis. A single 2.6 kb band was revealed in a wide range of tissues including liver, kidney, heart, gonad, spleen and intestine but not hypothalamus or cortex (222). Thus IGFBP-3 appears to be produced in a wide range of tissues and may be expected to be widely distributed in the extra-vascular space. This contrasts with limited evidence from Binou and Hossenlopp (305). These authors compared the distribution by ligand blotting of IGFBP in matched serum and lymph samples collected from the superficial duct (lower leg) of patients undergoing radiographical examination for carcinoma. The characteristic IGFBP-3 doublet was observed in lymph but in much reduced amounts compared with IGBP-1 and 2 and with IGFBP-3 in plasma. Similarly, IGF-II tracer binding followed by neutral pH/SEC failed to reveal a 150 kDa BP despite a major peak of bound radioactivity at 30-50 kDa. The authors concluded that the

150 kDa complexes of plasma do not readily cross the capillary barrier (305).

Nonetheless IGFBP-3 and IGF were observed in lymph. One possibility therefore for the failure to demonstrate a 150 kDa complex in lymph may be the absence of ALS. Sites of synthesis and tissue distribution of ALS have not yet been reported but may now be of prime importance in understanding function of the 150 kDa complex.

Evidence for the plasma 150 kDa BP complex consisting of an IGF-ALS-IGFBP-3 ternary complex is very convincing. Nonetheless several observations require further clarification. The functional significance of the glycosylation doublets of IGFBP-3 and ALS is one example (302). Another is the possibility that not all 150 kDa IGF-BP complexes contain ALS, will need to be examined, especially in the light of a 79 kDa IGFBP being identified in human plasma by Grant (223). Apparently 'free' binding sites at 150 kDa have been reported by Rechler (182).

Regulation

The 150 kDa IGF binding protein complex is considered GH-dependent. Moses and colleagues demonstrated that ^{125}I -MSA (rat IGF-II) bound specifically to a major 150 kDa BP in normal rat plasma with minor forms at 40-50 kDa (224). This reflected the distribution of endogenous IGF activity as determined using

a fibroblast DNA synthesis bioassay (225). By contrast sera from hypophysectomised rats revealed specific tracer binding to the 40-50 kDa BP with no detectable peak at 150 kDa. Endogenous IGF was also associated with the lower molecular weight species in hypophysectomised rat plasma (224, 225). The effect of GH replacement was also evaluated. The 150 kDa species was detectable after 1 day of replacement therapy and fully restored by 10 days. More recently physiological control of IGFBP-3 has been examined in the human by RIA. Thus IGFBP-3 immunoreactivity was found to be approximately doubled in adult acromegalic plasma compared with normal adults (6 $\mu\text{g/ml}$) and halved in GH-deficiency (322). Plasma IGFBP-3 shows little circadian rhythmicity, demonstrates strong developmental dependence, and is related to nutritional status, hepatic function and renal function (226, 227, 228). Levels are low in young children rising to a peak at 14-15 years, and decline slowly thereafter. Fasting was found to cause a modest decrease in IGFBP-3 after a delay of 24 h. A strong positive correlation between liver function and plasma IGFBP-3 was also observed, suggesting the liver as an important source of plasma IGFBP-3. Only limited sex-related differences were observed. IGFBP-3 levels in foetal blood have not been reported but tracer binding studies and IGF-I assay of the 150 kDa region on SEC indicate that 150 kDa concentrations are much reduced (306). One explanation for this may be that the hepatic GH receptor, which is itself developmentally regulated, is much reduced at that stage (57). IGF-I concentrations are also low in foetal blood despite high GH (147, 310). Recent evidence in the hypophysectomised rat suggests that IGF-I itself induces IGFBP-3. If this is so the apparent GH-dependence of the 150 kDa

complex may be more a reflection of the GH responsiveness of IGF-I than a direct stimulatory effect of GH on IGFBP-3 (308, 370). On the other hand regulation of ALS by GH appears to be more direct, allowing for subtle control of 150 kDa complex components (229, 230).

ALS demonstrates marked GH-dependence as examined by RIA (221). Thus concentrations were effectively doubled in a sample of acromegalic compared with normal human plasma ($\sim 20 \mu\text{g/ml}$) and suppressed by greater than four-fold in GH-deficient adults. Other situations and conditions affecting IGFBP-3 and ALS include diabetes in which IGFBP-3 is suppressed and chronic renal failure in which IGFBP-3 is elevated while ALS is suppressed (221, 231, 227). The discordance between IGFBP-3 and ALS in chronic renal failure may be one explanation for the stunted growth which results in that condition despite normal GH and IGF-I by RIA.

Several cell types including neonatal fibroblasts (232) and vascular endothelial cells (318) are known to produce IGFBP-3 in culture. Epidermal growth factor (232) and foetal calf serum were found to be stimulatory to BP production, but by contrast GH had no effect. IGFBP-3 was found at $\sim 50\text{kDa}$ in culture fluid with no evidence for production of ALS by these cells.

Recent evidence suggests that regulation of IGFBP-3 may occur at the post-translational level. Thus a specific pregnancy-associated proteolytic enzyme has been shown to cleave IGFBP-3 (343, 233, 344) and phosphorylation of BP by

mammalian cells has also been demonstrated *in vitro* (234). More recently the finding of proteolysis of IGFBP-3 in different body fluids suggested that the cleavage of IGFBP-3 is a common occurrence *in vivo* and may provide a mechanism for the direct regulation of IGFBP-3 levels and indirect regulation of IGF action. The discovery of IGFBP-3 proteolysis in the conditioned media of a human breast cancer cell line indicated that IGFBP-3 proteases may operate in the micro-environment at the cell level to regulate the actions of IGF or IGFBP-3 (371).

Little additional information on the influence of nutrition on IGFBP-3 is available. McCusker and colleagues have examined nutritional regulation of neo-natal porcine plasma BPs by ligand blot analysis. In that study marked nutritional dependence of the 42 and 39 kDa doublet (presumably analogous to the IGFBP-3, 47 and 53 kDa BPs of human plasma) was observed. The abundance of these species fell by approximately ten-fold over a 24 h period of feed withdrawal. Little information on nutritional regulation of IGFBP-3 in mature animals has been reported (235).

IGFBP-4, 5 and 6

In addition to IGFBP-1, 2 and 3 several other BPs have been described, including a 32-36 kDa protein isolated from a human osteosarcoma cell line (237), a second species derived from SV-40 transformed fibroblasts (236), and a BP derived from endothelial cells (318).

At the IGF symposium in San Francisco in 1991 (316), purification of three novel BPs was described from rat serum and porcine follicular fluid. Amino acid sequences for the species were inferred from cDNA clones isolated from rat and human libraries. One protein of 237 residues had essentially the same N-terminal sequence as the bone cell-derived protein (316) and was designated IGFBP-4 (172). A second protein was found to correspond to the species derived from conditioned media of SV-40 transformed fibroblasts (236), also sharing homology with a new species isolated from CSF (238) and human serum (239) and has been assigned IGFBP-6 (172, 319). More recently this BP has been reported to consist of 216 amino acids. A distinctive feature of IGFBP-6 is that it appears to lack several (2 and 4; human and rat respectively) of the 18 cysteine residues which are highly conserved in other BPs (319). Finally the third protein of 252 amino acids was also identified and designated IGFBP-5 (316). This protein has not previously been described and its physiological significance is as yet uncertain. A diagrammatic representation of the major IGFBPs is shown in Figure 1.3.

In 1987 Kiess and colleagues described an IGF-II BP in rat serum that is considerably larger than the 150 kDa complex (313). Subsequently this protein was shown to be immunologically related to the type-2 IGF receptor (Section 1.2) though slightly smaller with an estimated molecular mass of 210 kDa as opposed to the 220 kDa of the membrane receptor (155). The difference is

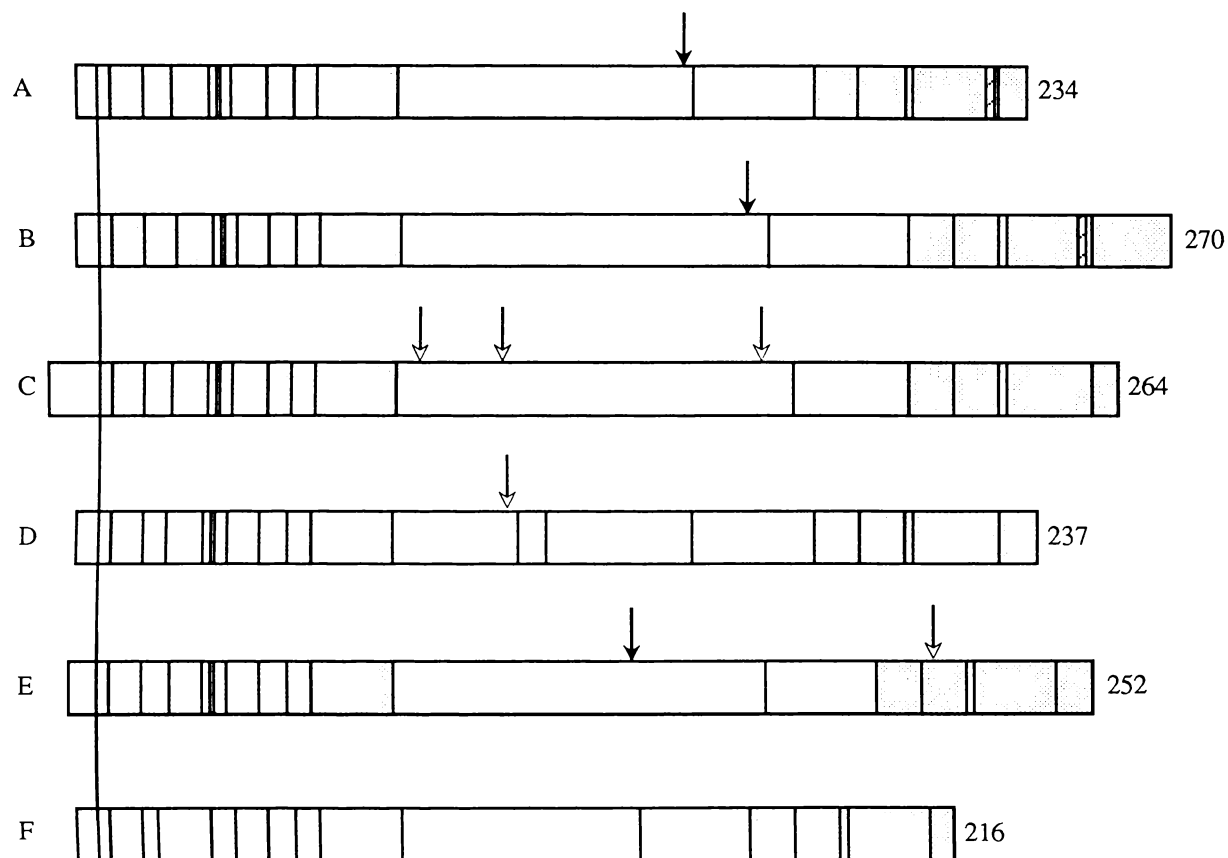


Figure 1.3 Schematic representation of insulin-like growth factor binding proteins 1 to 6. The diagrams labelled A-F represent human IGFBP-1, rat IGFBP-2 and human IGFBP-3 to 6 respectively. The binding proteins are aligned by the first of the 18 homologous cysteine residues, (represented by the vertical bars; hBP-4 has two additional cysteines and hBP-6 has two less). The shaded areas at the amino and carboxy terminals represent areas of high structural homology while the hatched areas in IGFBP-1 and 2 represent the locations of the Arg - Gly - Asp cell surface adherence recognition sequences. The open arrows (↓) mark the locations of putative N-glycosylation sites while the closed arrows (↓) represent potential heparin binding sites (refer sections 3.4 and 3.5). Additional sites for heparin binding may be located in the amino acid 180 - 210 region of the binding proteins which has relatively high basic amino acid content. The figures at the end of each structure represent the number of amino acid residues in each binding protein. Sequence data is from Shimasaki (316).

believed to be a short trans- membrane and cytoplasmic domain. The soluble type-2 receptor was shown to be homologous with the cation-independent mannose-6-phosphate receptor which appears to have a role in the targeting of lysosomal enzymes to lysosomes. The physiological significance of this duality has not been resolved (156).

The IGF-II/Man-6-P receptor is highly specific for IGF-II and has also been identified in human and porcine serum. Following initial observations of Butler and Gluckman (306) and more recently Hey (311), a high molecular weight IGF-II specific protein in foetal sheep serum has been determined to be the ovine equivalent of the soluble IGF-II/Man-6-P receptor and is also found in adult sheep serum (312).

Ovine/Bovine IGF Binding Proteins

IGFBP from sheep and cattle are less well described than from other species (particularly human and rat). The best characterised is the IGF-II-specific protein originally purified from conditioned media of foetal bovine kidney cells in culture (MDBK cells) and now designated IGFBP-2 (301, 171). An endothelial cell BP, purified by Bar and colleagues and distinct from other characterised IGFBP is also of bovine origin (318). In important work, Walton and co-workers reported the preliminary isolation of several ovine serum BPs (314). These included species migrating at 40 and 43 kDa on SDS-PAGE with the amino-terminal sequence of BP-3, a species of 35 kDa homologous to bovine

BP-2, and variants of 24 and 29 kDa BPs initially considered unique but now recognised as the ovine equivalents of IGFBP-4 (315).

A recent report describes developmental expression of IGFBP-1 and 2 in the sheep by mRNA hybridisation and immunocytochemistry (190, 210). IGFBPs are modulated according to metabolic status in the sheep (372).

Physiological Role of the IGFBPs

Our understanding of physiological functions of the IGFBPs are mostly based on circumstantial evidence and has, therefore, remained highly speculative. Agreement on functions of the IGFBPs can only be found in general terms: to determine bioavailability of IGFs (peripheral distribution/targeting/excretion or elimination) and to modulate biological actions of IGFs (stimulatory/inhibitory).

The IGFBPs appear to have evolved from a common ancestral protein demonstrating high homology and structural characteristics related to function including the N and C-terminal cysteine-rich binding domains and Arg-Gly-Asp cell adherence recognition sites (286). Emerging evidence suggests that the extracellular matrix constitutes a supportive micro-environment for cell growth and development, and recognition that the BPs contain the Arg-Gly-Asp sequence is suggestive of important regulatory functions for the BP at this level (329). Glycosaminoglycans are also constituents of the extra cellular matrix and

recent reports that some IGFBPs have affinity for the heparin-like compounds may be further evidence supporting roles for the BP at this level (317, 318, 320). Both stimulatory and inhibitory roles for the BPs are conceivable. For example, the cell surface adherence sequence in the BPs may facilitate presentation of IGF to the membrane receptor, or alternatively matrix and BPs may serve to buffer the cell from the effects of IGF produced in excess of requirements.

One important question relates to the great diversity of the IGFBPs. Functions for the BPs as transporters and/or local regulators of IGF are all very well but do not explain the need for so many distinct proteins. Increasingly the evidence suggests that they are products of different cell types (e.g. the endothelial cell BP, osteoclast BP (BP-4), and transformed fibroblast BP (BP-6) suggesting that they subserve specialised functions for the particular cell type from which they are derived. An implication of this is that IGF bound to each of these proteins may constitute functionally independent pools, an observation which would appear to hold for the major forms of IGFBP in the circulation (287).

Finally roles for the BPs independent of IGF are also possible. Examples have been reported that the endothelial cell BP of Bar and colleagues is a potent stimulator of glucose transport in microvessel endothelial cells (288), and rat IGFBP-2 apparently has an independent role in axonal guidance (289). Such roles may be a further plausible explanation as to why there are so many BPs.

The phenomenon of specific BPs adds a further level of complexity to

understanding the physiology of IGF. At present the BP family appears to be expanding at a greater rate than our understanding of precise functions for these proteins in facilitating IGF action.

Theories of GH Action in Stimulating Growth

Somatomedin Hypothesis of GH Action

Evidence for a crucial role of GH in post-natal somatic growth in mammals is overwhelming. Animals which lack growth hormone show stunted growth which can be restored by hormone replacement, and animals with excess GH display gigantism. Growth hormone has a wide range of metabolic actions (outlined in Section 1.1) which serve to make energy available for somatic growth and to stimulate the synthesis of a wide range of proteins and nucleic acids in multiple tissues. Foremost amongst these is the selective synthesis of somatomedin-C/IGF-I in multiple tissues.

Although the effect of GH on somatic growth is easily recognised, sites and cellular mechanisms of GH action are much less known. Historically, this must in part be due to the complexity and broad range of GH actions, but also to the apparent discrepancy between *in vitro* and *in vivo* effects. For example, while marked stimulatory effects of GH can be observed on musculoskeletal tissues *in vivo*, *in vitro* only slight, inconsistent growth-associated effects are observed

(reviewed by Daughaday in 66 and Isaksson in 5, 73). Such discrepancies plus the identification and characterisation of GH-dependent peptides in plasma (Somatomedin-C/IGF-I and others; see Section 1.2) derived primarily from liver led to the concept that GH exerts its effects on cell growth indirectly via these factors (72). Several bioassays require consideration when discussing the development of theories regarding GH action. The surgically prepared hypophysectomised rat is the classical animal model for investigating efficacy of growth-promoting agents (69, 67). Although some growth is obtained in such animals with insulin, and thyroxine and corticosteroids are required for maintenance, only GH replacement can fully restore growth (68).

GH increases weight gain and longitudinal bone growth in hypophysectomised rats. Increases in nose-tail length result primarily from thickening of the epiphyseal growth plates in bone ends by stimulation of chondrocyte proliferation. Determination of the width of the tibial growth plate in hypophysectomised rats was found to be a sensitive and specific bioassay for GH, providing dose-dependent responses over a wide range of hormone concentrations, and is still a major reference method for estimating GH activity *in vivo* (70). Despite the value of these methods they are whole body bioassays and it cannot be assumed that growth results from a direct action of GH on target tissues and not via an intermediate. An important modification of this method involved examining ³⁵S incorporation into cartilage chondroitin sulphate. *In vivo* experiments using hypophysectomised rats demonstrated that incorporation of this isotope was very dependent on growth hormone

replacement (240).

Cartilage is also convenient to maintain *in vitro* and this, together with the rapidity of the GH response *in vivo*, suggested that ^{35}S incorporation into cartilage could form the basis of a simple *in vitro* bioassay for GH. However growth hormone failed to stimulate ^{35}S incorporation *in vitro* (66). Sulphation activity was however identified in normal rat plasma and plasma from GH-treated hypophysectomised rats but not in plasma of untreated hypophysectomised rats. Thus in 1957 Salmon and Daughaday postulated that the growth promoting effects of GH on longitudinal bone growth are not direct but rather they are mediated by serum factors now known as somatomedins or IGFs (72, 71). It was suggested that GH acts initially on the liver to stimulate production of IGF-I and that the IGF circulates in blood to target tissues where it mediates GH actions on growth and development. The main basis for the theory was the failure of early attempts to demonstrate direct effects of GH on cartilage *in vitro*.

Support for the somatomedin hypothesis comes from a wide range of areas. Firstly, the IGF content of blood is much higher than that of other major tissues (338, 245, 244), and hepatic production of IGF-I can account for the bulk of blood-borne IGF-I (339, 241, 123, 128) raising the obvious question: what is the role of circulating IGF-I? Secondly a large body of circumstantial evidence suggests that growth and plasma somatomedin/IGF activity are closely correlated (reviewed in 242 and 243) at least in post-natal animals.

Further supportive evidence for IGF-I as the endocrine mediator of GH action emerged as recently as 1988 with experiments on the administration of IGF-I to hypophysectomised rats. Guler and colleagues reported that maximally effective doses of recombinant IGF-I and hGH administered via minipumps were almost equally effective at stimulating body weight gain (246). However the results are controversial. Other groups have reported only minor growth effects of IGF-I compared with GH (247, 248, 349). Furthermore the growth effects appear inappropriate. Thus longitudinal bone growth was less with IGF-I than achieved with GH and a disproportionate increase in visceral weight, particularly of spleen and kidney was observed (245, 246).

These early experiments are confounded by several observations. Firstly, hypophysectomy completely changes the profile of IGFBPs in plasma (224). The molecular form of infused IGF-I may therefore have been inappropriate. Secondly, the quality of IGF-I may be important. And thirdly, GH was administered continuously, not in a pulsatile fashion. Its bioeffectiveness may therefore have been reduced (247).

Similar growth stimulation experiments involving systemic administration of IGF-I have been performed in other laboratories using a variety of animal models (249, 250) and do not support the data of Guler. Thus while significant growth stimulation is certainly observed with IGF replacement in such animals, the velocity is much less than could be achieved with GH. Consequently the weight of current evidence and opinion suggests that exogenous IGF-I cannot effectively

replace GH as a growth stimulant. Recently Ohlsson and co-workers showed that GH but not IGF-I stimulates division of slowly dividing cells in the rat epiphysial plate, indicating that GH and IGF-I have different target cells (373).

The observations outlined above, together with an increasing body of evidence that GH has direct anabolic actions on target tissues, have forced a major re-evaluation of mechanism(s) by which GH stimulates post-natal growth (5, 73).

Dual Effector Theory of GH Action

Important work by Green and co-workers on basic mechanisms by which GH and IGF stimulate somatic growth has led to the emergence of an alternative theory of GH action - 'The Dual Effector Theory of Growth' (74).

In initial experiments Green produced good evidence that GH can exert direct effects on peripheral tissues. The hormone was found to induce the differentiation of a line of pre-adipose (3T3) cells into functional adipocytes (75). These 3T3 cells may be capable of synthesising IGF-I, but addition of IGF-I to the cells in culture did not induce differentiation as examined by the glycerophosphate dehydrogenase assay (251). Only GH possessed adipogenic activity (252). Thus it appears that GH exerts a regulatory effect on differentiation (253), and evidence suggests that this effect is not restricted to fat cells. A similar commitment to differentiation was also observed in other precursor cells of mesenchymal origin such as myoblasts (251). Physiological

concentrations of GH were found to activate the differentiation process which involves cell fusion and migration of nuclei from adjacent satellite cells to form myofibres (254). The differentiation process appears to proceed in two stages. Initial interaction of GH with precursor cells appears to result in a commitment to differentiation, possibly involving activation of the c-myc proto-oncogene (255). This is followed by local expression of IGF-I which is mitogenic and stimulates clonal expansion of the differentiating cells (74, 262). This model of GH action has been named the Dual Effector Theory. GH has subsequently been found to have direct effect on proliferation of haematopoietic progenitor cells, human T cells, smooth muscle and rat pancreatic β -cells. Isaksson has examined the Dual Effector Theory with respect to chondrogenesis and longitudinal bone growth (reviewed in 11). Longitudinal bone growth results from proliferation of chondrocytes in the epiphyseal growth plate. The growth plate has characteristic histological features representing chondrocytes at different stages of differentiation. These include a germinal cell layer of undifferentiated cells located close to the bony epiphysis, proliferative and hypertrophic layers and zones of degeneration and matrixogenesis (ossification) (257, 258). This highly organised architecture is in marked contrast to chondrocytes in culture which are heterogeneous with respect to differentiation and show little spatial organisation (259). Cultured chondrocytes also bind GH poorly but the hormone binds strongly to the prechondrocytes and young differentiating cells close to the growth plate (260). By contrast cells of the proliferative layer bind GH poorly but have abundant IGF-I receptors (261) and express IGF-I mRNA (134). IGF-I stimulates the clonal expansion of cells which have reached this stage of their

differentiation programme. Cells in the hypertrophic zone demonstrate functional maturation followed by ageing and ossification (257). These events have been carefully described by Isaksson and colleagues in recent reviews (5) and appear consistent with Green's Dual Effector Theory of Growth (74). This description of longitudinal bone growth does not however exclude the possibility that other factors may also be involved in the process.

Observations that GH can stimulate longitudinal bone growth by direct local administration (263, 264) and that IGF-I is produced locally in tissues in response to GH (134), together with observations of IGF-I receptors on proliferating chondrocytes (261), provide strong circumstantial evidence that it is locally produced growth factor, acting by autocrine/paracrine mechanisms, that provides the mitogenic stimuli. While the observations do not exclude a role for circulating (endocrine) IGFs in growth-related processes they do raise major questions about the involvement of the blood-borne hormone. General acceptance of the dual effector theory has led to gradual erosion of the original somatomedin hypothesis to the point where its basic validity has been questioned (337, 98).

In summary, the sequence of events by which GH is now believed to exert its stimulatory effect on longitudinal bone growth includes:

1. Stimulation of differentiation in chondrocyte progenitor cells involves GH acting via somatogenic receptors.

2. IGF-I gene expression is induced in the differentiating cells.
3. Limited clonal expansion of the differentiating cells is stimulated by locally produced IGF-I acting by autocrine/paracrine mechanisms.
4. Proliferation is followed by cellular hypertrophy, degeneration and endochondral ossification.

IGF Action

Factors which influence anabolic processes can be loosely classified as competence and progression (efficiency) factors as outlined in Section 1.4. The IGFs are potent stimulators of clonal expansion and cellular proliferation in a wide variety of tissues and as such may be considered progression factors (280, 165). A major feature of IGF is its mitogenic activity (165). Thus the IGFs stimulate DNA synthesis, as examined by ^3H -thymidine incorporation, in a wide variety of cell types including fibroblasts, chondrocytes, corneal endothelial cells, intestinal and mammary epithelium and hepatocytes (242, 243). Maximal mitogenic activity of IGF on such cells is, however, often less than with serum-supplemented media. Stiles and colleagues (280) have demonstrated that other factors acting synergistically with IGF-I are required for optimal effects. These are the 'competence' factors and include platelet-derived growth factor (PDGF). Such factors are apparently also necessary for optimal IGF activity in the classical IGF bioassays - the cartilage sulphate incorporation assay and the L6 myoblast protein synthesis assay.

In addition to stimulation of cellular proliferation and mitogenesis the IGFs also play important roles in differentiation of a wide variety of cell types including myoblasts, osteoblasts and thyroid cells. In this role they are arguably behaving as competence factors (243, 95).

Other observations suggesting specific anabolic roles for the IGFs include compensatory hypertrophy of the contralateral organ following nephrectomy (281) and adrenalectomy (282) in rats which are associated with local increases in IGF-I mRNA and immunoreactivity in the remaining glands, erythropoiesis (283), wound or nerve repair (284,285) and preferential retention of protein during starvation following exogenous IGF-I (265).

Role for GH Axis in Lactation in Ruminants

The lactation cycle consists of an extended phase of mammary growth and development (mammogenesis), initiation (lactogenesis) and maintenance (galactopoiesis) of milk secretion and finally cessation of milk production. Mammary function is regulated by a diverse range of factors but of particular interest in agricultural science are the factors controlling galactopoiesis. The ability of growth hormone to increase milk secretion in ruminants is now well established (266), however sites of action and mechanisms by which GH increases galactopoiesis are much less clear.

GH increases nutrient intake and feed conversion efficiency in lactating

ruminants and has a raft of effects on fat, carbohydrate and protein metabolism (discussed in Section 1.1) which serve to make nutrients available for lactation. Increased plasma FFA and glucose are, however, not always observed in lactating dairy cows following GH administration, making the lipolytic and diabetogenic actions of GH controversial in such animals. This may be due in part to energy balance in the treated animals and also to the rate of nutrient supply and uptake by the mammary gland (267). The literature on nutrient partitioning in lactating ruminants is reviewed in references 53 and 20. Central to nutrient supply is mammary blood flow because of high extraction rates (>50%) by the udder of essential substrates. Mammary blood flow is sensitive to environmental factors such as suckling and feed intake and is also significantly increased by GH treatment (268). This may be a second role for GH in the control of lactation. Nonetheless, it is unlikely that mammary blood flow alone can explain the stimulatory effect on GH on milk output. Increases in mammary nutrient supply by close arterial infusion do not induce milk yield responses of the same magnitude as responses to GH (268), and conversely increases in mammary blood flow using, for example, adenosine, comparable with those achieved with GH, do not increase milk production (269). Consequently the weight of evidence suggests that it is not mammary blood flow alone nor nutrient supply which controls milk synthesis/secretion but rather a co-ordinated effect on both of these parameters that may be one mechanism by which GH stimulates mammary function.

More direct effects of GH on lactation have also proven difficult to demonstrate.

Evidence suggests that GH does not act directly at the level of the gland (332, 270). Close arterial infusions of GH into lactating mammary glands of sheep and goats have proven ineffective at stimulating milk production (271). Attempts to identify somatotrophic receptors on mammary membranes have also been unconvincing (270). Tracer binding (4, 331) and mRNA hybridization (336) studies in several laboratories have failed to convincingly demonstrate a somatotrophic receptor in lactating mammary tissue. Thus the evidence suggests that if GH has stimulatory effects on lactation, in addition to those relating to substrate supply, then the effect would appear to be indirect and mediated by some other factor(s). One such factor may be plasma IGF-I.

Several lines of evidence suggest a role for IGF-I in mammary function. Firstly, abundant IGF type-1 and type-2 receptors have been identified in samples of both ovine and bovine mammary epithelial tissue undergoing lactation (272). Secondly, mammary tissue and milk from normal lactating animals contain immunoreactive IGF-I, and a marked increase in hormone concentration occurs with GH treatment (approximately 50% in milk and 2-3 fold in mammary tissue) (572). Glimm and colleagues have shown intense IGF-I immunofluorescence around individual alveoli of bovine mammary tissue. GH treatment caused an apparent increase in cytoplasmic IGF in epithelial cells, consistent with the hypothesis that IGF-I is internalised and thereby transferred into milk (331). The origin of mammary IGF-I is less certain. Small amounts of IGF-I mRNA have been identified in mammary tissue from normal lactating rats (273) and there is evidence for the synthesis of immunoreactive IGF-I by lactating bovine

mammary explants in culture (274, 275). However there are no reports of increased mammary production of IGF with GH treatment. This together with the failure to identify somatotrophic receptors in mammary tissue suggests that the GH-dependent increase in tissue IGF-I is not due to local synthesis. The data appear to encourage the notion that the stimulatory effect on GH on milk production is mediated by circulating IGF-I targetted to the mammary gland and thereby acting in the classical endocrine fashion.

Administration of GH to lactating ruminants markedly increases plasma concentrations of IGF-I (276). However, in experiments performed at Ruakura, systemic intravenous (i.v.) infusion of IGF-I into lactating goats had little effect on milk output despite the observation that circulating concentrations of IGF-I were similarly elevated in control animals which received GH and demonstrated a marked increase in milk production (333). Nonetheless Prosser and colleagues have recently reported stimulatory effects of IGF-I on milk production by close arterial infusion of hormone into the pudic artery of lactating goats (573). IGF-I was infused directly into one gland and the contralateral gland acted as a control, receiving exogenous hormone only after recirculation. IGF-I infusion resulted in a highly significant increase in milk secretion in the infused gland. Mammary blood flow and milk IGF-I concentrations were also increased. Only minor changes in milk fat and protein were observed with treatment, and maintenance of milk lactose concentrations indicated the permeability of the mammary epithelium was unaffected. Thus the balance of evidence from that experiment was that IGF has a direct stimulatory effect on milk secretion in the lactating

goat. Nonetheless it is possible the Prosser experiment was non-physiologic since close arterial infusion of IGF may effectively bypass plasma BPs, flooding the gland with 'free' IGF.

A potential role for plasma IGF-I as the mediator of GH's stimulatory effect on lactation will require further clarification. If the mechanism by which GH increases milk production is indeed indirect and mediated by circulating IGF then it is an example of IGF acting in the classical endocrine fashion and will provide a convenient model system for examining such actions.

1.5 Autocrine/Paracrine and Endocrine Mechanism of IGF Action

There was within the scientific community a considerable divergence of opinion between those that believe that IGFs are produced by the liver and then act as hormones via blood circulation to their target tissues, and those that believe that locally produced IGFs have mainly an autocrine/paracrine function.

IGF-I/somatomedin-C was first defined as the endocrine mediator of GH action. It was postulated that GH acted on the liver to produce IGF-I which promoted growth by a range of anabolic actions on peripheral tissues (72). The main basis of this theory was the failure of early attempts to demonstrate direct effects of GH on musculoskeletal tissues *in vitro*. More recently the original somatomedin hypothesis of GH action has been extensively revised and its validity questioned (337). A number of observations have suggested that IGF is produced and acts

locally in tissues by autocrine/paracrine mechanisms (5, 98, 133, 337). The widespread production of IGF-I and IGF-II has been supported by the studies of IGF-I mRNA expression in a number of tissues (132, 574, 128, 99, 134, 98, 273, 438, 546, 481, 262, 482). This has led to an alternative theory of GH action - the dual effector theory - originally proposed by Green and co-workers and extended to longitudinal bone growth by Isaksson (74, 5).

An important implication of the dual effector theory is that systemic IGF may have little or no direct role in the growth process and/or represents material produced in excess of paracrine/autocrine requirements en route for clearance. Nonetheless the liver is a major site of IGF synthesis (338) and does so in response to a variety of stimuli (particularly GH and increased nutritional intake). Evidence from the rat suggests that hepatic production of IGF-I is sufficient to maintain the relatively large amount of IGF-I (~50% of total body content) present in the circulation (339). This together with the observations that type 1 IGF receptors are virtually absent from adult liver (340) supports the contention that hepatic IGF-I is not acting locally within the liver but enters the circulation to fill some endocrine function.

The evidence for a autocrine/paracrine action of IGF-I does not exclude the possibility that circulating IGF, acting in the classical endocrine fashion, may fill specific functions in support of the growth process. These may include; whole body protein and glucose metabolism (163), erythropoiesis (283) and renal function (575).

Nevertheless, analysis on a molecular biological basis suggests that IGF-I production may be regulated by numerous factor at transcriptional, translational and post-translational steps (351, 501). It is noteworthy particularly that the complexity of IGF-I gene transcription results in a large number of different IGF-I mRNA transcripts (351, 565), which may be of different functional significance (351) and differentially expressed or regulated by nutrition or GH in various tissues (496, 552, 554, 566, 567). These genetic analysis implicates that IGF-I production and its regulatory control is obviously very complex.

It has been over 35 years since Salmon and Daughaday made the first observations of IGF-I action in stimulating cartilage growth *in vitro* (72), the mechanisms of IGF action still remain inconclusive. A summary of key evidence for autocrine/paracrine and endocrine roles for IGF-I is outlined below:

Evidence favouring a primarily autocrine/paracrine model of IGF-I action includes:

- GH receptors are widely distributed in musculoskeletal tissues (47)
- IGF-I immunoreactivity is present in multiple tissues and demonstrates developmental and physiological regulation (338, 341)
- IGF-I mRNA is widely distributed in tissues suggesting local production of IGF-I and demonstrates developmental and physiological regulation (132, 574, 128, 99, 134, 98, 273, 438, 546, 481, 482, 262)
- GH is widely believed to be a more effective growth stimulant than IGF-I

(245, 348)

- Growth stimulation by GH may occur without increasing plasma IGF-I (277)
- Immunoneutralisation of circulating IGF-I does not affect growth rate (347, 346)
- Plasma IGFBPs are historically believed to restrict tissue accessibility of IGF-I and inhibit bioactivity (323, 278)
-

Evidence in favour of an endocrine role for IGF-I includes:

- Abundance of GH receptors in liver and their developmental and physiologic regulation (57)
- Liver is the major site of IGF-I synthesis (338, 98, 245)
- Liver is the major source of circulating IGF-I (123, 339, 279)
- Liver lacks type-I IGF-I receptors suggesting that it is not the target for locally produced IGF-I (340)
- Liver contains a pituitary - and nutrition-dependent, high molecular form of IGF-I with immunological and biological properties similar to circulating IGF-I. Diaphragm, spleen and kidney contained no significant IGF bioactivity and 8, 17 and 32% of the IGF-I immunoreactivity of normal rat liver (499).
- Liver IGF-I mRNA, IGF-I production and plasma IGF-I were all significantly correlated with gain and mean GH peak amplitude in cattle (128).

- Serum IGF-I concentrations paralleled levels of the major hepatic IGF-I mRNA transcripts during periods of both fasting and refeeding in rats (542).
- Circulating IGF-I is distributed outside the vascular space into peripheral tissues (497).
- Blood has the highest concentration of IGF-I (338, 245)
- There is close correlation between plasma IGF-I and growth (reviewed in 98)
- Molecular biological basis for GH dependence at the level of hepatic IGF-I gene transcription (128, 99, 351)
- In the mammalian IGF-I gene, hepatic exon 2 may have a pivotal role in determining rates of growth in normal animals (496).
- Some evidence of specific physiological functions for circulating IGF-I (e.g, protein metabolism; 342, 265)
- The role of circulating IGF-I in stimulation of antler growth and ruminant lactation (350, 61).

The greatest density of IGF-I and (-II) binding in skeletal muscle was always observed associated with blood vessels (568).

1.6 Aim of the Thesis

Post-natal control of growth through the GH axis occurs via a number of hormones and their specific receptors. Among these, IGF-I is considered to play an important part.

Accumulating observations that IGF-I is produced and acts locally in multiple tissues have led to more attention being given to tissue IGF-I production and its regulatory control besides plasma levels of the hormone.

The aims of this thesis were to study IGF-I production in both hepatic and extra-hepatic tissue origin and its regulatory control through the GH axis, with a view to having some possible insight into relative contribution or importance of local versus hepatic production of IGF-I in postnatal sheep.

The specific objectives for each of the experimental chapter in this thesis can be summarized as follows:

- To investigate the effect of nutritional manipulation and local administration of GH on tissue and plasma IGF-I immunoreactivity, and to determine relationships between liver IGF-I and IGF-I mRNA levels in postnatal sheep (chapter three).
- To investigate both hepatic and extrahepatic production of IGF-I under the influence of GH, and to test relationships between tissue and plasma IGF-I response to GH administration in postnatal sheep (chapter four).
- To investigate plasma IGFBP-3 and other IGFBP response of postnatal sheep to administration of GH, and relationships between the changes in plasma IGFBP-3 and plasma/tissue levels of IGF-I (chapter five).

CHAPTER TWO : MATERIALS AND METHODS

2.1 Measurement of Metabolites in Plasma

To monitor nutritional status, plasma levels of glucose and non-esterified fatty acids were measured in the experimental animals.

Measurement of Glucose

Materials

Enzymes reagent: the Beckman Certified Glucose Oxidase Reagent is a special-purpose solution designed specially for, and formulated for optimum performance in, the Beckman Glucose Analyser 2.

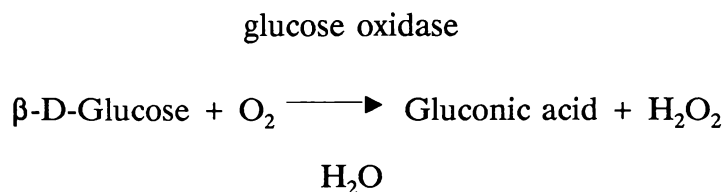
Active ingredients: 140 I.U./ml glucose oxidase (*Aspergillus niger*); 5% denatured ethanol; 10m mol/l potassium iodide. The enzyme reagent contains catalase and ammonium molybdate as catalytic reagents prepared in a phosphate buffer with thimerosal added as a preservative.

Beckman aqueous glucose standard: The analyser is calibrated with an aqueous standard which is formulated from D-glucose (150 ± 1 mg/100 ml) and 0.2% (w/v) benzoic acid as preservative.

Principle of Assay

The analyzer utilizes the Beckman-developed oxygen rate method, employing a Beckman Oxygen Electrode, Beckman Reagent, and an electronic system that measures the rate of change in oxygen consumption when a sample is injected into an enzyme reagent solution.

When sample is injected into the enzyme reagent solution, β -D-glucose from the sample combines with dissolved oxygen from the solution, according to the reaction:

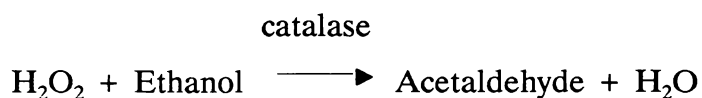


In the reaction, oxygen is consumed at the same rate as glucose reacts to form gluconic acid.

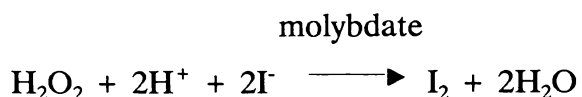
At all times during the reaction, the rate of oxygen consumption is directly proportional to the concentration of glucose then present in the reaction cup. The maximum observed rate, attained after a brief interval required for reagent mixing and system response, has been shown to be a direct measure of the concentration of glucose originally present in the sample at the time of injection. The analyzer measures and records this maximum rate, and permits the digital readout to be scaled, providing a number corresponding to the glucose

concentration in mg/dL.

Because oxygen consumption rather than peroxide formation is measured, the only requirement for peroxide is that it must be destroyed by a path not leading back to oxygen. The addition of ethanol to the reagent causes peroxide to be destroyed by catalase in the reaction mixture, without yielding oxygen, according to reaction:



To ensure destruction of the peroxide, iodide and molybdate are added to the enzyme reagent, causing reaction:



This reaction is effective even after catalase activity has diminished with storage.

The chemistry is free of interference from reducing substances and from agents used to prevent glycolysis or coagulation. The method does not depend on optical properties of a solution. The analyzer, therefore, will determine the concentration, even in uremic, icteric, turbid, or haemolyzed specimens.

Test Methodology

Plasma sample is manually pipetted into enzyme reagent in a cup containing an electrode that responds to oxygen concentration. Solid-state electronic circuitry determines the rate of oxygen consumption. A front-panel digital meter provides direct readout in milligrams of glucose per 100 milliliters, or may be changed to read millimoles-per-liter (Standard International Units) by changing the position of an electrical connector.

Measurement of Non-Esterified Fatty Acids (NEFA)

Materials

Extraction mixture: Isopropanol 40 parts; heptane, 10 parts; 0.5 M (1N) sulphuric acid 1 part.

Buffered phenol red stock: 1% solution of phenol red in 2.5% sodium barbitone.

Sodium barbitone stock: 2.5% sodium barbitone (0.25 g in 10 ml water).

Phenol Red Working Solutions

These were prepared one hour before use. The volumes of stock solution indicated were diluted to 100 ml with ethanol.

Solution	Stock phenol red (ml) %	Stock sodium barbitone (ml)	Final concentrations sodium barbitone
1	0.2		0.0050
2	0.4		0.0075
3	0.4		0.0100
4	0.5		0.0125
5	0.3	0.03	0.00835
6	0.3	0.07	0.00925
7	0.3	0.10	0.01000
8	0.3	0.15	0.01125
9	0.3	0.20	0.01250
10	0.4	0.01	0.01025
11	0.4	0.03	0.01075
12	0.4	0.05	0.01125
13	0.4	0.07	0.01175

Palmitic acid standards

Standards ranging from 160 to 1440 $\mu\text{mol/l}$ were prepared in heptane and extracted according to Baird *et al.* (376). Free fatty acids from both standards and plasma were extracted into heptane by the same method. Standards or plasma (1 ml) were mixed in test tubes with extraction mixture (5 ml), heptane (3 ml) and boiled distilled water (2 ml) and shaken vigorously for one and a half minutes. Heptane-saturated nitrogen was bubbled through each tube to remove volatile acids (especially carbon dioxide) which are present in greater amounts in plasma than in standards. The heptane phase was washed once with 4 ml 0.05% v/v sulphuric acid to remove lactic acid and an acetone-insoluble lipid (probably phospholipid) (376), and the extracts were stored at 4°C until analysis. Baird found plasma extracts stable for at least three days when stored at 4°C.

Principle of Assay

The method is based on the measurement of colour changes in phenol red barbitol buffer in heptane - ethanol. The fatty acids are first extracted manually from the plasma into heptane following Dole's extraction procedure (377), as modified by Trout *et al.* (378), and the extracts are then analyzed automatically using the colour reaction described by Mosinger (379). This involves the addition of sodium barbitol and phenol red in heptane-ethanol. Free diethyl-barbituric acid converts the alkaline phenol red into its yellow acid form and the optical density is measured.

Test Methodology

The manifold was the same as that described by Baird *et al.* (376). A stream of phenol red working solution was segmented with carbon dioxide-free air. After a steady baseline had been established the sample was introduced into the stream which was passed through a double mixing coil, the air was eliminated and the decrease of the red colour was recorded at 500 nm.

2.2 Estimation of Haemoglobin (Hb)

The aims of this thesis were to investigate tissue production or expression of IGF-1 and the regulatory control of IGF-1 through the GH axis. So, estimation of tissue levels of IGF-1 played an important part in this thesis. To exclude blood contamination, tissue IGF-1 was corrected for retained plasma IGF-1 using tissue and plasma haemoglobin levels as markers of retained plasma (437, 130).

Materials

TMB (3, 3', 5, 5'-tetramethylbenzidine): as chromogen (250 mg per vial, Sigma product, Catalog No. 527-2).

Hydrogen peroxide solution: as oxidant 0.3% (v/v) with stabilizer (Sigma product, catalog No. 527-4).

Acetic acid solution : for reconstitution of TMB with 50 ml acetic acid (glacial, 90%, v/v; Sigma product, catalog No. 527-6).

Haemoglobin Standard: With haemoglobin concentration of 45 mg/dl in human plasma (Sigma product, catalog No. 527-11).

Principle of Assay

The assay is based on the methods of Lijana & Williams (380) and Standefer &

Vanderjagt (381). Colorimetric determination of plasma or tissue extract Hb is based upon the catalytic action of Hb on the oxidation of TMB by hydrogen peroxide. Resulting rate of color formation is proportional to the Hb concentration of the test sample.

Test Procedure

1. To appropriately marked tubes, the following were added:

	Blank	Test	Standard
TMB solution	2.0 ml	2.0 ml	2.0 ml
Sample	-	0.01 ml	-
Hb standard (30 mg/dl)	-	-	0.01 ml
H ₂ O ₂ solution	2.0 ml	2.0 ml	2.0 ml

2. The tubes were mixed well and left to stand at room temperature (25°C) for 10 min.
3. The absorbance (A) of Test and Standard at 600 nm vs Blank as reference were measured.
4. The concentration of haemoglobin was calculated using following formula:

where C = concentration of Hb in Standard

$$Hb \text{ (mg/dL)} = \frac{A \text{ test}}{A \text{ standard}} \cdot C$$

2.3 Radioimmunoassay (RIA) for IGF-I

Materials

N-Met human IGF-I: human IGF-I with an added methionyl residue at the N-terminus, derived from bacteria using recombinant DNA technology, was kindly provided by Dr B. D. Burleigh, IMC/Pitman-Moore, Terre Haute, IN, USA (batches 742/44, and 2070/39). It was repurified in our lab (382).

Natural sequence human IGF-I: authentic human IGF-I derived from yeast using recombinant DNA technology, was gifted by Dr A. Skottner, Kabi Vitrum AB, Stockholm, Sweden (Batch y IGF-IDSQ 10/32835).

Primary antibody: an IGF-I antiserum, raised by repeated subcutaneous injection of rabbits with an egg albumin conjugate of IGF-I (443).

Secondary antibody: consisting of sheep or goat anti-rabbit antiserum ($1/_{60}$, v/v) and normal rabbit serum ($1/_{200}$, v/v) in polyethylene glycol (4%, w/v).

Bovine serum albumin (BSA): purified natural BSA (catalogue number

A 7030) was purchased from Sigma Chemical Company.

Radioiodination of IGF-1

Preparations of radioiodinated IGF-I were used extensively in the experimental work of this thesis as radioligands in RIA, IGF-I ligand blot analysis, and *in vitro* IGF-I binding activity study in biological fluids. Consequently there was a on-going requirement for high quality tracer preparations.

¹²⁵I-IGF-I preparations of high binding activity and stability have been produced by a wide range of procedures. The standard iodogen procedure proved a simple and convenient method resulting in tracer preparations of high activity by the criteria examined. Tracer prepared by the standard iodogen method compared very favourably with ¹²⁵I-IGF-I produced using Chloramine-T and LPO-GOD as reference methods. Iodination of IGF-I using Iodogen results in a product which shows 87.4% binding to ovine IGF plasma binding proteins (492). Iodinations were routinely performed by the iodogen method (outlined below).

Iodogen Method

Except where otherwise stated repurified N-Met IGF-I peak 2 peptide (382) was used for radioiodination. Radioiodination was achieved by a modification of the iodogen method (290). Iodogen (1, 3, 4, 6-tetrachloro-3 α , 6 α diphenyl glycoluril; Pierce and Warriner, NZ Ltd; 100

μl of a 100 $\mu\text{mol/l}$ solution in dichloromethane) was added to a polypropylene vial and the solvent evaporated. To perform an iodination, phosphate buffer (0.064 mol/l, pH 7.4, 20 μl), IGF-1 peptide ($\sim 3.5\mu\text{g}$, 0.5 nmol, 10 μl) and Na ^{125}I (0.5 mCi, ~ 0.25 nmol, 5 μl ; Cat No. IMS 30, 100 mCi/ml, Amersham International plc, Amersham, Bucks, UK) were added to the vial in order. The iodination was allowed to continue for 10 min before transferring the vial contents to a second vial containing phosphate buffer (0.064 mol/l, pH 7.4, 100 μl) for 5 min and addition of HCl (0.01 mol/l containing 0.15 mol/l NaCl and 0.1% w/v polypep (Sigma Chemical Co, 500 μl). Separation of radio-labelled peptide from unincorporated radioiodine was achieved by Sep-Pak chromatography. The potential specific activity of radio-labelled IGF-I prepared by this method was $\sim 143\mu\text{Ci}/\mu\text{g}$ (~ 1070 Ci/mmol), equivalent to approximately 1 molecule of ^{125}I per 2 molecules of IGF-I. Iodination mixtures were applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, MA, USA) which had been pre-equilibrated with HCl (0.01 mol/l) containing NaCl (0.15 mol/l, Solvent A) and washed with the same solvent (10 ml) to elute the unincorporated radioiodine. Fractions of 2 ml were collected. Adsorbed radio-labelled peptide was eluted using a stepwise gradient of ethanol (10-80% v/v in Solvent A). Samples (2 ml) of each solvent were applied and collected as separate fractions. Radioactivity in each fraction was estimated by gamma counting. Incorporation of radioiodine into peptide was estimated from the Sep-Pak elution profile as the proportion of radioactivity adsorbed

to the cartridge and eluted in the ethanolic fractions.

Separation of unincorporated ^{125}I from radio-iodinated IGF-I is illustrated in Figure 2.1. Reaction vial contents resulting from an iodination performed by the standard iodogen procedure were applied to a primed Sep-Pak and 'free' iodine recovered in the 'flow-through' fractions. Adsorbed peptide was eluted using ethanol and radioiodinated IGF-I (^{125}I -IGF-I) recovered mostly in the 40, 50 and 60% ethanolic fractions.

Incorporation of radioiodine into peptide was 94.6%. ^{125}I -IGF-I bearing fractions were stored at -20°C for experimentation.

Tracer storage

Radioiodinated IGF-I prepared by the standard iodogen method was stored at -20°C and its integrity examined over a 60 day period.

After the iodinations the radio-labelled peptide was purified by Sep-Pak chromatography as outlined in Section 2.3 and the peptide eluted in the 40% ethanolic fraction was used for experimentation.

Two procedures were used to examine structural/functional integrity of the radio-labelled IGF preparations. In the first, *in vitro* binding of ^{125}I -IGF-I preparations to high molecular weight binding sites in normal sheep plasma was examined using size exclusion chromatography (SEC). Full methodology is outlined in Section 2.4. Data are presented as the proportion of tracer which binds to such fractions as judged by a high

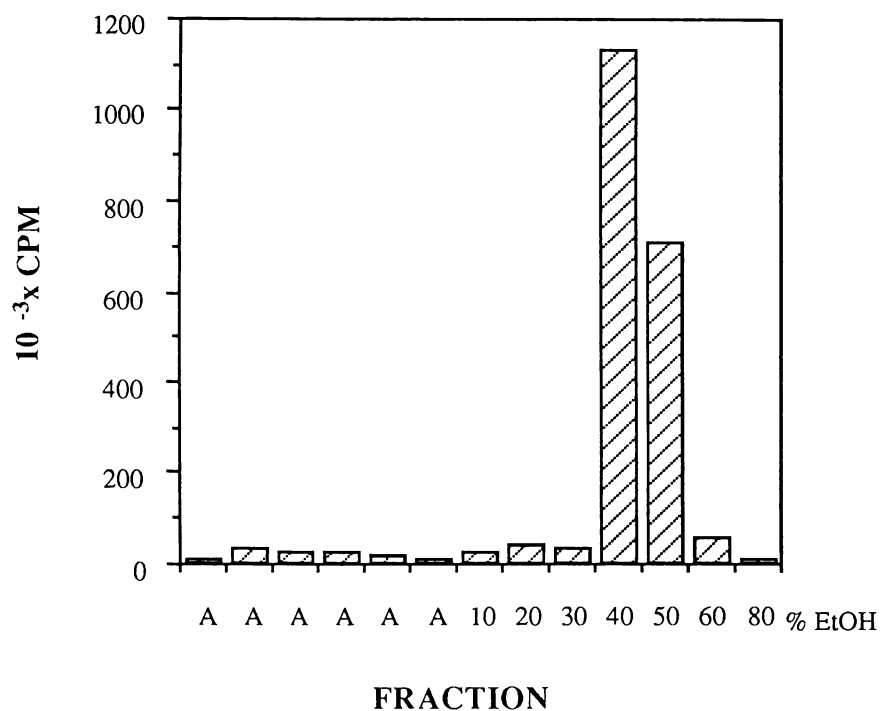


Figure 2.1 Purification of radiolabelled IGF-I on a reversed phase Sep Pak C₁₈ cartridge. Iodination mixtures were applied to a Sep Pak cartridge which had been pre equilibrated with HCl (0.01 mol/l) containing NaCl (0.15 mol/l ; Solvent A) and washed with the same solvent. Fractions of 2ml were collected. Adsorbed peptide was eluted using a step wise gradient of ethanol (10-80% v/v in solvent A). Radioactivity in each fraction was estimated by gamma counting.

molecular weight shift (earlier elution) on SEC. The second analysis involved examination of tracer binding to a reference IGF-I antiserum (AS-6; rabbit anti-somatomedin-C antiserum, Underwood & Van Wyk; courtesy National Hormone and Pituitary Program, National Institutes of Health). Buffer for all reagents was 0.064 mol/l sodium phosphate, pH 7.4 containing BSA (0.25% w/v) and sodium azide (0.02% w/v). Tracer was diluted to 20000 cpm per 200 μ l and incubated with excess reference antibody (1/100 initial dilution, 100 μ l) overnight at 4°C. Antibody-bound and free 125 I-IGF-I were separated by a modification of the polyethylene glycol precipitation method of Desbuquois (291). For this, bovine IgG (Sigma; 12 mg/ml, 50 μ l/tube) was added to each assay tube followed by polyethylene glycol 6000 solution (750 μ l, 20% w/v), the tube contents were mixed, centrifuged (2500 rpm, 4°C, 30 min) and the soluble free fraction aspirated to waste. Bound radioactivity was estimated by gamma counting. Assays were performed in quadruplicate and extra tubes included which contained tracer but not antibody in order to assess non-specific precipitation of 125 I-IGF-I. Data are expressed as percentage 125 I-IGF-I bound to excess antibody following correction for non-specific binding.

Antibody production

Primary and secondary antibodies for IGF-I assays described in the following section were generated 'in house'.

IGF-I Antiserum

Antisera to IGF-I were raised in sheep and rabbits to an egg albumin conjugate of N-Met IGF-I prepared using water soluble carbodiimide essentially as described by Goodfriend (292).

Conjugation Procedure

IGF-I (recombinant N-Met IGF-I; 5 mg in 1 ml of 0.003 mol/l HCl), muramyl dipeptide (MDP; adjuvant peptide; Sigma; 100 μ g in 0.0001 mol/l HCl, 0.1 ml) and egg albumin (10 mg in 0.5 ml of 0.15 mol/l NaCl; Sigma) were mixed, carbodiimide (EDC; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Bio Rad; 10 mg in 100 μ l distilled water) added and the mixture incubated for 3 h at room temperature. Activated sites remaining at the end of the coupling were blocked by the addition of β alanine (Sigma, 1 mg) and a further incubation period (1 h). The conjugate was resalted into NaCl (0.15 mol/l) on a small column of Sephadex G25 (1 x 10 cm; Pharmacia) diluted to 25 ml and stored in aliquots equivalent to 500 μ g of IGF-I for immunization.

Immunization Procedure

Antisera to IGF-I were raised in rabbits and sheep (5 of each) by immunization at four week intervals with conjugate (equivalent to 100 μ g IGF-I per rabbit and 200 μ g per sheep) emulsified in Freund's Complete Adjuvant (Sigma Chemical Co) for the primary immunization

(1 volume conjugate : 3 volumes adjuvant) and subsequently in incomplete adjuvant. Animals were injected with emulsion (2.0 ml per rabbit; 4 ml per sheep) at four subcutaneous or intramuscular sites. The animals were bled 10-14 days after each immunization and antisera assessed by means of antiserum dilution curves and evaluated for use in an IGF-I RIA.

Secondary Antiserum

Precipitating second antibodies were raised against rabbit IgG by immunization of sheep or goat with unconjugated rabbit IgG, Fc fragments (0.25 mg/animal; Jackson ImmunoResearch, PA, USA) emulsified in Freund's Adjuvant as outlined in "IGF-I Antiserum".

Secondary antibodies were characterized by their ability to precipitate complexes of the appropriate primary antibody, for example ^{125}I -IGF-I: rabbit anti-IGF-I/sheep anti-rabbit IgG Fc, in the presence of non-immuno serum (normal rabbit serum in this example) as carrier and polyethylene glycol 6000 (4% w/v).

IGF-I RIA for sheep plasma

The limited reagent IGF-I radioimmunoassay was applied in the experimental work of this thesis. It is a heterologous assay based on a rabbit anti-human IGF-I antiserum (R8) raised as described in Section 2.4, radioiodinated N-Met IGF-I as a tracer preparation and a second

antibody for the separation of bound and free fractions. This assay has been validated for the measurement of IGF-I in ruminant plasma using an acid-ethanol extraction modified from Daughaday (293).

As a limited reagent method, IGF-I RIA appears to be particularly susceptible to interference from other factors including the plasma IGFBPs. Although several mechanisms for this interference may be operative two major ones are apparent. The first involves binding of radio-labelled IGF-1 (^{125}I -IGF-I) to unoccupied binding sites on BPs from samples. This may decrease ^{125}I -IGF-I binding to antibody and result in apparent overestimates of IGF-I concentration since antibody bound ^{125}I -IGF-I is inversely proportional to hormone concentration. The second basis for interference may relate to steric considerations. Thus accessibility of IGF-I to antibody may be compromised if IGF-I is prebound to BP. Although information on the region specificity of BP binding to IGF is starting to emerge (Section 1.3), epitope specificity of most IGF-I antibodies is much less certain (294). Selected, region-specific antibodies which bind to IGF-I when the hormone is pre-bound to BP would be a big advantage for IGF-I immunoassay and may make possible non-extraction immunoassays in the future.

Currently, however, preliminary extraction of samples is employed to overcome BP interference. Two 'benchmark' methods have traditionally been employed. The first, employing gel chromatography on Sephadex

G75 under acidic conditions (295), effectively resolves IGF-I from high molecular weight binding proteins but is slow, time-consuming and subject to manipulative error. A second approach based on the use of acid-ethanol was originally described by Daughaday (293). The methodology, employing acid to dissociate BP-bound IGF-I and ethanol to precipitate the carrier proteins is widely used for the preparation of human plasma for IGF-I analysis but has proved to be problematic when applied to ruminant plasma and other fluids (296). Thus non-parallelism and poor recoveries are often observed. This is at least partly due to the presence of residual BP in ruminant fluid extracts but may also be exacerbated by the IGF-I antibody used for assay. Recently Breier and colleagues have reported a modification of the acid-ethanol extraction involving a cryoprecipitation step (AEC technique) which further reduces BP content of the extract and is suitable for use with ruminant plasma (297). However, the suitability of such extracts for IGF RIA remains dependent on binding characteristics of the antibody employed and may not be applicable to other fluids (for example lymph) with different constitution of BP, or to tissue extracts. The AEC technique was evaluated and validated for application in the analytical work of this thesis.

Materials

Assays were performed utilizing polystyrene tubes (Galanti Plastics, NZ Ltd; 4 ml) in Cole-Palmer tube racks and micro-pipettes (Finnpipette 0-

50 μ l, 50-200 μ l and 200-1000 μ l, and Eppendorf Combipet). Unless otherwise stated buffer for all assay reagents and dilutions was 0.064 mol/l sodium phosphate, pH 7.4 containing BSA (0.25 % w/v; Sigma), NaN_3 (0.02 % w/v; Sigma; PBSA) and Triton X-100 (0.02%, w/v; Sigma).

Radioiodination/Tracer Preparation

^{125}I -IGF-I was prepared by radioiodination of repurified N-Met IGF-I (peak 2 peptide; 382) by the iodogen method as described in Section 2.3. The 40% and 50% ethanolic fractions from the Sep Pak purification of radio-labelled IGF-I contained tracer of highest immunoreactivity. These were stored at -20°C and diluted to 20,000 cpm per 200 μ l in PBSA assay buffer before use.

Standard

Recombinant authentic sequence human IGF-I (Kabi Vitrum product) was already in solution at a concentration of 0.9 mg/ml supplied on dry ice. The aliquots were diluted to a 'working stock' concentration of 10 $\mu\text{g}/\text{ml}$ in PBSA buffer and stored at -70°C . Assay standards were prepared by further dilution of the 'working stock' to 100 ng/ml and then 30, 10, 3, 1, 0.3 and 0 ng/ml IGF-I in PBSA buffer. Assay standards were not stored but prepared and used immediately

Primary Antibody

Antibody for the standard IGF-I assay resulted from the immunization programme outlined in Section 2.3. Rabbit anti-IGF-I antibody (R8) was stored undiluted at -20°C and used for assay at an initial dilution of 1/1000.

Secondary Antibody

A solution consisting of sheep or goat anti-rabbit serum ($1/_{60}$, v/v) and normal rabbit serum ($1/_{200}$, v/v) in polyethylene glycol (4%, w/v) was used to achieve separation of bound and free antigen.

Sample Extraction by AEC Technique

Plasma sample (100 μ l) was diluted by assay buffer (400 μ l) then added with 2 ml cold acid-ethanol (12.5% 1 mol/l HCl - 87.5% absolute ethanol, v/v) in polystyrene tubes (4 ml), mixed and stored at 4°C for 30 min. Tubes were then centrifuges (2500 rpm; 4°C; 30 min), the soluble free fraction transferred to further tubes and neutralized by the addition of Tris base (0.855 mol/l; 1 ml). The extracts were then mixed, chilled at -20°C for 60 min, and centrifuged (2500 rpm; -5°C; 30 min). The supernatant was then transferred to further tubes without warming. Extracts were warmed to room temperature and mixed before assay.

Assay procedure

Assays were performed in duplicate or quadruplicate by incubation of

standard (0-100 ng/ml; 100 μ l) or sample extracts (100 μ l) with 125 I-IGF-I (20 000 cpm in 200 μ l) and antibody (R8, 1/1000 initial dilution; 100 μ l) for 24 h at 4°C. Samples were routinely assayed at 2-3 dilutions with controls. Extra tubes were included with each assay for the estimation of non-specific binding, total radioactivity and recovery of IGF-I from a normal sheep plasma pool. Separation of bound and free IGF-I was achieved using the secondary antibody solution (200 μ l/tube). After 60 min incubation at 4°C, bound antigen was sedimented by centrifugation (1000 g, 30 min, 4°C) and the soluble free fraction removed by aspiration. Radioactivity was estimated in an LKB 1260 multihead gamma counter and calculations performed using four-parameter logistics curve fitting software on a microcomputer (Either RIA Calc, Bromma, Sweden or AssayZap, Elsevier Biosoft, Cambridge, UK).

The minimal detectable dose of IGF-I was 2 fmol ($P < 0.05$, $n = 5$). Within- and between-assay coefficients of variation for a plasma quality control sample containing 9.3 nmol IGF-I/l were 3.9 and 9.1% respectively ($n = 5$). The mean recovery of IGF-I immunoreactivity from ovine plasma was $91.0 \pm 4.2\%$ (mean $\pm$ coefficient of variation). The assay demonstrated $< 1\%$ cross reactivity with IGF-II and $< 0.1\%$ with insulin.

2.4 In vitro IGF tracer binding/size exclusion chromatography

IGF-I Tracer Binding

The distribution of IGF immunoreactivity or IGF-I binding activity in sheep plasma samples was examined by equilibration with radio-labelled IGF-I preparations followed by size exclusion chromatography (SEC) at neutral pH. For this ^{125}I -IGF-I ($\sim 100\,000$ cpm) was added to plasma (0.5 ml) together with 50 μl of 0.064 mol/l sodium phosphate buffer, pH 7.4 containing NaN_3 (0.02% w/v) and incubated overnight at 4°C. Before chromatography samples were centrifuged in a microcentrifuge at 4°C (10 min; 2000 g).

IGF-I Binding activity in sheep plasma/SEC at pH 7.4

Except where stipulated all SEC was performed on an FPLC (Fast Protein Liquid Chromatograph) instrument (Pharmacia/LKB Biotechnology, Upsala, Sweden) fitted with a pre-packed Superose 12 column (HR 10/30, 1.0 x 30 cm; Pharmacia). Solvent for SEC was 0.064 mol/l sodium phosphate buffer, pH 7.4 containing NaN_3 (0.02% w/v). Samples of 200 μl were applied to the column and eluted with the same buffer at 0.5 ml/min and, following a 20 min delay period, fractions of 0.5 min (0.25 ml) collected. Radioactivity in eluted fractions was determined by gamma counting (384). The column was pre-equilibrated with 0.064 mol/l sodium phosphate buffer (pH 7.4) containing NaN_3 (0.02%, w/v), and calibrated before use using the following markers:

catalase, γ -globulin, bovine serum albumin, ovalbumin, soya bean trypsin inhibitor, IGF-I, insulin and ^{125}I with molecular weights of 247500, 165000, 67000, 43000, 21000, 7650, 5700 and 125 respectively (384). Format and programming of the FPLC system for SEC is attached at the end of this chapter (Appendix to chapter 2).

Equilibration of ^{125}I -labelled IGF-I with normal sheep plasma (NSP) followed by SEC revealed up to three peaks of radioactivity eluting with apparent molecular weights of 150 , 40-50 and 9 kDa in fractions 17, 23 and 33. These fractions corresponded to ^{125}I -labelled IGF-I complexed to 150 kDa, 40-50 kDa molecular weight binding proteins and to 'free' ^{125}I -labelled IGF-I (Fig. 2.2).

Thus the preliminary chromatography on the Superose 12 column provided a convenient system for separating the major forms of immunoreactivity of IGF-I or for monitoring the distribution/binding activity of IGF-I in ovine plasma. SEC was also useful for examining other ovine biological fluids and tissue extracts.

2.5 Validation of tissue extraction for IGF-I RIA

Introduction

Early studies of IGF regulation focused on the endocrine mechanisms

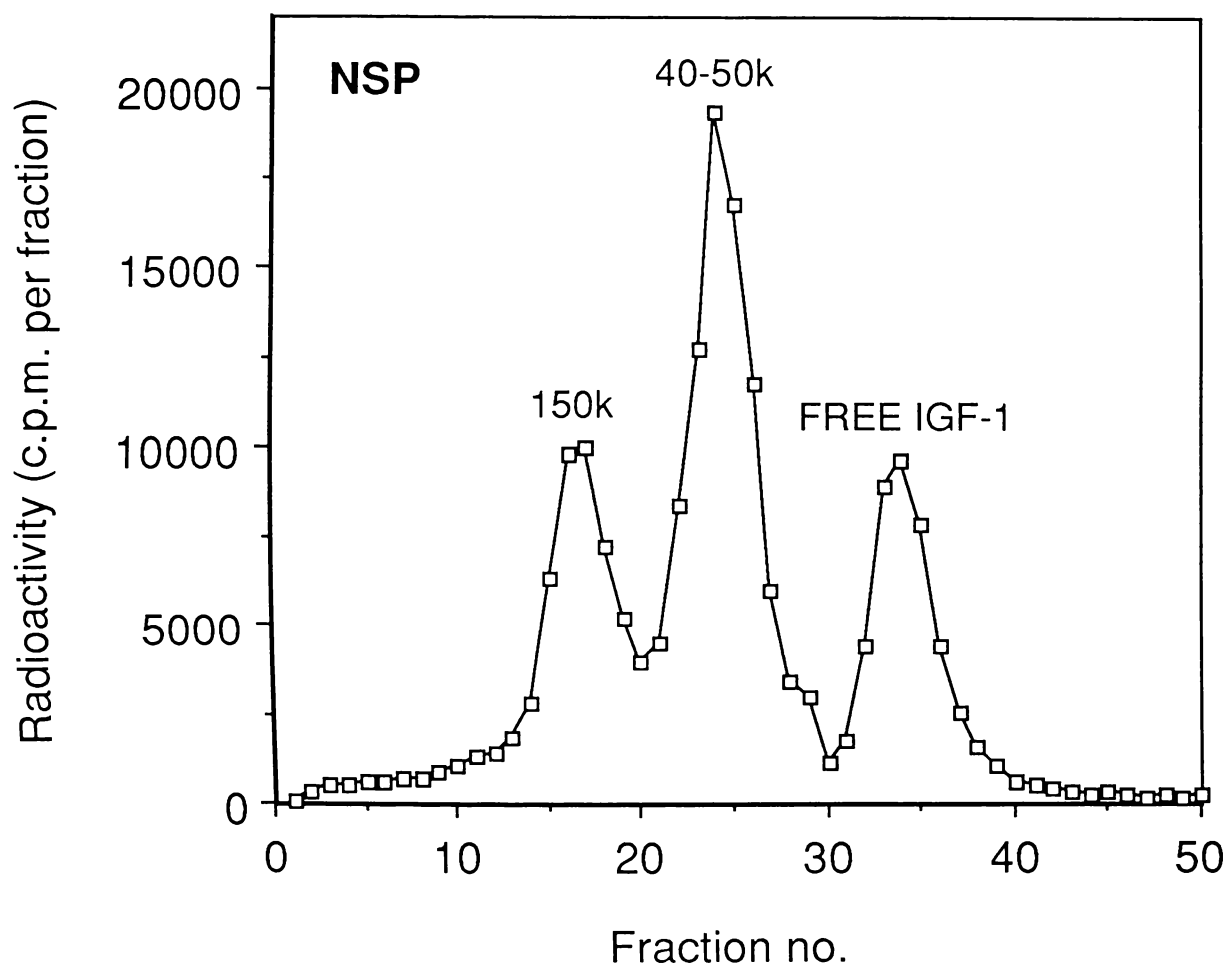


Figure 2.2 Superose 12 size exclusion chromatography of normal sheep plasma (NSP), collected from 6 month-ewe lambs by jugular venepuncture into vacutainers containing EDTA (15 mg). NSP was incubated with ^{125}I -labelled insulin-like growth factor-I (IGF-1) overnight at 4°C and subjected to chromatography using 0.064 mol/l phosphate buffer, pH 7.4, as solvent. The flow rate was 0.5 ml/min and, following a 20 min delay period, fractions at 0.5 min (0.25 ml) were collected. Radioactivity was detected in eluate fractions by gamma counting. The three peaks labelled 150K, 40-50K and free IGF-1 represent the expected positions of ^{125}I -IGF-1 complexed to high molecular weight binding sites of 150KDa, 40-50KDa and unbound ^{125}I -IGF-1.

that controlled the blood concentrations of IGFs (385, 386). Recently an autocrine/paracrine hypothesis has led to more attention being given to tissue IGFs.

To estimate tissue levels of IGFs the preliminary step is tissue extraction of IGFs. It is now well known that IGFs in blood and other biological fluids are complexed to BPs (387, 388). So, the objective of tissue extraction is the dissociation of the IGF-binding protein complex and precipitation of the IGF BPs, which result in interference in IGF-I RIA by binding of radio-labelled IGF-I (^{125}I -IGF-I) to unoccupied binding sites on IGFBPs from samples.

IGFBP complex can be dissociated from the BPs under acid conditions, the pH optimum being 3.6 (389-391). Although the dissociation of the IGF-I BP complex in plasma is most rapid at 37°C (391), tissues should be extracted at 4°C for relatively short times, because kidney and other tissues contain proteases that degrade IGF-I (392).

The technique used for extraction of IGF-I from sheep tissues is a modification of the method by D'Ercole and Underwood (130). The advantage of the modification is that it is faster, particularly when extracting a large number of tissue samples.

Tissue extraction

Tissue sampling

1. Sheep tissues were collected immediately after slaughter.
2. Dissection of tissues was delayed for approximately 30 s to allow exsanguination.
3. Tissue were promptly blotted on absorbent toweling to remove adherent blood, cut into small pieces and blotted.
4. Tissues were then flash frozen by submersion in liquid nitrogen.
5. Frozen tissues were stored at -20°C for up to 6 months, with no apparent reduction in IGF-1 content.

Extracting Tissues

1. Samples of frozen tissue (~0.5 g) were weighed in polystyrene centrifuge tubes. The tissue weight was recorded, and the tissue was placed on ice until the next step.
2. To iced tubes containing tissues, 1.0 mol/l acetic acid was added in a ratio of 5 ml for each gram of frozen tissue, then homogenized directly using a Polytron homogenizer (the resultant pH was 3.5 ~ 3.9).
3. Samples were incubated on ice for 2 hr.
4. Tubes are then centrifuged at 40 000 g for 30 min in a refrigerated centrifuge (4°C).
5. A measured volume of the supernatant, generally 0.5 ml, was then

removed for the IGF-I assay, and a second aliquot was saved for determination of Hb.

6. The IGF-I aliquot, but not the Hb aliquot, was then neutralized with 5 mol/l NaOH (about 12 μ l of NaOH/0.1 ml of extract resulted in a final pH between 6 and 8), and stored at -20°C until the time of assay. The Hb aliquot was assayed without prior neutralization, because Hb is precipitated by these conditions of neutralization.

Problems with separation of tissue homogenate

An initial problem encountered with separation of tissue homogenate, particularly muscle homogenate, was that it gelled after incubation of two hours, and no supernatant could be separated with initial centrifugation rate (1000 g, 10 min). This problem was overcome by increasing centrifugation rate to 40 000 g.

Validation

The AEC extraction technique provided a rapid and simple alternative to column chromatography on Sephadex G75 for blood plasma from a variety of species (297), but it had not been validated for IGF-I RIA in sheep tissues.

The efficiency of the AEC technique of extraction for sheep tissues was

evaluated by the following criteria:

1. Recovery of IGF-I immunoreactivity from tissue extracts.
2. Examination of BP in sample extracts
3. Parallelism of extract dilutions

Hodgkinson reported the parallelism of sheep fluid and tissue extract dilutions with repurified N-Met IGF-I peak 2 peptide standard (492), but the recovery of IGF-I immunoreactivity and IGF-I binding activity in sheep tissue extracts had not been examined.

Recovery of IGF-I immunoreactivity from sheep tissue extracts

Recovery of IGF-I was examined using tissue extracts 'spiked' with recombinant IGF-I (Kabi Vitrum). IGF-I (Kabi Vitrum) was added to tissue extracts to concentrations of 50 or 100 ng/ml of extract, incubated for 60 min at 4°C and subjected to AEC extractions then followed by IGF-I RIA. Results are outlined in Table 2.1. Recovery estimates ranged from ~84-94% and no evidence of a relationship between hormone concentration and recovery was observed. Despite this high recovery, there was a major limitation to the application of this sort of recovery experiment to tissues. Thus the experiment gave little indication of the efficiency of solubilization of endogenous IGF-I from

tissue but only estimates efficiency of extraction of IGF-I from the homogenate.

Data from the above recovery experiment indicated that residual BP after AEC extraction was not interfering by reducing the measurable peptide.

Table 2.1 Recovery of immunoreactive IGF-I in tissue extracts (n=3, per group; values are means)

Tissue extracts	IGF-I (Kabi Vitrum) added ng/ml extract	Actual IGF-I (ng/ml)	Measured IGF-I (ng/ml)	Recovery (%)	Recovery (mean of dose 1 & dose 2 $\pm$ % CV)
Liver	-	-	13.39	-	-
Liver	50 (dose 1)	63.39	51.66	81.50	85.84 $\pm$ 7.15
Liver	100 (dose 2)	113.39	102.25	90.18	
Kidney	-	-	16.52	-	-
Kidney	50 (dose 1)	66.52	54.55	82.00	83.84 $\pm$ 3.10
Kidney	100 (dose 2)	166.52	99.82	85.67	
Muscle	-	-	9.85	-	-
Muscle	50 (dose 1)	59.85	51.71	86.40	92.84 $\pm$ 9.81
Muscle	100 (dose 2)	109.85	109.06	99.28	
Heart	-	-	8.54	-	-
Heart	50 (dose 1)	58.54	45.83	78.29	88.00 $\pm$ 15.61
Heart	100 (dose 2)	108.54	106.07	97.72	
Spleen	-	-	7.19	-	-
Spleen	50 (dose 1)	57.19	51.80	90.58	93.62 $\pm$ 4.58
Spleen	100 (dose 2)	107.19	103.60	96.65	
Lung	-	-	17.73	-	-
Lung	50 (dose 1)	67.73	58.61	86.53	84.94 $\pm$ 2.64
Lung	100 (dose 2)	117.73	98.14	83.36	

Examination of IGF-I binding activity in sheep tissue extracts

The residual IGF-I binding activity of several tissue extracts was examined. For this, AEC extracts of sheep tissues were dried, using a vacuum centrifuge (Savant

Speed Vac. SVC100) attached to an ethanolic cold trap (-40°C), reconstituted with distilled water and the pH corrected to 7.0~7.4. These samples were incubated overnight at 4°C with ¹²⁵I-labelled IGF-I (100,000 cpm/200 µl tissue extract). The incubated samples were then centrifuged (2500 g, 4°C, 30 min) and subjected to SEC analysis.

Equilibration of ¹²⁵I-labelled IGF-I with normal sheep plasma (NSP) followed by SEC revealed up to three peaks of radioactivity eluting with apparent molecular weights of 150 , 40-50 and 9 kDa in fractions 17, 23 and 33. These fractions corresponded with ¹²⁵I-labelled IGF-I complexed with 150 kDa, 40-50 kDa molecular weight binding proteins and to 'free' ¹²⁵I-labelled IGF-I (Fig 2.3a).

Equilibration of ¹²⁵I-labelled IGF-I with AEC extracts of NSP and other sheep tissues followed by SEC revealed major peaks of radioactivity eluting only in the free IGF-I region. The major peak accounted for 79, 84, 83, 84, 77, 78 and 76% of recovered tracer in AEC extracts of NSP, liver, kidney, muscle, spleen, heart and lung respectively. Elution of ¹²⁵I-labelled IGF-I in the position expected for high molecular weight binding protein was found to be negligible for plasma and tissue AEC extracts (Fig. 2.3 b-h).

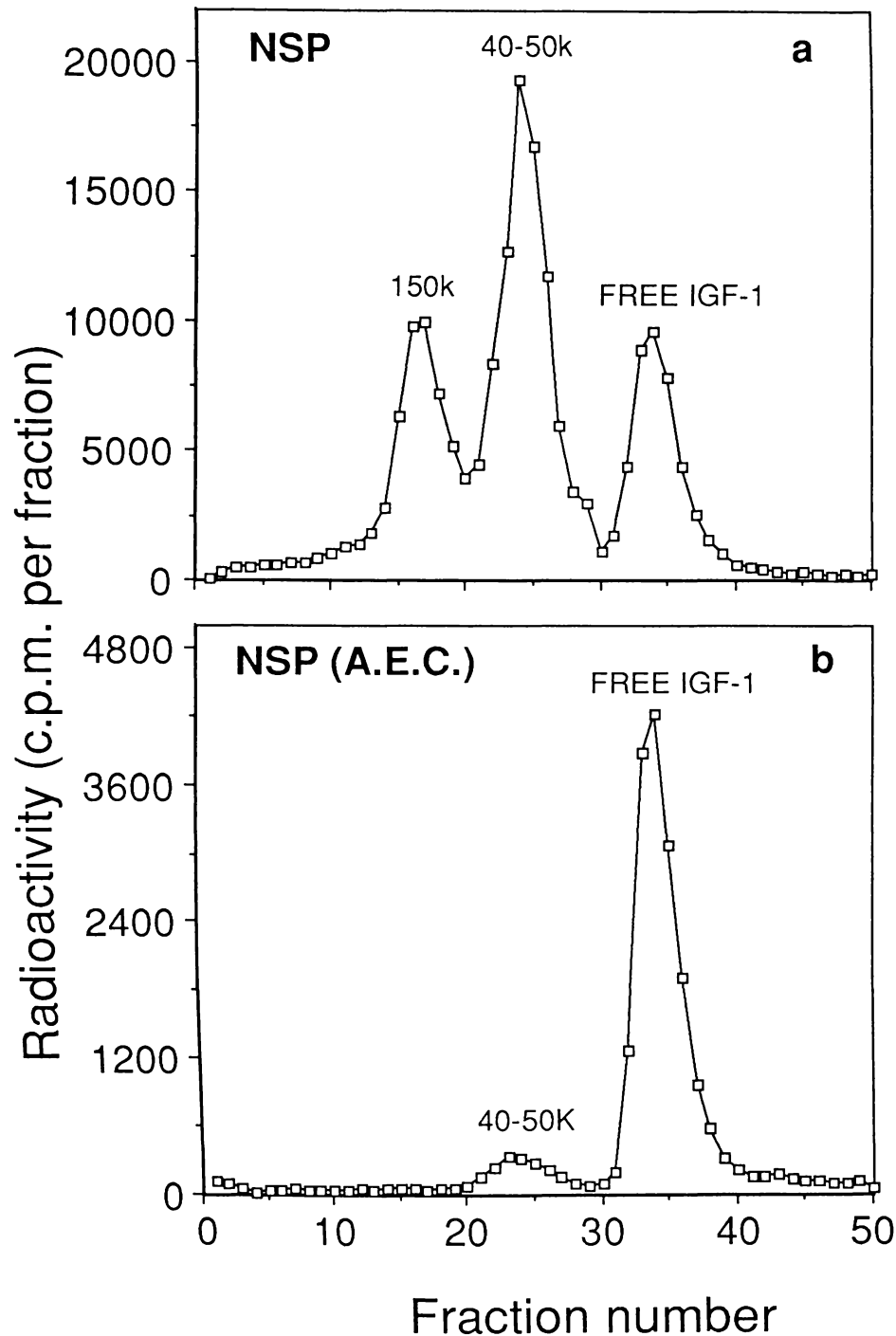


Figure 2.3a-b Size exclusion chromatography of normal sheep plasma (NSP) and acid-ethanol-cryo-precipitation (AEC) extracts of NSP following incubation with ^{125}I -labelled insulin-like growth factor-I (IGF-1). Equilibration of ^{125}I -labelled IGF-1 with NSP followed by size-exclusion chromatography revealed up to three peaks of radioactivity eluting with apparent molecular weights of 150, 40-50 and 9kDa in fractions 17, 23 and 33. These correspond to ^{125}I -labelled IGF-1 complexed to 150kDa, 40-50kDa molecular weight binding proteins and to 'free' ^{125}I -labelled IGF-1 (Fig. 2.3a). Equilibration of ^{125}I -labelled IGF-1 with AEC extracts of NSP followed by size-exclusion chromatography revealed major peaks of radioactivity eluting only in the free IGF-1 region (Fig. 2.3b).

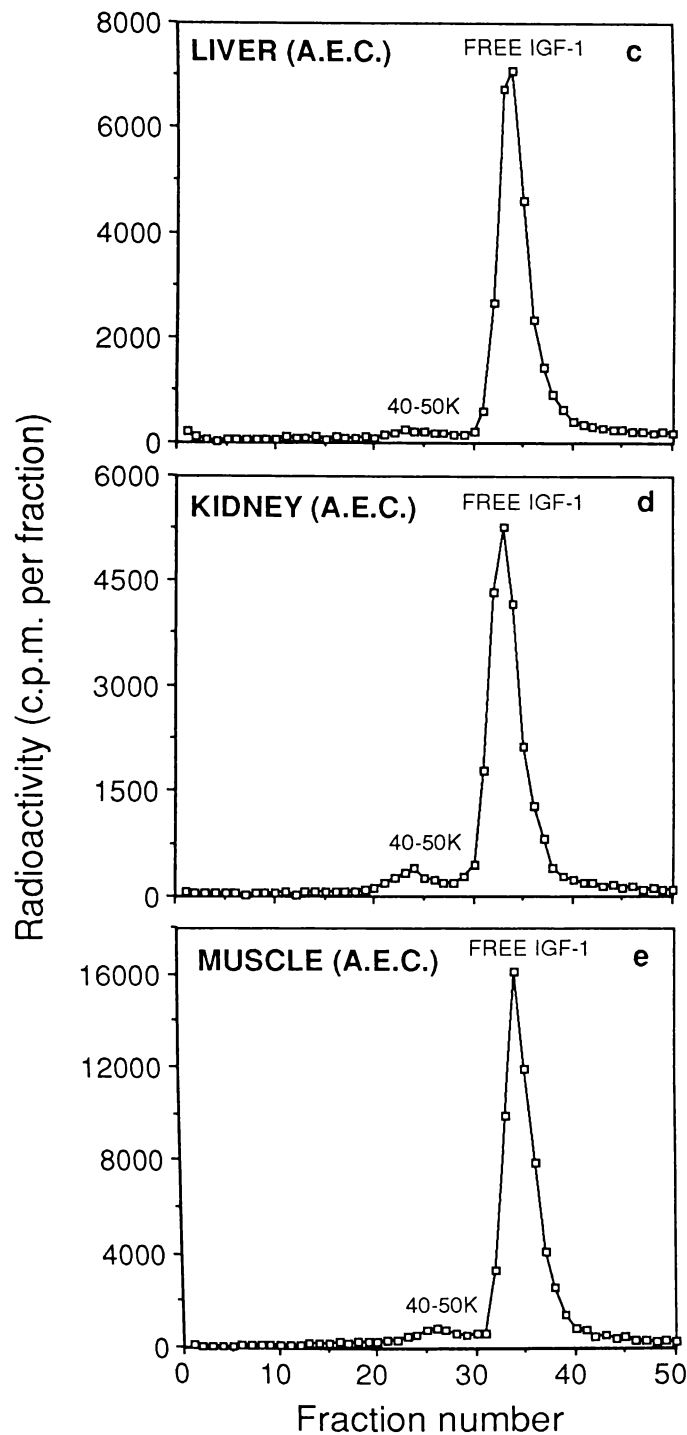


Figure 2.3c-e Size exclusion chromatography of acid-ethanol-cryo-precipitation (AEC) extracts of sheep tissues following incubation with ^{125}I -labelled insulin-like growth factor-I (IGF-1). Equilibration of ^{125}I -labelled IGF-1 with NSP followed by size-exclusion chromatography revealed up to three peaks of radioactivity eluting with apparent molecular weights of 150, 40-50 and 9kDa in fractions 17, 23 and 33. These correspond to ^{125}I -labelled IGF-1 complexed to 150kDa, 40-50kDa molecular weight binding proteins and to 'free' ^{125}I -labelled IGF-1 (Fig. 2.3a). Equilibration of ^{125}I -labelled IGF-1 with AEC extracts of other sheep tissues followed by size-exclusion chromatography revealed major peaks of radioactivity eluting only in the free IGF-1 region (Fig. 2.3c-e).

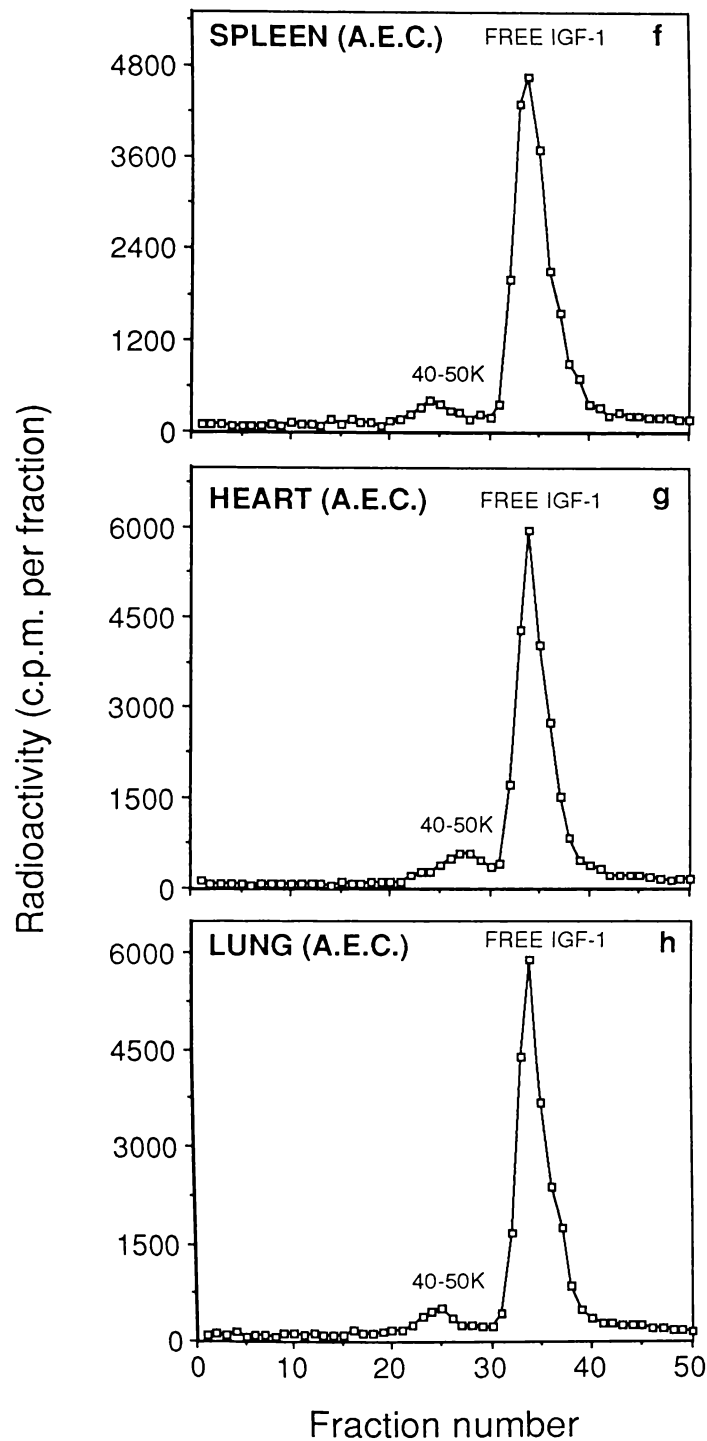


Figure 2.3f-h Size exclusion chromatography of acid-ethanol-cryo-precipitation (AEC) extracts of sheep tissues following incubation with ^{125}I -labelled insulin-like growth factor-I (IGF-1). Equilibration of ^{125}I -labelled IGF-1 with NSP followed by size-exclusion chromatography revealed up to three peaks of radioactivity eluting with apparent molecular weights of 150, 40-50 and 9kDa in fractions 17, 23 and 33. These correspond to ^{125}I -labelled IGF-1 complexed to 150kDa, 40-50kDa molecular weight binding proteins and to 'free' ^{125}I -labelled IGF-1 (Fig. 2.3a). Equilibration of ^{125}I -labelled IGF-1 with AEC extracts of other sheep tissues followed by size-exclusion chromatography revealed major peaks of radioactivity eluting only in the free IGF-1 region (Fig. 2.3f-h).

Conclusion

AEC technique provided a simple, convenient and reliable estimation of IGF-I immunoreactivity for sheep tissue extracts.

IGF-I RIA for tissue extracts

IGF-I RIA procedure for tissue extracts was the same as for plasma, which is outlined in the section 2.3.

2.6 IGF-I Ligand blot analysis

Modified Western Blotting Technique

The relative levels of IGFBPs in samples of plasma in this thesis were determined by a modification of the Western blotting technique described by Hossenlopp *et al* (393). Samples of plasma (50 μ l) were supplemented with 50 μ l of 5 x sample treatment buffer (0.5 mol/l Tris buffer pH 6.8, 1.25 ml; glycerol, 4 ml; MQ water, 4.75 ml; SDS, 0.5 g), 150 μ l of Tris buffer (0.05 M pH 7.5) and 2 μ l of bromophenol blue (0.03% w/v, in 50% ethanol) then boiled for 3 minutes. 10 μ l of each prepared sample was loaded onto a 4% stacking gel and electrophoresed through a 12% (v/v) polyacrylamide gel (1.5 x 80 x 55 mm gel containing 0.1% SDS, w/v) (353) at a constant voltage (150v) for 80 min at 4°C using Bio Rad Mini-Protein equipment (Bio-Rad Lab, Richmond, CA, USA). The gels were then equilibrated in pre-chilled transfer buffer [39 mmol/l glycine,

48 mmol/l Tris, 2% (v/v) methanol, MQ water, pH 9.0~9.4]and protein bands were electrophoretically transferred to nitrocellulose membranes (0.2 μ m, Bio-Rad Laboratories Pty Ltd, Auckland, NZ) at 35 mA/per gel for 2 hours at 4°C, using a 2117 + 250 Nova Blot Electrophoretic Transfer Kit (LKB-Producter AB, Sweden). Ligand blotting was performed by the procedure of Hossenlopp *et al.* (393). Briefly, the nitrocellulose membranes were washed at 4°C on a racking platform: in 1) Tris-saline buffer (15 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.4) containing 0.5 mg/ml sodium azide and 3% Nonidet P40 for 30 minutes; then 2) Tris-saline buffer plus 0.1% Tween-20 for 20 minutes. The membranes were then incubated individually with ¹²⁵I-IGF-I (400,000 cpm per membrane) in 20 ml of Tris-saline buffer containing 1% fatty-acid-free BSA and 0.1% Tween-20 overnight at 4°C on a racking platform. Following this incubation, the membrane were washed twice with Tris-saline buffer containing 0.1% Tween-20, three times with Tris-saline buffer alone (15 minutes per wash) then air dried and autoradiographed on x-ray film (Kodak X-Omat, XAR-20, Eastman Kodak, Rochester, NY) with intensifying screens at -70°C for 3-7 days.

Quantification of the autoradiograms were performed using scanning densitometry (Personal Desnitometer and Software : Image Quant Version 3.2, Molecular Dynamics Limited, USA). The relative levels of the bands were expressed as optical density (O.D).

IGF-I BPs in Adult Sheep Plasma

IGF-I BPs in adult sheep plasma were measured, then used as a convenient

protein marker in analysis of sheep plasma binding proteins in electrophoresis.

Samples of adult sheep plasma (ASP) were collected from Coopworth x Suffolk ewes by juglar venepuncture into vacutainers containing EDTA (15 mg): the animals were not pregnant, approximately 12 months of age and maintained on pasture. The plasma samples were subjected to the electrophoresis and blotting procedure described in the section of modified Western Blotting Technique.

IGF-I ligand blot analysis revealed four major classes of IGF BPs; a doublet band of 40-43 kDa, and single bands of 34 kDa, 30 kDa and 24 kDa.

The doublet band at 40-43 kDa could be analogous to the 45-50 kDa species characterized from ovine plasma by Walton and colleagues (314). Amino terminal sequence determination of the Walton polypeptide enabled its assignment as IGF BP-3. Again by analogy to the findings of Walton, the 35 kDa band appears to be the ovine equivalent of IGF-BP-2 and also has similar motility to bovine IGF BP-2 (327) while the 30 kDa and 24 kDa bands would be ovine IGF BP-1 (394) and IGF BP-4 respectively (334, 394) (Fig. 2.4). Recently the identities of the 35 and 30 kDa BPs were shown to be IGFBP-2 and IGFBP-1, respectively, by Western blotting using specific antisera against bovine IGFBP-2 and human IGFBP-1 (394). The identification of IGFBPs in sheep plasma was discussed more fully in section 5.4.

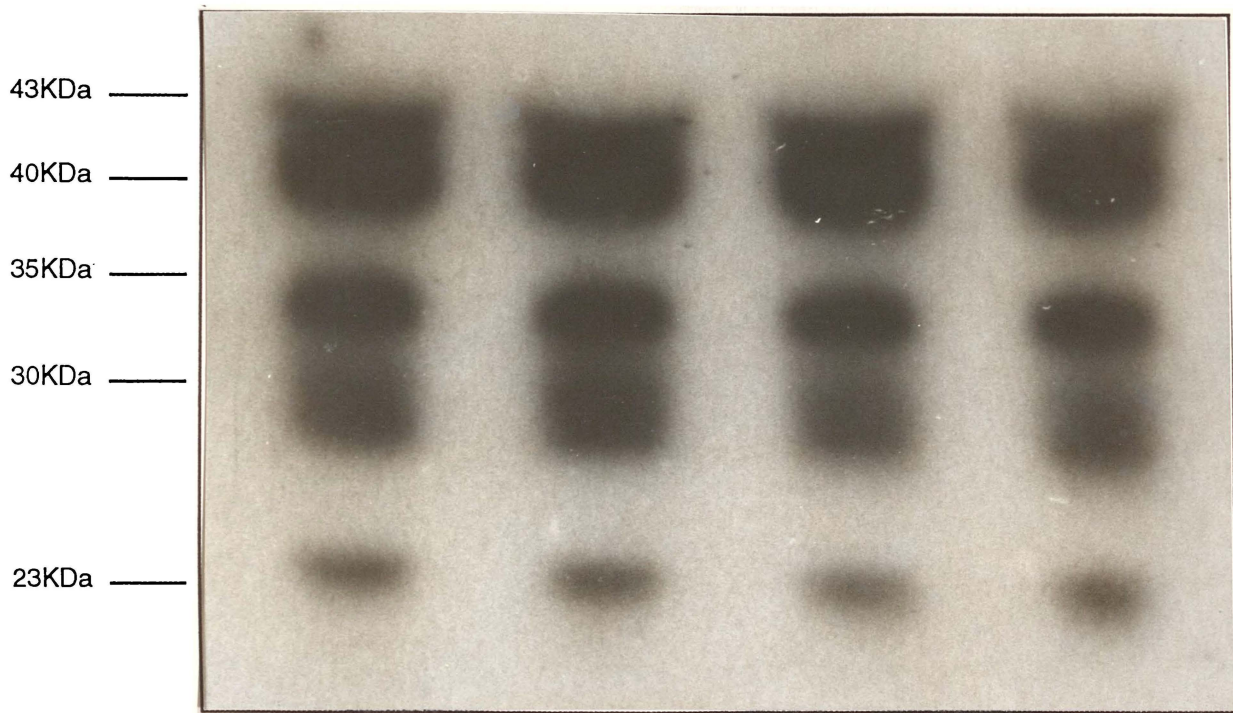


Figure 2.4 IGF-1 ligand blot analysis of adult sheep plasma (ASP), collected from 12 month-ewes. Samples were subjected to SDS/PAGE as described in section 2.8 and electrophoretically transferred to nitrocellulose membranes using a Bio Rad Semidry transfer cell, and incubated with ^{125}I -IGF-1 prepared as described in section 2.3. Membranes were then used to expose Kodak XAR x-ray films. Apparent molecular weights (KDa) of the bands as estimated by comparison of their electrophoretic mobilities with globular molecular weight markers (not presented) are shown on the left.

2.7 RIA for ruminant GH

Materials

Ovine GH (oGH): repurified NIH-oGH-14 (National Hormone and Pituitary Program, Baltimore, MD, USA) was used as standard, and radio-iodinated by the Iodogen Method as radiolabelled oGH tracer.

oGH standard solution: repurified NIH-oGH ($10 \mu\text{g}/\mu\text{l}$) was diluted with assay buffer in a range from 0.1 ng/ml to 200 ng/ml.

Rabbit anti-ovine GH antiserum: An antiserum was raised in rabbits by repeated subcutaneous injection of ovine GH (NIH-oGH-14, purified further by anion exchange and gel filtration chromatography) and used as first antibody (final dilution 1:13500, v/v).

Goat anti-rabbit antiserum: An antiserum was raised in goats to rabbit immunoglobulin (IgG Fc-1; Jackson Immuno Research Labs, PA), used at 1:60, v/v, in 4% PEG-6000 (poly ethylene glycol 6000).

Normal rabbit serum: used as a carrier at 1:120, v/v, in 4% PEG-6000.

BSA: purified natural bovine serum albumin (Sigma Chemical Company, catalogue number A7030).

Assay buffer: sodium phosphate buffer (0.064 mol/l, pH 7.4) with 0.25% BSA (PBSA).

GH Iodination

Radiolabelling of hormones in the Ruakura laboratory was performed using the Iodogen method (310) followed by purification on gel permeation column chromatography. Salacinski *et al.* (290) claim that iodination of GH and other proteins with Iodogen gives a product with greater ^{125}I incorporation and less iodination damage than iodination using either chloramine-T (538) or lactoperoxidase (395). Iodination of IGF1 using Iodogen results in a product which shows greater than 80% binding to ovine IGF plasma binding proteins (382, 383), indicating that there is also little damage to the conformation of IGF1 using this procedure.

The Iodogen method for iodination of IGF-I is detailed in Section 2.3. Here, briefly the following reagents were placed in the Iodogen vial; phosphate buffer (20 μl), GH (12 μg), and Na^{125}I (1 mCi, 10 μl). This was mixed with a pipette tip and allowed to react for ten minutes, with remixing after five minutes. The reaction was stopped by transferring the contents of the Iodogen vial to a second vial containing phosphate buffer (200 μl). Phosphate buffer with 0.25% BSA (PBSA) was added (200 μl) and the mixture applied to a Sephadex G100 column for purification. The column had earlier been equilibrated with PBSA and fractions were eluted with the same buffer. Aliquots of fractions were counted and the three fractions containing monomeric ^{125}I -GH were stored at -20°C for

use within three weeks. Specific activities of 73 $\mu\text{Ci}/\mu\text{g}$ GH (S. Hodgkinson, personal communication) was typically achieved using the procedure.

GH assay

Duplicate aliquots of sample and oGH standard (100 μl) were incubated with rabbit anti-oGH antiserum (100 μl) and ^{125}I -oGH (100 μl , 20,000 cpm) for four days at 4°C. The antiserum was used at a final dilution of 1:13,500 in phosphate buffer with sodium azide (0.1%) and BSA (0.25%). A mixture of goat anti-rabbit antiserum (1:60), normal rabbit serum (1:20) and 4% polyethylene glycol 6000 (PEG) in assay buffer was pre-incubated for four hours at room temperature and 200 μl added to the tubes which were then incubated for two hours at room temperature. Finally, 500 μl of PEG 6000 (4%) in assay buffer was added, the tubes centrifuged, the supernatant aspirated and the pellets gamma counted. Assay sensitivity was 1 ng/ml with an intra-assay variation of 1.3% and an inter-assay variation of 8.0%.

2.8 In-situ hybridisation

Introduction

In situ hybridisation is a technique that involves applying a labelled, complementary nucleic acid probe to tissue sections *in vitro*, resulting in hybridisation with specific RNA. The biggest advantage of *in situ* hybridisation is the ability to determine with high sensitivity cellular localisation of bound

probe indicating the localisation of RNA of interest.

In situ hybridisation was first introduced in 1969 (396, 397). RNA was isolated, labelled and then incubated with thin tissue squashes or cultured cells where it hybridised to denatured complementary DNA. Later developments enabled detection of specific RNA sequences by using denatured complementary DNA probes, complementary synthetic oligonucleotides (398, 399) or complementary single-stranded RNA probes synthesised *in vitro* using strong, specific promoters linked to a gene of interest and contained in a suitable plasmid (400, 401).

Single-stranded RNA probes (riboprobes) as used in this thesis have several advantages over alternative types of probe. Because the probes are single-stranded, there is not competition between re-annealing of denatured complementary strands and binding to target RNA. Therefore the amount of probe available for binding remains at a saturating level for maximum signal (400). Also, denaturation of probe prior to hybridisation becomes unnecessary with single-stranded RNA probes. RNA probes are free of template and vector sequences which could contribute to non-specific binding. In theory, cRNA-mRNA hybrids are more stable than corresponding cDNA-mRNA hybrids, enabling use of higher washing temperatures to reduce non-specific binding (402). Probes have a high specific activity since one quarter of the bases in each transcript are labelled (403). Lower background levels can be achieved with RNA probes since ribonuclease can be used as a post-hybridisation treatment to remove unhybridised, single-stranded RNA (404).

The choice of radioisotope was determined by the requirements for sensitivity, anatomical resolution and speed of development (405). ^{35}S was the label used in the present study, incorporated into the base uridine-5'-triphosphate to enable RNA synthesis. It has a relatively high energy emission (0.167 MeV) and good resolution (10-15 μm) (406). It is able to provide information on localisation at the cellular level due to its high specific activity, after a relatively short exposure period.

To investigate local production of IGF-1 in tissues in this thesis, mRNA levels in tissues were monitored by *in situ* hybridisation as an indicator of IGF-1 expression at the transcription level.

Materials

Markers for size determination

A mixture of DNA or RNA fragments of known molecular weight were used as markers to determine the size of DNA or RNA fragments of unknown molecular weight.

λ restricted with *Hind* III and *Eco*R I (BRL Bethesda Research Laboratories Life Technologies Inc., USA) was used, to obtain fragments of DNA for use, in agarose gel electrophoresis to determine the unknown molecular weight of RNA. λ restricted with *Hind* III and *Eco* RI yields the following fragments of DNA:

21.226 kb; 5.148 kb; 4.973 kb; 4,277 kb; 3.530 kb; 2.027 kb; 1.904 kb; 1.584 kb; 1.330 kb; 0.983 kb; 0.831 kb; 0.564 kb; 0.125 kb (407). Fragments from λ restricted with *Hind* III and *Eco* RI was end-labelled with [α - 32 P]-labelled 2'-deoxy-adenosine-5-triphosphate and used in agarose gel electrophoresis under denaturing conditions to check size and integrity of RNA transcripts used as mRNA probes (Fig. 2.5).

IGF-I cDNA probes

For *in situ* hybridization in tissue sections, 35 S-labelled UTP was incorporated into RNA transcribed from a 2,203 base pair *Sau*3A sequence containing part of the 5' coding region of a human IGF-I cDNA (a gift from Dr M. Jansen, University Hospital for Children and Youth, Utrecht, The Netherlands) cloned in the *Bam*HI site of a pGEM3 vector. The anti-sense (T) strand was transcribed from the pGEM T7 promoter, while the control sense (S) strand was transcribed from the pGEM SP6 promoter. The latter strand was used to probe tissue sections for non-specific hybridization. S and T probes were of equal size, each consisting mostly of one 0.2 kb radioactive transcript (Fig. 2.5). Homology of the probe to ovine IGF-I was 88% in the coding region.

Methods

Processing and Sectioning Tissue

The aim of fixation is to retain the maximum level of target RNA in tissue while maintaining optimal morphological detail and probe accessibility (408). Formalin crosslinks proteins and alkylates amino groups such as those in bases of single-

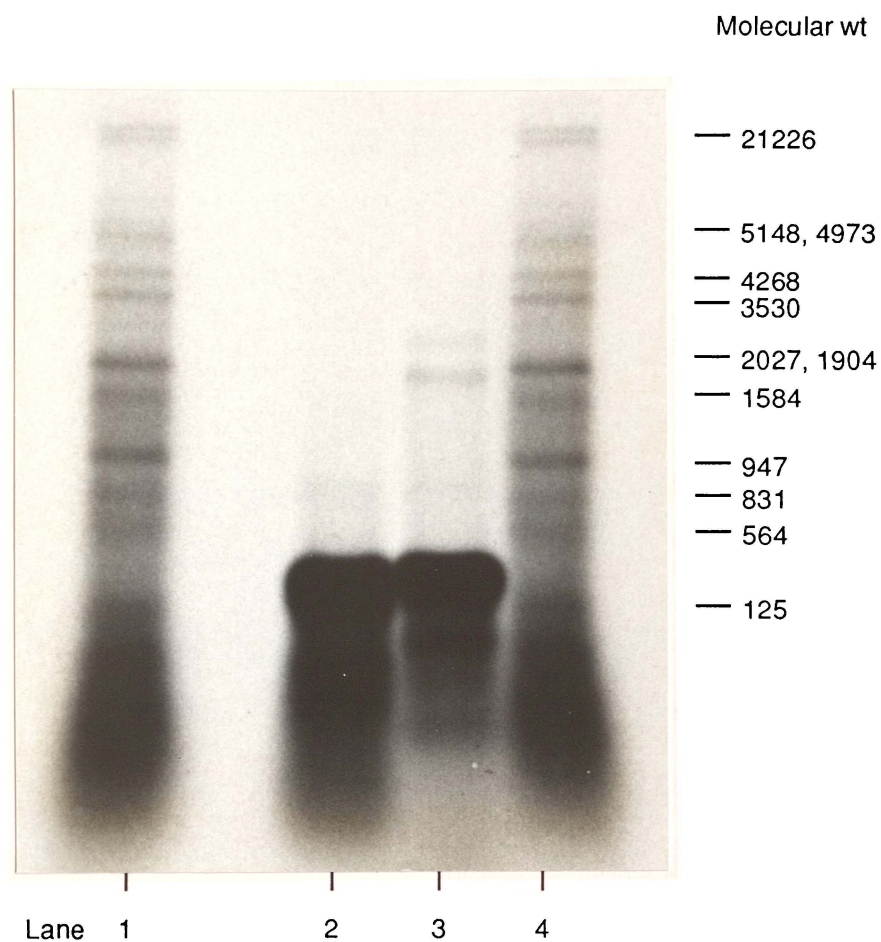


Figure 2.5 Autoradiography of RNA transcripts (probes for *in situ* hybridisation) following electrophoresis under denaturing conditions. Each batch of probe contains a heterogeneous population of transcripts, with the dominant band corresponding to full-length transcription from the template.

Lane 1 = end-labelled lambda marker cut with *Hind* III/*Eco* RI

Lane 2 = IGF-1 probe (antisense strand). Full-length transcript = 259bp

Lane 3 = IGF-1 probe (sense strand). Full-length transcript = 265bp

Lane 4 = end-labelled lambda marker cut with *Hind* III/*Eco* RI

stranded nucleic acids (409, 410). It is a fixative that provides a compromise between permeability and RNA retention in tissue (411).

Processing, embedding and sectioning of tissue was carried out by an experienced histologist. Tissue was embedded in an automated Shandon Elliott Duplex processor according to standard processing protocols (412). The procedure was as follows: dehydration at room temperature (70% ethanol, 1 hour; 96% ethanol, 1.5 hours; 99% ethanol, 1.5 hours) followed by clearing at room temperature (toluene, 1 hour; toluene, 1 hour) and impregnation at 60°C (paraffin wax, 3 hours; paraffin wax, 3 hours). Tissues were removed from the processor, placed into moulds and the moulds filled with molten (60°C) paraffin wax. The blocks were set at room temperature before sectioning. Where possible, two samples of each tissue were embedded, for cross and longitudinal section, in each paraffin block. Two blocks for each tissue sample were made to ensure ample tissue for these and future studies. Also, if one of the blocks were unsuitable for sectioning or it was lost, at least one other block of tissue was available.

Pre-cleaned glass slides were immersed in gelatin-chrome alum subbing solution (0.5% w/v gelatine, 0.05% w/v chromium potassium sulphate) at 4°C for 2 minutes (413). Slides were then thoroughly dried. Sections, 7 μ m thick, were cut on a microtome. Two adjacent sections were floated on each gelatin-subbed slide in diluted (20%) subbing solution. Sections were heat-stretched on a 60°C heat plate to remove wrinkles. Excess subbing solution was removed and then the sections were dried at 60°C and baked for 24 hours at 45°C (410).

Section Pre-treatment

The paraffin wax was removed from the sections by passing the slides through three changes of xylene for 10 minutes each. Rehydration was through an alcohol series of decreasing ethanol concentrations (100%, 100%, 95%, 70%) for 5 minutes each. The slides were rinsed in diethyl pyrocarbonate-treated MilliQ water for 2 minutes.

Mild deproteination was carried out to increase permeability for the probe and expose target RNA. Sections were placed in 0.2 mol/l hydrochloric acid for 10 minutes at room temperature to remove basic proteins (398, 414). After washing the sections in 2 x *SSC buffer* (3 M sodium chloride & 0.3 M sodium citrate, pH 7.0 with sodium hydroxide) for 30 minutes at room temperature, sections were treated with protease. *Proteinase K buffer* (200 mM Tris-HCl, pH 7.2; 2 mM CaCl_2) was preheated to 37°C. Proteinase K (30 U/ μl) (415) was added to the buffer prior to treating the sections. Slides were incubated for 15 minutes at 37°C in the proteinase K solution (398, 416, 414).

Acetylation of amino groups reduces non-specific electrostatic binding of the probe (417). To 0.1 mol/l triethanolamine (pH 8.0), 0.25% (v/v) acetic anhydride was added, mixed thoroughly and immediately poured over the slides. The half-life of the acetylation mixture that was formed was 1 minute (417). After 5 minutes, a further 0.25% (v/v) acetic anhydride was added, mixed thoroughly and incubated a further 5 minutes at room temperature. Sections were washed for 5 minutes in 2 x *SSC buffer* to remove traces of acetic

anhydride. Treatment of sections to test specificity of hybrid formation were carried out at this point (section 3.2). Dehydration was through an alcohol series of increasing ethanol concentrations (70%, 95%, 100%) for 5 minutes each. Slides were air-dried. A zone around each section was delimited with AdosTM rubber cement, effectively forming a well to hold the hybridisation buffer. Sections were then ready for hybridisation.

Hybridisation Method

In situ hybridization (ISH) was based on the procedure of Wilkins, *et al.*, (418) as modified by Kirk, Bass, Molenaar and Ord (unpublished observation, 1992). Duplicate slides were assayed for each animal. Two serial sections were probed per slide, one section with the antisense, T, probe, and one section with the control sense, S, probe. Samples were exposed to X-ray film (X-OMATE AR, KODAK, Rochester, NY, USA) for several days to determine relative amounts of probe hybridized per section. To estimate relative intensities of bound probe for ISH-probed sections, these images on developed X-ray film were digitized with a Hewlett Parkard Scanjet Scanner and quantitated on a Macintosh IIfx computer using the application Image (NIH version 1.37). For ISH quantitation, mean image densities (greyscale 1-255) were converted to optical density (OD) by calibration with a diffuse density 'step wedge' (KODAK), then the mean OD for each S control section was subtracted from the corresponding experimental T section. Tissue folding and edge artifacts on the sections were avoided during quantitation. The procedure has been calibrated (unpublished observation, Kirk *et al.*, 1992) by comparing relative muscle actin gene expression monitored by

ISH with dot blot muscle actin RNA-RNA hybridizations detected by scintillation counting.

Appendix to Chapter 2

Format and Programming of FPLC for SEC

Component List

1. Solvent (0.064 mol/l sodium phosphate, pH 7.4, containing NaN_3 (0.02% w/v)
2. Solvent delivery pump (P500)
3. Solven delivery pump
4. Gradient mixer
5. In line filter
6. Motor valve (MV7) for sample loading and injection
7. Sample loading pump
9. Superose 12 HR10/30 molecular exclusion chromatography coloumn
13. Fraction collector Frac 100
14. Controller unit LCC 500

FPLC Programme

Time	Command	Setting	Comment
0.0	Conc % B	50	Phosphate buffer in pumps A and B
0.0	Flow (ml/min)	0.5	Flow rate
0.0	Port. set	6.1	Fraction collector on
0.0	Valv. pos	1.1	Motor valve in load position
1.0	Flow C	1.0	Sample pump on (load loop)
1.5	Flow C	0	Sample pump off
2.5	Valv. pos	1.2	Inject sample
5.0	Valv. pos	1.1	
43.5	Port. set	6.0	Fraction collector off
55.0	Conc % B	50	
55.0	ml/min	0.5	
56.0	end		

Fraction collector 20 min wait period, 0.5 min per fraction

CHAPTER THREE : REGULATION OF PLASMA AND TISSUE IGF-1 IMMUNO - REACTIVITY BY NUTRITION AND GH IN POSTNATAL SHEEP.

3.1 Introduction

Over 35 years ago, Salmon & Daughaday (72) postulated that stimulation of skeletal growth by GH was mediated by a circulating growth factor, initially termed "sulfation factor". The basis for this hypothesis can be summarized as follows: 1) It was difficult to demonstrate *in vitro* effects of GH on growth related parameters, such as sulphate uptake in costal cartilage of hypophysectomized rats. 2) Addition of serum from hypophysectomized rats treated with GH had the capacity to stimulate sulphate uptake. Subsequent studies suggested more general stimulatory actions of these GH-dependent circulating peptides on anabolism and growth of various tissues (reviewed in 117). Since then, somatomedin C and IGF-I have been considered to be the endocrine mediators of GH action. Consequently most studies on the regulation of the somatomedins focused on their concentrations in serum.

It is now well established that the variation in plasma IGF-I depends on GH status (120, 55, 338, 429, 430, 483) and nutritional manipulation (117, 118, 431-436) in several species. In recent years, a number of observations have suggested that IGF-I is produced locally in multiple tissues (338, 437, 130). The widespread production of IGF-I and IGF-II has been confirmed with probes for the respective mRNA in the rats (273), human fetus (438), cattle (128) and sheep

(496). This has led to an alternative concept of GH/IGF-I action on tissues, with GH acting by stimulation of IGF-I production in target tissues: locally produced IGF-I then acts in an auto/paracrine manner on adjacent cells (439). Under such circumstances, tissue IGF-I and the relationship between tissue and plasma IGF-I regulation may better reflect alterations in somatomedin physiology.

An important observation, reported in 1984 by D'Ercole *et al.* (338) who detected immunoreactive IGF-I in extracts from numerous rat tissues of the rats, revealed that levels of IGF-I were markedly lower in tissues from hypophysectomized animals than in normal animals, and that the intraperitoneal administration of ovine GH to hypophysectomized rats caused tissue-extractable IGF-I to increase in kidney, liver, lung, heart and testes. The results demonstrated a strong GH-dependence of IGF-I in these rat tissues. However this report provided no information at transcriptional level. More recently, a study by Yahya, Bates and Millward (440) showed changes in tissue concentrations of IGF-I in rats in relation to the inhibition of bone and skeletal muscle proteoglycan synthesis following graded protein deficiency. Very little *in vivo* evidence is available on the effect of GH and nutrition on tissue IGF-I from farm animal species, which may be valuable in the manipulation of post-natal growth in such species.

The present study was designed to examine the effect of GH and nutrition on tissue and plasma IGF-I immunoreactivity, and to determine the relationship between the levels of liver IGF-I and liver IGF-I RNA in sheep.

To achieve the objective, in this study the following items were investigated in sheep that were fed or starved, with or without administration of GH:

1. Plasma levels of glucose and NEFA were measured as indicators of nutritional status in the experimental animals.
2. Circulating levels of ovine GH were measured, and the relationship between the plasma GH levels and nutritional status was tested.
3. Validation of the AEC technique for application to the measurement of IGF-I in sheep tissues by RIA.
4. Plasma IGF-I was extracted by the AEC technique, then estimated by RIA. Differences in plasma IGF-I levels between animals with different nutritional or hormonal status were compared.
5. Tissue IGF-I was solubilised then extracted using the AEC technique, and estimated by RIA. Tissue IGF-I was corrected for retained plasma IGF-I using tissue and plasma Hb levels. Differences in tissue IGF-I levels between animals with different nutritional or hormonal status were compared.
6. IGF-I mRNA levels in liver were measured by ISH, and correlations between liver IGF-I and IGF-I mRNA levels, in response to GH administration, were examined.

The study in this chapter was performed in 1990, at Growth Physiology, Ruakura Agricultural Research Centre, Hamilton, New Zealand.

3.2 Materials and Methods

Materials

Lyophilised GH (recombinantly derived hGH) was reconstituted as a stock solution of 10 mg/ml with distilled water. It was refrigerated at 4°C and used over a period of 5-7 day. The hGH (Met-hGH-G11-15-GH008DAX) was a gift from Genentech Inc. (South San Francisco, CA, USA).

Feed pellets were purchased from NRM Feeds Limited and were composed of lucerne (60%), barley (30%), linseed oil (5%), and molasses (5%). Protein content was between 15-17% (w/w).

IGF-I cDNA probes for ISH were prepared as described in Section 2.8.

Animals and Treatment

Twenty healthy 12-month old ewes were acclimatized indoors in individual pens on an *ad libitum* diet of lucerne pellets and water for a period of 3 weeks before the experiment. Weekly live-weights and daily feed intakes were recorded during this period. At the end of this pre-treatment period the sheep were then divided into groups (n=5 per group), balanced on live-weights and allocated to one of the following four treatments for five days.

Fed (FD): on the lucerne pellets at 3% of body weight per day: saline injected at 0.5 ml per day for 5 days.

Fasted (ST):	fasted: saline injected at 0.5 ml per day for 5 days.
Fed + hGH (FDGH):	on the lucerne pellets at 3% of body weight per day: hGH injected (0.15 mg/kg body weight per day) for 5 days.
Fasted + hGH (STGH):	fasted: hGH injected (0.15 mg/kg body weight per day) for 5 days.

Injections were delivered as a single, subcutaneous bolus in the neck at 09.00 am daily.

Animals were slaughtered 24 hours after the last daily treatment with hGH.

Blood Sampling

The sheep were fitted with indwelling catheters in the jugular vein and blood samples (5 ml) were collected at 15 min intervals from 09,00 to 14,00 h, for measurement of plasma concentrations of IGF-I, GH, glucose and NEFA during the experimental period. Samples were kept on ice and centrifuged under refrigeration as soon as possible. The plasma samples were then frozen at -20°C until assay. Catheters were flushed daily with saline which initially contained 25 000 IU heparin/l, then 15 000 IU heparin/l throughout sampling to keep the catheters patent.

Tissue processing

Samples of liver, kidney and muscle biceps femoris were collected immediately after slaughter. They were cut into small blocks (2-3g) for freezing in liquid nitrogen, and stored at -70°C for latter analysis. Tissue samples for measurement of IGF-I RNA were collected separately as follows. Within 15 min of slaughter, 1-2 g tissue pieces of liver per animal were fixed at room temperature in 10% formalin/phosphate buffered saline for 48 h (fixative was replaced after 24 h), dehydrated, wax-embedded and sectioned. At least two (7 μ m) sections per slide were placed onto gelatin-coated slides. Slides were placed in a 50°C oven overnight, then stored.

Tissue extraction

Tissue IGF-I was extracted using a modification of the procedure by D'Ercole and Underwood (130), which is outlined in Section 2.5. Briefly, samples of frozen sheep tissues were homogenized directly in ice-cold acetic acid, incubated on ice for 2 hours, centrifuged, then supernatant was removed for IGF-I assay and for determination of Hb. The aliquot for determining IGF-I was neutralized with NaOH and the aliquot for determining Hb was assayed without prior neutralization.

Assays

Measurement of glucose, NEFA and GH in plasma

During the period of treatment, the plasma levels of glucose. NEFA and GH were monitored to assess physiological stress, particularly of the fasting animals.

Glucose levels were measured using an automated glucose oxidase method (for methodology details refer to Section 2.1). NEFA concentrations were also measured by an automated system (441) (Section 2.1). Plasma GH levels were estimated by RIA (442) (Section 2.7).

In-situ hybridisation

Liver IGF-I mRNA levels were monitored by in-situ hybridisation. Methodology is described in Section 2.8.

IGF-I RIA

Tissue and plasma levels of IGF-I were measured using an 'in house' RIA (443) after the tissue and plasma samples were extracted by the AEC technique (297) (for assay procedure refer to Section 2.3).

Estimation of tissue-extractable IGF-I derived from entrapment of blood

To determine the contribution of IGF-I originating from entrapped blood to total tissue IGF-I, the blood content of tissues was estimated using haemoglobin analysis (437, 130).

Tissue Hb was determined in tissue extracts prior to neutralization by a quantitative colorimetric assay (Procedure No. 527, Sigma) using 3,3',5,5'-tetramethylbenzidine (TMB) as chromogen. The assay is based on the methods of Lijana and Williams (380) and Standefer and Vanderjagt (381). All reagents, including Hb standard (catalogue No. 527-30), were obtained from Sigma

Chemical Co. (the methodology is detailed in Section 2.2). The volume of serum that contaminated each extract was calculated as follows (437, 130):

$$ml\ serum/ml\ extract = \frac{[Hb]_{ex}}{[Hb]_{bl}} (1 - haematocrit)$$

where $[Hb]_{ex}$ is the experimentally determined weight of Hb (g) in 1 ml tissue extract, $[Hb]_{bl}$ is the concentration of Hb in 1 ml sheep blood, and haematocrit is expressed as a fraction (%). $[Hb]_{bl}$ and haematocrit values used for calculations were: the mean Hb concentration (g/ml) of sheep blood 0.1209 ± 0.0027 (mean $\pm$ SEM; $n=20$); the mean haematocrit of sheep blood $34.2 \pm 0.8\%$ (mean $\pm$ SEM; $n=20$) as determined in our laboratory.

The possible plasma IGF-I contribution to tissues studied was subtracted from the total extracted tissue IGF-I concentration.

Validation of AEC technique for measuring IGF-I in extracts of sheep tissues

Acid-ethanol (AE) extraction has been the 'standard' method for preliminary extraction of samples for IGF-I RIA. Recently the validity of this method for extraction of ruminant fluids has been questioned (296). We adopted the AEC extraction method of Breier *et al.* (297), which has been specifically validated for ruminant fluids. Here we extend that validation for the estimation of IGF-I in sheep tissue extracts.

Recovery of known amounts of purified IGF-I (Kabi Vitrum) from sheep tissue extracts, dose-response curves of the extracts and IGF-I binding activity of the extracts were evaluated (refer to Section 2.5). Based on this validation, AEC technique provided a simple, convenient and reliable estimation of IGF-I immunoreactivity for sheep tissue extracts.

Calculations of tissue IGF-I concentrations

Tissue IGF-I concentration was calculated as ng/per gram frozen tissue (130) or converted into S.I. units.

Contribution of IGF-I from blood contamination of tissues to total tissue IGF-I was estimated as described before in this section then subtracted from the total extracted tissue IGF-I.

Biometrics

Differences in IGF-I mRNA levels between GH-treated and untreated animals were analysed by the unpaired t-test, and correlations between IGF-I RNA and IGF-I were calculated by correlation analysis, using the Macintosh Statview (1987, Abacus concepts, Inc., Berkeley, CA, USA). Other data were analysed by analysis of variance (ANOVA) using Genstat 5 (Numerical Algorithms Group Ltd, Oxford, UK).

3.3 Results

Nutritional status in the experimental animals

Nutritional status was monitored on the basis of the blood concentrations of glucose and NEFA.

After five days of fasting, blood glucose levels were reduced ($P < 0.001$) in ST group, compared with FD group, as well as in STGH group ($P < 0.001$) compared with FDGH group. No significant difference in glucose concentrations was shown between FDGH and FD group, and between STGH and ST group five days after the initial GH injection (Fig. 3.1).

By contrast, blood NEFA levels were elevated in ST group ($P < 0.001$, compared with FD group) and in STGH group ($P < 0.05$, compared with ST group; $P < 0.001$, compared with FDGH group). No difference could be shown between FDGH and FD group (Fig. 3.2).

Hormonal status in the experimental animals

Plasma levels of oGH were measured during the period of the treatment to investigate the effect of nutritional status on GH release.

Starvation significantly elevated ($P < 0.001$) oGH levels in ST compared with FD group (Fig. 3.3).

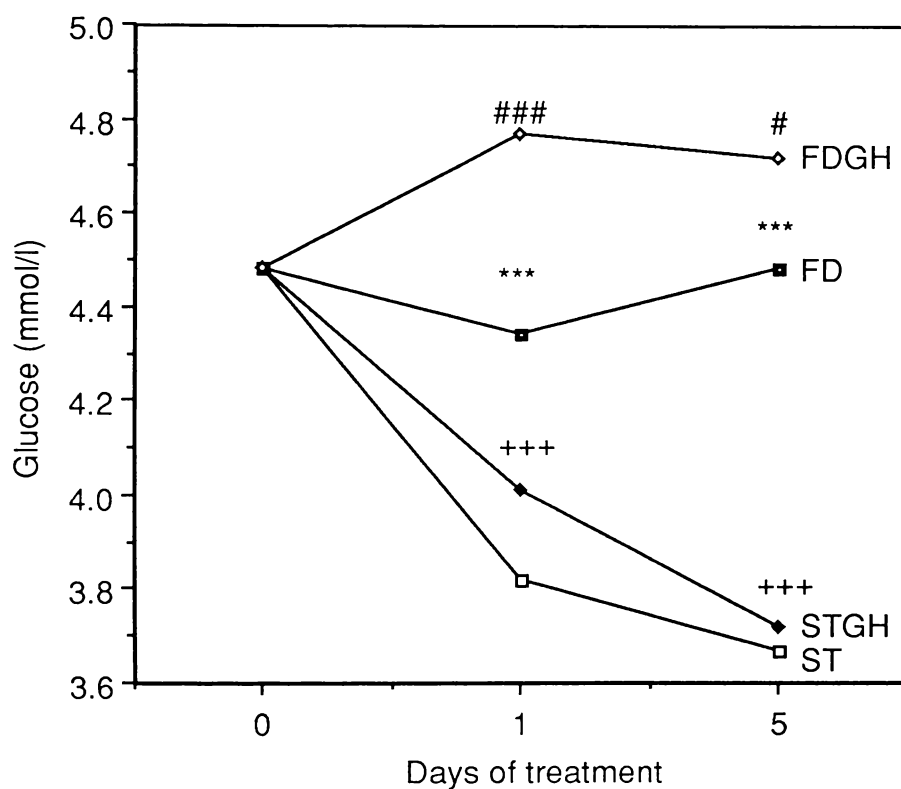


FIGURE 3.1 Effect of nutritional manipulation and GH treatment (hGH; 0.15 mg/kg bodyweight per day) on plasma levels of glucose (mmol/l) in 12-month-old ewes during a period of five-day-treatment. Four different treatment groups were as follows:

Fed (FD): on lucerne pellets at 3% of bodyweight per day and saline injected daily for five days

Fasted (ST): fasted and saline injected daily for five days

Fed + hGH (FDGH): on lucerne pellets at 3% of bodyweight per day and hGH injected daily for five days.

Fasted + hGH (STGH): fasted and hGH injected daily for five days.

$P < 0.05$ compared with FD; ### $P < 0.001$ compared with FD

*** $P < 0.001$ compared with ST; +++ $P < 0.001$ compared with FDGH for the significance of treatment as indicated by ANOVA.

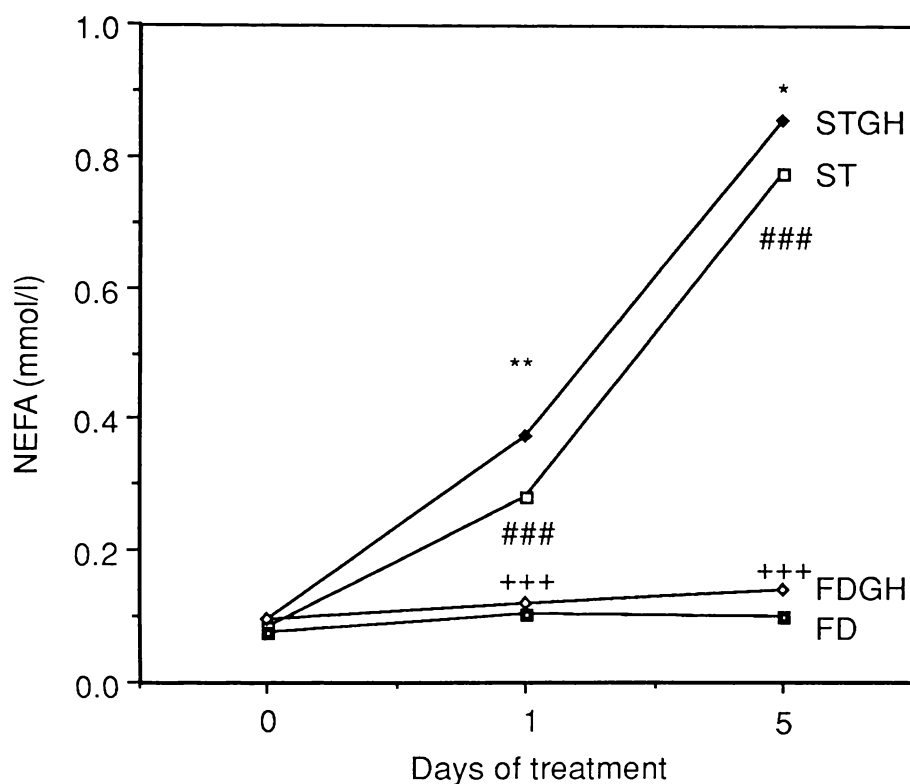


FIGURE 3.2 Effect of nutritional manipulation and GH treatment (hGH; 0.15 mg/kg bodyweight per day) on plasma levels of NEFA (mmol/l) in 12-month-old ewes during a period of five-day-treatment. Four different treatment groups were as follows:

Fed (FD): on lucerne pellets at 3% of bodyweight per day and saline injected daily for five days.

Fasted (ST):- fasted and saline injected daily for five days.

Fed + hGH (FDGH): on lucerne pellets at 3% of bodyweight per day and hGH injected daily for five days.

Fasted + hGH (STGH): fasted and hGH injected injected daily for five days.

* $P < 0.05$, ** $P < 0.01$ compared with ST; ** $P < 0.01$ compared with ST

$P < 0.001$ compared with FD; +++ $P < 0.001$ compared with STGH for the significance of treatment as indicated by ANOVA .

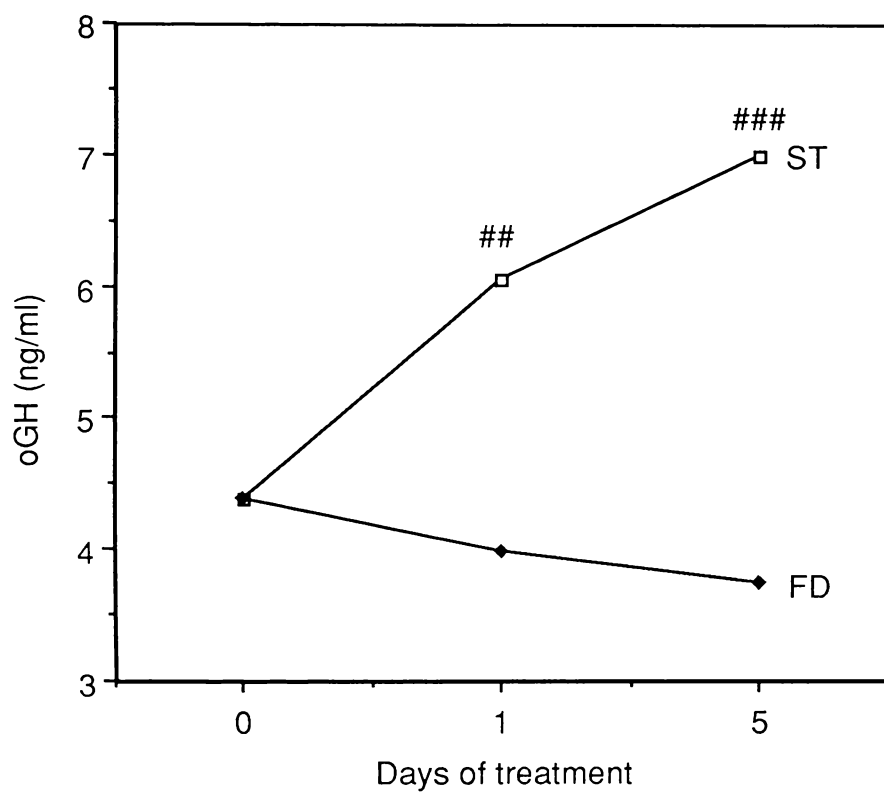


FIGURE 3.3 Effect of five-day-fasting on plasma levels of ovine GH (ng/ml) in 12-month-old ewes ($n=5$, per group). The different treatment groups were as follows:
Fed (FD): on lucerne pellets at 3% of bodyweight per day and saline injected daily for five days.
Fasted (ST): fasted and saline injected daily for five days

^{##} $P<0.01$ compared with FD; ^{###} $P<0.001$ compared with FD for the significance of treatment as indicated by ANOVA

Plasma and tissue IGF-I concentrations

Plasma IGF-I concentrations were higher ($P < 0.05$) in FD group than in ST group after five days of treatment. GH administration significantly increased IGF-I concentrations in plasma, but only in FD, not in ST group (Fig. 3.4).

Kidney IGF-I was significantly higher in FD compared with ST group ($P < 0.001$). IGF-I concentrations in muscle biceps femoris and liver were also higher in FD than in ST groups, although the differences were not statistically significant.

Tissue IGF-I was corrected for retained blood IGF-I using tissue and blood Hb levels (for methodology refer to Section 3.2). Data used for correction of tissue IGF-I are shown in Table 3.1.

No significant difference in IGF-I concentrations between GH-treated and untreated lambs was found in liver, kidney and muscle biceps femoris at either level of nutrition. IGF-I concentrations in plasma, liver, kidney and muscle biceps femoris after 5 days of treatment are shown in Table 3.2.

There were significant differences in IGF-I tissue concentrations between kidney and liver ($P < 0.001$) and kidney and muscle ($P < 0.001$) at a high level of nutrition, and between kidney and muscle ($P < 0.01$) at a low level of nutrition. No significant difference was found between kidney and liver at a low level of nutrition (Table 3.3).

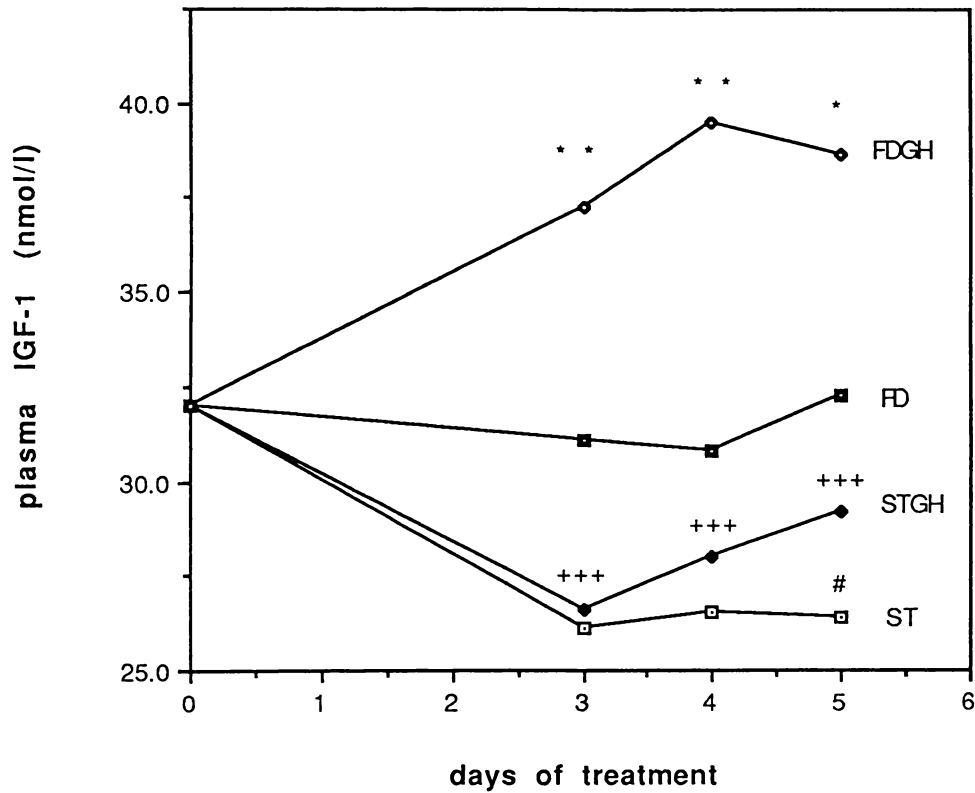


FIGURE 3.4 Effect of nutritional manipulation and GH treatment (hGH; 0.15 mg/kg body weight per day) on plasma levels of IGF-I in 12-month-old ewes during a period of five-day-treatment. Four different treatment groups were as follows:

Fed (FD): on lucerne pellets at 3% of bodyweight per day and saline injected daily for five days.

Fasted (ST): fasted and saline injected daily for five days.

Fed + hGH (FDGH): on lucerne pellets at 3% of bodyweight per day and hGH injected daily for five days.

Fasted + hGH (STGH): fasted and hGH injected daily for five days.

* $P < 0.05$, ** $P < 0.01$ compared with FD; # $P < 0.05$ compared with FD

+++ $P < 0.001$ compared with FDGH for the significance of treatment as indicated by ANOVA

TABLE 3.1 Contamination of tissues (Haemoglobin assay)

Tissue	Total IGF-I in plasma (ng/ml) or tissue (ng/g tissue)	Tissue blood content (ml/g tissue)	Contribution to total tissue IGF-I (ng/g tissue)	(%)
Plasma	246.8	-	-	-
Liver	142.4	0.0468	11.6	8.1
Kidney	213.4	0.0462	11.4	5.3
Muscle	129.8	0.0283	7.0	5.4
Spleen	130.1	0.0289	6.4	4.9
Heart	168.1	0.0277	6.8	4.0

Sheep tissue samples were collected from five healthy 12-month-old ewes. They were housed indoors and penned individually on a diet of lucerne pellets and water available ad libitum for a period of twenty-six days, then killed and tissue samples collected immediately after slaughter. Hb content in plasma and tissue extracts was estimated using the method outlined in section 2.2. Tissue and plasma IGF-I were measured by RIA (section 2.3).

TABLE 3.2 Effect of nutritional manipulation and GH treatment (hGH; 0.15 mg/kg per day) on plasma IGF-I (nmol/l) and tissue IGF-I (nmol/kg) concentrations in sheep after 5 days of treatment.

	IGF-I concentration (mean)				Significance			
	ST	STGH	FD	FDGH	SED	ST/STGH	FD/FDGH	ST/FD
Plasma	26.38	29.21	32.27	38.67	2.54	NS	*	*
Liver	17.30	20.20	18.62	19.22	2.27	NS	NS	NS
Kidney	20.32	18.93	27.90	24.93	2.27	NS	NS	***
M. biceps femoris	15.00	15.00	16.97	17.47	2.27	NS	NS	NS

NS, Not significant; *, $P < 0.05$; ***, $P < 0.001$ for the significance of treatments as indicated by analysis of variance (ANOVA)

Values are means; n=5 per group.

TABLE 3.3 Differences in IGF-I concentration (nmol/kg) in sheep tissues between two different levels of nutrition after five days of treatment. Values are means; n=5 per group.

	IGF-I concentration (nmol/kg)			Significance			
	Liver	Kidney	M.biceps femoris	SED	Kidney/Liver	Kidney/Muscle	Liver/Muscle
ST	17.30	20.32	15.00	1.67	NS	**	NS
FD	18.62	27.90	16.97	1.66	***	***	NS

NS, Not significant; *, $P < 0.05$; **, $P < 0.05$; ***, $P < 0.001$ for the significance of treatments as indicated (ANOVA)

Liver IGF-I mRNA levels monitored by ISH

Since IGF-I levels in liver showed a non-significant increase following GH treatment, IGF-I mRNA levels in liver sections were measured using ISH to check the observation at the RNA level. The sections probed all showed significant hybridization to the antisense human IGF-I ³⁵S-RNA probe but not to the sense probe (Fig. 3.5), and a positive control section of foetal muscle had higher IGF-I RNA levels as expected (576). When the X-ray film images of liver sections were analysed, images (OD) from GH-treated animals (mean $\pm$ SEM; 0.240 ± 0.014 , $n=10$) were found to be significantly elevated ($p<0.02$) relative to untreated animals (0.186 ± 0.016 , $n=10$).

Correlations between liver IGF-I and IGF-I mRNA levels

Liver IGF-I RNA levels (OD) were found to be positively correlated with liver IGF-I concentrations (ng/g tissue) in FD ($r=0.88$; $P<0.05$) and FDGH ($r=0.93$; $P<0.02$) lambs. No similar correlation could be shown in ST and STGH animals (Fig. 3.6).

3.4 Discussion

Influence of nutrition and GH on plasma and tissue IGF-I levels has been tested on an animal model where large differences between treatment groups were achieved. The effects of GH administration and long-term starvation were chosen as ways of achieving maximum differences.

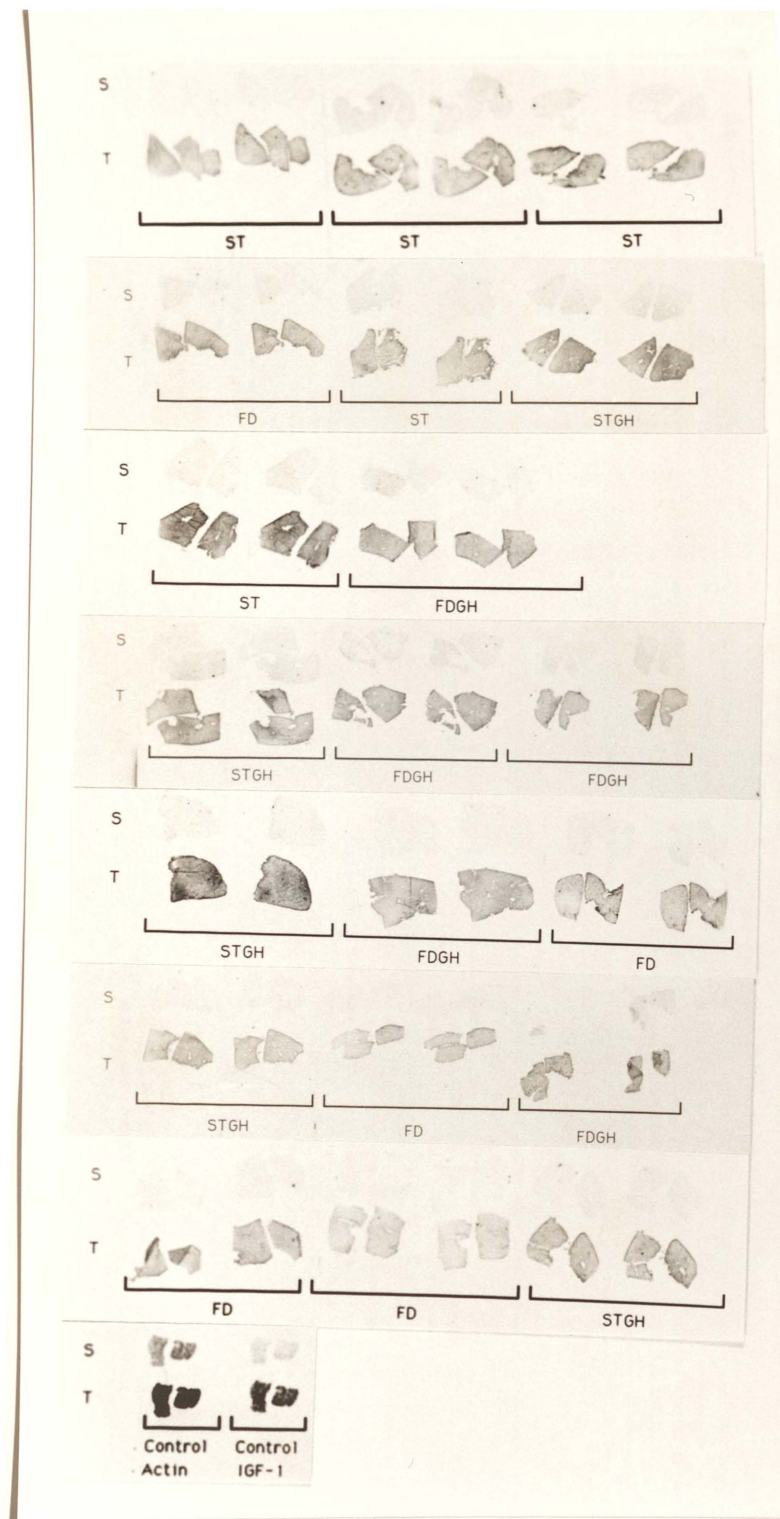


Figure 3.5 Autoradiography of ovine liver sections probed for IGF-1 RNA by in-situ hybridization in the four trial groups: Feeding (FD), Fasting (ST), Feeding and daily injection of GH (FDGH), Fasting and daily injection of GH as described in section 3.2. Two parallel sections were from 12-month-old ewes. IGF-1 RNA probes were transcribed from a pGm3 vector containing a 203-bp *Sau3A* fragment from exon E of a human IGF-1 cDNA. S indicates sense-strand probed sections while T indicates antisense-probed sections. Control sections are neonatal ovine muscle biceps femoris. Exposure time is 2.5 days. Images on the developed X-ray film were digitized with a Hewlett Packard Scanjet scanner and quantitated on a Macintosh IIci computer using the application Image (NIH version 1.37). For ISH quantitation, mean image densities (greyscale 1-255) were converted to OD.

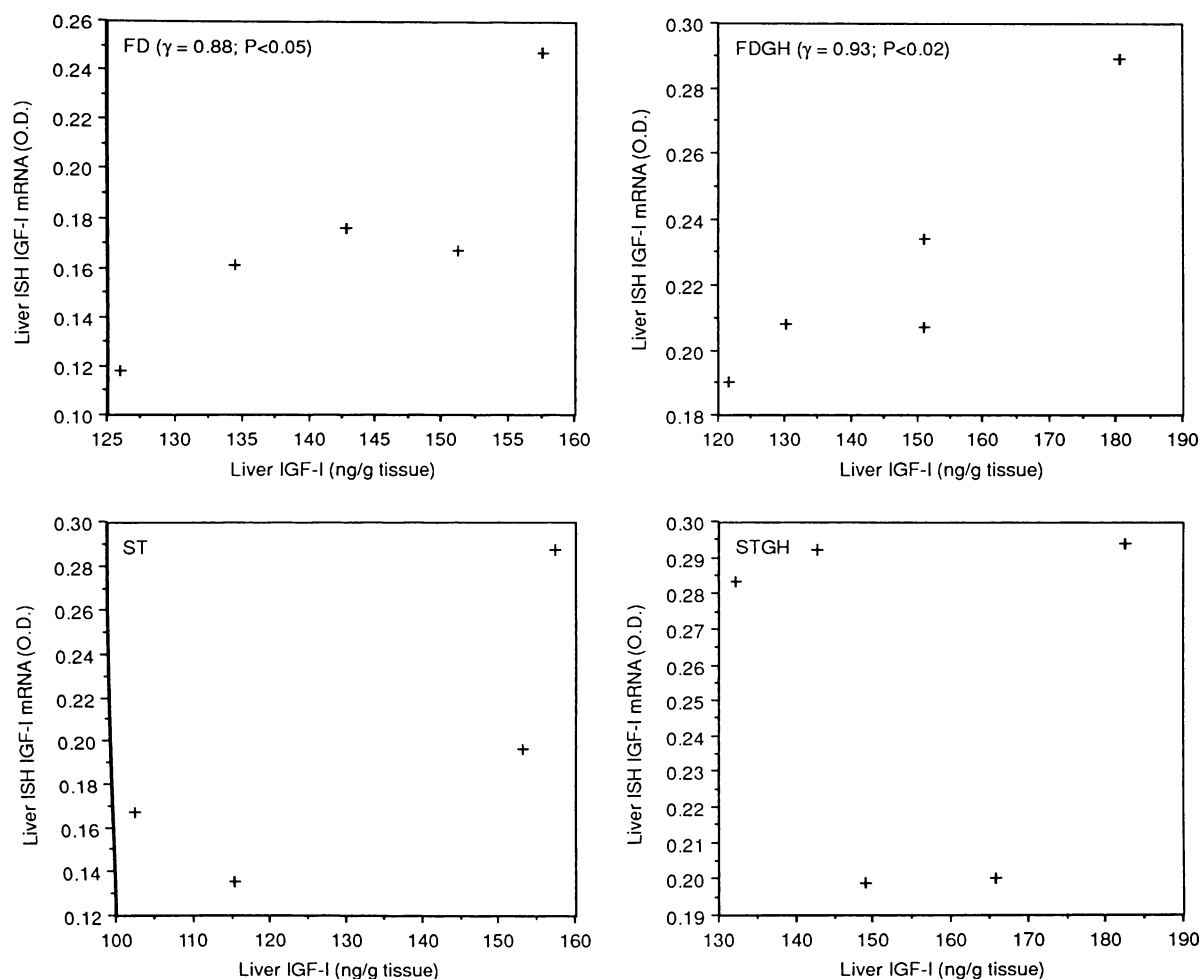


FIGURE 3.6 Correlation between IGF-I concentrations (ng/g liver) and the relative levels (O.D.) of IGF-I mRNA determined by ISH in 12-month-old ewes after five days of treatment. Four different treatment groups were as follows:

Fed (FD): on lucerne pellets at 3% of bodyweight per day and saline injected daily for five days

Fasted (ST): fasted and saline injected daily for five days

Fed + hGH (FDGH): on lucerne pellets at 3% of bodyweight per day and hGH injected daily for five days.

Fasted + hGH (STGH): fasted and hGH injected daily for five days.

The correlation analysis was done using the Macintosh Statview (1987; Abacus Concepts, Inc., Berkeley, CA, USA).

Nutritional status

Plasma concentrations of glucose and NEFA were used as indicators to monitor nutritional status in the animal model for this trial. Starvation caused dramatic decreases ($P < 0.001$) in plasma concentrations of glucose twenty-four hours after the treatment, and the differences in glucose concentrations between FD and ST groups remained highly significant ($P < 0.001$) in the following four days of the experimental period, which provided an extreme in nutritional status between the treatment groups as was expected.

Administration of GH, on the contrary, increased plasma concentrations of glucose ($P < 0.001$) within twenty-four hours of the treatment and the glucose levels remained significantly high in the following four days of the experimental period, but this GH-induced elevation of glucose was only seen at high (FD) planes of nutrition in the present study, not at low (ST) planes of nutrition. Possibly this resulted from antagonism of insulin in the fasted animals. The differences in glucose concentrations between FDGH and STGH animals during the period of treatment indicated an interaction of nutrition and GH on plasma metabolites.

The profile of plasma NEFA contrasted that of glucose. Starvation dramatically elevated plasma levels of NEFA during the period of treatment. Administration of GH also increased NEFA levels in plasma, but only at low (ST), not at high (FD), planes of nutrition. Highly significant ($P < 0.001$) differences in NEFA between STGH and FDGH animals showed an interaction between nutrition and

GH. These observations about NEFA suggest that GH may act more strongly as an anti-lipogenic or lipolytic agent for mobilising stored fatty acids under condition of malnutrition or starvation than under normal conditions.

Effect of fasting on plasma levels of GH

The obvious effect of fasting was an elevation in plasma GH concentrations in this study within twenty-four hours of withdrawing pellets. This became more pronounced by the final day of treatment (Fig. 3.4). Although some studies have shown no effect of fasting on plasma GH levels (32, 446-449), in which single or limited and infrequent samples were taken, accumulating observations have suggested that nutritional status plays a critical role in determining circulating GH concentrations, particularly in the ruminant.

During periods of nutritional deficit, elevated concentrations of circulating GH have been reported for several species including pigs (458), human (444, 445), sheep (450, 451, 456), domestic fowl (452), and lactating cows (453, 454).

Driver and Forbes (450) used a frequent blood sampling regime to determine GH profile, similar to the current experiment, and demonstrated episodic release of GH with peaks preceding and troughs in conjunction with food ingestion. The depressed GH concentrations associated with feeding have been suggested to occur as a result of reduced pituitary sensitivity to stimulation by GRH (455). Most animals showed no synchrony of release, but with fasting an increase in peak and trough levels was evident within a few hours (450). This corroborates

data presented from the current experiment where an increase in GH was detected on the first day of treatment. Driver and Forbes suggested that mean plasma concentrations may not be a valid measurement of GH, but on the basis of data in the current experiment, means appear to be acceptable in situations where sampling frequency is high and the experimental treatment is prolonged.

The Driver and Forbes study imposed a ten hour fast, while fasting in this experiment continued for 120 hours. Other studies have looked at periods of 48 or 72 hours fasting. Looking at all the studies, there was no evidence of a differential effect of short term or long term fasting on GH concentrations, in all cases GH levels increased after fasting.

In sheep the GH response to GRF is enhanced during restricted feeding compared with *ad libitum* feeding (456). This suggests an explanation for the enhanced GH secretion during diminished nutrition; reduced intake may lead to reduced sensitivity of GH receptors both peripherally and perhaps centrally and thus diminish negative feedback within the somatotrophic axis mediated in part via SRIF. The increase in GH concentrations at the lower planes of nutrition has been postulated to be an adaptation for mobilization of energy from adipose tissue to maintain basal metabolism (457, 459-461). Possible explanations are that it is due either to altered feedback through IGFs on GH secretion (462) or to altered metabolic clearance of GH (447).

It has been pointed out in a previous review (577) that the understanding and

interpretation of circulating hormone concentrations has to be seen in light of hormone metabolism. The situation for GH metabolism in the ruminant has been elucidated in part in a study by Trenkle (447). He reported an increase in half-life of injected GH from 20.3 to 31.9 min in fasted calves. In addition, the turnover and the metabolic clearance rate of GH were reduced significantly by starvation. This indicates that the high circulating GH concentrations observed in the underfed ruminant are not utilized fully and points to a partial refractoriness of target tissue, thus supporting the concept of malnutrition-induced GH resistance (465, 466). Malnutrition-induced resistance to GH is due at least in part to changes in GH receptor number (463), but post-receptor mechanisms may also be involved (464).

Nutritional and Hormonal regulation of IGF-I in plasma and tissues

In this study plasma IGF-I concentrations were decreased by starvation ($P < 0.05$), and were raised by GH ($P < 0.05$) only in fed animals. This is in agreement with previous reports on sheep (55, 355) and cattle (467, 468).

Plasma concentrations of IGF-I responded more slowly to fasting than did concentrations of GH (Fig.3.3). IGF-I concentrations were significantly lower by the last day of treatment (Fig. 3.4) although the divergence between treatment groups had begun earlier. Others working with fasted sheep have reported declining IGF-I concentrations by two days (469) and three days (449) of treatment. Unlike the report of an increased half-life of plasma GH with fasting (447), evidence suggests that there is no change in the half-life of plasma IGF-I

(469). This may be associated with the formation of stable IGF-plasma binding protein complexes in plasma, despite variations in the absolute levels of assayable IGF-I. There was no evidence of a treatment effect until the last day. Two other studies (449, 469) reported reductions of plasma IGF-I in the range of 35 to 45% compared with 18% in the current experiment. Freemark *et al.* (449) worked with pregnant ewes while Hodgkinson *et al.* (469) worked with wethers approximately three months younger than those in this experiment.

Using the Hodgkinson study for comparison, a sex difference in IGF-I sensitivity between pre-pubertal females and castrated males is unlikely. There may be an effect of age on sensitivity for both the onset and extent of IGF-I reduction with fasting (470). In a similar study with fasted rats, Thissen *et al.* (471) suggested less depression in IGF-I with advancing age, and the initiation of reduced IGF-I secretion was slower in mice which were almost fully grown than in younger mice (472). GH receptors may be important in the nutritional regulation of IGF-I secretion. GH binding to liver membrane preparations from sheep, while being regulated by plasma GH, is influenced to a greater extent by nutritional status of the animal (55). Under severe nutritional constraint such as fasting, the two endocrine responses may be independent and quite dissociated from their growth-promoting actions and interactions in well nourished animals. It has already been suggested that elevated GH may increase nutrient availability to the animal. Likewise, reduced IGF-I may act to depress non-essential metabolic and growth processes during nutritional deficit (466). Merimee *et al.* (466) have suggested that the substrates present during fasting e.g. free fatty acids and

ketones, may down-regulate IGF-I secretion. It has also been postulated that reduced IGF-I in rodents may be permissive in allowing tissue catabolism during fasting (356) and that the action of somatomedin inhibitors may contribute to altered tissue processes (473).

In addition to plasma IGF-I, in the current study, five days of fasting caused a 7% decrease in liver IGF-I, 27% decrease in kidney IGF-I and 12% in muscle IGF-I, but the magnitude of the decrease in liver and muscle IGF-I did not reach significant level. The possible explanation for why significance was not picked up for the decrease in liver or muscle IGF-I induced by fasting may be that there was high variation in tissue IGF-I between relatively small number (N=5, per group) of the experimental animals. Alternately, the age of the ewes in the present study may also play a part in the reduced response of IGF-1 (472). Nevertheless, the significant changes in plasma and tissue (kidney) IGF-I concentrations between ST and FD animals confirm the sensitivity of the GH-IGF-I axis to changes in nutrition (355, 474, 42).

The higher IGF-I concentration in kidney compared with liver and muscle in normal ewe lambs is consistent with the findings in normal male (338) and female (130) rats. The greatest IGF-I response to ovine GH from hypophysectomized rats also occurred in kidney (338). It seems that kidney IGF-I levels are sensitive to both GH and nutritional stimuli. This may be partly related to the kidney's excretory and metabolic function (338).

The liver has been considered to be the most important site of IGF-I synthesis, contributing about 55% of circulating IGF-I in rats (338). Although IGF-I concentration in liver in the present study did not show significant changes in response to GH administration, liver IGF-I RNA levels were significantly elevated in GH-treated lambs and positively correlated with IGF-I tissue levels on an individual animal basis. The elevated levels of liver IGF-I RNA in GH-treated lambs indicated that GH treatment increased expression of IGF-I RNA in sheep liver, and the positive correlation between liver IGF-I RNA and liver IGF-I, particularly in FDGH lambs, suggests that GH regulation occurred not only at the IGF-I RNA level but also at the level of tissue IGF-I.

The first step, in GH action, is the binding of the hormone to a specific cell receptor. The high affinity hepatic GH receptor, which may mediate the GH control of IGF-I, is particularly sensitive to nutritional manipulation in ruminants (54). The capacity of the high affinity GH receptor decreases with malnutrition and correlates positively with growth rate and plasma IGF-I (54). It is also known that IGF-I production is restricted by the amino acid and caloric supply (475). The dramatic difference in the correlations between liver IGF-I RNA and IGF-I levels from FDGH ($r=0.93$) and STGH ($r=0.07$) animals in this study indicates that the GH regulation of liver IGF-I is, to a large extent, dependent on nutritional status. So nutrition plays a critical role in the regulation of IGF-I production in sheep liver.

The IGF-I content of tissue extracts has previously been shown (338) to be

strongly GH-dependent. Intraperitoneal administration of ovine GH to hypophysectomized rats caused tissue-extractable IGF-I increase in kidney, liver, lung and heart and in each of those tissues the peak response occurred 12 h after ovine GH administration (338). In contrast, tissue IGF-I concentrations in lambs in the present study showed no significant increase with GH administered 24 h previously at either level of nutrition, although plasma levels of IGF-I were raised. It could be explained that circulating IGF-I is retained predominantly in a large complex of about 150 kDa, which is composed of an acid-labile non-IGF binding subunit (85 kDa), IGF binding protein-3 (IGFBP-3, 53 kDa) and IGF (476). The 150 kDa complex, which is not readily available to the tissues, carries most of the endogenous circulating IGF. The half-life of the 150 kDa complex in sheep has been shown to be approximately 550 min (469). Thus plasma responses of IGF-I may be more protracted than the short-term tissue responses where low molecular weight BPs predominant. Alternately, hypophysectomized rats with a lower basal level of tissue IGF-I might be expected to respond more strongly.

The lack of response to GH in tissues in this study may be associated with the time interval between GH treatment and tissue sampling, the dose of GH administered, or having insufficient numbers of animals per group, causing the variation in tissue IGF-I levels between animals. However, the aforementioned positive correlations between liver IGF-I and liver IGF-I RNA, together with the elevated tissue levels of IGF-I RNA in GH-treated animals, also suggest that tissue IGF-I levels are regulated, to some extent, differently from plasma levels.

3.5 Summary

The results in this study indicate that plasma and tissue IGF-I is regulated by nutrition and GH, with nutrition playing a critical role in the regulation of tissue and plasma IGF-I in normal lambs.

CHAPTER FOUR : GH STIMULATION OF TISSUE IGF-1 PRODUCTION IN POST-NATAL SHEEP

4.1 Introduction

For a long time it was believed that the IGF-I reaching peripheral tissues was ultimately of hepatic origin and arrived at its sites of action via the blood stream. The original somatomedin hypothesis proposed that GH, from the pituitary, exerts its effects by stimulating IGF-I release from the liver which then mediates the somatogenic actions in the target tissues (72, 478, 479, 480). Since then, the original somatomedin hypothesis of GH action has been extensively revised. Accumulating observations have suggested that IGFs are synthesized locally in many different tissues (338, 437, 130), and it has been postulated that the IGFs produced locally may be of greater physiological importance than those arriving from distant sources. The local production of IGFs is now well documented and has been confirmed with probes for the respective mRNAs (273, 438, 132). IGF-I mRNA levels have been shown to be regulated by GH in a number of tissues (481, 134, 262, 482). All this research work was conducted after an early important observation of multiple sites of IGF-I synthesis in rats, which was furnished by D'Ercole *et al.* in 1984 (338), who measured the somatomedin content of extracts from multiple tissues in normal and in hypophysectomized rats before and after the administration of growth

hormone. After correcting for the portion contributed by residual blood, the content of IGF-I in the tissues of hypophysectomized rats was strikingly lower than that in tissues of normal animals except in the brain. Furthermore, following the administration of growth hormone, the levels of IGF-I in tissue extracts rose many hours earlier than did the levels in blood. This study provided strong confirmation that IGF-I is formed by many tissues and not only by liver. This conclusion has been supported by the presence of mRNA's for the somatomedins in many peripheral tissues (273, 438, 132).

Nevertheless, multiple sites of tissue IGF-I production have not yet been investigated in intact farm animal species, which may reveal *in vivo* evidence for the mechanism by which GH regulates post-natal growth process in farm animals.

The present study was designed to examine IGF-I responsiveness to GH in liver as well as in other peripheral tissues, and to test relationships between tissue and plasma IGF-I response to GH administration in post-natal sheep.

To achieve the above objectives, in this experiment, the following items were investigated:

1. Circulating levels of GH during a 24 hr-period of experiment were

measured as an indicator of GH administration.

2. Circulating levels of glucose during a 24-hr-period of experiment were measured to monitor how they were affected by GH administration.
3. Circulating levels of IGF-I during a 24-hr-period of experiment were measured. The difference in levels of circulating IGF-I between GH-treated and non-treated animals was compared. The time course of circulating IGF-I responsiveness to GH was examined.
4. Tissue levels of IGF-I during the 24-hr-period of experiment were measured. The difference in levels of tissue IGF-I between GH-treated and non-treated animals was compared. The time course of tissue IGF-I responsiveness to GH was examined.
5. The relationship between tissue and plasma IGF-I response to GH treatment was examined.
6. Tissue IGF-I mRNA levels in liver, kidney and muscle biceps femoris were measured. The difference in tissue IGF-I mRNA levels between GH-treated and non-treated animals was examined.
7. Total levels of IGF-I in organ/muscle/plasma, and the distribution of the total levels of IGF-I in non-GH-treated animals were estimated.
8. Increase in total levels of IGF-I among organs in GH-treated animals were compared.

The studies in this chapter were performed in 1991 at Growth Physiology, Ruakura Agricultural Research Centre, Hamilton, New Zealand, and have been published in *Journal of Endocrinology*, Vol. 136, 217-224 (1993).

4.2 Materials and Methods

Materials

Lyophilised GH (recombinantly derived bovine GH, BST) was reconstituted as a stock solution of 10 mg/ml with saline. It was refrigerated at 4°C and used over a period of 7-10 days. The BST was kindly donated by American Cyanamid Company (Princeton, New Jersey).

Feed pellets were as described in Section 3.2. IGF-I cDNA probes for ISH to kidney, liver and muscle section were prepared as described in Section 2.8.

Animals and Treatment

Eighty-eight Dorset x Coopworth ewe lambs, 6 to 8 months of age were held in individual pens for a period of 3 weeks and fed a pelleted high protein (15-17%) diet *ad libitum* before the experiment. Weekly weights and daily feed intakes were recorded during this period.

The lambs were distributed into 11 groups which were balanced on body weight.

Seven groups were injected subcutaneously with a single dose of bovine

growth hormone (bGH), 0.25 mg/kg body weight, and serially killed at 3, 5, 7, 10, 13, 17 and 24 hours post injection. Control groups which had been injected with equivalent volumes of saline, were killed at 0, 5, 13 and 24 hours after injection.

Injection times were spread over the 24 hours prior to slaughter and adjusted so that all groups of animals could be killed within a period of 4 hours.

Blood sampling and tissue processing

Blood samples (10 ml, in EDTA) were collected as described in section 3.2 from all sheep prior to injection and also immediately prior to slaughter. Plasma was separated as soon as possible at 4°C after collection and stored at -20°C. Tissue samples of liver, kidney, spleen, heart and muscle biceps femoris were collected immediately after slaughter. They were processed as described in section 3.2.

Tissue Extraction

Tissues were extracted for determination of IGF-I and Hb using the procedure described in Section 2.5.

Assays

Plasma concentrations of GH

Plasma concentrations of GH were measured by RIA as described in detail in Section 2.7.

Plasma samples for measurement of GH were collected from five healthy ewe lambs (about six months of age, Dorset x Coopworth) at 0, 5, 7, 10, 13, 17 and 24 hr after one single dose injection of bGH (0.25 mg/kg body weight).

In-situ hybridisation (ISH) for monitoring tissue IGF-1 mRNA

In-situ hybridization was performed as detailed in Section 2.8 with the following changes to the quantitation procedure. Developed X-ray film images were scanned on a Molecular Dynamics (Sunneyvale, CA, USA) Personal Densitometer using the application ImageQuant. The digitised images were quantitated on a Macintosh IIfx computer using the application Image (NIH version 1.37). The mean image density from each S (control) section was subtracted from the corresponding value from the T (experimental) section to yield the net greyscale value.

IGF-1 RIA

Tissue and plasma levels of IGF-1 were measured using an 'in house' RIA (methodology was detailed in Section 2.3) after the tissue and plasma were extracted by the AEC technique (see Section 2.5). Tissue

IGF-1 in liver, kidney, spleen, heart and muscle biceps femoris was corrected for retained plasma IGF-1 using tissue and blood Hb analysis (as described in Section 3.2).

Calculations of tissue IGF-1 concentrations and total content of IGF-1 in organ, muscle and plasma.

Tissue IGF-1 concentrations were calculated as described in Section 3.2.

The total organ/muscle IGF-1 content (μg) was calculated as follows:
 $(\text{tissue IGF-1 concentrations ng/g}) \times (\text{total organ/muscle weight g}) / 1000$
 where the total organ weight measured in this trial (g) (mean $\pm$ SD, n = 32) was 608 ± 30 for liver, 103 ± 7 for kidney, 49 ± 6 for spleen and 151 ± 7 for heart. The total muscle weight was estimated as one third of sheep body weight (484). The average body weight of sheep in the trial was 28.8 ± 3.5 kg (mean $\pm$ SD, n=88). The IGF-1 concentration of muscle biceps femoris in control animals in this study has been used as an indicator of total skeletal muscle IGF-1 to facilitate calculations of total organ/muscle/plasma IGF-I distribution (%) in non-GH-treated lambs.

The increase in total organ IGF-1 (μg) was calculated as:
 $(\text{increase in tissue IGF-I concentration ng/g}) \times (\text{total organ weight g}) / 1000.$

The total circulating IGF-1 content (μg) in blood was calculated as:
(Plasma IGF-I concentration ng/ml) x (total volume of sheep blood ml)/1000.

The total blood volume was taken as 8% of sheep body weight (485).

Biometrics

Correlations between tissue and plasma IGF-1 were calculated using MINITAB (1989, Minitab Inc. PA16801, USA). Other data were analysed by analysis of variance (ANOVA) using Genstat 5 (Numerical Algorithms Group Ltd, Oxford, Oxon, UK).

4.3 Results

Circulating levels of GH: 24 h-profile of plasma GH

Circulating levels of GH during the 24 h-period of the experiment were measured as an indicator of administration of bGH.

GH concentrations measured in plasma rose significantly compared with the basal level (0 hour) and reached a peak in 5 hours post injection, then decreased gradually until returning to the basal level at 24 hours (Figure 4.1).

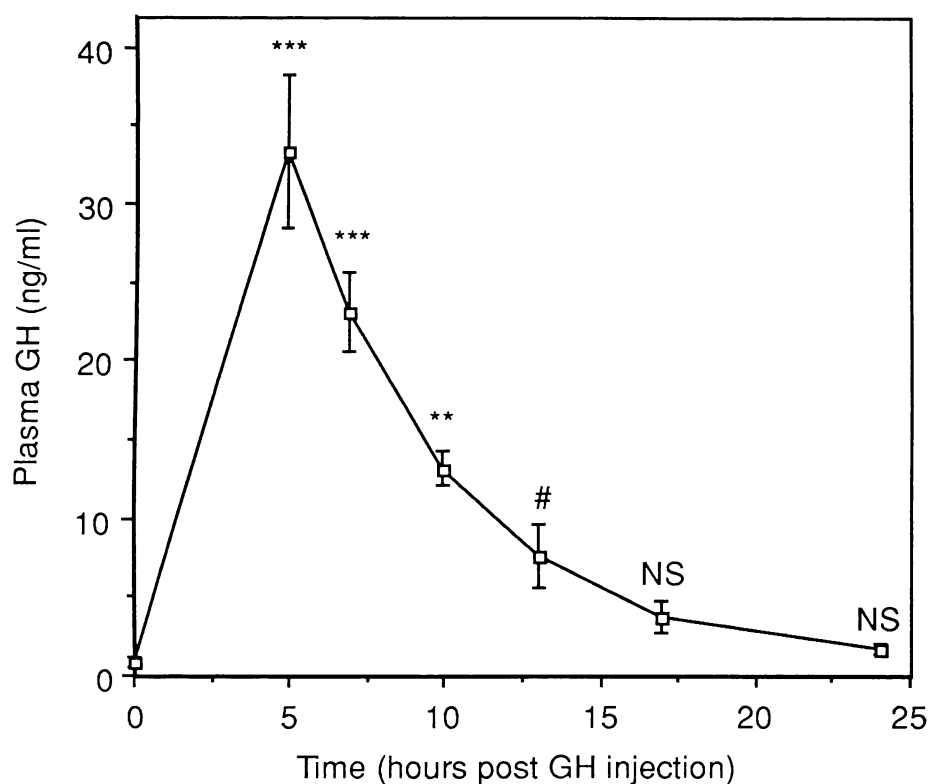


FIGURE 4.1 Twenty-four-hour-profile of circulating levels of growth hormone (GH). Plasma concentrations of GH were measured by RIA during the 24-hr-period of experiment as an indicator of administration of bGH (one single dose; 0.25 mg/kg bodyweight). Plasma samples for measurement of GH were collected from six-month-old Dorset x Coopworth ewes ($n=5$) at 0, 5, 7, 10, 13, 17 and 24 hr after GH injection. Significance of difference in GH concentrations was analysed by analysis of variance (ANOVA) using the Macintosh Statview (1987; Abacus Concepts, Inc., Berkeley, CA, USA).

$P<0.1$, ** $P<0.01$, *** $P<0.001$, NS not significant as compared with the basal level (0 hour).

Circulating levels of glucose; 24 h-profile of plasma glucose

Circulating levels of glucose during the 24 h-period of the experiment were measured as an indicator of the effect of GH administration on this metabolites in blood. Plasma concentrations of glucose rose significantly compared with basal level at 5 hours post GH injection and reached a peak at 10 hr, then decreased to the basal level at 24 hours (Figure 4.2).

Circulating levels of IGF-1: 24 h-profile of plasma IGF-1 response to GH

Plasma concentrations of IGF-1 were significantly elevated by 10 hours post GH injection compared with the control, and continued to rise to a maximum at 17 hours then declined at 24 hours. In contrast, plasma concentrations of IGF-I in control animals showed non-significant changes in the 24 h-period of the experiment (Figure 4.3).

Tissue levels of IGF-I: 24 h - profile of tissue IGF-1 response to GH

Tissue concentrations of IGF-1 were significantly increased compared with the control by 5 hours in liver and kidney, and by 7 hours in heart and spleen. They remained elevated until 13 hours after GH injection (Figure 4.4). By contrast, the muscle IGF-1 response to GH was

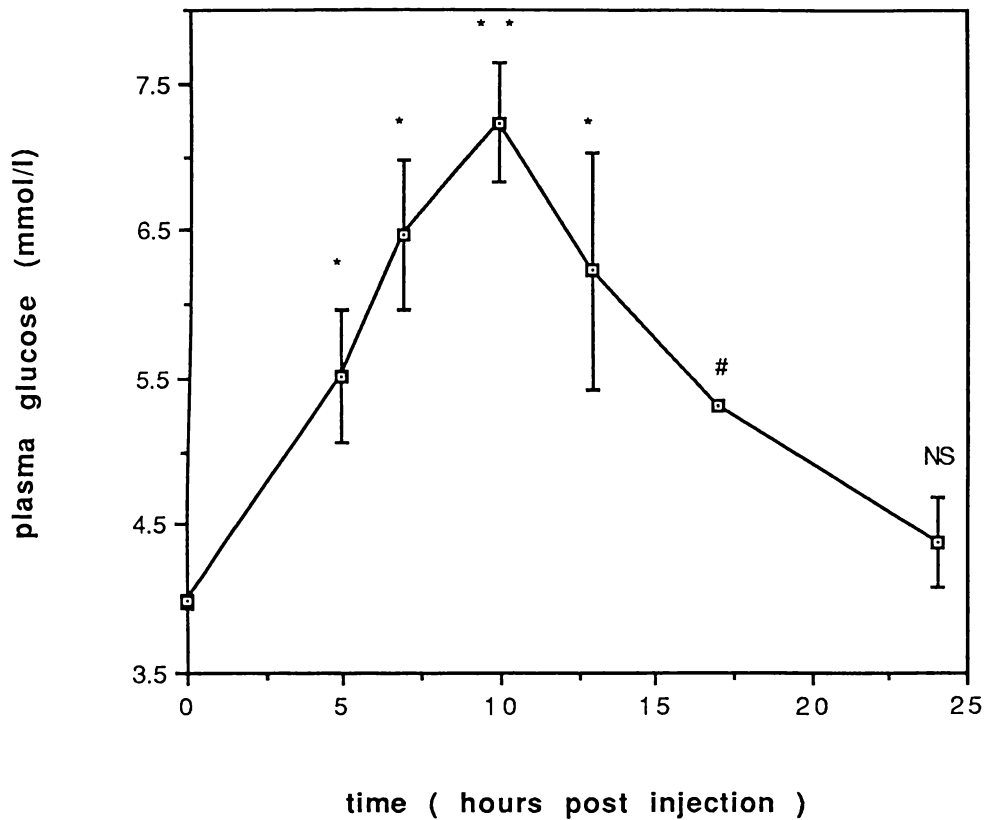


FIGURE 4.2 Twenty-four-hour-profile of circulating concentrations of glucose (mean $\pm$ SEM, n=3). Plasma concentrations of glucose were measured using an automated glucose oxidase method (for methodology refer to section 2.1) during the 24-h treatment period. Plasma samples were collected from six-month-old Dorset x Coopworth ewes (n=3) at 0, 5, 7, 10, 13, 17 and 24 h after GH injection. Significance of difference in glucose concentrations was analysed by analysis of variance (ANOVA) using the Macintosh Statview (1987; Abacus Concepts, Inc., Berkeley, CA, USA).

P<0.1, * P<0.05, ** P<0.01, NS not significant as compared with the basal level (0 hour).

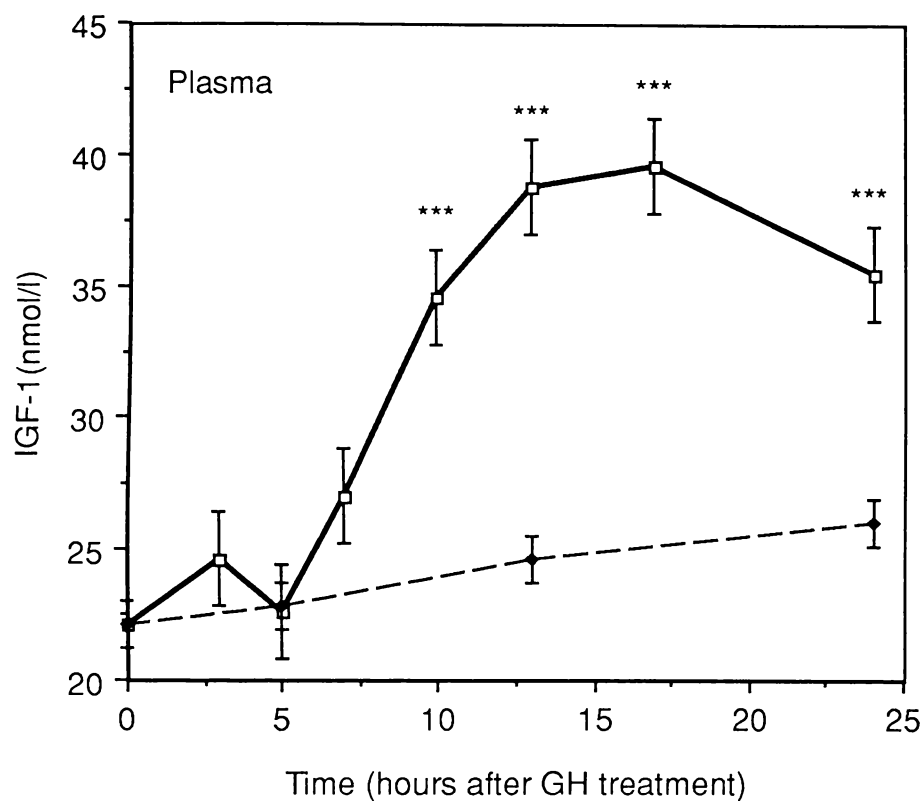


FIGURE 4.3 Plasma IGF-I (mean±SEM, n=8) response to bovine growth hormone (bGH) administration in six-month-old ewes. Seven groups of eight ewes were injected subcutaneously with bGH (—□—) (0.25 mg/kg bodyweight), another four control groups (—●—) were injected with equivalent volumes of saline and sacrificed at the time indicated. Blood samples (10 ml in EDTA) were collected from all ewes prior to injection and also immediately prior to slaughter. IGF-I concentrations (nmol/l) in plasma were measured by RIA. Significance of difference in IGF-I concentrations between GH-treated and non-treated animals was analysed by analysis of variance (ANOVA) using Genstat 5 (Numerical Algorithms Group Ltd, Oxford, Oxon, UK).

*** P<0.001 for the significance as indicated by ANOVA.

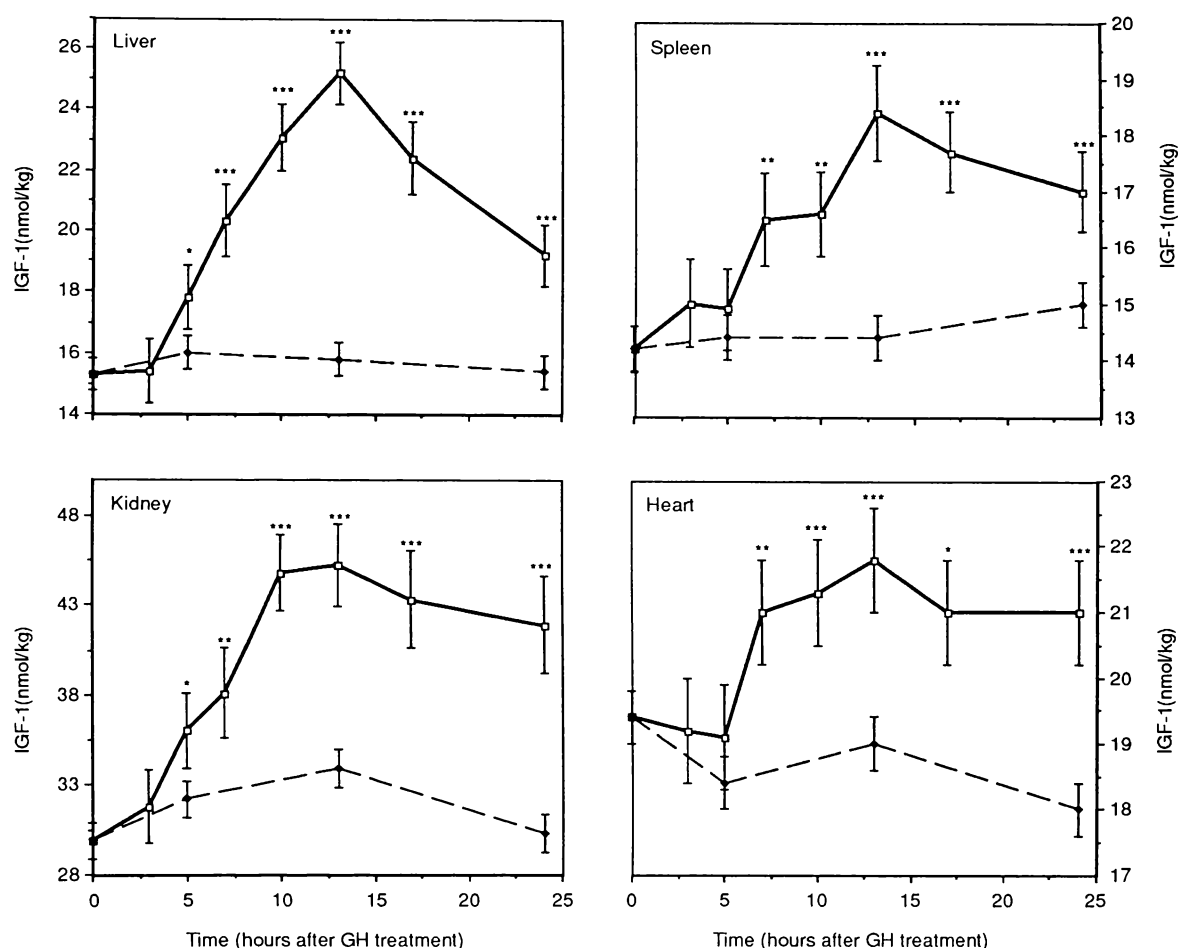


FIGURE 4.4 Tissue IGF-I response to bovine growth hormone (bGH) administration in six-month-old ewes. Seven groups of eight ewes were injected subcutaneously with bGH (—□—) (0.25 mg/kg bodyweight), another four control groups (—◆—) were injected with equivalent volumes of saline and sacrificed at the time indicated. Tissue samples were collected immediately after slaughter. They were cut into small blocks for freezing in liquid nitrogen, and stored at -70°C until IGF-I extraction. Tissue IGF-I concentrations in tissue extracts were measured by RIA after acid-ethanol-cryoprecipitation extraction. Significance of difference in IGF-I concentrations between GH-treated and non-treated animals was analysed by analysis of variance (ANOVA) using Genstat 5 (Numerical Algorithms Group Ltd, Oxford, Oxon, UK).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for the significance as indicated by ANOVA.

different from the above four tissues but similar to plasma. Muscle IGF-1 was elevated significantly by 10 hours post GH injection and reached a peak at 17 hours, then declined (Figure 4.5).

Concentrations of IGF-1 were maximal by 13 hours in liver, kidney, heart, spleen and by 17 hours in plasma and muscle. The rise in IGF-1 concentrations in liver, kidney, heart and spleen, in response to bGH, preceded the plasma IGF-1 response. Similarly the fall in IGF-1 concentrations was earlier in liver than in plasma. A decrease in IGF-1 concentrations could also be seen in kidney, spleen and heart after 13 hours, though the decrement in these tissues was not statistically significant.

IGF-1 concentrations in liver, kidney, spleen, heart and muscle in control animals did not show any significant fluctuation during the 24 h-period of the experiment.

Correlations between tissue and plasma IGF-1 concentrations

The positive correlations found between tissue IGF-1 and plasma IGF-I in GH-treated animals were as follows: liver ($r = 0.85$; $P < 0.007$), kidney ($r = 0.91$; $P < 0.002$), heart ($r = 0.87$; $P < 0.005$), spleen ($r = 0.94$; $P < 0.001$) and muscle ($r = 0.94$; $P < 0.001$).

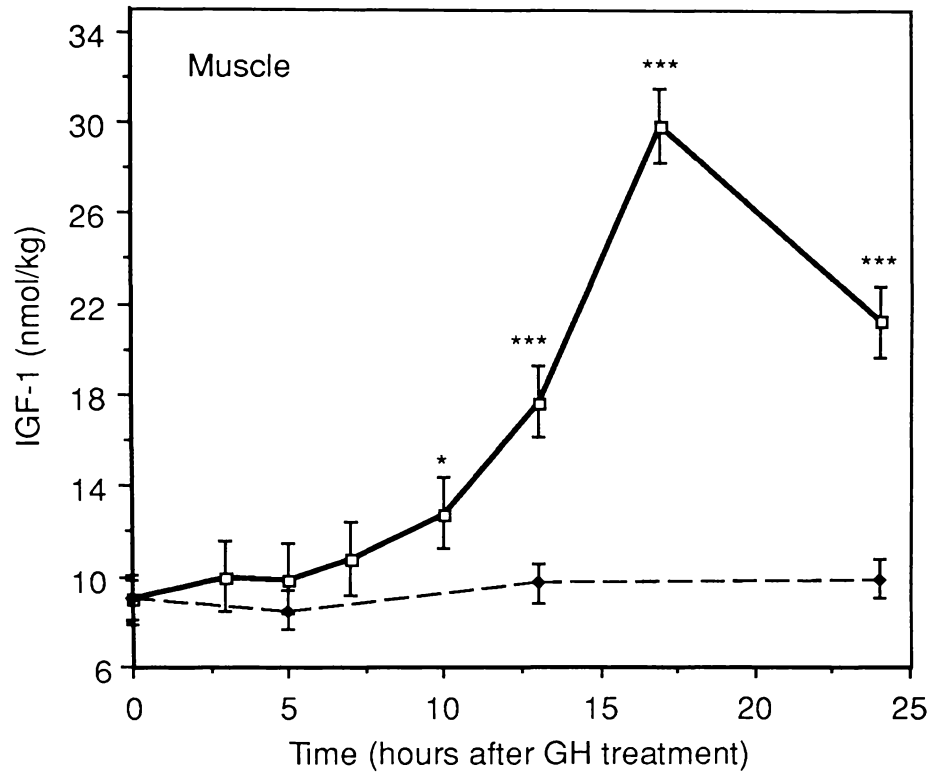


FIGURE 4.5 Muscle IGF-I response to bovine growth hormone (bGH) administration in six-month-old ewes. Animal treatment, tissue sample collection, IGF-I radioimmunoassay and biometrics were the same as indicated in Figure 4.4.

* $P < 0.05$; *** $P < 0.001$; *** $P < 0.001$ for the significance of difference between GH-treated (—□—) and non-treated animals (—♦—) as indicated by analysis of variance (ANOVA).

Basal levels and maximal increase in tissue and plasma IGF-1 concentrations in response to GH

The basal level of IGF-1 concentrations (in the control lambs) varied between tissues. The peak level of the increase in IGF-I concentrations (as % of control) in GH-treated lambs also varied between tissues. Kidney had the highest basal concentration of IGF-1, muscle biceps femoris had the lowest. However, the muscle had the highest increase in the peak concentration of IGF-1, followed by plasma and liver. Of the organs studied, liver had the highest increase in the peak concentration of IGF-I, and heart had the lowest (Table 4.1).

Basal levels of total IGF-1 in organ, muscle, plasma and the increase in total IGF-1 in organs after GH administration

Basal levels of total IGF-1 (μg) (mean $\pm$ SD, $n=32$) in organ, muscle, plasma and the (%) distribution amongst tissues in control animals were estimated as follows: 72.7 ± 2.0 for liver; 25.2 ± 0.8 for kidney; 5.4 ± 0.1 for spleen; 21.6 ± 0.4 for heart; 712.3 ± 62.4 for muscle; 420.7 ± 14.3 for plasma. Total muscle IGF-1 could account for about 56%; plasma for 34%; liver for 6% and other organs studied for 4% of total body IGF-I.

The increase in total organ content of IGF-1 (μg) (mean $\pm$ SD, $n=8$), 13 hours after GH administration, was 44.5 ± 2.2 in liver; 10.4 ± 0.7 in

TABLE 4.1 The basal levels of IGF-I concentrations (values are means $\pm$ SEM; n=32) in control and the peak concentrations of IGF-I (means $\pm$ SEM; n=8) in GH-treated sheep tissues after intravenous administration of bovine growth hormone: 0.25 mg/kg body weight. Tissue IGF-I concentrations are in nmol/kg (frozen tissue). Plasma IGF-I concentrations are in nmol/l.

Tissue	Control mean $\pm$ SEM n=32	Peak concentration mean $\pm$ SEM n=8	Significance compared with control	Increase in peak concentration (%) of control
Liver	15.6 $\pm$ 0.5	25.2 $\pm$ 1.0 (at 13 hr)	P<0.001	62
Kidney	32.0 $\pm$ 1.0	45.2 $\pm$ 2.3 (at 13 hr)	P<0.001	41
Spleen	14.5 $\pm$ 0.4	18.4 $\pm$ 0.8 (at 13 hr)	P<0.001	27
Heart	18.7 $\pm$ 0.4	21.8 $\pm$ 0.8 (at 13 hr)	P<0.001	17
Muscle	9.7 $\pm$ 0.8	29.9 $\pm$ 1.6 (at 17 hr)	P<0.001	208
Plasma	23.9 $\pm$ 0.9	39.6 $\pm$ 1.8 (at 17 hr)	P<0.001	66

IGF-I concentrations in tissue extracts or plasma were measured by radioimmunoassay after acid-ethanol-cryoprecipitation extraction. Significance of difference in IGF-I concentrations between GH-treated and non-treated animals was analysed by analysis of variance (ANOVA) using Genstat 5 (Numerical Algorithms Group Ltd, Oxford, Oxon, U.K.).

kidney; 1.5 ± 0.2 in spleen; and 3.5 ± 0.2 in heart. Liver had the greatest increase (Figure 4.6).

IGF-1 mRNA levels in liver, kidney and muscle

To assess local production or expression of IGF-1 in response to GH administration, IGF-1 mRNA in tissues was used as an indicator for changes at transcriptional level.

IGF-1 mRNA levels in liver, kidney and muscle all showed a trend of increase after GH administration (Figure 4.7). IGF-1 mRNA levels showed a 26% increase in liver, a 45% increase in kidney and a 29% increase in muscle 13 hours after GH administration, but the magnitude of the increases did not reach significant levels.

4.4 Discussion

Profiles of plasma GH and glucose in response to exogenous GH

Circulating levels of GH were used as an indicator to monitor the administration of GH. GH concentrations measured in plasma rose significantly and reached a peak at five hours post injection, then decreased to basal level at twenty-four hours. This indicated that the GH administration was available to the general circulation in the experimental animals.

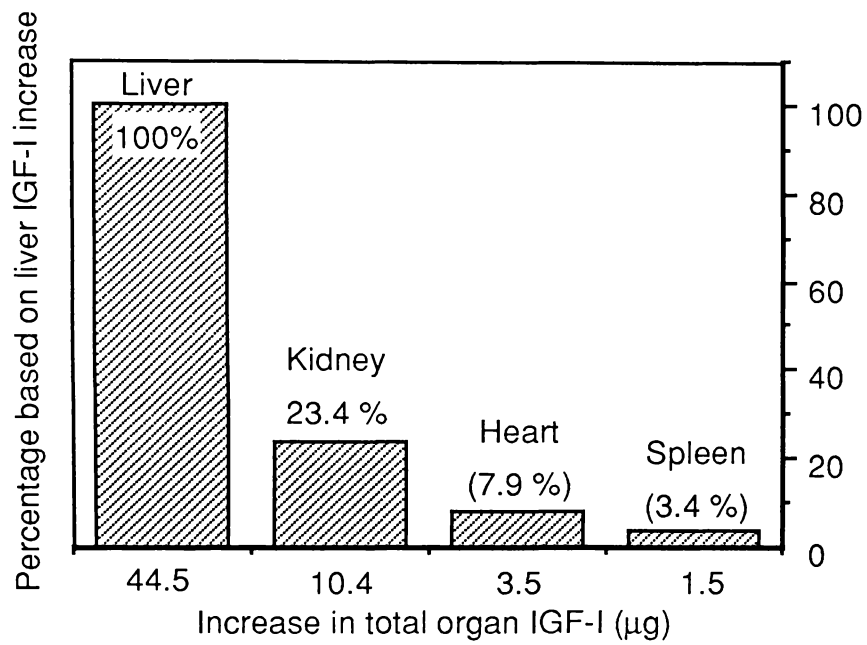


FIGURE 4.6 A comparison of the relative increases in total organ IGF-I 13 hours after administration of bovine growth hormone (0.25 mg/kg bodyweight). The increases in total organ IGF-I among different organs is expressed in μg along X axis in 'percent' of the increase of liver IGF-I along Y axis.

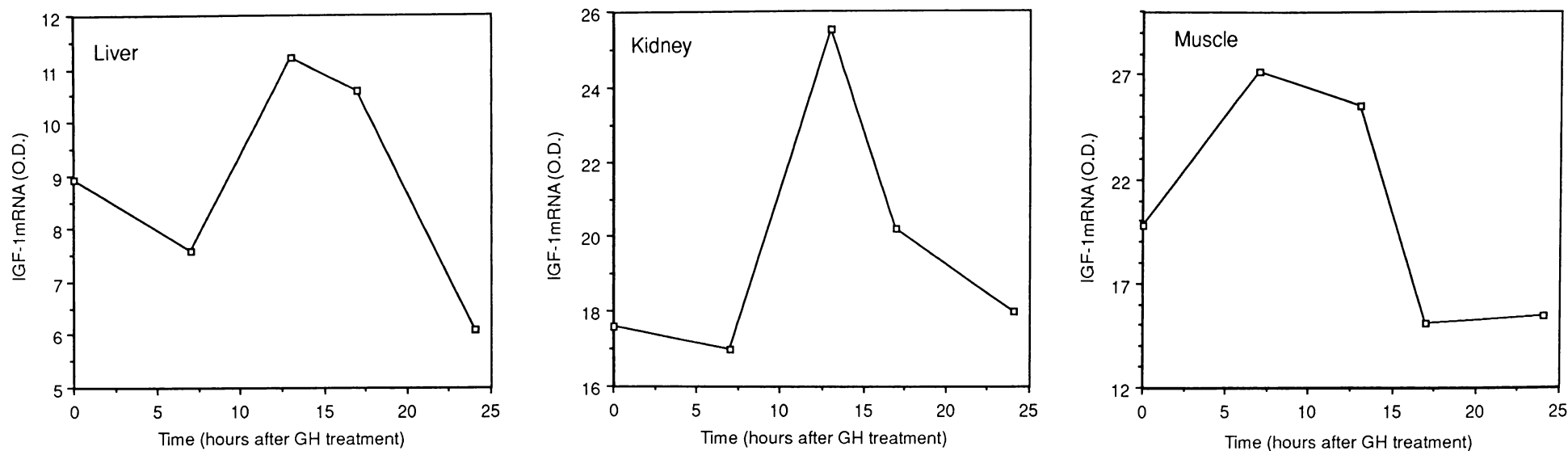


FIGURE 4.7 IGF-I mRNA levels in liver, kidney and muscle after GH administration (bGH; 0.25 mg/kg body weight; one single dose injection subcutaneously). Within 15 min of slaughter, 1-2 g tissue pieces of liver, kidney and muscle per animal (n=3 per group) were collected and treated as described in section 2.8. IGF-I mRNA levels were measured by in-situ hybridisation. Autoradiographic images on developed X-ray film were scanned by densitometry and quantitated on a Macintosh IICI computer using the application Image (NIH version 1.37). The mean image density from each S (control) section was subtracted from the corresponding value from the T (experimental) section. Relative levels of IGF-I mRNA were expressed by greyscale optical density (O.D.).

Circulating levels of glucose rose significantly after GH administration. This is in agreement with previous observations in non-lactating sheep, which showed apparent increases in plasma NEFA and glucose after treatment with bGH (486-488). Plasma concentrations of glucose in the present experiment reached maximal level at least five hours later than did plasma GH, indicating an effect of GH administration on glucose metabolism in normal lambs. It is well known that GH has important effects on carbohydrate metabolism. Following bolus administration acute insulin-like and prolonged (anti-insulin-like) diabetogenic effects are observed in several species as revealed by transient hypoglycemia followed by elevation of plasma glucose. The hyperglycemia is often associated with increased insulin concentrations, the classical manifestation of the insulin-resistant state (60, 65). Increased plasma concentrations of glucose may result in part from reduced insulin sensitivity and glucose uptake by peripheral tissues, particularly muscle, and also to increased hepatic glucose output in response to GH.

Tissue IGF-1 production in response to exogenous GH

A number of factors influence IGF-1 concentration in blood. Among these, GH has been shown to be of prime importance (120, 55, 338, 429, 430, 483) although many other factors have also been shown to be involved.

D'Ercole and his colleagues (338) found that in the rat not only serum but also tissue IGF-1 levels are strongly under GH control. The results from the present study revealed in sheep that all tissues studied have a significant increase in IGF-1 concentration in response to subcutaneous administration of bGH, indicating that tissue IGF-1 is, at least partly, dependant on GH in sheep.

In this study it was found that basal concentrations of IGF-1, in control animals, were highest in kidney, which may be related to excretory and metabolic functions of this organ (338). Conversely muscle biceps femoris had the lowest basal level of IGF-1.

Subcutaneous administration of bGH to normal sheep caused tissue IGF-1 concentration to increase in liver, kidney, heart and spleen. The time course of this response in these tissues preceded that of plasma by some hours. These time course observations together with the positive correlations between liver, kidney, spleen, heart with plasma IGF-1 suggest that locally produced IGF-1 is an important source of the growth factor in these tissues, and that IGF-1 derived from multiple sheep tissues contributes to plasma concentrations of the hormone. This is in agreement with D'Ercole's study in rats (338), and with studies by Han *et al.* (438, 489), Murphy *et al.* (273), Roberts *et al.* (481) and Isgaard *et al.* (262), who examined cellular localization, expression or regulation of IGF mRNA by GH in tissues. These studies have shown many different

tissues to be prominent sites of IGF synthesis.

Another interpretation for the time course observations from the present study could be that GH-stimulated hepatic IGF-1 was rapidly exported into blood and equally rapidly removed from blood by receptors on the peripheral tissues, which resulted in the increase of IGF-1 in the tissues without any paracrine/autocrine response. However, since the majority of the IGF-1 in serum is bound to the 150 KDa IGFBP complex (490), and the half life of the 150kDa IGFBP complex was estimated to be about 550 min in sheep plasma, the metabolic clearance is very slow (491, 492). It therefore seems unlikely that the results can be explained by the rapid loss of IGF-1 from plasma into tissues. By contrast, the muscle IGF-1 response to bGH was found to be much delayed, compared with the other tissues and plasma, but reached a significant level at the same time as plasma. This may indicate a late increase in local production of IGF-1 in the muscle. But the increase could also be at least partly accounted for by the slow transport of plasma IGF-1 into the muscle tissues.

IGF-1 mRNA levels measured by in-situ hybridization in the present study showed increases in liver (26%), kidney (45%), and muscle (29%) 13 hours after GH administration, suggesting local production of IGF-1, although the magnitude of the increases was not statistically significant. The lack of a significant induction of IGF-1 mRNA in liver, kidney or

muscle was surprising, given the increased levels of IGF-1 found in these tissues. This was particularly surprising in the case of liver, given that it is a location of presumed endocrine production of IGF-1. Increased hepatic IGF-1 mRNA production resulting from prolonged GH administration has been reported by Saunders *et al.* (493) for sheep, and by Grant *et al.* (494) for pigs. The magnitude of the reported increases were 2.5 to 5 fold, and approximately 3-fold, respectively. The treatment periods used in these studies were 10 weeks and 24 days, respectively, *ie.* much longer term than the treatment period used in the present study (24 hours or less), which could have a significant effect on determining the magnitude of the induction observed. It is presumed that the most significant reasons for failure to pick up an induction of IGF-1 mRNA, especially in liver tissue, stems from high inter-animal variation caused by insufficient numbers of tissues sampled ($n=3$ per group).

Transcriptional changes in IGF-1 have been documented in skeletal muscle as well, although the response appears to be considerably more variable than for liver. For instance, Schulster *et al.* (495) showed that GH-deficient dwarf rat extensor digitorum longus and gastrocnemius muscles had increased IGF-1 mRNA following a single dose of GH, but that soleus muscle failed to show such an induction of IGF-1 mRNA. Likewise, Grant *et al.* (494) reported decreased IGF-1 mRNA in muscles of GH-treated pigs relative to controls. More recent research in sheep by Pell *et al.* (496) revealed that GH treatment only stimulated

significant increases in expression from class-2 IGF-1 mRNA transcripts, which may be far more sensitive than the class-1 IGF-1 mRNA transcripts in intact animals (about class-1 and class-2 IGF-I mRNA transcripts, please refer to Section 6.1). Muscle IGF-1 gene expression was at least 20-fold less than that for the liver and consisted mainly of class-1 transcripts. Muscle total IGF-1 mRNA was insensitive to GH treatment. It would thus appear that the response in muscle IGF-1 mRNA to administered GH in our study is more in keeping with the findings of other researchers for farm animal species. The relatively large increase in peak concentrations of IGF-1 in muscle from the present study, 17 hours after administration of bGH, follows the rise in plasma IGF-1, which may represent, to some extent, the influence of IGF-1 circulating from plasma. In a previous study on the infusion of recombinant N-Met IGF-1 from plasma into tissues and lymph (497), it was found that muscle fibres, not connective tissue, had the highest percentage of recombinant N-Met IGF-1, indicating that the IGF-1 in muscle fibres had originated from plasma, and thus suggesting a possible endocrine role for IGF-1 in muscle fibres but not necessarily for muscle connective tissues. Since the connective tissue, which was shown to have the highest percentage of locally produced IGF-1 (497), can only account for a small portion of muscle, muscle from this age of sheep may possibly have low levels of production of IGF-1. So, IGF-1 originating from blood could contribute more to the total level of IGF-1 in muscle than that locally produced.

If the distribution of IGF-1 in the muscle biceps femoris is similar to that in all skeletal muscle then it could be estimated that more than half of the total body IGF-1 is present in muscle, which could be mostly originated from blood.

Nevertheless, results in this study do not exclude either local or plasma-origin IGF-1 being present in the muscle. To evaluate precisely the contribution of the relative importance of local vs plasma-origin IGF-1 in the tissues is technologically difficult. Whether IGFs, however, are effective in an endocrine or paracrine/autocrine manner is not necessarily mutually exclusive. Both actions may occur, with the relative importance of each depending on numerous factors (374).

Another interesting point in this study is to compare the possible contributions of IGF-1 derived from different sheep tissues to circulating IGF-1. The liver has been considered the major site of IGF synthesis (72, 338, 498, 499) and evidence from rats suggests that hepatic production of IGF-1 is sufficient to maintain a relatively large amount of plasma IGF-1, about 50% of total body content, present in the circulation (339). The current study has permitted estimation of total organ content of IGF-1 and the response of this parameter to GH treatment. Total liver IGF-1 was raised by $44 \pm 0.4 \mu\text{g}$ (mean $\pm$ SEM; $n=8$) in response to bGH, which is 4-fold more than the corresponding increase in kidney, 13-fold more than that in heart and 30-fold more

than that in spleen. These results support the traditional concept that liver is a major contributor of circulating IGF-1, although the data in this study suggested that IGF-1 is synthesised in multiple tissues in sheep.

Finally the distribution of the organ/muscle/plasma IGF-1 in control groups of lambs showed that less than 6% of the total body IGF-1 is retained in liver, which is considered to be the major source of IGF-1 synthesis, and that about one third of the total body IGF-1 is retained in blood. This reflects that there is little storage of IGF-1 in the cells where they are produced (478, 11) and it enters the circulation, possibly, in support of the growth process (500, 163, 283).

4.5 Summary

The present study indicates that in the sheep liver is a major contributor to circulating IGF-1, and that local production of IGF-1 is stimulated by GH in liver as well as in a number of other tissues. IGF-1 found in the muscle biceps femoris appears to follow the changes in circulating levels. This may imply an endocrine role for part of the IGF-1 present in muscle. The relative contribution or importance of local vs plasma-origin IGF-1 in skeletal muscle remains to be established.

CHAPTER FIVE : EFFECT OF GH ADMINISTRATION ON PLASMA LEVELS OF IGFBPs IN POST-NATAL SHEEP

5.1 Introduction

Insulin-like growth factors (IGF-I and IGF-II) are potent metabolic, mitogenic, and differentiating peptides, which are synthesized in many tissues in both foetus and adult (502). They act locally or after circulating to distant targets. In different contexts they may stimulate growth, differentiation, or the expression of differentiated functions by acting through IGF-I or possibly IGF-II receptors. IGF-I and IGF-II in plasma and other biological fluids occur bound to specific high affinity IGF binding proteins (IGFBPs) (503-505), of which six have been purified and cloned (506). The IGFBPs facilitate IGF transport in biological fluids and modulate IGF activity at the cellular level, thereby exerting regulatory effects on intermediary metabolism and growth (95, 357).

Among these BPs, IGFBP-3 is the major plasma carrier of both IGF-I and II, representing an important storage pool of hormone (507, 322). The most distinctive property of IGFBP-3 is that it combines with an acid-labile non-binding subunit (ALS) to form the predominant 150 kDa IGFBP complex in adult serum (303). Concentrations of ALS in human plasma are GH-dependent (221). Plasma IGFBP-3 levels vary with

developmental stage (508, 509, 322, 510) and GH/IGF-I (511-513) but show little diurnal or acute metabolic regulation (502, 514). It has been shown that long-term GH treatment altered IGFBP-3 serum levels in cows (515) and in sheep (443). The presence of IGFs or insulin has been demonstrated to influence IGFBP accumulation in the conditioned medium of many cell lines, and *in vivo*, IGFs and insulin influence serum IGFBP levels (193, 197, 516-523, 230) although reports are often contradictory. Thus, many aspects of IGFBP regulation remain unclear.

The present study was designed to examine changes in plasma IGFBP-3 and other IGFBP in post-natal sheep in response to administration of bovine growth hormone (bGH), and possible relationships between the changes in plasma IGFBP-3 and plasma/tissue levels of IGF-I.

The GH dependence of IGFBP may provide a mechanism by which the pituitary controls circulating levels and tissue availability of IGF; changes in the plasma concentrations of individual IGFBPs may reflect changes in the transcription, translation, uptake and/or degradation of IGFBPs by individual organs, tissues, and cells (357, 233, 524, 344, 525, 358, 526). The possible relationships between the changes in plasma IGFBP and plasma/tissue levels of IGF-I may reveal further information about the regulation of IGFBP.

To achieve the objective of the current study the following items were

investigated:

1. Plasma levels of GH were measured as an indicator of GH administration.
2. Plasma levels of IGFBP-3 after GH administration were measured using IGF-I ligand blot analysis. Differences between GH-treated and non-treated animals were examined for significance. The time course of IGFBP-3 response to GH was examined.
3. Plasma levels of other IGFBPs after GH administration were measured using IGF-I ligand blot analysis. Differences between GH-treated and non-treated animals were examined for significance. The time course of each IGFBP response to GH was examined.
4. Circulating IGF-I was extracted by the AEC technique and estimated by RIA. Differences between GH-treated and non-treated animals were examined for significance. The time course of plasma IGF-I response to GH was examined.
5. Tissue IGF-I was extracted by the AEC technique and estimated by RIA. Tissue IGF-I was corrected for retained plasma IGF-I using tissue and plasma Hb levels. Differences in tissue IGF-I between GH-treated and non-treated animals were examined for significance. The time

course of tissue IGF-I response to GH was examined.

6. The relationship between plasma IGFBP-3 response to GH and tissue/plasma IGF-I response to GH was examined.

The studies in this chapter were performed in 1992, at Growth Physiology, Ruakura Agricultural Research Centre, Hamilton, New Zealand.

5.2 Materials and Methods

Materials

Lyophilised GH (recombinantly derived bovine GH, BST) was prepared as described in Section 4.2 and used the next day. The BST was kindly donated by American Cyanamid Company (Princeton, New Jersey).

Feed pellets were as described in Section 3.2.

Animals and Treatment

Fifty-four mixed-breed ewe lambs, about six months of age, were held in individual pens for a period of three weeks and fed a pelleted high protein diet *ad libitum* before the experiment. Weekly weights and daily feed intakes were recorded during this period.

Forty-eight lambs were distributed into six groups (n=8, per group)

which were balanced on body weight. Three groups were injected subcutaneously with one single dose of bovine growth hormone (bGH; 0.25 mg/kg body weight) then serially killed at 5, 17 and 24 hours post injection. Three control groups of lambs, injected with equivalent volumes of saline, were killed at 0, 13 and 24 hours.

10 ml blood samples, in EDTA, were collected from all lambs immediately prior to slaughter. The plasma was separated as soon as possible at 4°C after collection and stored at "-20°C".

Samples (2~3g) of liver and kidney were collected, immediately after death, and snap-frozen in liquid nitrogen. Tissue samples were stored at "-20°C" until assayed. The frozen samples of both plasma and tissues were used to measure the levels of IGF-I.

Another six lambs were divided into two groups, A and B (n=3, per group). Group A was treated with bGH (one single dose, sc, 0.25 mg/per kg body weight); Group B was injected with saline. Plasma samples, collected from group A and B at 5, 17 and 24 hour post injection, were stored at -20°C after separation. These plasma samples were used for measurement of IGFBPs.

Tissue Extraction

Tissues were extracted for determination of IGF-I. The procedure was

as described previously (Section 2.5). Briefly samples of frozen sheep tissue were homogenized in ice-cold acetic acid (1 mol/l) using 4°C. The supernatant was removed and neutralized with 5 mol NaOH/l to a final pH between 6 and 8, then centrifuged at 10,000 g for 15 min at 4°C. The supernatant was stored at -20°C until the time of IGF-I assay.

Assays

GH RIA

Plasma concentrations of GH were measured by RIA, which was described in detail in Section 2.7. Plasma samples for measurement of GH were collected from three Dorset x Coopworth ewe lambs (six month old) at 0, 5, 17 and 24 h after one single dose injection of bGH (0.25 mg/kg body weight).

IGF-I RIA

IGF-I in plasma and tissue extracts was measured by RIA, which was detailed in Section 2.3. Briefly, buffer for assay reagents was 0.064 mol/l phosphate, pH 7.4 containing bovine serum albumin (0.25%; w/v), sodium aside (0.02%, w/v) and Triton X-100 (0.02%, w/v) all reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

An amino-terminal methionyl IGF-I preparation (IMC, Pitman/Moore, Terre Haute, IN, USA) radioiodinated by the Iodogen method (290) was used as tracer. An IGF-1 antiserum, raised by repeated subcutaneous

injection of rabbits with an egg albumin conjugate of this preparation, was used as the first antibody. Highly purified recombinant natural sequence IGF-I (courtesy of Dr A. Skottner, Kabi Vitrum, Stockholm, Sweden) was used as the standard. A solution consisting of sheep anti-rabbit serum (1/20, v/v) and normal rabbit serum (1/60, v/v) in polyethylene glycol (4%, w/v) was used as the second antibody. Radioactivity was estimated in an LKB 1260 gamma counter.

Tissue extracts and plasma samples were subjected to the AEC procedure before RIA.

IGF-I Ligand Blot Analysis

The relative levels of IGFBPs in samples of plasma, collected from Groups A and B, were determined by a modification of the Western blotting technique described in detail in Section 2.6. Briefly, 10 μ l of each prepared plasma sample was loaded onto a 4% stacking gel and subjected to electrophoresis through a 12% (v/v) polyacrylamide gel at a constant voltage (150 v) for 80 min at 4°C using Bio Rad Mini-Protean equipment. The protein bands were electrophoretically transferred to nitrocellulose membranes for ligand blotting (393).

Quantification of the autoradiograms was performed using scanning densitometry. The relative levels of the bands were expressed as optical density (O.D.).

Biometrics

Differences in tissue or plasma IGF-I levels and in plasma IGFBPs levels between GH-treatment and untreated lambs were analysed by analysis of variance (ANOVA) using Macintosh Statview (1987; Abacus concepts, Inc., Berkeley, CA, USA).

5.3 Results

Circulating levels of GH

Plasma concentrations of GH rose significantly compared with basal level (0 hour) and reached the highest level measured in 5 hours post injection, then decreased to basal level after 17 hours (Figure 5.1).

Tissue and Plasma IGF-I response to GH administration

A significant increase in IGF-I concentrations in liver, kidney and plasma was shown in response to a single-dose administration of bGH (Table 5.1). Tissue and plasma levels of IGF-I in control groups of lambs showed no significant change during the 24 hours of experimental period (Figure 5.2).

IGFBPs in sheep plasma

Ligand blot analysis of the sheep plasma from all of the animals revealed four major classes of IGFBPs with the capacity to bind ¹²⁵I-IGF-I; a double band of 40-43 kDa, and bands of 34 kDa, 30 kDa and

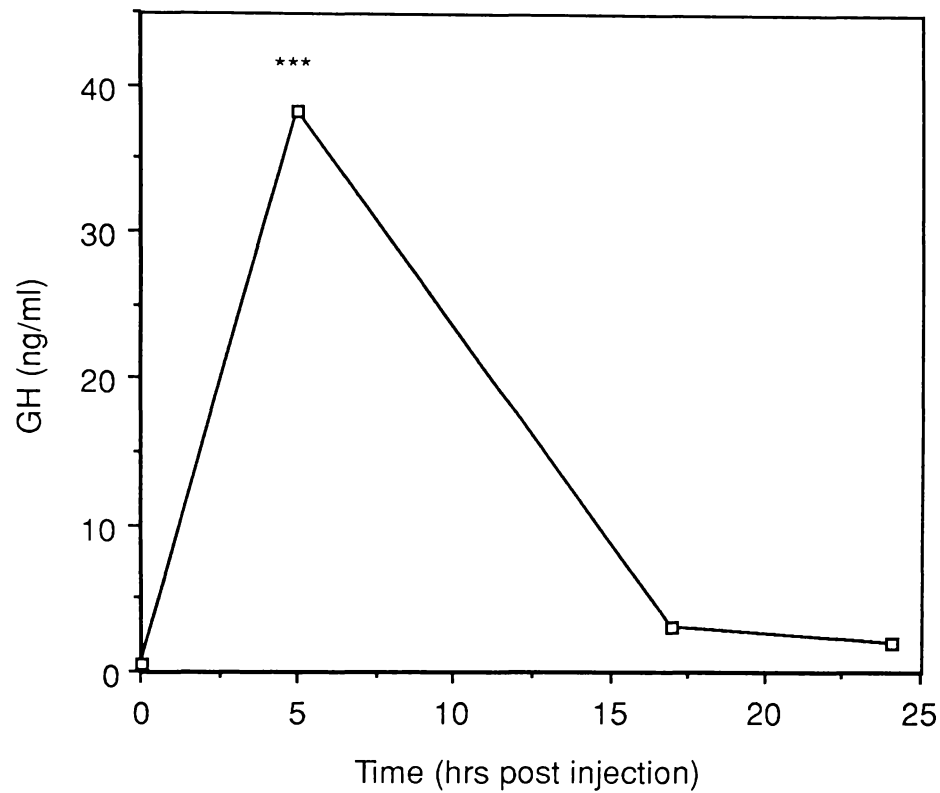


FIGURE 5.1 Plasma levels of growth hormone (GH).

Plasma concentrations of GH were measured by RIA during a 24 hr-period of experiment as an indicator of administration of bGH (one single dose; 0.25 mg/kg body weight). Plasma samples were collected from six-month-old Dorset x Coopworth ewes (n=3) at 0, 5, 17 and 24 hr after GH injection. Significance of difference in GH concentrations was analysed by analysis of variance (ANOVA) using the Macintosh Statview (1987, Abacus Concepts, Inc., Berkeley, CA, USA).

*** $p < 0.001$ as compared with the basal level (0 hour).

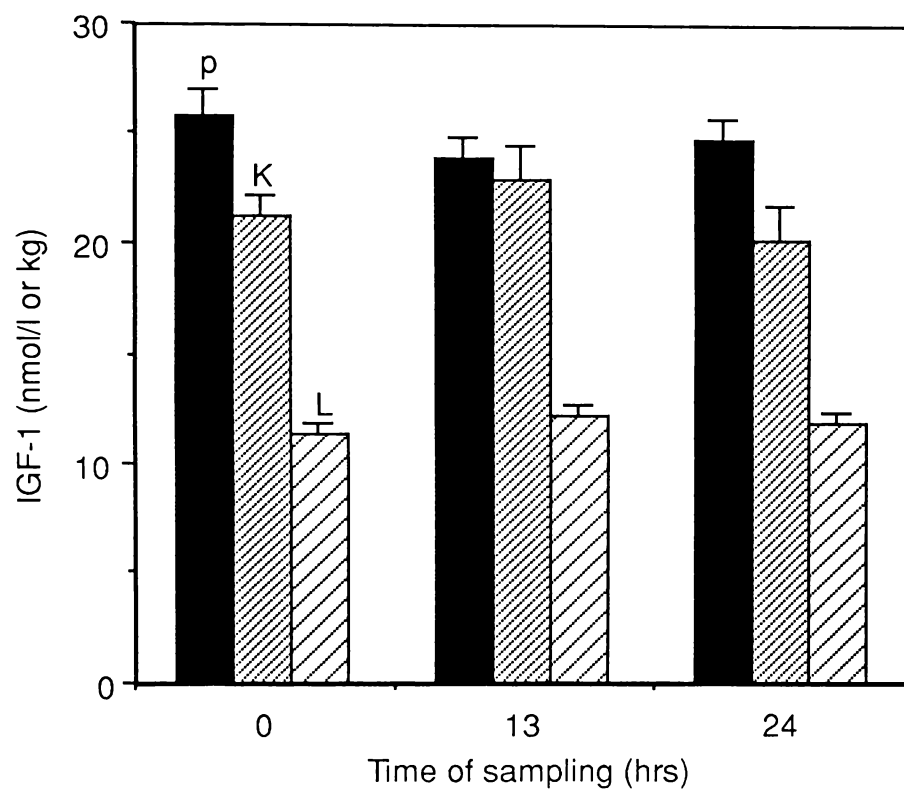


FIGURE 5.2 Tissue (nmol/kg) and plasma (nmol/l) IGF-I concentrations (mean $\pm$ SEM, $n=8$) in control groups of Dorset $\times$ Coopworth ewes (six-month-old) at different sampling times. (P, plasma; K, kidney; L, liver).

TABLE 5.1 IGF-1 concentrations (means $\pm$ SEM; n=8) in sheep tissue (nmol/kg) and plasma (nmol/l) 0, 5, 17 and 24 hours after s.c. administration of bovine growth hormone (0.25 mg/kg body weight).

	Time (hrs) after growth hormone injection			
	0	5	17	24
Liver	11.33 $\pm$ 0.53	15.41 ^{**} $\pm$ 0.95	19.88 ^{***} $\pm$ 1.10	17.35 ^{***} $\pm$ 0.95
Kidney	21.18 $\pm$ 1.03	26.76 [*] $\pm$ 2.30	41.46 ^{***} $\pm$ 2.10	27.62 [*] $\pm$ 2.10
Plasma	25.8 $\pm$ 1.16	26.25 $\pm$ 1.40	38.74 ^{***} $\pm$ 1.50	35.80 ^{***} $\pm$ 1.50

Significance of differences between "0" (control) and other time groups after growth hormone injection were determined by analysis of variance (ANOVA) using the Macintosh Statview (1987, Abacus Concepts, Inc., Berkeley, CA, USA).

* p<0.05, ** p<0.01, *** p<0.001 as indicated by ANOVA.

24 kDa. By analogy with the human, rat and bovine IGFBPs, and from earlier characterisation studies (314, 315, 214, 213, 304, 527, 301, 237, 528), these would appear to be the ovine equivalents of IGFBP-3, IGFBP-2, 30 kDa IGFBP and IGFBP-4 respectively (Figure 5.3). Identification of these IGFBPs is discussed in Section 5.4.

Plasma IGFBP response to GH administration

It was observed that the increase in tissue and plasma IGF-I levels was accompanied by an increase in plasma levels of IGFBP-3. Plasma IGFBP-3 in GH-treated lambs, in a similar manner to plasma IGF-I, reached significantly high levels by 17 h and remained high at 24 h (Figures 5.4 a, b and 5.5).

The concomitant increase in plasma levels of IGFBP-3 and plasma/tissue levels of IGF-I is plotted in Figure 5.6.

By contrast, the response to GH administration in other IGFBPs was different from that in IGFBP-3. No significant change could be seen in plasma levels of the 30 kDa IGFBP. A decrease in IGFBP-2 levels was found 24 h post GH injection (Figure 5.4 a, b). The IGFBP-4 response to GH administration varied among the lambs. GH-treated lambs #1 and #2 (Figure 5.4b) showed a similar decrease, but GH-treated lamb #3 (Figure 5.4a) had no change in plasma levels of IGFBP-4 (Figure 5.7).

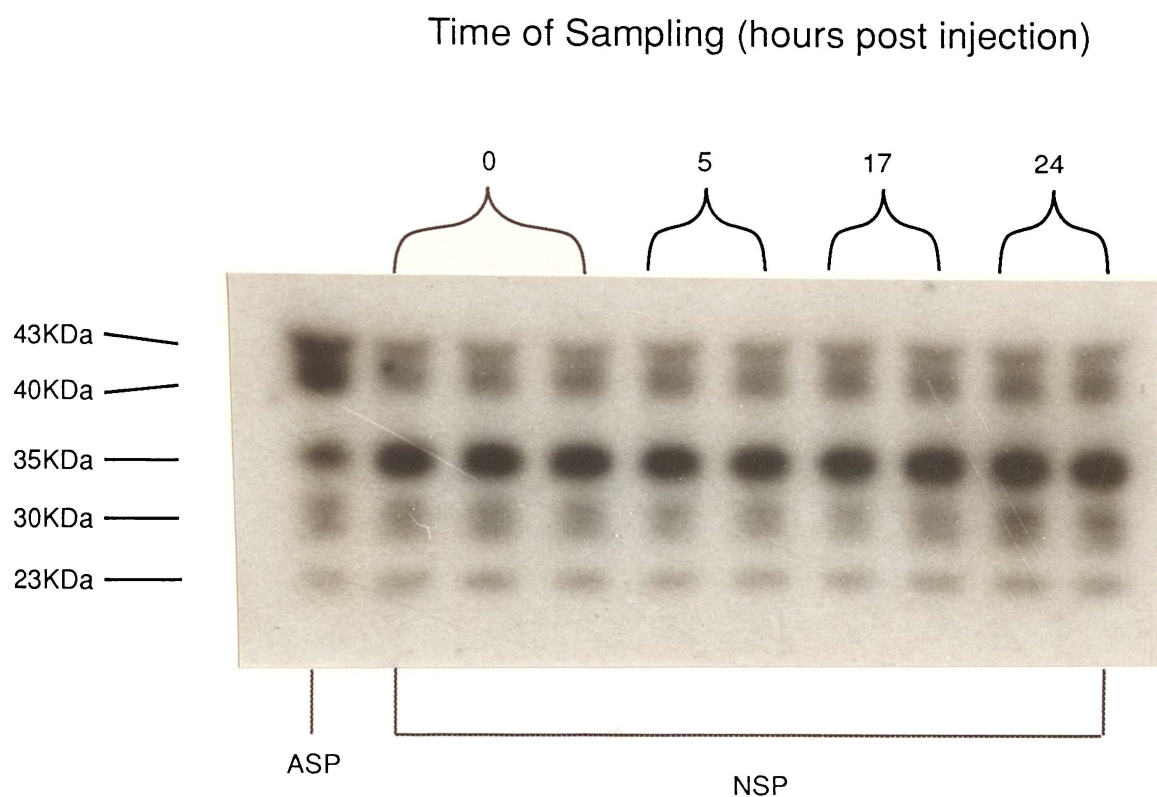


Figure 5.3 IGF-1 ligand blot analysis of normal sheep plasma (NSP), collected from a control ewe (6 months of age) 0, 5, 17 and 24h after one single-dose injection of saline. Samples were subjected to SDS/PAGE as described in section 2.8 and electrophoretically transferred to nitrocellulose membranes using a Bio Rad Semidry transfer cell, and incubated with ^{125}I -IGF-1 prepared as described in section 2.3. Membranes were then used to expose Kodak XAR x-ray films. Apparent molecular weights (KDa) of the bands as estimated by comparison of their electrophoretic mobilities with globular molecular weight markers (not presented) are shown on the left. Other two control ewes (6 months of age) showed similar results. ASP: adult sheep plasma (12 months of age) used as a reference.

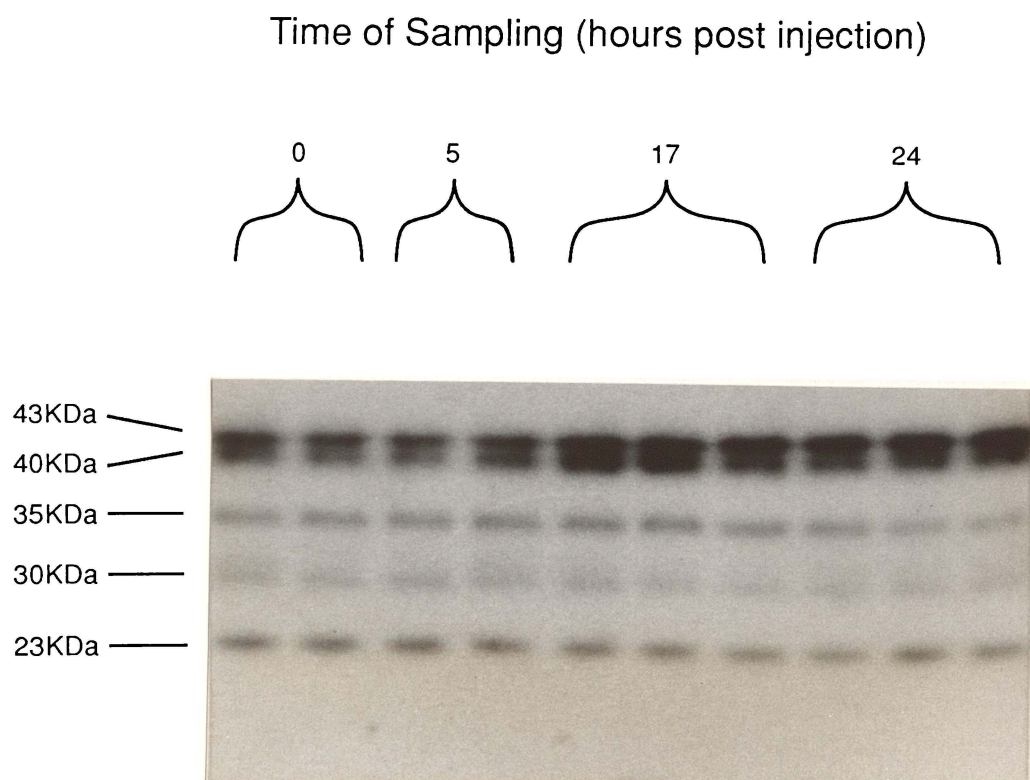


Figure 5.4a IGF-1 ligand blot analysis of sheep plasma, collected from a representative 6-month-ewe (lamb #3) 0, 5, 17 and 24h after GH administration. Samples were subjected to SDS/PAGE as described in section 2.8 and electrophoretically transferred to nitrocellulose membranes using a Bio Rad Semidry transfer cell, and incubated with ^{125}I -IGF-1 prepared as described in section 2.3. Membranes were then used to expose Kodak XAR x-ray films. Apparent molecular weights (KDa) of the bands as estimated by comparison of their electrophoretic mobilities with globular molecular weight markers (not presented) are shown on the left.

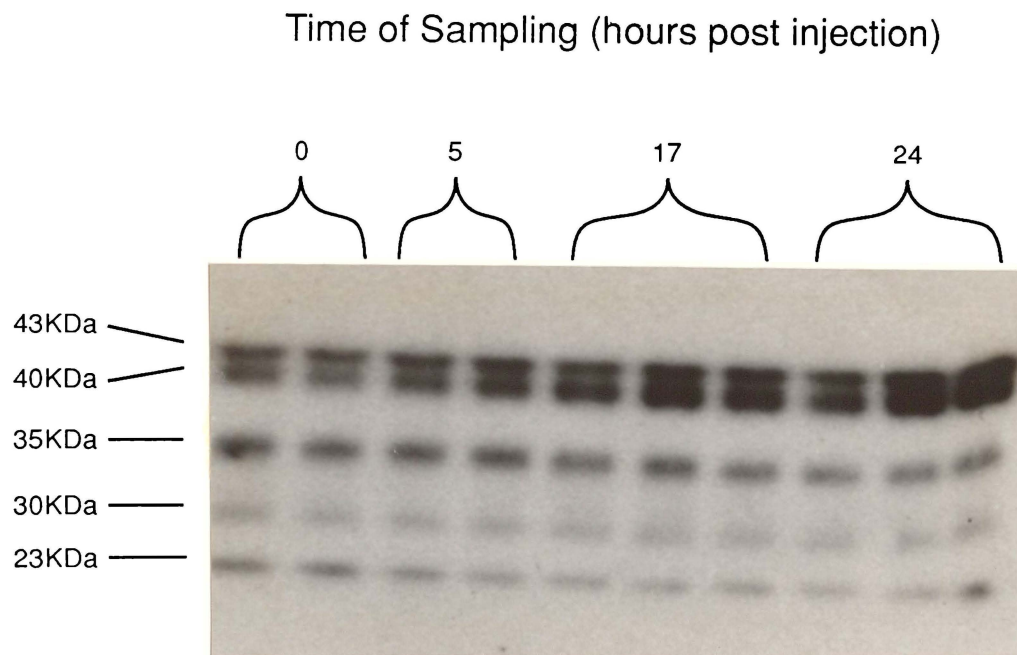


Figure 5.4b IGF-1 ligand blot analysis of sheep plasma, collected from a 6-month-ewe (lamb #2) 0, 5, 17 and 24h after GH administration. Samples were subjected to SDS/PAGE as described in section 2.8 and electrophoretically transferred to nitrocellulose membranes using a Bio Rad Semidry transfer cell, and incubated with ^{125}I -IGF-1 prepared as described in section 2.3. Membranes were then used to expose Kodak XAR x-ray films. Apparent molecular weights (KDa) of the bands as estimated by comparison of their electrophoretic mobilities with globular molecular weight markers (not presented) are shown on the left. Another 6-month-ewe (lamb 1#) showed similar results.

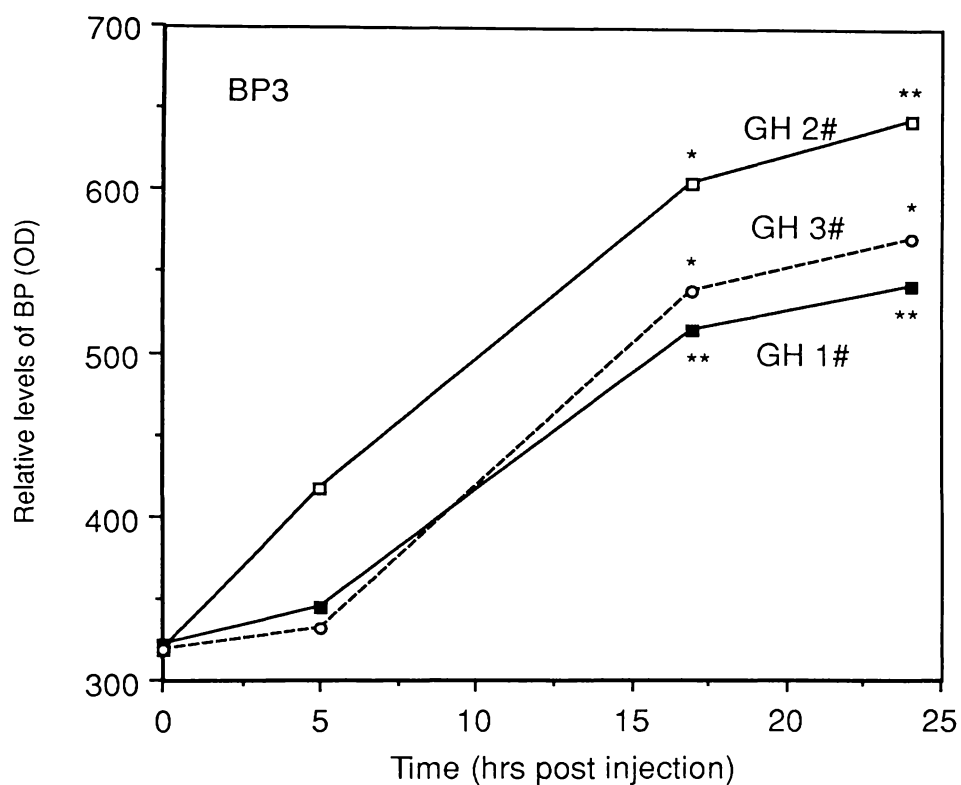


FIGURE 5.5 Plasma IGFBP-3 response to GH administration.

Plasma samples taken from GH-injected (bGH; 0.25 mg/kg body weight) Dorset x Coopworth ewes (six-month-old) at various intervals were analysed by IGF-1 ligand blot analysis using ^{125}I -IGF-1. Relative levels of IGFBP were quantified by densitometry and expressed as optical density (OD). GH 1#, GH 2# and GH 3# represent GH-treated ewe 1#, 2# and 3# respectively.

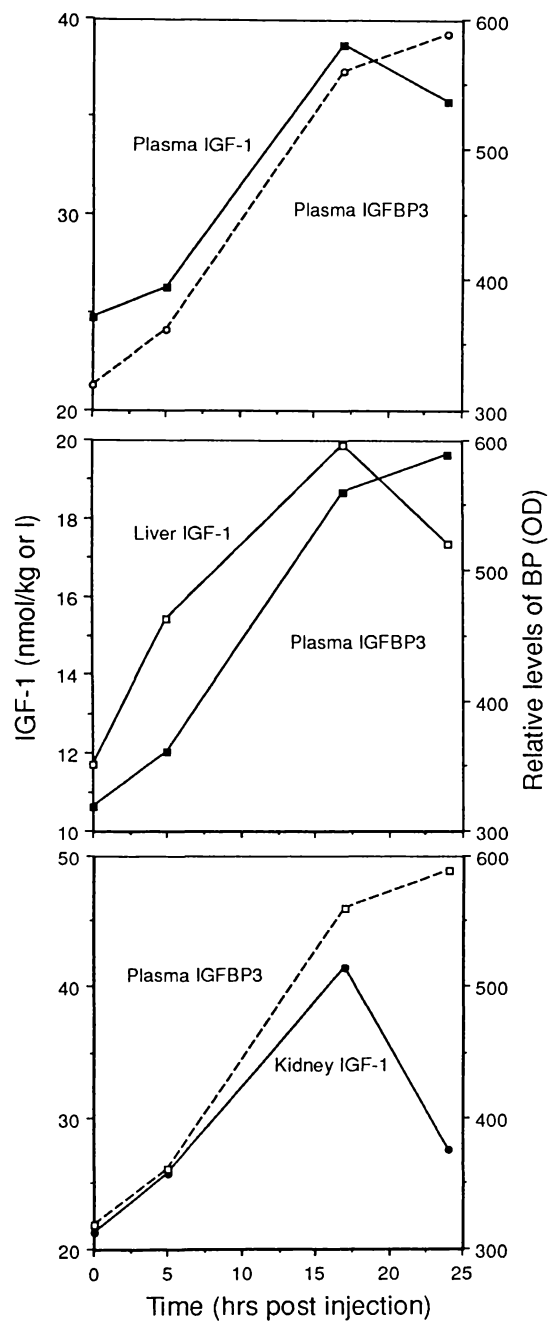


FIGURE 5.6 Concomitant increase in plasma/tissue IGF-1 (mean, $n=8$) and plasma IGFBP-3 (mean, $n=3$) in response to GH administration. Plasma/tissue IGF-1 was measured by RIA. Plasma IGFBP-3 was analysed by IGF-1 ligand blot analysis using ^{125}I -IGF-1. Relative levels of BP were quantified by densitometry.

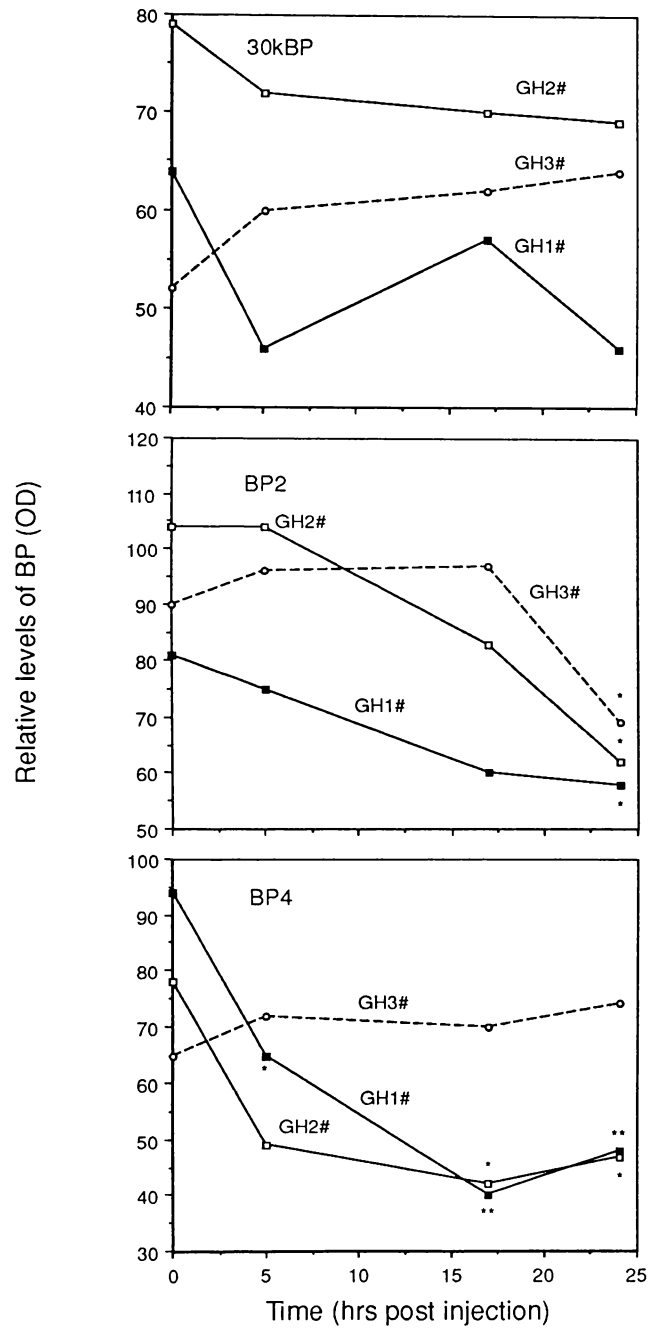


FIGURE 5.7 Plasma IGFBP response to GH administration (bGH; 0.25 mg/kg body weight). Plasma samples taken from GH-injected Dorset x Coopworth ewes (six-month-old) at various intervals were analysed by IGF-1 ligand blot analysis using 125 I-IGF-1. Relative levels of IGFBP were quantified by densitometry and expressed as optical density (OD). GH 1#, GH 2# and GH 3# represent GH-treated ewe 1#, 2# and 3# respectively.

Basal levels of plasma IGFBPs in non-GH-treated lambs (Group B - control) remained relatively stable under the experimental conditions (Figures 5.3 and 5.8). It was found in control lambs that IGFBP-2 levels were higher than IGFBP-3, as opposed to the situation in adult sheep plasma (ASP in figure 5.3) collected from 12 month-old-ewes maintained on pasture.

5.4 Discussion

Identification of IGFBPs in sheep plasma

In important work, Walton *et al.* (314) reported that three distinct species of IGFBPs were isolated from ovine plasma as follows:

The species migrating at 40 and 43 kDa on SDS-PAGE are ovine IGFBP-3 since they share homology with human (214), rat (213) and porcine (304) forms IGFBP-3:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ovine:	G	A	X	I	V	X	A	X	P	V	V	F	R/C	I	X...
Rat:	G	A	G	A	V	G	A	G	P	V	V	R	C	D	P....
Human:	G	A	S	S	G	G	L	G	P	V	V	R	C	E	P....
Porcine:	G	K	G	A	V	G	A	G	P	V	V	R...			

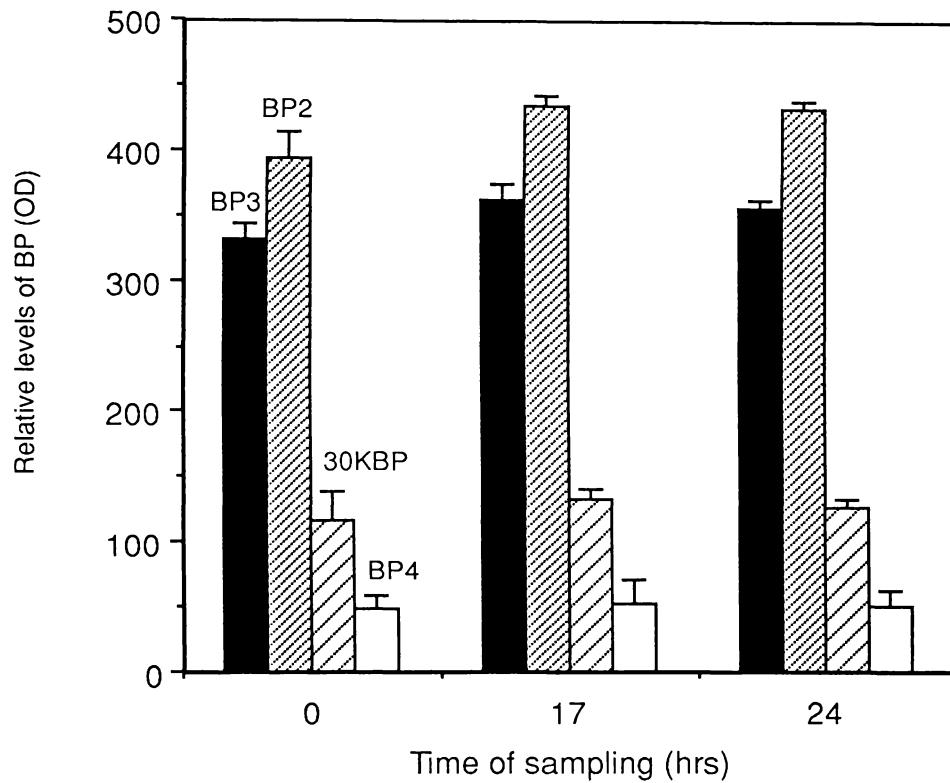


FIGURE 5.8 Basal levels of plasma IGF-BPs in control ewe lambs (mean $\pm$ SEM, n=3). Plasma samples taken from three six-month-old ewes at various intervals during a 24 hr-period were measured by IGF-1 ligand blot analysis using ^{125}I -IGF-1. IGF-BP levels were quantified by densitometry and expressed as optical density (OD).

The 35 kDa species is ovine IGFBP-2, sharing homology with the rat (527) and bovine (301) forms of IGFBP-2:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ovine:				F	R	L/C	P	P	X	T	P	E	D	L	A	A
Bovine:	E	V	L	F	R	C	P	P	X	T	P	E	D	L	A	A
Rat:	E	V	L	F	R	C	P	P	C	T	P	E	R	L	A	A
	17	18	19	20	21	22	23	24	25	26	27	28	29			
Ovine:	D/C	X	P	P	P	X	G	A	A	G	X	P	G...			
Bovine:	C	G	P	P	P	G	A	A	A	G	A	P	G...			
Rat:	C	G	P	P	P	D	A	P	C	A...						

The 24 and 29 kDa ovine IGFBPs share sequence homology with a human bone cell-derived IGFBP (237):

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Ovine:	D	E	A	I	H	C/S	P	P	L	N	E	E	E	G	A	A/R	R
Human:	D	E	A	I	H	C	P	P	E	S	E	A	K	L	A.....		

The variants of 24 and 29 kDa were initially considered unique but are now recognised as the ovine equivalents of IGFBP-4 (315).

Recently, McLellan and co-workers (528) identified five species of IGFBPs, in fetal sheep plasma, molecular sizes of 24 kDa (IGFBP-1), 34 kDa (IGFBP-2) and two IGFBP-3 species of 38 kDa (non-glycosylated) and 41 kDa (glycosylated). The identities of the 30 kDa BP appeared to be IGFBP-1 by Western blotting using specific antisera against human IGFBP-1. However the assignation of the 30 kDa species as BP-1 was

not affirmatively convincing, since the basis for the assignment by the McLellan group was only immuno-blotting using an anti-human IGFBP-I antibody, given the homology between the IGFBPs and the paucity of precise specificity data for the antibody, it is not excluded that the antibody used by these workers could also recognize some other protein (eg. IGFBP-5 or IGFBP-6). Moreover the levels of the 30 kDa IGFBP in the present study showed no diurnal fluctuation and no response to GH administration in contrast with previous observations that the levels of IGFBP-I demonstrated marked diurnal rhythmicity (192, 324), with variations of up to 20-fold in concentration during a 24 h-period (324), and that IGFBP-I is inversely related to GH secretory status (537, 359, 579). It is therefore recommended that the 30 kDa IGFBP species in the current study stands by itself until further confirmation, rather than an uncertain assignation as IGFBP-I.

Based on the above characterization or identification studies of IGFBPs (314, 315, 214, 213, 304, 527, 301, 237, 324), the four major classes of ovine IGFBPs bound to ^{125}I -IGF-I in the present study would appear to be the ovine equivalents of IGFBP-3, IGFBP-2, 30 kDa IGFBP and IGFBP-4 respectively.

IGFBP response to administration of GH

GH has been shown to be one of the most potent factors stimulating IGF-I production *in vivo* (55, 338, 529) and it is also involved in the

induction of a wide range of proteins, filling structural, storage and regulatory functions (11). Amongst these, IGFs and their BPs are of major important in the control of growth and development.

IGFBP-3, the most abundant IGFBP in plasma, is the acid-stable binding subunit of the 150 kDa GH-dependent BP complex (303). IGFBP-3 may inhibit or potentiate IGF actions (502, 514). It has a strong tendency to associate with the cell surface, which lowers its affinity for IGF-I, promoting release of IGF-I from an inactive complex (530).

IGFBP-3 was formerly called the GH-dependent IGFBP, because its presence in serum correlated with GH secretory status (214). Its serum concentrations are also dependent upon IGF-I (531). Specifically, when IGF-I is infused into hypophysectomized rats, IGFBP-3 is induced. Likewise, normal rats fed a protein-deficient diet have reduced IGFBP-3 levels that can be restored to normal with IGF-I infusion, but not by GH administration (532).

In the present study, IGFBP-3 levels in plasma were significantly elevated after GH administration. This is in agreement with previous observations in normal or hypophysectomized rats as assessed by ligand blotting (531, 532).

The concomitant increase in plasma levels of IGFBP-3 and the increase in tissue/plasma levels of IGF-I in the present study suggests a dependence of IGFBP-3 on GH/IGF-1. Defining the precise *in vivo* regulatory influences of each is difficult. Cecilia Camacho-Hubner and co-workers (370) showed in transgenic mice that IGF-I can induce IGFBP-3 independently of GH, indicating that IGF-I is an important variable controlling IGFBP-3 serum levels. In their work, additional evidence for the key role of IGF-I in IGFBP-3 regulation came from the findings that IGFBP-3 blood concentrations were elevated above normal in the mice over-expressing IGF-I, despite suppression of GH secretion in these mice presumably as a result of negative feedback by IGF-I overexpression (533), and that IGFBP-3 serum levels correlated strongly with those of IGF-I when data from all groups of mice were analysed (370).

In the present study, the time course of IGFBP-3 response to GH matched well with that of plasma/tissue IGF-I induced by GH administration. This supports the concept that IGF-I expression mediates GH's stimulation of circulating IGFBP-3 concentrations (370).

Nonetheless, these studies do not exclude a role for GH (direct or indirect) in the regulation of IGFBP-3. In the case of IGFBP-3, IGF-I and GH appear to be working in concert to increase the IGF-binding capacity of serum. In contrast, responses to GH from other IGFBPs in

the present study were shown to be different from that of IGFBP-3. Plasma levels of IGFBP-2 were found to be decreased ($P < 0.05$) 24 hours after GH treatment. This is in general agreement with the findings in pig (326) and in cows (515, 360), which showed that IGFBP-2 was suppressed by GH administration and increased by hypophysectomy or in negative energy balance. In the present study, the plasma concentration of IGFBP-2 in control ewe lambs exceeded that of IGFBP-3 (Figure 5.3). The reverse pattern between IGFBP-3 and IGFBP-2, compared with ASP, may result from some undernourishment caused possibly by changes in the animals' living environment. Alternatively a different developmental stage may also play a part; IGFBP-2 plasma concentrations in the rat, pig, rhesus monkey, and human are highest in late foetal and early post-natal life and subsequently decline with age (95, 199, 534-536).

The 30 kDa IGFBP in the current study showed no response to GH treatment in contrast with previous observations of IGFBP-I (400, 360, 537, 539); further characterization or identification remains to be investigated.

IGFBP-4, in the present study, seemed to show a down-regulation by GH administration but the results between the lambs varied. Two of the GH-treated lambs showed a decrease in the plasma concentrations of IGFBP-4, but another one gave no response to GH administration. This

needs further clarification. Little *in vivo* information so far is available about the biological regulation of IGFBP-4. IGFBP-4 has been reported to be increased in conditioned media in the presence of IGF-I in several cell systems (539, 238), but decreased in human decidual cells (517). Recently more *in vitro* work revealed that IGFBP-4 production was inhibited by IGF-I in sheep (540) and human (541) fibroblast cell line, but stimulated by IGF-I in a human epidermal squamous cell carcinoma line (541).

Obviously, regulatory control of IGFBPs is a very complicated process, depending on numerous factors. This is so particularly in the case of plasma, since it is a potpourri of IGFBPs synthesized and degraded by many different tissues. So, changes in the plasma concentrations of IGFBPs in response to hormonal (or other) stimuli could represent a combination of the possible changes in the transcription, translation, uptake, and/or degradation of IGFBPs from different organs, tissues and cells.

To improve further *in vivo* investigation of hormonal control of IGFBPs, more experimental animals per group, longer treatment period of GH administration and estimation of IGFBP mRNA expression in tissues (especially liver) are recommended.

5.5 Summary

Results from this study showed that administration of bGH induced a concomitant increase in tissue/plasma IGF-I and plasma levels of IGFBP-3, as well as a decrease in IGFBP-2, indicating a role for GH in regulation of circulating levels and tissue availability of IGF-I in sheep by affecting IGF-I and its binding proteins.

CHAPTER SIX : GENERAL DISCUSSION

It has been become increasingly apparent over recent decades that a complex and integrated process such as growth is not solely controlled by any one variable. Somatic growth represents the outcome of interactions between the genetic potential of an individual to grow and environmental factors, prominent amongst these is nutrition. GH, the IGFs, and their respective receptors appear to play important and inter-related roles in co-ordinating these interactions, and as such they are considered as one hormonal system (GH axis) controlling normal postnatal development. In this thesis, the concept of growth is considered in its broad sense as developmental processes.

Although it is well known that GH has a profound influence on post-natal growth in most species, particularly its potential in improving productivity in farmed livestock, major questions remain regarding precise mechanisms by which the GH axis acts. A large number of external (*ie.* nutrition, season, temperature, *etc*) and internal (*ie.* genetic, sex, hormonal, *etc*) stimuli impinge on this axis. Experiments in this thesis were conducted to address specific questions of the influence of nutritional status (a powerful external stimulus) or GH (a prominent internal stimulus) treatment on tissue and plasma IGF-I immunoreactivity (Chapter Three), stimulation of hepatic and extra-hepatic production of IGF-I (Chapter Four), and responsiveness of circulating IGFBP (Chapter Five), with a view to investigating IGF-I production of both hepatic and extra-hepatic origin and its regulatory control through the GH axis; and to having some possible

insight into relative contribution or importance of local versus hepatic production of IGF-I in post-natal sheep. The role of GH and IGF receptors is obviously important in mediating the aforementioned interactions, but has not been investigated in the experiments of this thesis.

Chapters One and Two of this thesis serve as a general introduction to the GH axis and a materials/methods section respectively, while Chapters Three, Four and Five describe experimental investigations.

6.1 Nutritional and GH regulation of tissue IGF-I production

Although the effects of nutrition and GH on circulating IGF-I have been well established (120, 55, 338, 117, 118, 429-436, 483) the production and regulation of tissue IGF-I is inconclusive. It is well-known that nutrition is a potent regulator of IGF-I synthesis in post-natal life, and this regulation of IGF-I may be an important means by which nutrition regulates growth. However, the molecular mechanisms involved in regulation of IGF synthesis by nutrition have not been fully elucidated. Since the structure of the IGF-I gene allows for multiple levels of regulation at transcriptional, translational and post-translational steps, the regulatory control of tissue IGF-I production is obviously very complex.

It is molecular biology that provided access for investigating IGF physiology at a molecular basis. In adult fasting rats, it has been reported that the abundance of hepatic IGF-I mRNA transcripts decreased and rebounded after 24 h of re-

feeding (542, 543), and the IGF-I mRNA levels of major transcripts were in parallel with serum IGF-I levels during periods of both fasting or re-feeding (542). Lowe *et al.* (544) demonstrated in fasting rats that total IGF-I mRNA levels were decreased in liver as well as in other peripheral tissues, including lung, kidney, muscle, stomach brain and testis. These results indicate that nutrition regulates IGF-I synthesis at least at the mRNA level, verifying the central role of nutrition in growth regulation. Nevertheless, it should be kept in mind that mRNA concentrations do not always reflect the rate of translation (*ie.* production) of the protein. It is therefore important to investigate the regulatory changes of IGF-I at the protein level as well. Yahya *et al.* (440) showed nutritionally-induced changes in tissue concentrations of IGF-I in rats in relation to the inhibition of bone and muscle growth and proteoglycan synthesis by protein deficiency. In this experiment, plasma concentrations of IGF-I fell markedly in the protein-deficient rats in which growth was inhibited. At the same time, concentrations of IGF-I were minimally influenced in bone, but decreased in muscle by protein deficiency. Results from Chapter Three of this thesis also revealed that IGF-I is regulated by nutrition in a tissue-specific manner. Five-day starvation significantly depressed IGF-I levels in plasma and kidney, but had only minimal effect on muscle IGF-I concentrations, suggesting the possibility of differential regulation of IGF-I in different tissues by nutrition in sheep.

GH, on the other hand, has been shown to be of great potential in regulation of IGF-I synthesis. Roberts *et al.* (545) demonstrated an increase in abundance of

all species of IGF-I mRNA in liver of hypophysectomized rats following daily subcutaneous injections of hGH for 10 days. Moreover, Murphy *et al.* (546, 255) demonstrated peak expression of IGF-I mRNA in the liver and kidney of hypophysectomized rats 6-12 hours after GH administration.

The expression of the IGF-I gene and its regulation by GH in *lit/lit* mice have been studied by Mathews *et al.* (547) using the solution hybridization technique. The highest concentration of IGF-I mRNA is found in the liver, but there are also detectable levels in several other tissues. In addition, infusion of rGH for 9 days results in a 10-fold increase in the IGF-I mRNA in the liver. Results from Chapter Three of this thesis showed a marked effect of GH on hepatic concentrations of IGF-I mRNA in 12 month-old-sheep, in agreement with the previous observations in castrated pigs (494) and 9 week-old-lambs (556). Hypothetically, increased levels of IGF-I mRNA can be due to an increased rate of transcription or increased mRNA stability. However, nuclear run-on assays on *lit/lit* mice showed that GH regulation of IGF-I mRNA abundance was at least partially at the transcriptional level and not only due to increased mRNA stability (547). In Chapter Three, the increased levels of hepatic IGF-I mRNA were found to be positively correlated with liver IGF-I protein concentrations, confirming that GH regulation occurred not only at the level of IGF-I mRNA but also at the level of IGF-I protein in the sheep. This observation is further strengthened by the results of *in vivo* investigations of GH-induced tissue IGF-I production in postnatal sheep from Chapter Four of this thesis, which demonstrated a strong GH-dependence of tissue IGF-I in sheep liver, kidney,

spleen, heart and skeletal muscle in response to GH administration. The time course of induction of IGF-I protein was in general agreement with the report by D'Ercole *et al.* (338) for tissue immunoreactive IGF-I in hypophysectomized rats after the intraperitoneal administration of ovine GH.

It was reported previously that the response of plasma IGF-I concentrations to GH injection is blunted in protein-malnourished and fasted rats as compared with fed controls (548, 549), suggesting that such nutritional alterations can result in a GH-resistant state. The dramatic difference in the correlations between liver IGF-I mRNA and IGF-I protein levels from FDGH ($r = 0.93$) and STGH ($r = 0.07$) lambs in the study of Chapter Three indicates that the GH regulation of hepatic IGF-I expression is, to a large extent, dependent on nutritional status. Nutrition therefore plays a critical role in the interaction with GH for regulation of tissue IGF-I production.

Our understanding of the regulatory control of IGF-I production has been greatly enhanced by more detailed genetic analysis. In mammalian species studied, IGF-I is encoded by a single-copy gene and the mature circulating peptide of 70 amino acids contains A and B domains, as originally described for insulin (550, 84). The mature form of IGF-I, however, retains the C peptide and also contains a short carboxyterminal extension, termed the D domain. Both cDNA and genomic sequences suggested the presence of an unexpected additional carboxyterminal E peptide, not originally found in circulating IGF-I. In the case of human, rat and mouse IGF-I, two different E peptides are encoded by

alternately spliced IGF-I mRNAs (550, 85, 553). These E peptides are presumably cleaved off during the processing and maturation of pre-pro-IGF-I.

Of particular interest is the alternate splicing of exons at the 5' end of the gene. Two major classes of mRNA transcripts have been identified in hepatic and non-hepatic tissues, each coding for the same mature peptide but containing different 5'-untranslated regions, derived from transcription initiation at different transcription start sites in exons 1 and 2 (551). The two classes of mRNA transcripts are designated class 1 and class 2 respectively (555). The class 2 transcripts are expressed primarily in liver, whereas the class 1 transcripts are the major transcripts found in all tissues, including liver. The effect of GH on choice of transcription initiation sites was studied in hypophysectomized rats. A marked decrease in the class 2 transcripts was seen in liver following hypophysectomy, with a 10-fold increase after repeated GH injections (552). In contrast, the steady-state levels of the class 1 mRNA transcripts in all tissues studied were significantly less affected by these manipulations. Developmental regulation of these transcripts has similarly been investigated. A post-natal increase in the class 1 transcripts was found to begin before the GH-dependent increase in serum IGF-I, *ie.* prior to post-natal day 20 in the rat. Interestingly, the class 2 transcripts began to increase only between post-natal days 20 and 30, corresponding to the period of the GH-dependent increase in circulating IGF-I and longitudinal growth (554).

Recent research in sheep (496) has further revealed that exon 1 and 2 were

expressed in liver but to a variable extent. Increased dietary energy and protein-induced increased expression from both the class 1 and 2 mRNA transcripts, but the percent increase was at least five-fold greater for the class 2 than class 1 mRNA. GH treatment only stimulated significant increases in expression from the class 2 transcripts. Moreover, muscle IGF-I gene expression was at least 20-fold less than that for the liver and consisted mainly of class 1 transcripts. Muscle total IGF-I mRNA was insensitive to changed nutritional status or to GH treatment (496). These data indicated that exon 2 may be far more sensitive than exon 1 in response to nutritional or GH stimuli in intact animals, and that the relative abundance of these two different mRNA species may vary between liver and non-hepatic tissues. The human IGF-I cDNA probe used in the present study detects all these forms of IGF-I mRNA. It is possible therefore that the differentiated regulation by nutrition or GH is also related to differences in the relative abundance of these different mRNA transcripts but it remains to be investigated. However these genetic analysis suggested differential regulation of IGF-I production in a tissue-specific manner, and possibly further on a 'exon-specific' basis in tissue as well.

Based upon these genetic investigations, the significant increase of IGF-I mRNA and IGF-I protein levels in tissues from the present study appears to be more a reflection of increased expression of the IGF-I class 2 mRNA species induced by GH or nutritional manipulation, but this needs further confirmation.

6.2 Role for IGF; Paracrine/Autocrine or Endocrine?

IGF-I was originally considered to be exclusively of hepatic origin and to reach its sites of action via the blood stream.

The possibility of extra-hepatic production of Sm/IGFs came into focus when Atkison *et al.* (557) and Clemmons *et al.* (558) demonstrated that cultured fibroblasts can synthesize Sm/IGF-like peptides. In addition, it has been demonstrated that IGF-I is produced by several mouse tissues in culture (559), as well as by cultured porcine smooth muscle cells (560). D'Ercole *et al.* (338) showed that GH-treatment *in vivo* increases extractable IGF-I in peripheral tissues, including kidney, liver, heart, lung and testes of hypophysectomized rats. This increase in tissue IGF-I levels occurs prior to an elevation of circulating IGF-I, suggesting that IGF-I is locally synthesized (338). A GH-dependent IGF-I immunoreactivity in the growth plate of hypophysectomized rats was reported also by Nilsson *et al.* (561), indicating a local synthesis of IGF-I in cartilage. The findings in Chapter Four of this thesis that GH administration *in vivo* induced a significant elevation of IGF-I in both hepatic and extra-hepatic tissues, and that the rise in tissue IGF-I concentrations (except muscle: see later) preceded the plasma IGF-I response, were in general agreement with D'Ercole's observations (338), suggesting that a local synthesis of IGF-I in sheep tissues under the influence of GH. This result is in favour of a paracrine/autocrine mechanism also in sheep tissues. However, measurement of immunoreactive IGF-I cannot distinguish between locally produced and serum-delivered IGF-I. Therefore, the

possibility of GH-stimulated uptake of circulating IGF-I, *eg.* due to increased receptor concentration, can not be excluded. However, the findings in Chapters Three and Four that there is GH-dependent IGF-I production mRNA in both hepatic and extra-hepatic sheep tissues support the concept of local IGF-I production. Moreover, Wroblewski *et al.* (562) demonstrated that IGF-I immunoreactivity is located almost exclusively in the endoplasmic reticulum and Golgi system in the cytoplasm of epiphyseal chondrocytes, further indicating a local synthesis of IGF-I. Key evidence favouring an autocrine/paracrine model of IGF-I action is listed in Section 1.4.

The results of several studies suggest that locally produced IGF-I is of importance for the growth promoting effect of GH. Addition of monoclonal antibodies to IGF-I to the medium of cultured smooth muscle cells or human fibroblasts blocks the effects of hGH on cell replication (563, 560). Moreover, the stimulatory effect of locally administered GH on longitudinal bone growth is completely abolished if antibodies to IGF-I are co-infused with GH (564).

Although it is not conclusive, yet now widely accepted that the principal actions of IGF-I in growth and development occur close to its site of synthesis by autocrine/paracrine mechanisms. Evidence supporting this contention has led to a major re-evaluation and gradual erosion of the original somatomedin hypothesis of GH action and raises important questions about the biological significance of circulating IGF-I. Nonetheless arguments supporting local production and action of IGF-I do not exclude the possibility of classical

endocrine roles for the hormone. Key evidence favouring an endocrine role for IGF-I remains still firm (Section 1.4). For instance, Goldstein and Phillips (499) reported that rat liver contains a pituitary- and nutrition-dependent, high molecular weight form of IGF-I exhibiting both immune- and bio-activity parallel to that of recombinant and circulating IGF-I. In contrast, diaphragm, spleen and kidney contained no significant IGF bio-activity, and only 8, 17 and 32% of IGF-I immunoreactivity of normal liver respectively. The rat liver appears to contain 50% of the IGF-I in the circulation, and the fluctuations in hepatic extract IGF-I immunoreactivity paralleled change in serum IGF-I during fasting and refeeding of rats. Results from Chapter Four of this thesis revealed that total liver IGF-I was raised by 44 μg after administration of GH, which is 4-fold more than the corresponding increase in kidney, 13-fold more than that in heart and 30-fold more than that in spleen. There is no doubt that liver is a major contributor of circulating IGF-I, although data in this study suggested that IGF-I is synthesised in multiple sheep tissues as well. The current study also showed that one third of the total body IGF-I content is retained in sheep plasma. This is in general agreement with the data of Goldstein and Phillips above and previous estimations, by D'Ercole *et al.* (338) and by Schwander *et al.* (339), that hepatic production of IGF-I is sufficient to maintain a relatively large amount of plasma IGF-I present in the circulation (about 50% of total body content in rat). It is very hard to explain why is such a large amount of plasma IGF-I produced if it is en route for clearance only? Does not circulating IGF-I have any biological significance in growth and development at all?

It was interesting to find out in Chapter Four that the muscle IGF-I response to GH reached significance at the same time as circulating IGF-I, which may represent a late response to local production but may also implicate a possible endocrine role at least for part of the IGF-I present in muscle. This implication is supported by Hodgkinson's study on the infusion of recombinant N-Met-IGF-I from plasma into tissues and lymph in sheep (497), and by the study of Pell and co-worker on tissue IGF-I expression in response to changed growth hormone and nutritional status at transcriptional level in sheep (496) (refer to section 4.4).

Furthermore, data from Chapter Five of this thesis demonstrated that GH not only stimulates IGF-I production but also plays a pivotal role in regulation of circulating levels and tissue availability of IGF-I (*ie.* tissue distribution of IGF-I) by affecting IGF-I and its binding proteins. This reflects the possibility of the typical classic endocrine mechanism by which GH regulates growth or development via blood stream.

However, results in favour of an endocrine role for IGF-I in this thesis do not contradict those in favour of an autocrine/paracrine fashion in the same study. Data in Chapter Four, moreover, may suggest a possibility for both models in the muscle, although the relative contribution or importance of each is not certain. As discussed before, evidence of a molecular biological basis for autocrine/paracrine versus endocrine IGF-I suggests that there is alternate splicing of exons at the 5' end of the IGF-I gene producing two major classes of mRNA transcripts in hepatic and non-hepatic tissues. While each transcript was

found to encode the same mature peptides, they were found to have distinct transcription initiation sites and one transcript was predominantly expressed in liver and the other in a wide range of non-hepatic tissues and liver as well. Hypophysectomy was associated with a marked decrease in the liver-specific transcript while GH was found to induce it. Developmental regulation of the transcripts was also observed with appearance of the liver-specific species coinciding with the onset of GH dependence around post-natal day 20-30 in the rat. In contrast, the other transcript was found to be significantly less affected by GH, but regulated by a number of other factors, such as oestrogen in ovary, placenta and bone; gonadotropins in ovary and testes; and insulin in the aorta (501). Based on these and related observations it has been proposed that the liver-specific transcript, regulated primarily by GH, encodes 'endocrine' IGF while the other encodes 'autocrine/paracrine' growth factor (351). It appears that the two different transcripts of IGF-I mRNA may be of different functional significance. However, they both are expressed in all tissues, suggesting that neither autocrine/paracrine nor endocrine modes of IGF action is exclusive, but rather both occur and are necessary for normal growth and development.

Since IGF-I gene transcription, translation or post-translation modification can be regulated by a number of factors on a tissue-specific basis, the relative contribution or importance of local versus liver production of the growth factor would be closely related to this integrated and very complex regulatory system, depending upon numerous factors.

Conclusion

The results of the present study are compatible with the concept of both local and classic central production of IGF-I under the influence of GH, and therefore do not exclude either an autocrine/paracrine or an endocrine mechanism for IGF-I action. The relative importance or contribution of each may be related to a tissue-specific fashion of IGF-I gene expression which is differentially regulated by GH, nutrition and numerous other factor at multiple levels. Particularly at the transcriptional step the complexity of the gene transcription and large number of different IGF-I mRNA species have received considerable attention, the precise evaluation relies on future study.

REFERENCES

- 1 Widdowson, E.M. (1980) Definitions of growth. *In*: Lawrence, T.L.J. ed. Studies in the Agricultural and Food Sciences. Growth in animals. Butterworths, London. pp 1-10.
- 2 Cheek, D.B. (1975) Fetal and postnatal cellular growth: Hormones and Nutrition. Wiley, New York.
- 3 Gluckman, P.D. (1986) The role of pituitary hormones, growth factors and insulin in the regulation of fetal growth. *In*: Clark, J.R. ed. *Oxford Reviews of Reproductive Biology*, Clarendon Press, Oxford. 8: 1-11.
- 4 Gluckman, P.D., Breier, B.H. and Davis, S.R. (1987) Physiology of the somatotrophic axis with particular reference to the ruminant. *J. Dairy Sci.* 70: 442-448.
- 5 Isaksson, O.G.P., Lindahl, A., Wilson, A. and Isgaard, J. (1987) Mechanisms of the stimulatory effect of growth hormone on longitudinal bone growth. *Endocr. Rev.* 8(4): 426-438.
- 6 Li, C.H. Evans, H.M. and Simpson, M.E. (1945) Isolation and properties of the anterior hypophyseal growth hormone. *J. Biol. Chem.* 159: 353-366.
- 7 Wallis, M. (1978) The chemistry of pituitary growth hormone, prolactin and related hormones and its relationship to biological activity. *In*: Weinstein, B. ed. Chemistry and Biochemistry of Amino Acids, Peptides and Proteins: a Survey of Recent Developments. Marcel Dekker, New York. 5: 213-320.
- 8 Li, C.H. and Papkoff, H. (1956) Preparation of prolactins and growth hormones from human and monkey pituitary glands. *Science* 124: 1293-1294.

- 9 Lewis, U.J., Singh, R.N.P., Tutwiler, G.F., Sigel, M.B., Vanderlaan, E.F. and Vanderlaan, W.P. (1980) Human growth hormone: a complex of proteins. *Rec. Prog. Horm. Res.* **36**: 477-504.
- 10 Lewis, U.J. (1984) Variants of growth hormone and prolactin and their post-translational modifications. *Ann. Rev. Physiol.* **46**: 33-42.
- 11 Wallis, M. (1988) Mechanism of action of growth hormone. *In*: Cooke, B.A., King, R.G.B. and van der Molan, H.J. eds. Hormones and their Actions, Part III. Elsevier Science Publications, BV (Biomedical Div.) pp 265-294.
- 12 Nicoll, C.S., Mayer G.L. and Russell, S.M. (1986) Structural features of prolactins and growth hormones that can be related to their biological properties. *Endocr. Rev* **7**(2): 169-203.
- 13 Lesniak, M.A. and Roth, J. (1976) Regulation of receptor concentration by homologous hormone: effect of human growth hormone on its receptor in IM-9 lymphocytes. *J. Biol. Chem.* **251**(12): 3720-3729.
- 14 Wallis, M. (1989) Species specificity and structure/function relationships of growth hormone. *In*: Heap, R.B., Prosser, C.G. and Lamming, G.E. eds. Biotechnology of Growth Regulation. Butterworths, London. pp 3-14.
- 15 Abdel-Meguid, S.S., Sheih, H.S., Smith, W.W., Dayringer, H.E., Violand, B.N. and Bentle, L.A. (1987) Three dimensional structure of a genetically engineered variant of porcine growth hormone. *Proc. Natl. Acad. Sci. USA* **84**: 6434-6437.
- 16 Guyda, H., Colle, R.F. and Hardy, J. (1973) Histologic, ultrastructural and hormonal characterization of a pituitary tumor secreting both hGH and prolactin. *J. Clin. Endocrinol.* **36**: 531-547.
- 17 Fiddes, J.C., Seeburg, P.H., DeNoto, F.M., Hallewell, R.A., Baxter, J.D. and Goodman, H.M. (1979) Structure of genes for human growth

- hormone and chorionic somatomammotropin. *Proc. Natl. Acad. Sci. USA* **76**(9): 4294-4298.
- 18 Miller, W.L. and Eberhardt, N.L. (1988) Structure and evolution of the growth hormone gene family. *Endocr. Rev.* **2**(2): 97-128.
 - 19 Linzer, D.I.H. and Nathans, D. (1984) Nucleotide sequence of a growth related mRNA encoding a member of the prolactin-growth hormone family. *Proc. Natl. Acad. Sci.* **81**: 4255-4259.
 - 20 Frankenne, F., Scippo, M., Van Beeumen, J., Igout, A. and Hennen, G.(1990) Identification of placental human growth hormone as the growth-V gene expression product. *J. Clin. Endocrinol. Metab.* **71**(1): 15-18.
 - 21 Tannenbaum, G.S. and Martin, J.B. (1976) Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology* **98**: 562-570.
 - 22 Bassett, J.M. (1974) Diurnal patterns of plasma insulin, growth hormone, corticosteroid and metabolite concentrations in fed and fasted sheep. *Austr. J. Biol. Sci.* **27**: 167-181.
 - 23 Rawlings, S.R. and Mason, W.T. (1989) Modulation of growth hormone release: from CNS to the secretory event. *In*: Heap, R.B., Prosser, C.G. and Lemming, G.E. eds. *Biotechnology in Growth Regulation*. Butterworths, London. pp 35-46.
 - 24 Robinson, I.C.A.F. and Clark, R.G. (1987) The secretory pattern of GH and its significance for growth in the rat. *In*: Isaksson, O.G.P., Binder, C., Hall, K. and Hokfelt, B. eds. *Growth Hormone - Basic and Clinical Aspects* Elsevier Science Publishers (Biomedical Division) pp 109-127.
 - 25 Reichlen, S. (1983) Somatostatin. *In*: Kreiger, D.T., Brownstein, M. and Martin, J.B. eds. *Wiley, New York*. pp 711-752.

- 26 Patel, Y.C. and Srikant, C.B. (1986) Somatostatin-mediation of adenohipophyseal secretion. *Ann. Rev. Physiol.* **48**: 551-567.
- 27 Tannenbaum, G.S. (1985) Physiological role of somatostatin in regulation of pulsatile growth hormone secretion. *In*: Patel, Y.C. and Tannenbaum, G.S. eds. *Advances in Experimental Medicine and Biology*. Vol 188, Somatostatin. Plenum, New York. pp 229-259.
- 28 Guillemin, R., Brazeau, P., Bohlen, P., Esch, F., Ling N. and Wehrenberg, W.B. (1982) Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. *Science* **218**: 585-587.
- 29 Ling, N., Esch, F., Bohlen, P., Brazeau, P. and Wehrenberg, W.B. (1984) Isolation, primary structure and synthesis of human hypothalamic somatocrinin: growth hormone releasing factor. *Proc. Natl. Acad. Sci. USA* **81**: 4302-4306.
- 30 Gelato, M.C. and Merrian, G.R. (1986) Growth hormone releasing hormone. *Ann. Rev. Physiol.* **48**: 568-591.
- 31 Wehrenberg, W.B., Baird, A., Zeytin, F., Bohlen, P., Ling, N., Ying, S. and Guillemin, R. (1985) Physiological studies with somatocrinin, a growth hormone releasing factor. *Ann. Rev. Pharmacol. Toxicol.* **26**: 463-483.
- 32 McAtee, J.W. and Trenkle, A. (1971) Effect of feeding, fasting and infusion of energy substrates on plasma growth hormone levels in cattle. *J. Anim. Sci.* **33**(3): 612-616.
- 33 Trenkle, A. (1989) Influence of feeding on growth hormone secretion and response to growth hormones releasing factor in sheep. *J. Nutr.* **119**: 61-65.
- 34 Hart, I.C.P., Chadwickote, Coert A., James, S. and Simmonds, A.D. (1985) Effect of different growth hormone releasing factors on the concentrations of growth hormone, insulin and metabolites in the

- plasma of sheep maintained in positive and negative energy balance. *J. Endocrinol.* **105**: 113-119.
- 35 Moseley, W.M., Alaniz, G.R., Claflin, W.H. and Krabill, L.F. (1988) Food intake alters the serum growth hormone response to bovine growth hormone releasing factor in meal fed Holstein steers. *J. Endocrinol.* **117**: 253-259.
- 36 Brenton, B., Chabanet, C. and Pelletier, J. (1987) Influence of photoperiods and protein diet on growth hormone secretion in rams. *Proc. Soc. Exp. Biol. Med.* **185**: 312-317.
- 37 Lormore, M.J., Muller, C.D., Reaver, D.R. and Griel, L.C. (1990) Early lactation responses of dairy cows fed diets high in energy and protein. *J. Dairy Sci.* **73**: 3237-3247.
- 38 Abribat, T., Lapierre, H., Dubreuil, P., Pelletier, G., Gaudreau, P., Brazeau, P. and Petitderc, D. (1990) Insulin-like growth factor-I concentration in Holstein female cattle: variations with age, stage of lactation and growth hormone releasing factor administration. *Dom. Anim. Endocrinol.* **7**(1): 93-102.
- 39 Davis, S.R., Farr, V.C., Gluckman, P.D. and Breier, B.H. (1989) A note on annual variation of plasma insulin-like growth factor-I concentration and growth rates of Jersey heifers differing in genetic merit for milk production. *Anim. Prod.* **48**: 467-470.
- 40 Davis, S.C., Ohlson, D.L., Kindt, J. and Aufinson, N.S. (1977) Episodic growth hormone secretion in sheep: relationship to gonadal steroid hormones. *Am. J. Physiol.* **233**: E519.
- 41 Keeler, D.G., Smith, V.G., Coulter, G.H. and King, G.J. (1979) Serum growth hormone concentration in Hereford and Angus calves: effect of breed, sire, sex, age, age of dam and diet. *Can. J. Anim. Sci.* **59**: 367-382.

- 42 Breier, B.H., Bass, J.J., Butler, J.H. and Gluckman, P.D. (1986) The somatotrophic axis in young steers: influence of nutritional status on pulsatile release of growth hormone and circulating concentrations of insulin-like growth factor-I. *J. Endocrinol.* **111**: 209-215.
- 43 Gluckman, P.D., Mueller, P.L., Kaplan, S.L., Rudolph, A.M. and Grumbach, M.M. (1979) Hormone ontogeny in the ovine fetus. I. Circulating growth hormone in mid and late gestation. *Endocrinology* **104**(1): 162-168.
- 44 Gluckman, P.D. (1984) Functional maturation of the neuroendocrine system in the perinatal period: Studies of the somatotrophic axis in the ovine fetus. *J. Dev. Physiol.* **6**: 301-322.
- 45 Page, M.D., Dieguez, C. and Scanlon, M.F. (1989) Neuroregulation of growth hormone secretion. *In*: Heap, R.B., Prosser, C.G. and Lamming, G.E. eds. *Biotechnology in Growth Regulation*. Butterworths, London. pp 47-56.
- 46 Yamashita, S. and Melmed, S. (1986) Insulin-like growth factor-I action on rat anterior pituitary cells: suppression of growth hormone secretion and messenger ribonucleic acid levels. *Endocrinology* **118**(1): 176-182.
- 47 Waters, M.J., Spencer, S.A., Leung, D., Hammonds, R.G., Cachianes, G., Henzel, W.J., Wood, W.J., Barnard, R., Quirk, P. and Hamlin, G. (1989) Growth hormone receptors and binding proteins. *In*: Heap, R.B., Prosser, C.G. and Lamming, G.E. eds. *Biotechnology in Growth Regulation*. Butterworths, London. pp 15-26.
- 48 Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J. and Wood, W.I. (1987) Growth hormone receptor and serum binding protein: Purification,

- cloning and expression. *Nature* **330**: 537-543.
- 49 Hughes, J.P. and Friesen, H.G. (1985) The nature and regulation of the receptors for pituitary growth hormone. *Ann. Rev. Physiol.* **47**: 469-482.
- 50 Foster, C.M., Shafer, J.A., Rozsa, F.W., Wang, X., Lewis, S.B., Renken, D.A., Natale, J.E., Schwartz, J. and Carter-Su, C. Growth hormone promoted tyrosyl phosphorylation of growth hormone receptors in murine 3T3-F442A fibroblasts and adipocytes. *Biochemistry* **27**: 326-334.
- 51 Barnard, R. and Waters, M.J. (1986) Serum and liver cytosolic growth hormone-binding proteins are antigenically identical with liver membrane 'receptor' types 1 and 2. *Biochem. J.* **237**: 885-892.
- 52 Gluckman, P.D., Butler, J.H. and Elliot, T.B. (1983) The ontogeny of somatotrophic binding sites in ovine hepatic membranes. *Endocrinology* **112**(5): 1607-1612.
- 53 Breier, B.H., Gluckman, B.D., McCutcheon, S.N. and Davis, S.R. (1991) Physiological responses to somatotropin in the ruminant. *J. Dairy Sci.* **74**(Suppl. 2): 20-34.
- 54 Breier, B.H., Gluckman, P.D. and Bass, J.J. (1988) The somatotrophic axis in young steers: influence of nutritional status and oestradiol-17B on hepatic high and low affinity somatotrophic binding sites. *J. Endocrinol.* **116**: 169-175.
- 55 Bass, J.J., Oldham, J.M., Hodgkinson, S.C., Fowke, P.J., Sauerwein, H., Molan, P., Breier, B.H. and Gluckman, P.D. (1991) Influence of nutrition and bovine growth hormone on hepatic GH binding, insulin-like growth factor-I and growth of lambs. *J. Endocrinol.* **128**: 181-186.
- 56 Rao, A.J. and Ramachandran, J. (1977) Growth hormone and the

- regulation of lipolysis. *In*: Li, C.H. ed. Hormonal Proteins and Peptides, Vol IV. Academic Press, London. pp 43-61.
- 57 Gluckman, P.D. and Breier, B.H. (1989) Regulation of the growth hormone receptor. *In*: Heap, R.B., Prosser, C.G. and Lamming, G.E. eds. Biotechnology in Growth Regulation. Butterworths, London. pp 27-34.
- 58 Hart, I.C. and Johnsson, I.D. Growth hormone and growth in meat producing animals. *In*: Buttery, P.J., Lindsay, D.B. and Haynes, N.B. eds. Control and Manipulation of Animal Growth. Butterworths, London. pp 135-159.
- 59 Vernon, R.G. and Flint, D.J. (1989) Role of growth hormone in the regulation of adipocyte growth and function. *In*: Heap, R.B., Prosser, C.G. and Lamming G.E. eds. Biotechnology in Growth Regulation. Butterworths, London. pp 3-14.
- 60 Etherton, T.D., Sorensen, M.T., Coleman M.E. and Chaudhuri, S. (1989) Mechanisms by which somatotropin alters muscle growth and adipose tissue accretion. *Proceedings of the 42nd Annual Reciprocal Meat Conference, Guelph, Canada.* pp 83-89.
- 61 Breier, B.H., Gluckman, P.D., McCutcheon, S.N. and Davis, S.R. (1990) Physiological responses to somatotropin in the ruminant. *J. Dairy Sci.* **73**: 1-15.
- 62 Johnson, I.D. and Hart, I.C. (1986) Manipulation of milk yield with growth hormone. *In*: Haresign, W. and Cole, D.J.A. eds. Recent Advances in Animal Nutrition. Butterworths, London. Ch 8.
- 63 Bauman, D.E. and McCutcheon, S.N. (1986) The effects of growth hormone and prolactin on metabolism. *In*: Milligan, L.P., Grovum, W.L. and Dobson, A. eds. Control of Digestion and Metabolism in Ruminants. Prentice Hall, Englewood Cliffs, New Jersey. Ch 23.

- 64 Korner, A. and Hogan, B.C.M. (1972) *In: Growth and growth hormone* (Peak, A. and Muller, E.E., eds). *Excerpta Medica Amsterdam* pp 98-105.
- 65 Davidson, M.B. (1987) Effect of growth hormone on carbohydrate and lipid metabolism. *Endocr. Rev.* **8(2)**: 115-131.
- 66 Daughaday, W.H. (1989) A personal history of the origin of the somatomedin hypothesis and recent challenges to its validity. *Perspect. Biol. Med.* **32(2)**: 194-211.
- 67 Smith, P.E. (1927) The disabilities caused by hypophysectomy and their repair. *JAMA* **83**: 158-161.
- 68 Grosbeck, M.D., Parlow, A.F. and Daughaday, W.F. (1987) Stimulation of supranormal growth in prepubertal, adult plateaued and hypophysectomized female rats by large doses of rat growth hormone: physiological effects and adverse consequences. *Endocrinology* **120**: 1963-1975.
- 69 Greenspan, F.S., Li, C.H., Simpson, M.E. and Evans, H.M. (1949) Bioassay of hypophyseal growth hormone: the tibia test. *Endocrinology* **45**: 455-463.
- 70 Cheek, D.B. and Hill, D.E. (1974) Effect of growth hormone on cell and somatic growth. *In: Knobil, E. and Sawyer, W.H. eds. Handbook of Physiology. American Physiological Society, Bethesda, Md. Section 7, Vol 4, Part 2, pp 159-185.*
- 71 Denko, C.W. and Bergenstal, D.M. (1975) The effect of hypophysectomy and growth hormone on ³⁵S fixation in cartilage. *Endocrinology* **57**: 76-86.
- 72 Salmon, W.D. and Daughaday, W.H. (1957). A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J. Lab. Clin. Med.* **49(6)**: 825-836.

- 73 Isaksson, O.G.P., Eden, S. and Jansson, J.O. (1985) Mode of action of pituitary growth hormone on target cells. *Ann. Rev. Physiol.* **47**: 483-499.
- 74 Green, H., Morikawa, M. and Nixon, T. (1985) A dual effector theory of growth hormone action. *Differentiation* **29**: 195-198.
- 75 Green, H., Zekulak, K. and Dijan, P. (1987) On the action of growth hormone as revealed by the study of adipose conversion. *In*: Isaksson, O., Binder, C., Hall, K. and Hokfelt, B. eds. *Growth Hormone Basic and Clinical Aspects*. Elsevier, Amsterdam. pp 307-320.
- 76 Daughaday, W.H., Hall, K. and Raben, M.S. (1972) Somatomedin: a proposed designation for the 'sulfation factor'. *Nature* **235**: 107-109.
- 77 Pierson, R.W.Jr. and Temin, H.M. (1972) The partial purification from calf serum of a fraction with multiplication-stimulating activity from chicken fibroblasts in cell culture and with non-suppressible insulin-like activity. *J. Cell Physiol.* **79**: 319-392.
- 78 Dulak, N.C. and Temin, H.M. (1973) Multiplication-stimulating activity for chicken embryo fibroblasts from rat liver cell-conditioned medium: a family of small polypeptides. *J. Cell Physiol.* **81**: 153-160.
- 79 Froesch, E.R., Burgi, H., Ramseier, E.B., Bally P. and Labhart, A. (1963) Antibody-suppressible and non-suppressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precision and specificity. *J. Clin. Invest.* **42**(11): 1816-1834.
- 80 Humbel, R.E.(1984) Insulin-like growth factors, somatomedins, and multiplication stimulating activity *In*: Li, C.H. ed. *Hormonal*

Proteins and Peptides, Vol 12, Academic Press, London. pp 57-79.

- 81 Rinderknecht, E. and Humbel, R.E. (1978) The amino acid sequence of human insulin-like growth factor-I and its structural homology with proinsulin. *J. Biol. Chem.* **253**(8): 2769-2776.
- 82 Rinderknecht, E. and Humbel, R.E. (1978) Primary structure of insulin-like growth factor-II. *FEBS Lett.* **89**: 283-286.
- 83 Rubins, J.S., Mariz, I., Jacobs, J.W., Daughaday, W.H. and Bradshaw, R.A. (1982) Isolation and partial sequence analysis of rat basic somatomedin. *Endocrinology* **110**: 734-740.
- 84 Shimatsu, A. and Rotwein, P. (1987) Mosaic evolution of the insulin-like growth factors: organization, sequence and expression of the rat insulin-like growth factor-I gene. *J. Biol. Chem.* **262**: 2894-2900.
- 85 Bell, G.I., Stempien, M.M., Fong, N.M. and Rall, L.B. (1986) Sequences of liver cDNA encoding two different mouse insulin-like growth factor-I precursors. *Nucleic Acid Res.* **14**: 7873-7882.
- 86 Honegger, A. and Humbel, R.E. (1986) Insulin-like growth factors-I and -II in fetal and adult bovine serum. Purification, primary structures and immunological crossreactivities. *J. Biol. Chem.* **261**(2): 569-575.
- 87 Francis, G.L., McNeil, K.A., Wallace, J.C., Ballard, F.J. and Owens, P.C. (1989) Sheep insulin-like growth factors-I and II: sequences, activities and assays. *Endocrinology* **124**(3): 1173-1183.
- 88 Tavakkol, A., Simmen, F.A. and Simmen, R.C.M. (1988) Porcine insulin-like growth factor-I: complementary deoxyribonucleic acid cloning and uterine expression of messenger ribonucleic acid encoding evolutionarily conserved IGF-I peptides. *Mol. Endocrinol.* **2**: 674-681.
- 89 Dawe, S.R., Francis, G.L., McNamara, P.J., Wallace, J.C. and Ballard, F.J. (1988) Purification, partial sequence and properties of chicken

- insulin-like growth factors. *J. Endocrinol.* **117**: 173-181.
- 90 Marquardt, H., Todaro, G.J., Henderson L.E. and Oroszlan, S. (1981)
Purification and primary structure of a polypeptide with
multiplication stimulating activity from rat liver cell cultures. *J.*
Biol. Chem. **256**(13): 6859-6865.
 - 91 Stempien, M.M., Fong, N.M., Rall, L.B. and Bell, G.I. (1986) Sequence
of a placental DNA encoding the mouse insulin-like growth
factor-II precursor. *DNA* **5**: 357-361.
 - 92 Jansen, M., van Schaik, F.M.A., Richert, A.T., Bulloch, B., Woods, D.E.,
Gabbay, K.M., Nussbaum, A.L., Sussenbach, J.S. and Van den
Brande, J.L. (1983) Sequence of cDNA encoding human
insulin-like growth factor-I precursor. *Nature* **306**: 609-611.
 - 93 Rotwein, P (1986) Two insulin-like growth factor messenger RNAs are
expressed in human liver. *Proc. Natl. Acad. Sci. (USA)* **83**: 77-81.
 - 94 Irminger, J.C., Rosen, K.M., Humbell, R.E. and Villa Komaroff, L. (1987)
Tissue-specific expression of insulin-like growth factor-II mRNAs
with distinct 5' untranslated regions. *Proc. Natl. Acad. Sci. (USA)*
84: 6330-6334.
 - 95 Sara, V.R. and Hall, H. (1990) Insulin-like growth factors and their
binding proteins. *Physiol. Rev.* **70**(3): 591-614.
 - 96 Rotwein, P., Pollock, K.M., Didier, D.K. and Krivi, G.G. (1986)
Organization and sequence of the human insulin-like growth
factor-I gene. Alternative RNA processing produces two
insulin-like growth factor-I precursor peptides. *J. Biol. Chem.*
261:4828-4832.
 - 97 Bell, G.I., Gerhard, D.S., Fong, N.M., Sanchez-Pescador, R. and Rall,
L.B. (1985) Isolation of the human insulin-like growth factor
genes: insulin-like growth factor-II and insulin genes are

- contiguous. *Proc. Natl. Acad. Sci. (USA)* **82**:6450-6454.
- 98 Daughaday, W.H. and Rotwein, P. (1989) Insulin-like growth factors I and II. Peptide messenger ribonucleic acid and gene structures, serum and tissue concentrations. *Endocr. Rev.* **10**(1): 68-91.
 - 99 Simmen, F.A. (1991) Expression of the insulin-like growth factor-I gene and its products: complex regulation by tissue specific and hormonal factors. *Dom. Anim. Endocrinol.* **8**(2): 165-178.
 - 100 Zumstein, P.P., Luthi, C. and Humbel, R.E. (1985) Amino acid sequence of a variant proform of insulin-like growth factor-II. *Proc. Natl. Acad. Sci. (USA)* **82**: 3169-3172.
 - 101 Blum, W.F., Ranke, M.B. and Bierich, J.R. (1986) Isolation and partial characterization of 6 somatomedin-like peptides from human plasma Cohn fraction IV. *Acta Endocrinol. (Copenhagen)* **111**: 271-284.
 - 102 Blum, W.F., Ranke, M.B., Lechner, B. and Bierich, J.R. (1987) The polymorphic pattern of somatomedins during human development. *Acta Endocrinol. (Copenhagen)* **116**: 445-451.
 - 103 Sara, V.R., Carlsson-Skwirut, C., Andersson, C., Hall, E., Sjogren, B., Holmgren, A. and Jornvall, H. (1986) Characterization of somatomedins from human fetal brain: identification of a variant form of insulin-like growth factor-I. *Proc. Natl. Acad. Sci. (USA)* **83**: 4904-4907.
 - 104 Carlsson-Skwirut, C., Jornvall, H., Holmgren, A., Andersson, C., Bergman, T., Lundquist, G., Sjogren, B. and Sara, V.R. (1986) Isolation and characterization of variant IGF-I and IGF-II from adult human brain. *FEBS Lett.* **201**: 46-50.
 - 105 Buell, G., Schulz, M.F., Selzer, G., Chollet, A., Moura, N.R., Semon, D., Escanez, S. and Kawashima, E. (1985) Optimizing the expression

- in *E. coli* of a synthetic gene encoding somatomedin-c/IGF-I. *Nucleic Acid Res.* **13**: 1923-1938.
- 106 Marston, F.A.O. (1986) Purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem J.* **240**: 1-12.
- 107 Meng, H., Burleigh, B.D. and Kelly, G.M. (1988) Reduction studies on bacterial recombinant somatomedin-c/insulin-like growth factor-I. *J. Chromatogr.* **443**: 183-192.
- 108 Gellerfors, P., Axelsson, K., Helander, A., Johansson, S., Kenne, L., Lindvuist, S., Pavlu, B., Skottner, A. and Frykland, L. (1989) Isolation and characterization of a glycosylated form of human insulin-like growth factor-I produced by *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264**(19) 11444-11449.
- 109 Jasper, H. Martinez, A. and Heinrich, J. (1983) Somatomedin activity in patients with growth retardation due to hypopituitarism or familial-constitution growth delay. *In*: Insulin-like growth factors/somatomedins. Basic Chemistry, Biology, Clinical Importance. Spencer, E.M. Ed. de Gruyter, Berlin pp 259-262.
- 110 Rudman, D.G., Kutner, M.H., Blackston, D.R., Cushman, R.A., Bain, R.P. and Patterson, J.H. (1981) Children with normal-variant short stature: treatment with human growth hormone for 6 months. *N. Engl. J. Med.* **305**: 123-131.
- 111 Daughaday, W.H., Cryer, P.E. and Mariz, I.K. (1983) Somatomedin and GH measurements in acromagaly. *In*: Insulin-like growth factors/Somatomedins. Basic Chemistry, Clinical Importance, Spencer E.M. Ed. de Gruyter, Berlin pp 277-284.
- 112 Copeland, K.C., Underwood, L.E. and Van Wyk, J.J. (1980) Induction of immunoreactive somatomedin-c in human serum by growth hormone: dose response relationships and effect on

- chromatographic profiles. *J. Clin. Endocrinol. Metab.* **50**: 690-697.
- 113 Hua, K.M., Bass, J., Ord, R., Hodgkinson, S., Spencer, G and Molan, P.
(1991) Regulation of ovine plasma and tissue IGF-I by nutrition and GH. *Proceedings of the New Zealand Society of Endocrinology*, **Abstract 18**.
- 114 Bala, R.M., Lopatoka, J., Leung, A., McCoy, E. and 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. *J. Clin. Endocrinol. Metab.* **52**: 508-512.
- 115 Sibergeld, A., Litwin, A., Brudris, S., Varsana, I. and Laron, Z. (1986)
Insulin-like growth factor-I in healthy children, adolescents and adults as determined by a radioimmunoassay specific for the synthetic 53-70 region. *Clin. Endocrinol. (Oxford)* **25**: 67-72.
- 116 Rosenfeld, R.L. and Furlanetto, R. (1985) Physiologic induction of puberty with testosterone or estradiol increases plasma somatomedin-C. *J. Pediatr.* **107**: 415-421.
- 117 Phillips, L.S. and Vassilopoulou-Sellin, M.D. (1979) Nutritional regulation of somatomedin. *Am. J. Clin. Nutr.* **32**: 1082-1096.
- 118 Unterman, T.G., Vazquez, R.M., Slas, A.J., Martyn, P.A. and Phillips, L.S.
(1985) Nutrition and somatomedin XIII. Usefulness of somatomedin-C in nutritional assessment. *Am. J. Med.* **78**: 228-234.
- 119 Phillips, L.S. and Drawski, A.T. (1977) Nutrition and somatomedin III. Diabetic control, somatomedin and growth in rats. *Diabetes* **26**: 864-869.
- 120 Pell, J.M., Blake, L.A., Elcock, C., Hathorn, D.T., Jones, A.R., Morrell, D.J. and Simmonds, A.D. (1987) Effect of growth hormone on

- IGF-I concentrations, body composition and growth in lambs. *J. Endocrinol.* **112 (Suppl.) Abstract 63.**
- 121 Maes, M., Ketelslegers, J. and Underwood, L.E. (1986) Low serum somatomedin-C in insulin-dependent diabetes and malnutrition: evidence for a post-receptor mechanism. *Endocrinology* **118**: 377-382.
 - 122 Kogawa, M., Takano, K., Hizuka, N., Asakawa, K. and Shizume, K. (1982) Effect of GH and insulin on generation of somatomedin by perfused rat liver. *Endocrinol. Jpn.* **29**: 141-147.
 - 123 Scott, C.D. and Baxter, R.C. (1986) Production of insulin-like growth factor-I and its binding protein in rat hepatocytes cultured from diabetic and insulin-treated diabetic rats. *Endocrinology* **119(5)**: 2346-2351.
 - 124 Phillips, L.S. and Scholz, T.D. (1982) Nutrition and somatomedin IX. Blunting of insulin-like activity by a specific inhibitor in diabetic rat serum. *Diabetes* **31**: 97-106.
 - 125 Kuffer, A.D. and Herington, A.C. (1984) Partial purification of a specific inhibitor of the insulin-like growth factors by reversed phase HPLC. *J. Chromatogr.* **336**: 87-92.
 - 126 Ooi, G.T. and Herington, A.C. (1986) Covalent cross-linking of insulin-like growth factor-I to a specific inhibitor from human serum. *Biochem. Biophys. Res. Commun.* **137(1)**: 411-417.
 - 127 Phillips, L.S., Goldstein, S., Klein, J.D., Sulinski, J.M., Wang, Y., Alam, S., El-Kabbi, I.M., Ziemer, D.C., Pollet, R.J. and Stults, J. (1990) Uremic serum IGF/insulin inhibitor. *Proceedings of the 72nd Annual Meeting of The American Endocrine Society, Atlanta, Ga., Abstract 574.*
 - 128 Hannon, K., Gronowski, A. and Trenkle, A. (1991) Relationship of liver

- and skeletal muscle IGF-I mRNA to plasma GH profile production of IGF-I by liver, plasma IGF-I concentrations and growth rates of cattle. *Proceedings of the Society for Experimental Biology and Medicine* **196**: 155-163.
- 129 Andersson, J., Billig, H. and Frykland, L. (1986) Localization of IGF-I in adult rats. Immunohistochemical studies. *Acta Physiol. Scand.* **126**: 311-312.
- 130 D'Ercole, A.J. and Underwood, L.E. (1987) Estimation of tissue concentrations of somatomedin-C/insulin-like growth factor-I. *Methods in Enzymology* **146**: 227-233.
- 131 Hansson, H.A., Nilsson, A. and Isgaard, J. (1988) Immunohistochemical localization of insulin-like growth factor-I in the adult rat. *Histochem.* **89**: 403-410.
- 132 Lund, P.K., Moats-Staats, B.M. and Hynes, M.A. (1986) Somatomedin-C/insulin-like growth factor-I and IGF-II mRNAs in rat fetal and adult tissues. *J. Biol. Chem.* **261**: 14539-14544.
- 133 Hill, D.J. and Han, V.K.M. (1991) Paracrinology of growth regulation. *J. Dev. Physiol.* **15**: 91-104.
- 134 Isgaard, J., Moller, C., Isaksson, O.G.P., Nilsson, A., Mathews, L.S. and Norstedt, G. (1988) Regulation of insulin-like growth factor messenger ribonucleic acid in rat growth plate by growth hormone. *Endocrinology* **122**: 1515-1520.
- 135 Emler, L.A. and Schalch, D.S. (1987) Nutritionally-induced changes in hepatic insulin-like growth factor-I (IGF-I) gene expression in rats. *Endocrinology* **120**: 832-834.
- 136 Lowe, J.L.Jr., Adams, M., Werner, H., Roberts, C.T. and Le Roith, D. (1989) Regulation by fasting of rat insulin-like growth factor-I and its receptor: effects of gene expression and binding. *J. Clin. Invest.*

84: 619-626.

- 137 Cohen, K.L. and Nissley, S.P. (1975) Comparison of somatomedin activity in rat serum and lymph. *Endocrinology* **97**: 654-658.
- 138 Breier, B.H., Gallaher, B.W., Milsom, S., Gibson, C. and Gluckman, P.D. (1990) Solutions of potential problems and pitfalls of the radioimmunoassay for insulin-like growth factor-I (IGF-I): measurement of IGF-I in plasma and milk. *Proceedings of the New Zealand Society of Endocrinology. Auckland. Abstract 48.*
- 139 Backstrom, M., Hall, H. and Sara, V. (1984) Somatomedin levels in cerebrospinal fluid from adults with pituitary disorders. *Acta Endocrinol. (Copenhagen)* **107**: 171-178.
- 140 Baxter, R.C., Martin, J.L. and Handelsman, D.J. (1984) Identification of human semen insulin-like growth factor-I/somatomedin-C immunoreactivity and binding protein. *Acta Endocrinol. (Copenhagen)* **106**: 420-427.
- 141 Adashi, E.Y., Resnich, C.E., Hernandez, E.R., Hurwitz, A., Roberts, C.T., Le Roith, D. and Rosenfeld, R. (1991) The intraovarian IGF-I system. In: Spencer, E.M. ed. Modern Concepts of Insulin-Like Growth Factors. *Proceedings of the Second International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, Ca. Elsevier, New York.* pp 265-267.
- 142 Hizuka, N., Takano, K., Asakawa, K., Mijykawa, M., Horikawa, R. and Shizume, K. (1987) Demonstration of insulin-like growth factor-I in urine. *J. Clin. Endocrinol. Metab.* **64**: 1309-1312.
- 143 Collier, R.J., Ganguli, S., Menke, P.T., Buonomo, F.C., McGrath, M.F., Knotts, C.E. and Krivi, G.G. (1989) Changes in insulin and somatostatin receptors and uptake of insulin, IGF-I and IGF-II during mammary growth, lactogenesis and lactation. In: Heap,

- R.B., Prosser, C.G. and Lamming, G.E. eds. *Biotechnology in Growth Regulation*. Butterworths, London. pp 153-163.
- 144 Davis, S.R., Gluckman, P.D., Hart, I.C. and Henderson, H.V. (1987) Effects of injecting growth hormone or thyroxine on milk production and blood plasma concentrations of insulin-like growth factors I and II in dairy cows. *J. Endocrinol.* **114**: 17-24.
- 145 Merimee, T.J., Grant, M. and Tyson, J.E. (1984) Insulin-like growth factors in amniotic fluid. *J. Clin. Endocrinol. Metab.* **59**: 752-755.
- 146 Daughaday, W.H., Parker, K.A., Borowsky, S., Trivedi, B. and Kapdia, M. (1982) Measurement of somatomedin related peptides in fetal, neonatal and maternal rat serum by IGF-I RIA and IGF-II RRA and MSA RRA after acid-ethanol extraction. *Endocrinology* **110**: 575-581.
- 147 Gluckman, P.D., Johnson-Barrett, J.J., Butler, J.H., Edgar, B.W. and Gunn, T.R. (1983) Studies of insulin-like growth factors I and II by specific radioligand assays in umbilical cord blood. *Clin. Endocrinol. (Oxf.)* **19**: 405-411.
- 148 Rechler, M.M. and Nissley, S.P. (1985) The nature and regulation of receptors for insulin-like growth factors. *Ann. Rev. Physiol.* **47**: 425-442.
- 149 Czech, M.P. (1989) Signal transmission by the insulin-like growth factors. *Cell* **59**: 235-238.
- 150 Yarden, Y. and Ullrich, A. (1988) Growth factor receptor tyrosine kinases. *Ann. Rev. Biochem.* **57**: 443-478.
- 151 Roth, R.A. (1988) Structure of the receptor for insulin-like growth factor-II: the puzzle amplified. *Science* **239**: 1269-1271.
- 152 Alexandrides, T., Moses, A.C. and Smith, R.J. (1989) Developmental expression of receptors for insulin, insulin-like growth factor-I and

- IGF-II in rat skeletal muscle. *Endocrinology* **124**(2): 1064-1076.
- 153 Soos, M.A. and Siddle, K. (1989) Immunological relationships between receptors for insulin and insulin-like growth factor-I. *Biochem J.* **263**: 553-563.
- 154 Nishimoto, I., Ohkuni, Y., Ogata, E. and Kojima, I. (1987) Insulin-like growth factor-II increases cytoplasmic free calcium in competent Balb/c 3T3 cells treated with epidermal growth factor. *Biochem. Biophys. Res. Commun.* **142**: 275-286.
- 155 Kiess, W., Blichenstaff, G.D., Sklar, M.M., Thomas, C.L., Nissley, S.P. and Sahagian, G.G. (1988) Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose-6-phosphate receptor. *J. Biol. Chem.* **263**: 9339-9344.
- 156 Morgan, D.D., Edman, J.C., Standring, D.N., Fried, V.A., Smith, M.C., Roth, R.A. and Rutter, W.J. (1987) Insulin-like growth factor-II receptor is a multifunctional binding protein. *Nature (Lond.)* **329**: 301-307.
- 157 Zapf, J., Hauri, L., Waldvogel, M. and Froesch, E.R. (1986) Acute metabolic effects and half-lives of intravenously administered insulin-like growth factors-I and II in normal and hypophysectomized rats. *J. Clin. Invest.* **77**: 1768-1775.
- 158 Zapf, J., Schoenle, E. and Froesch, E.R. (1978) Insulin-like growth factors-I and II: some biological actions and receptor binding characteristics of two purified constituents of non-suppressible insulin-like activity in human serum. *Eur. J. Biochem.* **87**: 285-296.
- 159 Zapf, J., Froesch, E.R. and Humbel, R.E. (1981) The insulin-like growth factors: chemical and biological characterization and aspects of their possible physiological roles. *Curr. Top. Cell Regul.* **19**:

257-309.

- 160 Salmon, W.D. and DuVall, M.R. (1970) In vitro stimulation of leucine incorporation into muscle and cartilage protein by a serum fraction with sulfation activity: differentiation of effects from those of growth hormone and insulin. *Endocrinology* **87**: 1168-1180.
- 161 Poggi, C., LeMarchand-Bustel, Y. Zapf, J., Froesch, E.R. and Freychet, P. (1979) Effects and binding of IGF-I on the isolated soleus muscle of lean and obese mice. Comparison with insulin. *Endocrinology* **105**: 723-730.
- 162 Meuli, C., and Froesch, E.R. (1975) Effects of insulin and of NSILA on the perfused rat heart and glucose uptake, lactate production and efflux of 3-deoxy-methyl glucose. *Eur. J. Clin. Invest.* **5**: 93-99.
- 163 Jacob, R., Barrett, E., Plewe, G., Fagin, K.D. and Sherwin, R.S. (1989) Acute effects of insulin-like growth factor-I on glucose and amino acid metabolism in the awake fasted rat. *J. Clin. Invest.* **83**: 1717-1723.
- 164 Cascieri, M.A., Saperstein, R., Hayes, N.S., Green, B.G., Chicchi, G.G., Applebaum, J. and Bayne, M.L. (1988) Serum half-life and biological activity of mutants of human insulin-like growth factor-I which do not bind to serum binding proteins. *Endocrinology* **123(1)**: 373-381.
- 165 Ballard, F.J., Francis, G.L., Bagley, C.J., Szabo, L. and Wallace, J.C. (1989) Effects of insulin-like growth factors on protein metabolism: why are some molecular variants more potent? *Biochem. Soc. Symp.* **55**: 91-104.
- 166 Ross, M., Francis, G.L., Szabo, L., Wallace, J.C. and Ballard, F.J. (1989) Insulin-like growth factor binding proteins inhibit the biological activities of IGF-I and II but not des(1-3)IGF-I. *Biochem. J.* **258**:

267-272.

- 167 Gillespie, C., Read, L.C., Bagley, C.J. and Ballard, F.J. (1990) Enhanced potency of truncated insulin-like growth factor-I (des(1-3)IGF-I) relative to IGF-I in lit/lit mice. *J. Endocrinol.* **127**: 401-405.
- 168 Henry, D.P., Bowsher, R.R., Cerimele, B.J. and Nyhert, E.H. (1990) The administration of recombinant-derived insulin-like growth factor-II (IGF-II) to normal human subjects. *Proceedings of the 72nd Annual Meeting of The American Endocrine Society, Atlanta, Ga.* **Abstract 1150.**
- 169 Lewis, K.J., Molan, P.C., Bass, J.J. and Gluckman, P.D. (1988) B: The lipolytic activity of low concentrations of insulin-like growth factors in ovine adipose tissue. *Endocrinology* **122**(6): 2554-2557.
- 170 Zapf, J., Waldvogel, M. and Froesch, E.R. (1975) Binding of non-suppressible insulin-like activity to human serum: evidence for a carrier protein. *Arch. Biochem. Biophys.* **168**: 638-645.
- 171 Drop, S.L.S. and Hintz, R.L. (1989) On the nomenclature of the IGF binding proteins. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins*. Excerpta Medica, New York. pp v-vii.
- 172 Ballard, F.J., Baxter, R.C., Binoux, M., Clemmons, D.R., Drop, S.L.S., Hall, K., Hintz, R.L., Ling, N., Rechler, M.M., Rutanen, E.M., Schwander, J.C., Spencer, E.M. and Zapf, J. (1991) On the nomenclature of the IGFBPs. *In*: Spencer, E.M. ed. *Modern Concepts of Insulin-Like Growth Factors. Proceedings of the Second International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, Ca.* pp 731-732.
- 173 Chochinov, R.H., Mariz, I.K., Hajek, A.S. and Daughaday, W.H. (1977) Characterization of a protein in mid-term human amniotic fluid

- which reacts in the somatomedin-C radioreceptor assay. *J. Clin. Endocrinol. Metab.* **44**: 902-908.
- 174 Brinkman, A., Groffen, C., Kortleve, D.J., Geurts Van Kessel, A. and Drop, S.L.S. (1988) Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IBP-I). *EMBO J.* **7**: 2417-2423.
- 175 Koistinen, R., Kalkkinen, N., Huhtala, M.L., Soppala, M., Bohn, H. and Rutanen, E-M. (1986) Placental protein 12 is a decidual protein that binds somatomedin and has an identical N-terminal amino acid sequence to somatomedin-binding protein from human amniotic fluid. *Endocrinology* **118**: 1375-1378.
- 176 Baxter, R.C., Martin, J.L. and Wood, M.H. (1987) Two immunoreactive binding proteins for insulin-like growth factors in human amniotic fluid: relationship to fetal maturity. *J. Clin. Endocrinol. Metab.* **65**: 423-431.
- 177 Lee, Y-L., Hintz, R.L., James, P.M., Lee, P.D.K., Shively, J.E., and Powell, D.R. (1988) Insulin-like growth factor binding protein complementary deoxyribonucleic acid from human Hep G2 hepatoma cells: predicted protein sequence suggests an IGF binding domain different from those of IGF-I and IGF-II receptors. *Mol. Endocrinol.* **2**: 404-411.
- 178 Bell, S.C. and Keyte, J.W. (1988) N-terminal amino acid sequence of human pregnancy-associated endometrial alpha1-globulin, an endometrial insulin-like growth factor binding protein: evidence for two small molecular weight IGF binding proteins. *Endocrinology* **123**: 1202-1204.
- 179 Koistinen, R., Huhtala, M.L., Stenman, U.H. and Seppala, M. (1987) Purification of placental protein PP-12 from human amniotic fluid

- and its comparison with PP-12 from placenta by immunological, physicochemical and somatomedin binding properties. *Clin. Chim. Acta* **164**: 293-303.
- 180 Drop, S.L.S., Kortleve, D.J. and Guyda, H. (1984) Isolation of a somatomedin binding protein from pre-term amniotic development of a radioimmunoassay. *J. Clin. Endocrinol. Metab.* **59**(5): 899-907.
- 181 Pova, G., Engberg, G., Jornvall, H. and Hall, K. (1984) Isolation and characterization of a somatomedin binding protein from mid-term human amniotic fluid. *Eur. J. Biochem.* **144**: 199-204.
- 182 Rechler, M.M., Yang, Y.W.H., Brown, A.L., Orlowski, C.G. and Ooi, G.T. (1989) Molecular characterization of insulin-like growth factor binding proteins in the rat. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins. Excerpta Medica International Congress Series, New York.* pp 133-142.
- 183 Huhtala, M. L., Koistinen, R., Palomaki, P., Partanen, P., Bohn, H. and Seppala, M. (1986) Biologically active domain in somatomedin binding protein. *Biochem. Biophys. Res. Commun.* **141**: 263-270.
- 184 Brinkman, A., Kortleve, D.J., Groffen, C.A.H. and Drop, S.L.S. (1989) The C-terminal region of the low molecular weight IGF binding protein, IGFBP-1 is essential for IGF-I binding. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins. Excerpta Medica International Congress Series, New York.* pp 11-20.
- 185 Forbes, B., Szabo, S., Baxter, R.C. and Ballard, F.J. (1988) Classification of the insulin-like growth factor binding proteins into three distinct categories according to their binding specificities. *Biochem. Biophys. Res. Commun.* **157**(1): 196-202.
- 186 Bagley, C.J., May, B.L., Szabo, L., McNamara, C.J., Ross, M., Francis, G.L., Ballard, F.J. and Wallace, J.C. (1989) A key functional role

- for the insulin-like growth factor-I N-terminal pentapeptide. *Biochem. J.* **259**: 665-671.
- 187 Cubbage, M.L., Suwanichkul, A. and Powell, D.R. (1989) Insulin-like growth factor binding protein-3. Organisation of the human chromosomal gene and demonstration of promoter activity. *Mol. Endocrinol.* **3**: 846-851.
- 188 Hill, D.J., Han, V.K.M., Clemmons, D.R., Camacho-Hubner, C. and Wang, J.A. (1989) Expression, distribution, release and biological action of insulin-like growth factor binding proteins during development. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins. Excerpta Medica, New York.* pp 55-62.
- 189 Julkunen, M., Koistinen, R., Aalto-Setälä, K., Seppälä, M., Janne, O.A. and Kentula, K. (1988) Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue expression of its mRNA. *FEBS Lett* **236**: 295-302.
- 190 Bassett, N.S. and Challis, J.R.G. (1991) Localization and expression of insulin-like growth factors (IGFs) and IGF binding proteins in the ovine placenta. *Proceedings of the 73rd Annual Meeting of the American Endocrine Society, Washington D.C.* **Abstract 452.**
- 191 Lewitt, M.S. and Baxter, R.C. (1991) Insulin-like growth factor binding protein-I. A role in glucose counter-regulation? *Mol. Cell Endocrinol.* **79**: C147-C152.
- 192 Cotterill, A.M., Cowell, C.T., Baxter, R.C., McNeil, D. and Silinik, M. (1988) Regulation of growth hormone-independent insulin-like growth factor binding protein in human plasma. *J. Clin. Endocrinol. Metab.* **67**: 882-887.
- 193 Suikkari, A.M., Koivisto, V.A., Rutanen, E-M., Yhi-Jarvinen, H.,

- Karonen, S-L. and Seppala, M. (1988) Insulin regulates the serum levels of low molecular weight insulin-like growth factor binding protein. *J. Clin. Endocrinol. Metab.* **66**: 266-272.
- 194 Hall, K., Lundin, G. and Pova, G. (1988) Serum levels of the low molecular weight form of insulin-like growth factor binding protein in healthy subjects and patients with growth hormone deficiency, acromegaly and anorexia nervosa. *Acta Endocrinol.* **118**: 321-326.
- 195 Conover, C.A. and Lee, P.D.K. (1990) Insulin regulation of insulin-like growth factor-binding protein production in cultured HepG2 cells. *J. Clin. Endocrinol. Metab.* **70**: 1062-1067.
- 196 Brismar, K., Gutniak, M., Pova, G., Werner, S. and Hall, K. (1988) Insulin regulates the 35 kDa IGF binding protein in patients with diabetes mellitus. *J. Endocrinol. Invest.* **11**: 591-594.
- 197 Lewitt, M.S. and Baxter, R.C. (1989) Regulation of growth hormone-independent insulin-like growth factor binding protein (BP-28) in cultured human fetal liver explants. *J. Clin. Endocrinol. Metab.* **69**: 246-252.
- 198 Knauer, P.J., Wagner, F.W. and Smith, G.L. (1981) Purification and characterization of multiplication stimulating activity (MSA) carrier protein. *J. Supramol. Struct. Cell Biochem.* **15**: 177-191.
- 199 Romanus, J.A., Terrell, J.E., Yang, Y.W-H., Nissley, S.P. and Rechler, M.M. (1986) Insulin-like growth factor carrier proteins in neonatal and adult rat serum are immunologically different: demonstration using a new radioimmunoassay for the carrier protein from BRL-3A rat liver cells. *Endocrinology* **118**: 1743-1758.
- 200 Lyons, R.M. and Smith, G.L. (1986) Characterization of multiplication stimulating activity (MSA) carrier protein. *Mol. Cell Endocrinol.* **45**: 263-270.

- 201 Brown, A.L., Chiarotti, L., Orlowski, C.C., Mehlman, T., Burgess, W.H., Ackerman, E.T., Bruni, C.B. and Rechler, M.M. (1989) Nucleotide sequence and expression of a cDNA encoding a fetal rat binding protein for insulin-like growth factors. *J. Biol. Chem.* **264**: 5148-5154.
- 202 Binkert, C., Landwehr, J., Mary, J., Schwander, J. and Heinrich, G. (1989) Cloning, sequence analysis and expression of a cDNA encoding a novel insulin-like growth factor binding protein (IGFBP-2). *EMBO J.* **8**: 2497-2502.
- 203 Lamson, G., Pham, H., Youngman, O., Ocrant, I., DeLeon, D., Donovan, S., Schwander, J. and Rosenfeld, R.G. (1989) Expression of the 3 kDa (hBP-31) and 30 kDa (rBP-30) binding proteins in human and rat tissues. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins*. Excerpta Medica, New York. pp 143-150.
- 204 Tseng, L.Y.H., Brown, A.L., Yang, Y.W.H., Romanus, J.A., Orlowski, C.C., Taylor, T. and Rechler, M.M. (1989) The fetal rat binding protein for insulin-like growth factors is expressed in the choroid plexus and cerebrospinal fluids of rats. *Mol. Endocrinol.* **3**(10): 1559-1567.
- 205 Ooi, G.T., Orlowski, C.C., Brown, A.L., Becker, R.E., Unterman, T.G. and Rechler, M.M. (1990) Different tissue distribution and hormonal regulation of messenger RNAs encoding rat insulin-like growth factor binding proteins 1 and 2. *Mol. Endocrinol.* **4**(2): 321-328.
- 206 Hossenlopp, P., Seurin, D., Segovia-Quinson, B. and Binoux, M. (1986) Identification of an insulin-like growth factor binding protein in human cerebrospinal fluid with selective affinity for IGF-II. *FEBS*

Lett. **208**: 439-444.

- 207 Zapf, J., Schmid, L., Guler, H.P., Binz, K., and Froesch, E.R. (1989) Effects of growth hormone, insulin and IGF-I treatment on IGF carrier proteins in hypophysectomized and diabetic rats: comparison with fasting and refeeding. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins*. Excerpta Medica, New York. pp 151-158.
- 208 Rechler, M.M. (1991) Regulation of rat IGFBP-1 and IGFBP-2 gene expression. *Proceedings of the Second International IGF Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, Ca.* **Abstract p 19.**
- 209 Schwander, J.C., Mary, J.L., Landwehr, J., Boni, M., Margot, J and Binkert, C. (1989) Regulation of the mRNA of an insulin-like growth factor binding protein in the rat. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins*. Excerpta Medica, New York. pp 125-132.
- 210 Delahanty, R.J.D., Nygard, K. and Han, V.K.M. (1991) Ovine insulin-like growth factor binding protein-2: characterization of its cDNA structure and mRNA expression in adult sheep tissues. *Proceedings of the Second International IGF Symposium, San Francisco, Ca.* **Abstract D45.**
- 211 Daughaday, W.H., Ward, A.P., Goldberg, A.C., Trivedi, B. and Kapadia, M. (1982) Characterization of somatomedin binding on human serum by ultracentrifugation and gel filtration. *J. Clin. Endocrinol. Metab.* **51**: 12-19.
- 212 Baxter, R.C., Martin, J.L., Tyler, M.I. and Howden, M.E.H. (1986) Growth hormone-dependent insulin-like growth factor binding protein from human plasma differs from other human IGF binding

- proteins. *Biochem. Biophys. Res. Commun.* 139: 1256-1261.
- 213 Baxter, R.C. and Martin, J.L. (1987) Binding proteins for insulin-like growth factors in adult rat serum. Comparison with other rat binding proteins. *Biochem. Biophys. Res. Commun.* 147: 408-415.
- 214 Wood, W.I., Cachianes, G., Henzel, W.J., Winslow, G.A., Spencer, S.A., Hellmiss, R., Martin, J.L. and Baxter, R.C. (1988) Cloning and expression of the growth hormone-independent insulin-like growth factor binding protein. *Mol. Endocrinol.* 2(12): 1176-1185.
- 215 Albiston, A.L. and Herington, A.C. (1990) Cloning and characterization of the growth hormone dependent insulin-like growth factor binding protein (IGFBP-3) in the rat. *Biochem. Biophys. Res. Commun.* 166(2): 892-896.
- 216 Mukku, V.R., Courter, R.G., Gorman, C., Parker, M., Larsen, B. and Wood, W.I. (1989) Expression, purification and characterization of recombinant BP-53, the GH-dependent IGF binding protein. *In: Drop, S.L.S. and Hintz, R.L. eds. Insulin-Like Growth Factor Binding Proteins. Excerpta Medica, New York.* pp 65-70.
- 217 Ooi, G.T., Aguilar, M., Hearn, M.T.W., and Herington, A.C. (1989) Relationship of a low molecular weight (16K) IGF inhibitor/binding protein to the native IGF binding proteins of human serum. *In: Drop, S.L.S., and Hintz, R.L. eds. Insulin-Like Growth Factor Binding Proteins. Excerpta Medica, New York.* pp 231-238.
- 218 Wilkins, J.R. and D'Ercole, A.J. (1985) Affinity labelled plasma somatomedin-C/IGF-I binding protein: evidence of growth hormone-dependence and subunit structure. *J. Clin. Invest.* 75: 1350-1358.
- 219 Furlanetto, R.W. (1980) The somatomedin-C binding protein: evidence

- for a heterologous subunit structure. *J. Clin. Endocrinol. Metab.* **51**: 12-19.
- 220 Hintz, R.L. and Lin, F. (1980) Somatomedin plasma binding proteins. *In*: Pecile, A. and Muller, E. eds. Growth Hormone and Other Biologically Active Peptides. *Excerpta Medica, Amsterdam.* pp 133-140.
- 221 Baxter, R.C. (1989) The acid-labile alpha-subunit of the 140K IGF-binding protein complex - circulating levels in health and disease. *In*: Drop, S.L.S. and Hintz, R.L. eds. Insulin-Like Growth Factor Binding Proteins. *Excerpta Medica, New York.* pp 83-92.
- 222 Shimasaki, S., Koba, A., Mercado, M., Shimanaka, M. and Ling, W. (1989) Complementary DNA structure of the high molecular weight rat insulin-like growth factor binding protein (IGFBP-3) and tissue distribution of its mRNA. *Biochem. Biophys. Res. Commun.* **165(2)**: 907-912.
- 223 Grant, M.B., Russell, B., Harwood, H.J. and Merimee, T.J. (1987) Separation of the insulin-like growth factor binding proteins in plasma and purification of the larger molecular weight species. *J. Clin. Endocrinol. Metab.* **64**: 1060-1065.
- 224 Moses, A.C., Nissley, S.P., Cohen, K.L. and Rechler, M.M. (1976) Specific binding of a somatomedin-like polypeptide in rat serum depends on growth hormone. *Nature (London)* **263**: 137-140.
- 225 Moses, A.C., Nissley, S.P., Pasamani, J., White, R.M. and Rechler, M.M. (1979) Further characterization 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**: 536-546.

- 226 Blum, W.F., Ranke, M.B., Kietzmann, K., Sauggel, E., Zeisel, H.J. and Bierich, J.R. (1990) A specific radioimmunoassay for the growth hormone (GH)-dependent somatomedin binding protein: its use for diagnosis of GH deficiency. *J. Clin. Endocrinol. Metab.* **70**: 1292-1298.
- 227 Ranke, M.B., Blum, W.F. and Frisch, H. (1989) The acid-stable subunit of the insulin-like growth factor binding protein (IGFBP-3) in disorders of growth. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins. Excerpta Medica, New York.* pp 103-113.
- 228 Blum, W.F. and Ranke, M.B. (1991) Plasma IGFBP-3 as clinical indicators. *In*: Spencer, E.M. ed. *Modern Concepts of Insulin-Like Growth Factors. Proceedings of the Second International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, Ca.* Elsevier, New York. pp 381-393.
- 229 Scott, C.D. and Baxter, R.C. (1991) Synthesis of the acid-labile subunit of the growth hormone-dependent insulin-like growth factor binding protein complex by rat hepatocytes in culture. *Biochem. J.* **275**(2): 441-446.
- 230 Martin, J.L. and Baxter, R.C. (1990) Production of an insulin-like growth factor (IGF)-inducible IGF binding protein by human skin fibroblasts. *Endocrinology* **127**: 781-788.
- 231 Blum, W.F., Ranke, M.B., Kietzmann, K., Tonshoff, B. and Mehls, O. (1989) Excess of IGF binding proteins in chronic renal failure: evidence for relative GH resistance and inhibition of somatomedin activity. *In*: Drop, S.L.S. and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins. Excerpta Medica, New York.* pp 93-102.
- 232 Martin, J.L. and Baxter, R.C. (1988) Insulin-like growth factor binding

- proteins produced by human skin fibroblasts: immunological relationship to other human IGFBPs. *Endocrinology* **123**(4): 1907-1915.
- 233 Davenport, M.L., Clemmons, D.R., Miles, M.V., Camacho-Hubner, C., D'Ercole, A.J. and Underwood, L.E. (1990) Regulation of serum insulin-like growth factor-I and IGF binding proteins during rat pregnancy. *Endocrinology* **127**: 1278-1285.
- 234 Mukku, V.R. and Chu, H. (1990) Phosphorylation of insulin-like growth factor binding proteins by mammalian cells. *Proceedings of the 72nd Annual Meeting of the American Endocrine Society, Atlanta, Ga. Abstract 190.*
- 235 McCusker, R.H., Campion, D.R., Jones, W.K. and Clemmons, D.R. (1989) The insulin-like growth factor binding proteins of porcine serum: endocrine and nutritional regulation. *Endocrinology* **125**(1): 501-509.
- 236 Martin, J.L., Willetts, K.E. and Baxter, R.C. (1990) Purification and properties of a novel insulin-like growth factor binding protein from transformed human fibroblasts. *J. Biol. Chem.* **265**: 4124-4130.
- 237 Mohan, S., Bautista, C.M., Wergedal, J. and Baylink, D.J. (1989) Isolation of an inhibitory insulin-like growth factor binding protein from bone cell-conditioned medium: a potential local regulator of IGF action. *Proc. Natl. Acad. Sci. (USA)* **86**: 8338-8342.
- 238 Roghani, M., Hossenlopp, P., Lepage, P., Balland, A. and Ling, N. (1989) Isolation from human cerebrospinal fluid of a new insulin-like growth factor binding protein with selective affinity for IGF-II. *FEBS Lett.* **255**(2): 253-258.
- 239 Zapf, J., Kiefer, M., Merryweather, J., Masiarz, F., Bauer, D., Born, W.,

- Fischer, J.A. and Froesch, E.R. (1990) Isolation from adult human serum of four insulin-like growth factor binding proteins and molecular cloning of one that is increased by IGF-I administration and in extrapancreatic tumor hypoglycaemia. *J. Biol. Chem.* **265**: 14892-14898.
- 240 Murphy, W.R., Daughaday, W.H. and Hartnett, C. (1956) The effect of hypophysectomy and growth hormone on the incorporation of labeled sulfate into tibial, epiphyseal and nasal cartilage in the rat. *J. Lab. Clin. Med.* **47**:715-722.
- 241 Vassilopoulou-Sellin, R., Phillips, L.S., Oyedeki, C.O. and Samaan, N.A. (1984) Metabolic regulation of somatomedin activity in rat liver. *J. Endocrinol.* **101**: 257-261.
- 242 Van Wyk, J.J. (1984) The somatomedins: biological actions and physiological control mechanisms. *In*: Li, C.H. ed. *Hormonal Proteins and Peptides*, Vol XII. Academic Press, New York. pp 82-125.
- 243 Froesch, E.R., Schwander, S.J. and Zapf, F.J. (1985) Actions of insulin-like growth factors. *Ann. Rev. Physiol.* **47**: 443-467.
- 244 Hannsson, H.A. (1990) Insulin-like growth factor-I: aspects of its cell localization and dynamic changes. *Current Medical Literature: Growth and Growth Factors* **5**(1): 3-10.
- 245 Skottner, A., Clark, R.G., Frykland, L., Robinson, I.C.A.F. (1989) Growth responses in a mutant dwarf rat to human growth hormone and human insulin-like growth factor-I. *Endocrinology* **124**(5): 2519-1526.
- 246 Guler, H.P., Zapf, J., Scheiwiller, E. and Froesch, E.R. (1988) Recombinant human insulin-like growth factor-I stimulates growth and has distinct effects on organ size in hypophysectomized rats.

- Proc. Natl. Acad. Sci (USA)* **85**: 4889-4893.
- 247 Robinson, I.C.A.F. and Clark, R.G. (1989) Growth promotion by growth hormone and insulin-like growth factor-I in the rat. *In*: Heap, R.P., Prosser, C.G. and Lamming, G.E. eds. *Biotechnology in Growth Regulation*. Butterworths, London. pp 129-140.
- 248 Skottner, A., Clark, R.G., Robinson, I.C.A.F. and Frykland, L. (1987) Recombinant insulin-like growth factor-I. Testing the somatomedin hypothesis in hypophysectomized rats. *J. Endocrinol.* **112**: 123-132.
- 249 Phillips, A.F., Persson, B., Hall, K. et.al. (1988) The effects of biosynthetic insulin-like growth factor-I supplementation on somatic growth, maturation and erythropoiesis in the neonatal rat. *Pediatr. Res.* **23**: 298-305.
- 250 Carlsson, L.M.S., Clark, R.G. and Skottner, A. (1989) Growth hormone and growth in diabetic rats: Effects of insulin-like growth factor-I infusion. *J. Endocrinol.* **122**: 661-670.
- 251 Nixon, B.T. and Green, H. (1984) Growth hormone promotes the differentiation of myoblasts and pre-adipocytes generated by azacytidine treatment of 10T1/2 cells. *Proc. Natl. Acad. Sci. (USA)* **81**: 3429-3432.
- 252 Nixon, B.T. and Green, H. (1984) Contribution of growth hormone to the adipogenic activity of serum. *Endocrinology* : 527-532.
- 253 Morikawa, M., Nixon, T. and Green H. (1982) Growth hormone and the adipose conversion of 3T3 cells. *Cell* **29**: 783-789.
- 254 Florini, J.R. (1987) Hormonal control of muscle growth. *Muscle and Nerve* **10**: 577-598.
- 255 Murphy, L.J., Bell, G.I. and Friesen, H.G. (1987) Growth hormone stimulates sequential induction of c-myc and insulin-like growth

- factor-I expression in vivo *Endocrinology* **120**: 1806-1812.
- 256 Jansson, J.O., Eden, S. and Isaksson, O. (1985) Sexual dimorphism in the control of growth hormone secretion. *Endocr. Rev.* **6**(2): 128-150.
- 257 Sissons, H. (1961) The growth of bone. *In*: Bouine, G.H. ed. The biochemistry and physiology of bone. Academic Press, NY pp 443-474.
- 258 Kember, H.F. and Sissons, H. (1976) A quantitative histology of the human growth plate. *J. Bone Joint Surgery* **58B**: 426-438.
- 259 Trippel, S.B., Van Wyk, J.J., Foster, M.B. and Svoboda, M.E. (1982) Characterization of a specific somatomedin-c receptor on isolated bovine growth plate chondrocytes. *Endocrinology* **112**: 2128-2136.
- 260 Barnard, R., Haynes, K.M., Werther, G.A. and Waters, M.J. (1988) The ontogeny of growth hormone receptors in the rabbit tibia. *Endocrinology* **122**(6): 2562-2569.
- 261 Trippel, S.B., Chernausk, S.D., Van Wyk, J.J., Moses, A.C. and Mankin, H.J. (1988) Demonstration of type I and type II somatomedin receptors on bovine growth plate chondrocytes. *J. Orthopaed. Res.* **6**: 817-826.
- 262 Isgaard, J., Nilsson, A., Vihmank, K. and Isaksson, O.G.P. (1989) Growth hormone regulates the level of insulin-like growth factor-I mRNA in rat skeletal muscle. *J. Endocrinol* **120**: 107-112.
- 263 Isaksson, O., Jansson, J-O. and Gause, I.A.M. (1982) Growth hormone stimulates longitudinal bone growth directly. *Science* **216**: 1237-1239.
- 264 Russell, S.M. and Spencer, E.M. (1985) Local injections of human or rat growth hormone or of purified human somatomedin-c stimulate unilateral tibial epiphyseal growth in hypophysectomised rats. *Endocrinology* **116**: 2563-2567.

- 265 Ballard, F.J., Tomas, F.M., Read, L.C., Knowles, S.E., Owens, P.C.,
Lemmey, A.B., Martin, A.A., Wells, J.R.E., Wallace, J.C. and
Francis, G.L. (1991) Effects of IGF-I and IGF analogs on growth
during catabolic states in rats. *In: Spencer, E.M. ed. Modern
Concepts of Insulin-Like Growth Factors. Proceedings of the
Second International Symposium on Insulin-Like Growth
Factors/Somatomedins, San Francisco, Ca., Elsevier, New York.* pp
617-628.
- 266 Bauman, D.E., Eppard, P.J., DeGeeter, M.J. and Lanza, G.M. (1985)
Responses of high-producing dairy cows to longterm treatment
with pituitary somatotropin and recombinant somatotropin. *J.
Dairy Sci.* **68**: 1352-1362.
- 267 Tyrrell, H.F., Brown, A.C.G., Reynolds, P.J., Haaland, G.L., Bauman,
D.E., Peel, C.J. and Steinhour, A.T. (1988) Effect of bovine
somatotropin on metabolism of lactating dairy cows: energy and
nitrogen utilization as determined by respiration calorimetry. *J.
Nutr.* **118**: 1024-1030.
- 268 Davis, S.R., Collier, R.J., McNamara, J.P., Head, H.H. and Sussman, W.
(1988) Effects of thyroxine and growth hormone treatment of
dairy cows on milk yield, cardiac output and mammary blood flow.
J. Anim. Sci. **66**: 70-79.
- 269 Prosser, C.G. and Davis, S.R. (1992) Milking frequency regulates the
milk yield response of goats to external pudic artery infusion of
IGF-I. *J. Endocrinol* submitted..
- 270 Keys, J.E. and Dijane, J. (1988) Prolactin and growth hormone binding
in mammary and liver tissue of lactating cows. *J. Rec. Res.* **8**:
731-750..
- 271 McDowell, J.H., Hart, I.C. and Kirby, A.C. (1987) Local intra-arterial

- infusion of growth hormone into the mammary glands of sheep and goats: effects on milk yield, composition and plasma hormones. *Aust. J. Biol. Sci.* **40**: 181-.
- 272 Collier, R.J., Ganguli, S., Menke, P.T., Buonomo, F.C., McGrath, M.F., Knotts, C.E. and Krivi, G.G. (1989) Changes in insulin and somatomedin receptors and uptake of insulin, IGF-I and IGF-II during mammary growth, lactogenesis and lactation. *In*: Heap, R.B., Prosser, C.G. and Lamming, G.E. eds. *Biotechnology in Growth Regulation*. Butterworths, London. pp 153-163.
- 273 Murphy, L.J., Bell, G.I. and Friesen, H.G. (1987) Tissue distribution of insulin-like growth factor-I and II messenger ribonucleic acid in the adult rat. *Endocrinology* **120**: 1279-1282.
- 274 Campbell, P.G., Skaar, T.C., Vega, J.R. and Baumrucker, C.R. (1991) Secretion of insulin-like growth factor-I (IGF-I) and IGF binding proteins from bovine mammary tissue in vitro. *J. Endocrinol.* **128**: 219-228.
- 275 Shamay, A., Cohen, N., Niwa, M. and Gerther, A. (1988) Effect of insulin-like growth factor-I on deoxyribonucleic acid synthesis and galactopoiesis in bovine undifferentiated and differentiated mammary tissue in vitro. *Endocrinology* **123**: 804-809.
- 276 Davis, S.R. and Hodgkinson, S.C. (1991) The time course of milk yield and hormonal responses following GH injection of hourly milked goats. *Proceedings of the New Zealand Society of Animal Production*. **Abstract**.
- 277 Orlowski, C.C. and Chernausek, S.D. (1988) Discordance of serum and tissue somatomedin levels in growth hormone-stimulated growth in the rat. *Endocrinology* **123**: 44-49.
- 278 Knauer, D.J. and Smith, G.L. (1980) Inhibition of biological activity of

- multiplication stimulating activity by binding to its carrier protein. *Proc. Natl. Acad. Sci. (USA)* **77**: 7252-7256.
- 279 Uthne, K. and Uthne, T. (1972) Influence of liver resection and regeneration on somatomedin (sulphation factor) activity in sera from normal and hypophysectomized rats. *Acta Endocrinol. (Copenhagen)* **71**: 255-264.
- 280 Stiles, C.D., Capone, G.T., Scher, C.D., Antoiades, H.N., Van Wyk, J.J. and Pledger, W.J. (1979) Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proc. Natl. Acad. Sci. (USA)* **76**: 1279-1283.
- 281 Stiles, D., Sosenko, I.R.S., D'Ercole, A.J. and Smith, T. (1985) Relation of kidney tissue to somatomedin-c/insulin-like growth factor-I to post nephrectomy renal growth in the rat. *Endocrinology* **117**: 2397-2401.
- 282 Jackson, S., Hodgkinson, S., Estivariz, F.E. and Lowry, P.J. (1990) IGF-I and II in two models of adrenal growth. *Proceedings of the VIIIth International Congress on Hormonal Steroids. Le Hague Abstract*.
- 283 Kurtz, A., Matter, R., Echardt, K.U. and Zapf, J. (1990) Erythropoiesis, serum erythropoietin, and serum IGF-I in rats during accelerated growth. *Acta Endocrinol. (Copenhagen)* **122**: 323-328.
- 284 Steenfos, H., Hunt, T. and Spencer, E. (1989) Insulin-like growth factor has a major role in wound healing. *Proceedings of the Surgical Forum* 68-70.
- 285 Hansson, H.A., Dahlen, L.B., Danielsen, N., Frykland, L., Nachemsen, A.K., Polleryd, P., Rozell, B., Skottner, A. and Lundberg, G. (1986) Evidence indicating trophic importance of IGF-I in regenerating peripheral nerves. *Acta Physiol. Scand.* **126**: 609-614.
- 286 Powell, D.R., Suwanichkul, A. and Cubbage, M.L. (1989) The 25 kDa

- insulin-like growth factor binding protein: analysis of chromosomal gene structure and demonstration of promoter activity. *In*: Drop, S.L.S., and Hintz, R.L. eds. *Insulin-Like Growth Factor Binding Proteins. Excerpta Medica, New York.* pp 3-9.
- 287 Drop, S.L.S., Brinkman, A., Kortleve, D.J., Groffen, L.A.H., Schuller, A. and Zwarthoff, E.L. (1991) The evolution of the insulin-like growth factor binding protein family. *In*: Spencer, E.M. *Modern Concepts of Insulin-Like Growth Factors. Proceedings of the Second International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, Ca. Elsevier, New York.* pp 311-328.
- 288 Bar, R.S., Booth, B.A., Boes, M. and Dake, B.L. (1989) Insulin-like growth factor binding proteins from vascular endothelial cells: purification, characterization and intrinsic biological activities. *Endocrinology* **125**(4): 1910-1920.
- 289 Wood, T.L., Brown, A.L., Rechler, M.M. and Pintar, J.E. (1990) The expression pattern of an insulin-like growth factor (IGF)-binding protein gene is distinct from IGF-II in the midgestational rat embryo. *Mol. Endocrinol.* **4**(8): 1257-1263.
- 290 Salacinski, P.R., McLean, P.C., Sykes, J.E., Clement-Jones, V.V. and Lowry, P.J., (1981). Iodination of proteins, glycoproteins and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3a, 6a-diphenyl glycoluril (iodogen). *Analyt. Biochem.* **117**:136-146.
- 291 Desbuquois, B. and Aurbach, G.D. (1971) Use of polyethylene glycol to separate free and antibody-bound hormones in radioimmunoassay. *J. Clin. Endocrinol. Metab.* **33**: 732-738.
- 292 Goodfriend, T.L., Levine, L. and Fasman, G.D. (1964) Antibodies to bradykinin and angiotensin. A use for carbodiimides in

- immunology. *Science* **144**: 1344-1346.
- 293 Daughaday, W.H., Mariz I.K. and Blethen, S.L. (1980) Inhibition of access of bound somatomedin to membrane receptor and immunobinding sites: a comparison of radioreceptor and radioimmunoassay of somatomedin in native and acid-ethanol-extracted serum. *J. Clin. Endocrinol. Metab.* **51**(4): 781-788..
- 294 Tamura, K., Kobayashi, M., Suzuki, M., Ishii, Y., Koyama, S., Yamada, H., Hashimoto, M., Niwa, R. and Shibayama, F. (1990) Enzyme-lined immunosorbent assay for human insulin-like growth factor using monoclonal and polyclonal antibodies with defined epitope recognition. *J. Endocrinol.* **125**: 327-335.
- 295 Horner, J.M., Liu F. and Hintz, R.L. (1978) Comparison of [¹²⁵I]somatomedin A and [¹²⁵I]somatomedin C radioreceptor assays for somatomedin peptide content in whole and acid-chromatographed plasma. *J. Clin. Endocrinol. Metab.* **47**(6): 1287-1295.
- 296 Mesiano, S., Young, I.R., Browne C.A. and Thorburn, G.D. (1988) Failure of acid-ethanol treatment to prevent interference by binding proteins in radioligand assays for the insulin-like growth factors. *J. Endocrinol.* **119**: 453-460.
- 297 Breier, B.H., Gallaher, B.W. and Gluckman, P.D. (1991) Radioimmunoassay for insulin-like growth factor-I: Solutions to some potential problems and pitfalls. *J. Endocrinol.* **128**: 347-357.
- 298 Martin, J.L. and Baxter, R.C. (1986) Insulin-like growth factor-binding protein from human plasma: purification and characterization. *J. Biol. Chem.* **261**(19): 8754-8760..
- 299 Ballard, F.J. (1982) Regulation of protein accumulation in cultured cells.

Biochem. J. **208**: 275-287.

- 300 Tamura, K., Yamada, H., Ishii, Y., Koyama, S., Asada, T., Heriai, H., Kobayashi, M., Niwa, H., Shibayama, F. and Iwai, M. (1988) Structure and biological characteristics of hIGF-I/SmC disulphide bond isomers produced in the refolding process and preferential refolding method. *In: 8th International Congress of Endocrinology, Kyoto, Japan. Abstract 23-18-040.*
- 301 Szabo, L., Mottershead, D.G., Ballard, F.J. and Wallace, J.C. (1988) The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-1 for binding. *Biochem. Biophys. Res. Comm.* **151**(1): 207-214.
- 302 Baxter, R.C., (1988) Characterization of the acid-labile subunit of the growth hormone-dependent insulin-like growth factor binding protein complex. *J. Clin. Endocrinol. Metab.* **67**(2): 265-272.
- 303 Baxter, R.C. and Martin, J.L. (1989) Structure of the Mr 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity-labeling. *Proc. Natl. Acad. Sci. USA* **86**: 6898-6902.
- 304 Walton, P.E., Baxter, R.C., Burleigh, B.D. and Etherton, T.D. (1989) Purification of the serum acid-stable insulin-like growth factor binding protein from the pig (*Sus scrofa*). *Comp. Biochem. Physiol.* **92B**(3): 561-567.
- 305 Binoux, M. and Hossenlopp, P. (1988) Insulin-like growth factor (IGF) and IGF-binding proteins: comparison of human serum and lymph. *J. Clin. Endocrinol. Metab.* **67**(3): 509-514.
- 306 Butler, J.H. and Gluckman, P.D. (1986) Circulating insulin-like growth factor-binding proteins in fetal, neonatal and adult sheep. *J. Endocrinol.* **109**: 333-338.

- 307 Gluckman, P.D., Ball, R., Bassett, N. and Breier, B. (1987) Growth hormone secretion and action: A maturational perspective. *Proceedings of the 30th Annual Meeting of the Endocrine Society of Australia, Sydney.* pp S1-17.
- 308 Zapf, J., Hauri, C., Waldvogel, M., Futo, E., Hasler, H., Binz, K., Guler, H.P., Schmid C. and Froesch, E.R. (1989). Recombinant human insulin-like growth factor-I induces its own specific carrier protein in hypophysectomized and diabetic rats. *Proc. Natl. Acad. Sci. USA* **86**: 3813-3817.
- 309 Romanus, J.A., Tseng, Y, Yemg, Y.H. and Rechler, M.M. (1989) The 34kDa insulin-like growth factor binding protein in human cerebrospinal fluid and the A673 Rhabdo myosarcoma cell line are human homologues of the rat BRL3A binding protein. *Biochem. Biophys. Res. Comm.* **163**: 875-881.
- 310 Gluckman, P.D. and Butler, J.H. (1983) Parturition-related changes in insulin-like growth factors-I and II in the perinatal lamb. *J. Endocrinol.* **99**: 223-232.
- 311 Hey, A.W., Browne, C.A. and Thorburn, G.D. (1987) Fetal sheep serum contains a high molecular weight insulin-like growth factor binding protein that is acid-stable and specific for IGF-II. *Endocrinology* **121**(6): 1975-1984.
- 312 Gelato, M.C., Rutherford, C., Stark, R.I. and Daniel, S.S. (1989) The insulin-like growth factor-II/mannose-6-phosphate receptor is present in fetal and maternal sheep serum. *Endocrinology* **124**(6): 2935-2943.
- 313 Kiess, W., Greenstein, L.A., White, R.M., Lee, L., Rechler, M.M. and Nissley, S.P. (1987) Type II insulin-like growth factor receptor is present in rat serum. *Proc. Natl. Acad. Sci. USA* **84**: 7720-7724.

- 314 Walton, P.E., Grant, P., Owens, J.A., Wallace, J.C. and Ballard, F.J. (1990) Isolation and characterization of ovine plasma IGF binding proteins 1, 2 and 24 kDa species. *Proceedings of the 72nd Annual Meeting of The American Endocrine Society, Atlanta, Ga.* **Abstract 180.**
- 315 Walton, P.E., Grant, P.A., Seamark, R.F., Francis, G.L., Ross, M., Wallace, J.C. and Ballard, F.J. (1991) Characterization and binding properties of the 24/29 kDa IGF binding protein and IGFBP-3 from ovine plasma. *In: Spencer, E.M. ed., Modern Concepts of Insulin-Like Growth Factors. The Second International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, Ca. Elsevier, New York.* **Abstract C56.**
- 316 Shimasaki, S., Shimonaka, M, Zhang, H.P. and Ling, N. (1991) Isolation and molecular characterization of three novel insulin-like growth factor binding proteins (IGFBP-4, 5 and 6). *In: Spencer, E.M. ed., Modern Concepts of Insulin-like Growth Factors. Proceedings of the Second International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, Ca. Elsevier, New York.* **Abstract C 54.**
- 317 Simonescu, N. (1983) Cellular aspects of transcapillary exchange. *Physiol. Rev.* **63(4)**: 1536-1579.
- 318 Bar, R.S., Harrison, L.C., Baxter, R.C., Boes, M., Dake, B.L., Booth, B. and Cox, A. (1987) Production of IGF-binding proteins by vascular endothelial cells. *Biochem. Biophys. Res. Comm.* **148(2)**: 734-739.
- 319 Shimasaki, S., Gao, L., Shimonaka, M. and Ling, N. (1991) Isolation and molecular cloning of insulin-like growth factor binding protein-6.

Mol. Endocrinol. **5**(7): in press.

- 320 Baxter, R.C. (1990) Glycosaminoglycans inhibit formation of the 140kDa insulin-like growth factor binding protein complex. *Biochem. J.* **271**: 773-777.
- 321 Bassett, N.S., Breier, B.H., Hodgkinson, S.C., Davis, S.R., Henderson, H.V. and Gluckman, P.D. (1990) Plasma clearance of radiolabelled IGF-I in the late gestation ovine fetus. *J. Dev. Physiol.* **14**: 73-79.
- 322 Baxter, R.C. and Martin, J.L. (1986) Radioimmunoassay of the growth hormone-dependent insulin-like growth factor binding protein in human plasma. *J. Clin. Invest.* **78**: 1504-1512.
- 323 Cohen, K.L. and Nissley, S.P. (1976) The serum half-life of somatomedin activity: evidence for growth hormone-dependence. *Acta Endocrinol.* **83**: 243-258.
- 324 Baxter, R.C. and Cowell, C.T. (1987) Diurnal rhythm of the growth hormone-independent binding protein for insulin-like growth factors in human plasma. *J. Clin. Endocrinol. Metab.* **65**: 432-440.
- 325 Holly, J.M.P., Biddlecombe, R.A., Dunger, D.B., Edge, J.A., Amiel, S.A., Howell, T.R., Chard, T., Rees, L.H. and Wass, J.A.H. (1988) Circadian variation of GH-independent IGF-binding protein in diabetes mellitus and its relationship to insulin. A new role for insulin? *Clin. Endocrinol.* **29**: 667-675.
- 326 McCusker, R.H., Cohick, W.S., Busby, W.H. and Clemmons, D.R. (1990) Control of insulin-like growth factor binding protein-2 (IGFBP-2) levels in porcine sera by fasting and growth hormone status. *J. Anim. Sci. Vol 69 (Suppl. 1)* **Abstract 140**, p 285.
- 327 Cohick, W.S., Busby, W.H., Clemmons, D.R. and Bauman, D.E. (1990) Insulin-like growth factor binding proteins in serum of lactating

cows treated with recombinant N-methionyl bovine somatotropin.

J. Anim. Sci. Vol 69 (Suppl 1) Abstract 208, p 315.

- 328 Bass, J.J., Hodgkinson, S.C., Breier, B.H., Carter, W. and Gluckman, P.D. (1991) Effect of bovine somatotrophin on insulin-like growth factor-I, insulin, growth and carcass composition of lambs. *Livestock Prod. Sci.* (In Press).
- 329 Ooi, G.T. (1989) At the cutting edge. Insulin-like growth factor binding proteins (IGFBPs): more than just 1, 2, 3. *Mol. Cell Endocrinol.* 71: C39-C43.
- 330 Holly, J.M.P., Smith, C.P., Dunger, A.B., Edge, J.A., Biddlecombe, R.A., Williams, A.J.K., Howell, T.R., Chard, T., Savage, M.O., Rees, L.H. and Wass, J.A.H. (1989) Levels of the small insulin-like growth factor binding protein are strongly related to those of insulin in prepubertal and pubertal children but only weakly so after puberty. *J. Endocrinol.* 121: 383-387.
- 331 Glimm, D.R., Baracos, V.E. and Kennelly, J.E. (1988) Effect of bovine somatotrophin on distribution and immunoreactive insulin-like growth factor-I in lactating bovine mammary tissue. *J. Dairy Sci.* 71: 2923-2935.
- 332 Gertler, A., Cohen, N. and Maoz, A. (1983) Human growth hormone but not ovine or bovine growth hormone exhibits galactopoietic prolactin-like activity in organ culture from lactating bovine mammary gland. *Mol. Cell Endocrinol.* 33: 169-182.
- 333 Davis, S.R., Gluckman, P.D., Hodgkinson, S.C., Farr, V.C., Breier, B.H. and Burleigh, B.D. (1989) Comparison of the effects of administration of recombinant bovine growth hormone or N-methionyl insulin-like growth factor-I to lactating goats. *J. Endocrinol.* 123: 33-39.

- 334 Hossenlopp, P. Seurin, D., Segovia-Quinson, S. and Binoux, M. (1986)
Analysis of serum insulin-like growth factor binding proteins using
western blotting: use of the method for titration of the binding
proteins and competitive binding studies. *Analyt. Biochem.* **154**:
138-143.
- 335 Bar, R.S., Boes, M., Clemmons, D.R., Busby, W.H., Sandra, A., Dake,
B.L. and Booth, B.A. (1990) Insulin differentially alters
transcapillary movement of intravascular IGFBP-1, IGFBP-2 and
endothelial cell IGF binding proteins. *Endocrinology* **127**: 497-499.
- 336 Glimm, D.R., Baracos, V.E. and Kennelly, J.E. (1990) Molecular
evidence for the presence of growth hormone receptors in bovine
mammary gland. *J. Endocrinol.* **126**: 125-128.
- 337 Holly, J.M.P. and Wass, J.A.H. (1989) Insulin-like growth factors:
autocrine, paracrine or endocrine? New perspectives of the
somatomedin hypothesis in the light of recent developments. *J.*
Endocrinol. **122**: 611-618.
- 338 D'Ercole, A.J., Stiles, A.D. and Underwood, L.E. (1984) Tissue
concentrations of somatomedin-c: further evidence for multiple
sites of synthesis and paracrine or autocrine mechanisms of action.
Proc. Natl. Acad. Sci. (USA) **81**: 935-939.
- 339 Schwander, J.C., Hauri, C., Zapf, J. and Froesch, E.R. (1983) Synthesis
and secretion of insulin-like growth factor and its binding protein
by the perfused rat liver: dependence on growth hormone status.
Endocrinology **113**: 297-305.
- 340 Caro, J.F., Poulos, J., Hoop, O., Portes, W.F., Flickinger, E.G. and Sinha,
M.K. (1988) Insulin-like growth factor-I binding to hepatocytes
from human liver, human hepatoma and normal regenerating and
fetal rat liver. *J. Clin. Invest.* **81**: 976-981.

- 341 D'Ercole, A.J., Hill, D.J., Strain, A.J. and Underwood, L.E. (1986) Tissue and plasma somatomedin-C/insulin-like growth factor-I concentrations in the human fetus during the first half of gestation. *Pediat. Res.* **20**(3): 253-255.
- 342 Gluckman, P.D., Douglas, R.S., Ambler, G.R., Breier, B.H., Hodgkinson, S.C., Koea, J.B. and Shaw, J.H.K. (1991) The endocrine role of insulin-like growth factor-I. *Acta Paediatr. Scand. (Suppl.)* **372**: 97-105.
- 343 Hossenlopp, P., Segovia, B., Lassarre, C., Roghani, M., Bredon, M. and Binoux, M. (1990) Evidence of enzymatic degradation of insulin-like growth factor binding proteins in the '150K' complex during pregnancy. *J. Clin. Endocrinol. Metab.* **71**: 797-905.
- 344 Giudice, L.C., Farrell, B.M., Pham, H., Lamson, G. and Rosenfeld, R.G. (1990) Insulin-like growth factor binding proteins in maternal serum throughout gestation and the puerperium: effects of pregnancy-associated serum protease activity. *J. Clin. Endocrinol. Metab.* **71**: 806-816.
- 345 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. *J. Endocrinol.* **127**: 383-390.
- 346 Spencer, G.S.G., Hodgkinson, S.C. and Bass, J.J. (1991) Passive immunization against insulin-like growth factor-I (IGF-I) does not inhibit growth in dwarf rats. *Endocrinology* **128**(4): 2103-2109.
- 347 Kerr, D.E., Laarveld, B. and Mans, J.G. (1990) Effects of passive immunization of growing guinea pigs with an insulin-like growth factor-I monoclonal antibody. *J. Endocrinol.* **124**: 403-415.
- 348 Schoenle, E., Zapf, J., Hauri, C., Steiner, T. and Froesch, E.R. (1985)

- Comparison of in vivo effects of insulin-like growth factors-I and II and of growth hormone in hypophysectomized rats. *Acta Endocrinol. (Copenhagen)* **108**: 167-174.
- 349 Van Buul-Offers, S., Ueda, I. and Van der Brande, J.L. (1986) Biosynthetic somatomedin-c (Sm-C/IGF-I) increases the length and weight gain of small dwarf mice. *Pediatr. Res.* **20**: 825-827.
- 350 Suttie, J.M., Gluckman, P.D., Butler, J.H., Fennessy, P.H., Carson, I.D. and Lass, F.J. (1985) Insulin-like growth factor-I: antler stimulating hormone. *Endocrinology* **116**: 846-848.
- 351 LeRoith, D. and Roberts, C.T. (1991) At the cutting edge: insulin-like growth factor-I (IGF-I): a molecular basis for endocrine versus local action. *Mol. Cell Endocrinol.* **77**: C57-C61.
- 352 Walker, D.G., Simpson, M.E., Asling, C.W. and Evans, H.M. (1950) Growth and differentiation in the rat following hypophysectomy at six days of age. *Anat. Rec.* **106**: 539-544.
- 353 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
- 354 Van Obberghen-Schilling, E. and Pouyssegur, J. (1983) Mitogen potentiating action and binding characteristics of insulin and insulin-like growth factors in chinese hamster fibroblasts. *Exp. Cell. Res.* **147**: 369-378.
- 355 Bass, J.J., Davis, S.R., Peterson, A.J., Gluckman, P.D., Butler, J. and Gray M. (1984) Effect of different diets and starvation on concentrations of somatomedins and other growth controlling hormones in wether lambs. *Proc. Aust. Soc. Endocrinol.* **27** (Suppl.) p 15.
- 356 O'Sullivan, U., Gluckman, P.D., Breier, B.H., Wooddall, S., Siddiqui, R.A. and McCutcheon, S.N. (1989) Insulin-like growth factor-I (IGF-I)

- in mice reduces weight loss during starvation. *Endocrinology* **125**: 2793-2794.
- 357 Clemmons, D.R. (1991) Insulin-like growth factor binding proteins: roles in regulating IGF physiology. *J. Dev. Physiol.* **15**: 105-110.
- 358 Hill, D.J., Clemmons, D.R., Wilson, S et.al. (1989) Immunological distribution of one form of insulin-like growth factor binding protein and IGF peptides in human fetal tissues. *J. Mol. Endocrinol.* **2**: 31-38.
- 359 Pova, G., Roovete, A. and Hall, K. (1984) Cross-reaction of serum somatomedin-binding protein in a radioimmunoassay developed for somatomedin-binding protein isolated from human amniotic fluid. *Acta Endocrinol.* **107**: 563-570.
- 360 Vicini, J.L., Buonomo, F.C., Collier, R.J. and Clemmons, D.R. (1990) Response of insulin-like growth factor binding protein-2 (IGFBP-2) to sometribove (bST). *J. Anim. Sci. Vol 68 (Suppl 1)* **Abstract 128**, p 280.
- 361 Smith, P.E. (1954) Continuation of pregnancy in Rhesus monkeys (macaca mulatta) following hypophysectomy. *Endocrinology* **55**: 655-64.
- 362 Reid, J.D. (1960) Congenital absence of the pituitary gland. *J. Pediatr.* **56**: 658-64.
- 363 Rimoin, D.L., Merimee, T.J., McKusick, V.A. (1966) Growth-hormone deficiency in man: An isolated, recessively inherited defect. *Science* **152**: 1635-7.
- 364 Palmiter, R.D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Posenfeld, M.G., Birnberg, N.C., Evans, R.M. (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* **300**: 611-5.

- 365 Li, C.H., Dixon, J.S. (1971) Human pituitary growth hormone. XXXII. The primary structure of the hormone: *Revision. Arch. Biochem Biophys* **146**: 233-6.
- 366 Niall, H.D. (1971) Revised primary structure for human growth hormone. *Nature New Bio.* **230**: 90-1.
367. Schuller, A.G.P., Lindenbergh-Kortleve, D.J., De Boer, W.I., Zwarthoff, E.C., and Drop, S.L.S. (1992) Epitope mapping of a monoclonal antibody against IGFBP-I, which functionally interferes with IGF binding. *Proceedings of 2nd International IGFBP workshop*, Opio, Coted'Azur, France, 27-30 August. **Abstract 9.**
- 368 Cohick, W.S., Busby, W.H., Clemmons, D.R. and Bauman, D.E. (1990) Insulin-like growth factor binding proteins in serum of lactating cows treated with recombinant N-methionyl bovine somatotopin. *J. Ani. Sci.* *68 (suppl. 1)*. **Abstract 208.**
- 369 Vicini, J.L., Buonomo, F.C., Collier, R.J., Clemmons, D.R. (1990) Response of insulin-like growth factor binding protein-2 (2GFBP-2) to sometribove (bST, USAN) and stage of lactation in dairy cows. *J. Anl. Sci.* *68 (suppl. I)*. **Abstract 128.**
- 370 Camacho-Hubner, C., Clemmons, D.R., D'Ercole, A.J., (1991) Regulation of insulin-like growth factor (IGF) binding proteins in transgenic mice with altered expression of growth hormone and IGF-I. *Endocrinology* **129**: 1201-1206.
- 371 Lamson, G., Cohen, P., Gargosky, S., Giudice, L.C., Müller, H.L., Wilson, Y Oh K., Hintz, R.L., and Rosenfeld, R.G. (1992) Proteolysis of IGFBP-3 may be a common regulatory mechanism of IGF action in vivo. *Proceedings of 2nd International IGFBP workshop*, Opio, Cote d'Azur, France. 27-30 August. **Abstract 28.**
- 372 Hodgkinson, S.C., Bass, J.J. and Gluckman, P.D. (1991) Plasma IGF-I

- binding proteins in sheep: Effect of recombinant growth hormone treatment and nutritional status. *Domestic Animal Endocrinology* **8** (3): 343-351.
- 373 Ohlsson, C., Nilsson, A., Isaksson, O. and Lindahl, A. (1992) Growth hormone but not insulin-like growth factors-1 increases cell divisions in the germinal cell layer of the rat tibia growth plate. *Ninth International Congress of Endocrinology*. Nice, France. **Abstract P-08.01.061**.
- 374 Daughaday, W.H. (1989) A personal history of the origin of the somatomedin hypothesis and recent challenges to its validity. *Perspectives in Biology and Medicine* **32** (2): 194-211.
- 375 Smith, P.E. (1926) Ablation and transplantation of the hypophysis in the rat. *Anat. Rec.* **32**: 221.
- 376 Baird, J.D., Black, M.W., and Faulkner, D.E. (1967) Semiautomated method for the determination of free fatty acids in plasma. *Journal of Clinical Pathology*, **20**: 905-909.
- 377 Dole, V.P. (1956) A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *Journal of Clinical Investigation* **35**: 150-154.
- 378 Trout, D.L., Estes, E.H. Jr., and Friedberg, S.J. (1960) Titration of free fatty acids of plasma: a study of current methods and a new modification. *J. Lipid Res.* **1**: 199.
- 379 Mosinger, F. (1965) Photometric adaptation of Dole's microdetermination of free fatty acids. *Journal of Lipid Research* **6**: 157-159.
- 380 Lijana, R.C. and Williams, M.C. (1979) Tetramethylbenzidine - a substitute for benzidine in hemoglobin analysis. *Journal of Laboratory Clinical Medicine* **94**: 266-276.

- 381 Standefer, J.C. and Vanderjagt, D. (1977) Use of tetramethylbenzidine
in plasma hemoglobin assay. *Clinical Chemistry* **23**: 749-751.
- 382 Hodgkinson, S.C., Napier, J.R., Davis, S.R., Patel, B. and Gluckman, P.D.
(1989) Binding protein, radioreceptor and biological activities of
recombinant methionyl insulin-like growth factor-I variants. *Mol.
Cell. Endocrinol.* **66**: 37-44.
- 383 Harel, Z. and Tannenbaum, G.S. (1992) Synergism between insulin-like
growth factor-I and -II in central regulation of pulsatile growth
hormone secretion. Ninth International Congress of
Endocrinology, Nice, France. **Abstract P-08.01.050**
- 384 Hodgkinson, S.C., Moore, L.G., Napier, J.R., Davis, S.R., Bass, J.J. and
Gluckman, P.D. (1989) Characterization of insulin-like growth
factor binding proteins in ovine tissue fluids.
- 385 Clemmons, D.R. (1984) Factors controlling blood concentrations of
somatomedin C. *Journal of Clinical Endocrinology and Metabolism*
13: 113-143.
- 386 Clemmons, D.R. and Van Wyk, J.J. (1989) Structural and functional
analysis of insulin-like growth factors. *British Medical Bulletin* **45**:
415-430.
- 387 Rechler, M.M., Nissley, S.P. Insulin-like growth factors In: Sporn M.B.,
Roberts A.B. (eds) *Peptide Growth Factors and Their Receptors*
I. Berlin. *Springer-Verlag*. 1990: 263.
- 388 Rechler, M.M. (1992) Insulin-like growth factor binding proteins.
Vitamins & Hormones (in press).
- 389 Hintz, R.L. (1984) Clinics in Endocrinology and Metabolism (W.H.
Daughaday, eds.) p. 31: Saunders, London.
- 390 Smith, G.L. (1984) Somatomedin carrier proteins. *Mol. Cell. Endocrinol.*
34: 83-89.

- 391 Chatelain, P.G., Van Wyk, J.J., Copeland, K.C., Blethen, S.L. and Underwood, J.E. (1983) Effect of *in vitro* action of serum proteases or exposure to acid on measurable immunoreactive somatomedin C in serum. *J. Clin. Endocrinol. Metab.* **56**: 376.
- 392 D'Ercole, A.J., Decedue, C.J., Furlanetto, R.W., Underwood, J.E. and Van Wyk, J.J. (1977) Evidence that somatomedin-C is degraded by the kidney and inhibits insulin degradation. *Endocrinology* **101**: 577.
- 393 Hossenlopp, P., Seurin, D., Segovia-Quinson, S. and Binoux, M. (1986) Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies. *Analyt. Biochem.* **154**: 138-143.
- 394 McLellan, K.C., Hooper, S.B., Bocking, A.D., Delhanty, P.J.D., Phillips, I.D., Hill, D.J. and Han, V.K.M. (1990) Prolonged hypoxia induced by the reduction of maternal uterine blood flow alters insulin-like growth factor-binding protein-1 (IGFBP-1) and IGFBP-2 gene expression in the ovine fetus. *Endocrinology* **131**: 1619-1628.
- 395 Marchalonis, J.J. (1969) An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochemical Journal* **113**: 299-305.
- 396 Gall, J.G. and Pardue, M.L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proceedings of the National Academy of Sciences USA* **63**: 378-383.
- 397 John, H.A., Birnstiel, M.L., Jones, K.W. (1969) RNA-DNA hybrids at the cytological level. *Nature* **223**: 582-587.
- 398 Brahic, M. and Haase, A.T. (1978) Detection of viral sequences of low

- reiteration frequency by *in situ* hybridization. *Proceedings of the National Academy of Sciences USA* **75** (12): 6125-6129.
- 399 Hudson, P., Penschow, J., Shine, J., Ryan, G., Niall, H. and Coghlan, J. (1981) Hybridization histochemistry: Use of recombinant DNA as a "Homing Probe" for tissue localization of specific mRNA populations. *Endocrinology* **108** (1): 353-356.
- 400 Cox, K.H., DeLeon, D.V., Angerer, L.M. and Angerer, R.C. (1984) Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Developmental Biology* **101**: 485-502.
- 401 Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from
- 402 Wetmur, J.G., Ruyechan, W.T. and Douthart, R.J. (1981) Denaturation and renaturation of penicillium chrysogenum mycophage double-stranded ribonucleic acid in tetraalkylammonium salt solutions. *Biochemistry* **20**: 2999-3002.
- 403 Gibson, S.J. and Polak, J.M. (1990) Principles and applications of complementary RNA probes. In *in situ* hybridization. Principles and Practice J.M. Polak and J. O'D. McGee (eds). Oxford University Press, Incorporated, Oxford: 81-94.
- 404 Pintar, J.E. and Lugo, D.I. (1987) Localization of peptide hormone gene expression in adult and embryonic tissue. In *in situ* hybridization. Applications to Neurobiology K.L. Valentino, J.H. Eberwine and J.D. Barchas (eds). Oxford University Press, Incorporated, New York: 179-196.
- 405 Tecott, L.H., Eberwine, J.G., Barchas, J.D. and Valentino, K.L. (1987) Methodological considerations in the utilization of *in situ*

- hybridization. In *in situ* hybridization. Applications to Neurobiology K.L. Valentino, J.H. Eberwine and J.D. Barchas (eds). Oxford University Press, Incorporated, New York: 3-24.
- 406 Penschow, J.D. (1990) *In situ* hybridization: 2-21.
- 407 Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. (1982) Nucleotide sequence of bacteriophage λ DNA. *Journal of Molecular Biology* **162**: 729-773.
- 408 Höfler, H., (1990) Principles of *in situ* hybridization. In *In Situ Hybridization. Principles and Practice* J.M. Polak and J.O'D. McGee (eds). Oxford University Press, Incorporated, Oxford: 15-29.
- 409 Haase, A., Brahic, M., Stowring, L. and Blum, H. (1984) Detection of viral nucleic acids by *in situ* hybridization. In *Methods in Virology*. Academic Press, Incorporated VII: 189-226.
- 410 Zeller, R., (1989) Fixation, embedding, and sectioning of tissues, embryos, and single cells. In *Current Protocols in Molecular Biology* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. More, J.G. Seidman, J.A. Smith and K. Struhl (eds). John Wiley and Sons, Incorporated, New York **2**: 14.1.1-14.1.8.
- 411 Pfeifer-Ohlsson, S., Goustin, A.S., Rydnert, J., Wahlström, T., Bjersing, L., Stehelin, D. and Ohlsson, R. (1984) Spatial and temporal pattern of cellular myc oncogene expression in developing human placenta: Implications for embryonic cell proliferation. *Cell* **38**: 585-596.
- 412 Humason, G.L. (1979) Part I Basic procedures and Part II specific staining methods. In animal tissue techniques. W.H. Freeman and Company, W.H. Freeman and Company, San Francisco: 1-384.
- 413 Gall. J.G. and Pardue, M.L. (1971) Nucleic acid hybridization in cytological preparations. In *Methods and Enzymology* L.

- Grossman and K. Moldave (eds.). Academic Press, Incorporated, New York **XXI**: 470-480.
- 414 Gee, C.E. and Roberts, J.L. (1983) Laboratory methods. *In situ* hybridization histochemistry: A technique for the study of gene expression in single cells. *DNA* **2** (2): 157-163.
- 415 Ebeling, W., Hennrich, N., Klockow, M., Metz, H. Lang (1974) Proteinase K from *Tritirachium album* Limber. *European Journal of Biochemistry* **47**: 91-97.
- 416 Angerer, L.M. and Angerer, R.C. (1981) Detection of poly A+ RNA in sea urchin eggs and embryos by quantitative in situ hybridization. *Nucleic Acids Research* **9** (12): 2819-2840.
- 417 Hayashi, S., Gillam, I.C., DeLaney, A.D. and Tener, G.H. (1978) Acetylation of chromosome squashes of *drosophila melanogaster* decreases the background in autoradiographs from hybridization with [¹²⁵I]-Labelled RNA. *The Journal of Histochemistry and Cytochemistry* **26** (8): 677-679.
- 418 Wilkins, R.J., Molenaar, A.J., Ohlsson, R., Reeve, A.E., Yun, K. and Becroft, D.M.O. (1989). Wilm's tumorigenesis, insulin-like growth factor II gene expression, and blocked differentiation. *In Cancer Cells/7: Molecular Diagnostics of Human Cancer*, pp 321-326. Ed. M Furth and M. Greaves. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- 419 Clemmons, D.R. and Van Wyk, J.J. (1981) Somatomedin: physiological control and effects on cell proliferation. Ch. 5. Tissue growth factors. R. Basearga, ed. Handbook Exp. Pharmacol. Springer Verlag Berlin. pp 161.
- 420 Clemmons, D.R. and Van Wyk, J.J. (1984) Factors controlling blood concentration of somatomedin. *C. Clin. Endocrinol. Metabol.* **13**:

113.

- 421 Canalis, E., Centrella, M., and McCarthy, T.L. (1991) Regulation of insulin-like growth factor-II production in bone cultures. *Endocrinology* **129**, p 145.
- 422 Shapiro, B., Waligora, K. and Pinstone, B.L. (1978) Generation of somatomedin activity in response to growth hormone and insulin from isolated perfused livers of normal and protein-malnourished rats. *J. Endocrinol.* **79**: 369.
- 423 Gluckman, P.D. and Butler, J.H. (1985) Circulating insulin-like growth factor-I and -II concentrations are not dependant on pituitary influences in the midgestation fetal lambs. *J. Dev. Physiol.* **7**: 441.
- 424 Gluckman, P.D., Bassett, N.S. and Butler, J.H. (1986) The somatotrophic axis in the fetus and newborn. In: *Perinatal Endocrinology*. E. Albrecht (ed) Perinatol. Press, Ithaca. pp 1-20.
- 425 Spencer, G.S.C., Hill, D.J., Garssen G.J., MacDonald, A.A. and Colenbrander, B. (1983) Somatomedin activity and growth hormone levels in body fluid of the fetal pig: effect of chronic hyperinsulinaemia. *J. Endocrinol.* **96**: 107.
- 426 Phillips, L.S. and Unterman, T.G. (1984) Somatomedin activity in disorders of nutrition and metabolism. *J. Clin. Endocrinol. Metab.* **13**: 145.
- 427 Phillips, L.S. and Vassilopoulou-Sellin, R. (1980) Somatomedins (part I and II). *New England J. Med.* **302**: 271 and 438.
- 428 Baxter, R.C., Bryson, J.M. and Turtle, J.R. (1980) Somatogenic receptors of rat liver: regulation by insulin. *Endocrinology* **107**: 1176.
- 429 Clemmons, D.R. and Van Wyk, J.J. (1984) Factors controlling blood concentrations of somatomedin C. *Journal of Clinical Endocrinology and Metabolism* **13**: 113-143.

- 430 Clemmons, D.R. (1989) Structural and functional analysis of insulin-like growth factors. *British Medical Bulletin* **45**: 465-480.
- 431 Clemmons, D.R., Seek, M.M. and Underwood, L.E. (1985) Supplemental essential amino acids augment the somatomedin-C/insulin-like growth factor I response to refeeding after fasting. *Metabolism* **34**: 391.
- 432 Clemmons, D.R. and Van Wyk, J.J. (1984) Factors controlling blood concentration of somatomedin C. *Clin. Endocrinol. Metabol.* **13**: 113.
- 433 Isley, W.L., Underwood, L.E. and Clemmons, D.R. (1983) Dietary components that regulate serum somatomedin-C concentrations in humans. *J. Clin. Invest.* **71**: 175.
- 434 Isley, W.L., Underwood, L.E. and Clemmons, D.R. (1984) Changes in plasma somatomedin-C in response to ingestion of diets with variable protein and energy content. *J. Parenter. Ent. Nutr.* **8**: 407.
- 435 Phillips, L.S., Orawski, A.T., Belosky, D.C. (1978) Somatomedin and nutrition. IV. Regulation of somatomedin activity and growth cartilage activity by quantity and composition of diet in rats. *Endocrinology* **103**: 121.
- 436 Phillips, L.S. and Unterman, T.G. (1984) Somatomedin activity in disorders of nutrition and metabolism. *J. Clin. Endocrinol. Metab.* **13**: 145.
- 437 D'Ercole, A.J., Hill, D.J., Strain, A.J. and Underwood, L.E. (1986) Tissue and plasma somatomedin-C/insulin-like growth factor-I concentrations in the human fetus during the first half of gestation. *Pediatric Research*, **20**: 253-255.
- 438 Han, V.K.M., Lund, P.K., Lee, D.C. and D'Ercole, A.J. (1988) Expression of somatomedin/insulin-like growth factor messenger

- ribonucleic acids in human fetus: identification, characterization and tissue distribution. *Journal of Clinical Endocrinology and Metabolism* **66**: 422-429.
- 439 Underwood, L.E., D'Ercole, A.J., Clemmons, D.R. and Van Wyk, J.J. (1986) Paracrine functions of somatomedin. In *Clinics in Endocrinology and Metabolism: Paracrine control*, Vol. 15, pp 59-77. Ed. P. Franchimont. London: W.B. Saunders.
- 440 Yahya, Z.A.H., Bates, P.C. and Millward, D.J. (1990) Responses to protein deficiency of plasma and tissue insulin-like growth factor-I levels and proteoglycan synthesis rates in rat skeletal muscle and bone. *Journal of Endocrinology* **127**: 497-503.
- 441 Crane, B. and Lane, C. (1977) Modified semi-automated method for free fatty acids in serum. *Journal of Clinical Pathology* **30**: 754-757.
- 442 Spencer, G.S.G., Bass, J.J., Hodgkinson, S.C., Edgley, W.H.R. and Moore, L.G. (1991) Effect of intracerebroventricular injection of IGF-I on circulating growth hormone concentrations in the sheep. *Domestic Animal Endocrinology* **8**: 155-160.
- 443 Hodgkinson, S.C., Bass, J.J. and Gluckman, P.D. (1991) Plasma IGF-I binding proteins in sheep: Effect of recombinant growth hormone treatment and nutritional status. *Domestic Animal Endocrinology* **8**: 343-351.
- 444 Merimee, T.J. and Fineberg, S.E. (1974) Growth hormone secretion in starvation: A reassessment. *Journal of Clinical Endocrinology and Metabolism* **39**: 385-386.
- 445 Ho, K.Y., Veldhuis, J.D., Johnson, M.L., Furlanetto, R., Evans, W.S., Alberti, K.G.M.M., Thorner, M.O. (1988) Fasting enhances growth hormone secretion and amplifies the complex rhythms of growth hormone secretion in man. *Journal of Clinical Investigation*

81: 968-975.

- 446 Wallace, A.L.C. and Bassett, J.M. (1970) Plasma growth hormone concentrations in sheep measured by radioimmunoassay. *Journal of Endocrinology* **47**: 21-36.
- 447 Trenkle, A. (1976) Estimates of the kinetic parameters of growth hormone metabolism in fed and fasted calves and sheep. *Journal of Animal Science* **43** (5): 1035-1043.
- 448 Van Maanen, M.C., McCutcheon, S.N. and Purchas, R.W. (1989) Plasma metabolite and hormone concentrations in southdown ram hoggets from lines divergently selected on the basis of backfat thickness. *New Zealand Journal of Agricultural Research* **32**: 219-226.
- 449 Freemark, M., Comer, M., Mularoni, T., D'Ercole, A.J., Grandis, A. and Kodack, L. (1990). Placental lactogen receptors in maternal sheep liver: Effects of fasting and refeeding. *American Journal of Physiology* **258** (21): E338-E346.
- 450 Driver, P.M. and Forbes, J.M. (1981) Episodic growth hormone secretion in sheep in relation to time of feeding, spontaneous meals and short term fasting. *Journal of Physiology* **317**: 413-424.
- 451 Bassett, J.M., Weston, R.H., and Hogan, J.P. (1971) Dietary regulation of plasma insulin and growth hormone concentrations in sheep. *Australian Journal of Biological Sciences* **24**: 321-330.
- 452 Harvey, S., Scanes, C.G., Chadwick, A. and Bolton, N.J. (1978) Influence of fasting, glucose and insulin on the levels of growth hormone and prolactin in the plasma of the domestic fowl (*gallus domesticus*). *Journal of Endocrinology* **76**: 501-506.
- 453 Hart, I.C., Bines, J.A., Morant, S.V. and Ridley, J.L. (1978) Endocrine control of energy metabolism in the cow: comparison of the levels of hormones (prolactin, growth hormone, insulin and thyroxine)

- and metabolites in the plasma of high and low yielding cattle at various stages of lactation. *J. Endocrinol.* **77**: 333.
- 454 Hove, K. and Bloom, A.K. (1973) Plasma insulin and growth hormone in dairy cows; diurnal variation and relation to food intake and plasma sugar and acetoacetate levels. *Acta Endocrinol.* **73**: 289.
- 455 Trenkle, A., (1989) Influence of feeding on growth hormone secretion and response to growth hormone-releasing factor in sheep. *Journal of nutrition* **119**: 61-65.
- 456 Hart, I.C., Chadwick, P.M.E., Coert, A., James, S. and Simmonds, A.D. (1985) Effect of different growth hormone-releasing factors on the concentrations of growth hormone, insulin and metabolites in the plasma of sheep maintained in positive and negative energy balance. *J. Endocrinol.* **105**: 113.
- 457 Bauman, D.E. and Currie, W.B. (1980) Partitioning of nutrients during pregnancy and lactation: A review of mechanism involving homeostasis and homeorhesis. *J. Dairy Sci.* **63**: 1514.
- 458 Atinmo, T., Baldijao, C., Houpt, K.A., Pond, W.G. and Barnes, R.H. (1978) Plasma levels of growth hormone and insulin in protein malnourished vs. normal growing pigs in response to arginine or glucose infusion. *J. Anim. Sci.* **46**: 409.
- 459 Bines, J.A., Hart, J.L. and Morant, S.V. (1980) Endocrine control of energy metabolism in the cow: the effect on milk yield and levels of some blood constituents of injecting growth hormone and growth hormone fragments. *Br. J. Nutr.* **43**: 179.
- 460 Bines, J.A. and Hart, I.C. (1982) Metabolic limits to milk production, especially roles of growth hormone and insulin. *Journal of Dairy Science*, **65**: 1375-1389.
- 461 Bauman, D.E., McCutcheon, S.N., Steinhour, W.D., Eppard, P.J. and

- Sechen, S.J. (1985) Sources of variation and prospects for improvement of productive efficiency in the dairy cow: a review. *J. Anim. Sci.* **60**: 583.
- 462 Berelowitz, N., Szabo, M., Frohman, L.A., Firestone, S. and Chu, L. (1981) Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. *Science* **212**: 1279.
- 463 Maes, M., Underwood, L.E., Ketelslegers, J.M. (1983b) Plasma somatomedin-C in fasted and refed rats: close relationship with changes in liver somatogenic but not lactogenic binding sites. *J. Endocrinol.* **97**: 243.
- 464 Maes, M., Underwood, L.E., Ketelslegers, J.M. (1986) Low serum somatomedin-C in insulin-dependent diabetes: evidence for a postreceptor mechanism. *Endocrinology* **118**: 377.
- 465 Phillips, L.S. and Young, H.S. (1976) Nutrition and somatomedin. 1. Effect of fasting and refeeding on serum somatomedin activity and cartilage growth activity in rats. *Endocrinology* **99**: 304.
- 466 Merimee, T.J., Zapf, J., Froesch, E.R. (1982) Insulin-like growth factors (IGFs) in pygmies and subjects with pygmy trait: characterization of the metabolic actions of IGF-I and IGF-II in man. *J. Clin. Endocrinol. Metab.* **55**: 1081.
- 467 Elsasser, T.H., Rumsey, I.S. and Hammond, A.C. (1989) Influence of diet on basal and growth hormone-stimulated plasma concentrations of IGF-I in beef cattle. *Journal of Animal Science* **67**: 128-141.
- 468 McShane, T.M., Schillo, K.K., Boling, J.A., Bradley, N.W. and Hall, J.B. (1989). Effects of recombinant DNA-derived somatotropin and dietary energy intake on development of beef heifers. I. Growth

- and puberty. *Journal of Animal Science* **67**: 2230-2236.
- 469 Hodgkinson, S.C., Davis, S.R., Burleigh, B.D., Henderson, H.V. and
Gluckman, P.D. (1987) Metabolic clearance rate of insulin-like
growth factor-I in fed and starved sheep. *Journal of Endocrinology*
115: 233-240.
- 470 Gluckman, P.D. and Butler, J.H. (1983) Parturition-related changes in
insulin-like growth factors-I and -II in the perinatal lamb. *Journal*
of Endocrinology **99**: 223-232.
- 471 Thissen, J.P., Triest, S., Maes, M., Underwood, L.E. and Ketelslegers,
J.M. (1990) The decreased plasma concentration of insulin-like
growth factor-I in protein-restricted rats is not due to decreased
numbers of growth hormone receptors on isolated hepatocytes.
Journal of Endocrinology **124**: 159-165.
- 472 McKnight, B.J. and Goddard, C. (1989) The effect of food restriction on
circulating insulin-like growth factor-I in mice divergently selected
for high or low protein or fat to body mass ratios. *Comparative*
biochemistry and physiology **92A (4)**: 565-569.
- 473 Phillips, L.S., Goldstein, S. and Gavin, J.R. (1988) Nutrition and
somatomedin XVI: Somatomedins and somatomedin inhibitors in
fasted and refed rats. *Metabolism* **37 (3)**: 209-216.
- 474 Bass, J.J., Gluckman, P.D., Fairclough, R.J., Peterson, A.J., Davis, S.R.
and Carter, W.D. (1987) Effect of nutrition and immunisations
against somatostatin on growth and insulin-like growth factors in
sheep. *Journal of Endocrinology* **112**: 27-31.
- 475 Clemmons, D.R., and Van Wyk, J.J. (1981) Somatomedin: physiological
control and effects on cell proliferation. In *Handbook of*
Experimental Pharmacology. Vol. **57**, pp 161-208 Ed. R. Baserga,
Berlin Heidelberg New York: Springer.

- 476 Baxter, R.C. and Martin, J.L. (1989) Binding proteins for the insulin-like growth factors and structure and function. *Progress in Growth Factors Research* 1: 49-68.
- 477 Evans, H.M. and Long, J.A. (1921) The effect of the anterior lobe administered intraperitoneally upon growth maturity, and oestrus cycles of the rat. *Anat. Rec.* 21: 62.
- 478 Preece, M.A. and Holder, A.T. (1982) The somatomedins: a family of serum growth factors. In: *Recent Advances in Endocrinology and Metabolism* 2: 47-72. Ed. O'Riordan, J.L.H., Churchill-livingstone, New York.
- 479 Humbel, R.E. (1984) Insulin-like growth factors, somatomedins and multiplication stimulating activity. In: *Hormonal proteins and peptides*. XII: 57-79. (Li, C.H. ed) Academic Press, New York.
- 480 Froesch, E.R., Schmid, C., Schwander, J. and Zapf, J. (1985) Actions of insulin-like growth factors. *Annual Review of Physiology* 47: 443-467.
- 481 Roberts, C.T., Lasky, S.R., Lowe, W.L., Seaman, W.T. and LeRoith, D. (1987) Molecular cloning of rat insulin-like growth factor I complementary deoxyribonucleic acids; differential messenger ribonucleic acid processing and regulation by growth hormone in extrahepatic tissues. *Molecular Endocrinology* 1: 243-248.
- 482 Nilsson, A., Carlsson, B., Isgaard, J., Isaksson, O.G.P., and Rymo, L. (1989) Regulation by GH of insulin-like growth factor-I mRNA expression in rate epiphyseal growth plate as studied with in situ hybridization. *Journal of Endocrinology* 125: 67-74.
- 483 Owens, P.C., Johnson, R.J., Campbell, R.G. and Ballard, F.J. (1989) 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.

- 484 Fourie, P.D., Kirton, A.H. and Jury, K.E. (1970) Growth and development of sheep II. Effect of breed and sex on the growth and carcass composition of the Southdown and Romney and their cross. *New Zealand J. of Agricultural Research* **13**: 753-770 New Zealand Publication No. 516.
- 485 Dukes, H.H. (1974) The physiology of domestic animals. Sixth Edition, Chapter II Blood, p 61. Ed. H.H. Dukes, Comstock Publishing Company Inc. Ithaca, New York.
- 486 Hart, I.C., Blake, L.A., Chadwick, P.M.F., Payne, G.A. and Simmonds, A.D. (1984) The heterogeneity of bovine growth hormone: extraction from the pituitary of components with different biological and immunological properties. *Biochem. J.* **218**: 573.
- 487 Hart, I.C., Chadwick, P.M.E., Coert, A., James, S. and Simmonds, A.D. (1985a) Effect of different growth hormone-releasing factors on the concentrations of growth hormone, insulin and metabolites in the plasma of sheep maintained in positive and negative energy balance. *J. Endocrinol.* **105**: 113.
- 488 Hart, I.C., Chadwick, P.M.E., James, S. and Simmonds, A.D. (1985b) Effect of intravenous bovine growth hormone and human pancreatic growth hormone-releasing factor on milk production and plasma hormones and metabolites in sheep. *J. Endocrinol.* **195**: 189.
- 489 Han, V.K.M., D'Ercole, A.J. and Lund, P.K. (1987) Cellular localization of somatomedin (insulin-like growth factor-I) messenger RNA in the human fetus. *Science* **236**: 193-197.
- 490 Pova, G., Hall, K. and Collins, V.P. (1989) Studies on somatomedin binding proteins. In: Proc. Adv. in Growth Hormone and Growth

- Factor Research, Milano, 1987, edited by E.E. Muller, D. Cocchi, and V. Locatelli. New York: Springer-Verlag, 1989, p. 121-132.
- 491 Hodgkinson, S.C., Davis, S.R., Burleigh, B.D., Henderson, H.V. and Gluckman, P.D. (1987) Metabolic clearance rate of insulin-like growth factor-I in fed and starved sheep. *J. Endocr* **115**: 233-240.
- 492 Hodgkinson, S.C. (1991) Insulin-like growth factors & their binding proteins in post-natal ruminants. PhD thesis. University of Auckland, Auckland, New Zealand.
- 493 Saunders, J.C., Dickson, M.C., Pell, J.M. and Gilmour, R.s. (1991) Expression of a growth hormone-responsive exon of the ovine insulin-like growth factor-I gene. *Journal of Molecular Endocrinology* **7**: 233-240.
- 494 Grant, A.L., Helferich, W.G., Kramer, S.A., Merkel, R.A. and Bergen, W.G. (1991) Administration of growth hormone to pigs alters the relative amount insulin-like growth factor-I mRNA in liver and skeletal muscle. *Journal of Endocrinology* **130**: 331-338.
- 495 Schulster, D., Moreland, B.M. and Chiong, M. (1991) In vivo effect of growth hormone on mRNA for IGF-1A and IGF-1B in muscles of dwarf rats. *Journal of Endocrinology* **129**: (supplement).
- 496 Pell, J.M., Saunders, J.C. and Gilmour, R.S. (1993) Differential regulation of transcription initiation from insulin-like growth factor-I (IGF-I) leader exons and of tissue IGF-I expression in response to changed growth hormone and nutritional status in sheep. *Endocrinology* (in press).
- 497 Hodgkinson, S.C., Spencer, G.S.G., Bass, J.J., Davis, S.R. and Gluckman, P.D. (1991) Distribution of circulating insulin-like growth factor-I into tissues. *Endocrinology* **129** (4): 2085-2093.
- 498 Matthews, L.S., Norstedt, G. and Palmiter, R.D. (1986) Regulation of

- insulin-like growth factor 1 gene expression by growth hormone.
Proc. Natl. Acad. Sci. (USA) **83**: 9343-9347.
- 499 Goldstein, S., and Phillips, L.S. (1991) Extraction and
 nutritional/hormonal regulation of tissue insulin-like growth factor
 1 activity. *J. of Biochem.* **266**: 14725-14731.
- 500 Guler, H.P., Eckardt, K.U., Zapf, J., Bauer, C. and Froesch, E.R. (1989)
 Insulin-like growth factor-I increases glomerular filtration rate and
 renal plasma flow in man. *Acta Endocrinologica* **121**: 101-106.
- 501 Murphy, L.J., Murphy, L.C. and Friesen, H.G. (1987) Estrogen induces
 insulin-like growth factor-I expression in the rat uterus. *Mol.*
Endocrinol. **1**: 445-450.
- 502 Rechler, M.M., Nissley, S.P. (1990) Insulin-like growth factors In: Sporn,
 M.B., Roberts, A. B. (eds) *Peptide Growth Factors and Their*
Receptors I. Berlin. Springer-Verlag. **263**.
- 503 Clemmons, D.R. (1990) Insulin-like growth factor binding proteins.
Trends Endocrinol. Metab. **1**: 412-417.
- 504 Rosenfeld, R.G., Lamson, G., Pham, H., Oh, Y., Conover, C., DeLeon,
 D.D., Donovan, S.M., Ocrant, I. and Giudice, L. (1990) Insulin-
 like growth factor-binding proteins. *Recent Prog. Horm. Res.*, **46**:
 99-163.
- 505 Ooi, G.T., (1990) Insulin-like growth factor-binding proteins (IGFBPs);
 more than just 1, 2, 3. *Mol. Cell. Endocrinol.* **71**: C39-C43.
- 506 Shimasaki, S., Gao, L., Shimonaka, M. and Ling, N. (1991) Isolation and
 molecular cloning of insulin-like growth factor-binding protein-6.
Mol. Endocrinol. **5**: 938-948.
- 507 Daughaday, W.H., Ward, A.P., Goldberg, A.C., Trivedi, B. and Kapadia,
 M. (1982) Characterization of somatomedin binding on human
 serum by ultracentrifugation and gel filtration. *J. Clin. Endocrinol.*

Metab. **51**: 12-19.

- 508 Donovan, S.M., OH., Y., Pham, H. and Rosenfeld, R.G. (1989)
Ontogeny of serum insulin-like growth factor binding proteins in
the rat. *Endocrinology* **125**: 2621-2627.
- 509 McCusker, R.H., Campion, D.R. and Clemmons, D.R. (1988) The
ontogeny and regulation of 31,000 molecular weight insulin-like
growth factor-binding protein in fetal porcine plasma and sera.
Endocrinology **122**: 2071-2079.
- 510 Liu, F., Powell, D.R., Styne, D.M., and Hintz, R.L. (1991) Insulin-like
growth factors (IGFs) and IGF-binding proteins in the developing
rhesus monkey. *K. Clin. Endocrinol. Metab.* **72**: 905-911.
- 511 Zapf, J., Schmid, C., Kiefer, M., Guler, II-P. Froesch, E.R. (1991) *In*
vivo models of IGF binding protein (IGFBP) regulation with
possible relevance to their physiological roles. In: Spencer E.M.
(ed) *Modern Concepts of Insulin-like Growth Factors*. New York.
Elsevier Science Publ. Co. **591**.
- 512 Camacho-Hubner, C., Clemmons, D.R., D'Ercole, A.J. (1991) Regulation
of insulin-like growth factor (IGF) binding proteins in transgenic
mice with altered expression of growth hormone and IGF-I.
Endocrinology **129**: 1201-1206.
- 513 Kirk, E., Neely and Rosenfeld, R.G. (1992) Insulin-like growth factors
(IGFs) reduce IGF-binding protein-4 (IGFBP-4) concentration and
stimulate IGFBP-3 independently of IGF receptors in human
fibroblasts and epidermal cells. *Endocrinology* **130**: 985-993.
- 514 Rechler, M.M. (1992) Insulin-like growth factor binding proteins.
Vitamins & Hormones (in press).
- 515 Cohick, W.S., Busby, W.H., Clemmons, D.R. and Bauman, D.E. (1990)
Insulin-like growth factor binding proteins in serum of lactating

cows treated with recombinant N-methionyl bovine somatotropin.

J. Anl. Sci. (suppl. 1) **Abstract 208.**

- 516 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. *J. Endocrinol.* **123**: R17-R20.
- 517 Thrailkill, K.M., Clemmons, D.R., Busby, W.H., Handwerger, S. (1990) The differential regulation of insulin-like growth factor binding protein secretion from human decidual cells by IGF-I, insulin and relaxin. *J. Clin. Invest.* **86**: 878-883.
- 518 Hill, D.J., Camacho-Hubner, C., Rashid, P., Strain, A.J., Clemmons, D.R. (1989) Insulin-like growth factor (IGF)-binding protein release by human fetal fibroblasts: dependency on cell density and IGF peptides. *J. Endocrinol.* **122**: 87-89.
- 519 Snyder, D.K., Clemmons, D.R. (1990) Insulin-dependent regulation of insulin-like growth factor binding protein-1. *J. Clin. Endocrinol. Metab.* **71**: 1632-1636.
- 520 Conover, C.A., Lee, P.D.K. (1990) Insulin regulation of insulin-like growth factor binding protein production in cultured HepG2 cells. *J. Clin. Endocrinol. Metab.* **70**: 1062-1067.
- 521 Zapf, J., Schmid, C., Guler, H.P., Waldvogel, M., Hauri, C., Futo, E., Hossenlopp, P., Binoux, M., Froesch, E.R. (1990) Regulation of binding proteins for insulin-like growth factors (IGF) in humans. *J. Clin. Invest.* **86**: 952-961.
- 522 Smith, E.P., Dickson, B.A., Chernausk, S.D. (1990) Insulin like growth factor binding protein-3 secretion from cultured rat Setoli cells: dual regulation by follicle stimulating hormone and IGF-I.

Endocrinology **127**: 2744-2751.

- 523 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.
- 524 Delhanty, P.J.D., Nygard, K., and Han, V.K.M. (1991) The coordinated expression of insulin-like growth factor II (IGF II) and IGF binding protein-2 (IGFBP-2) genes during ovine development. Program of the *73rd Annual Meeting of The Endocrine Society*, Washington D.C., p. 143 (abstract).
- 525 Hill, D.J., Camacho-Hubner, C., Rashid, P., Strain, A.J. and Clemmons, D.R. (1989a) Insulin-like growth factor (IGF)-binding protein release by human fetal fibroblasts: Dependency on cell density and IGF peptides. *J. Endocrinol.* **122**: 87-98.
- 526 Phillips, I.D., Delhanty, P.J.D., Hill, D.J. and Han, V.K.M. (1991) Ontogeny of insulin-like growth factor binding protein (IGF-BP1) mRNA expression in sheep. *J. Endocrinol.* **129** (suppl) 209 (abstract).
- 527 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**: 7, 1053-1060; 55 ref.
- 528 McLellan, K.C., Hooper, S.B., Bocking, A.D., Delhanty, P.J.D., Phillips, I.D., Hill, D.J. and Han, V.K.M. (1992) Prolonged hypoxia induced by the reduction of maternal uterine blood flow alters insulin-like growth factor-binding protein-1 (IGFBP-1) and IGFBP-2 gene expression in the ovine fetus. *Endocrinology* **131**: 1619-1628.

- 529 Hua, K.M., Hodgkinson, S.C., Molan, P.C. and Bass, J.J. (1992) Growth hormone stimulates tissue insulin-like growth factor-I production in sheep. *Proceedings of New Zealand Society of Endocrinology, Annual Conference. Abstract 19.*
- 530 McCusker, R.H., Camacho-Hubner, C., Bayne, M.L., Cascieri, M.A., Clemmons, D.R. (1990) Insulin-like growth factor (IGF) binding to human fibroblast and glioblastoma cells: the modulating effect of cell released IGF binding proteins (IGFBPs). *J. Cell. Physiol.* **144**: 244-253.
- 531 Zapf, J., Hauri, C., Waldvogel, M., Futo, E., Hiasler, H., Binz, K., Guler, H.P., Schmid, C., Froesch, E.R. (1989) Recombinant human insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats. *Proc. Natl. Acad. Sci. USA* **86**: 3813-3817.
- 532 Clemmons, D.R., Thissen, J.P., Maes, M., Ketelslegers, J.M., Underwood, L.E. (1989) Insulin-like growth factor-I (IGF-I) infusion into hypophysectomized or protein-deprived rats induces specific IGF-binding proteins in serum. *Endocrinology* **125**: 2967-2972.
- 533 Matthews, L.S., Hammer, R.E., Behringer, R.R., D'Ercole, A.J., Bell, G.I., Brinster, R.L., Palmiter, R.D. (1988). Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* **123**: 2827-2833.
- 534 Donovan, S.M., OH, Y., Pham, H. and Rosenfeld, R.G. (1989) Ontogeny of serum insulin-like growth factor binding proteins in the rat. *Endocrinology* **125**: 2621-2627.
- 535 Lee, C.Y., Bazer, F.W., Etherton, T.D., and Simmen, F.A. (1991) Ontogeny of insulin-like growth factors (IGF-I and IGF-II) and IGF-binding proteins in porcine serum during fetal and postnatal

- development. *Endocrinology* **128**: 2336-2344.
- 536 Clemmons, D.R., Snyder, D.K. and Busby, W.H. (1991) Variables controlling the secretion of insulin-like growth factor binding protein-2 in normal human subjects. *J. Clin. Endocrinol. Metab.* **73**: 727-733.
- 537 Hardonein, S., Hossenlopp, P., Segovia, B., Seurin, D., Portolan, G., Lasarre, C., Binoux, M. (1987) Heterogeneity of insulin-like growth factor binding proteins and relationships between structure and affinity I. Circulating forms in man. *Eur. J. Biochem.* **170**: 121-132.
- 538 Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963) The preparation of ^{131}I -labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**: 114-123.
- 539 Conover, C.A., Liu, F., Powell, D., Fosenfeld, R.G., Hintz, R.L. (1989) Insulin-like growth factor binding proteins from cultured human fibroblasts: characterization and hormonal regulation. *J. Clin. Invest.* **83**: 852-859.
- 540 Fowlkes, J. and Freemark (1992) Developmental regulation of insulin-like growth factor binding protein production: studies in fetal, postnatal and pregnant sheep. *J. Cellular Physiology* **152**: 19-27.
- 541 Neely, E.K., Rosenfeld, R.G. (1992) Insulin-like growth factors (IGFs) reduce IGF-binding protein-4 (IGBF-4) concentration and stimulate IGFBP-3 independently of IGF receptors in human fibroblasts and epidermal cells. *Endocrinology* **130**: 985-993.
- 542 Emler, C.A. and Schalch, D.S. (1987) Nutritionally-induced changes in hepatic insulin-like growth factor 1 gene expression in rats. *Endocrinology* **120**, 832-834.
- 543 Straus, D.S. and Takeinoto, C.D. (1990) Effect of fasting on IGF-I and

- growth hormone receptor mRNA levels and IGF-I gene transcription in rat liver. *Molecular Endocrinology*, **4**: 91-100.
- 544 Lowe, W.L. Jr., Adamo, M., Werner, H., Roberts, C.T. Jr. and LeRoith, D. (1989) Regulation by fasting of rat insulin-like growth factor I and its receptor. *The Journal of Clinical Investigation, Inc.* **84**: 619-626.
- 545 Roberts, C.T. Jr., Brown, A.L., Graham, D.E., Seelig, S., Berry, S., Gabbay, K.H., Rechler, M.M. (1986) Growth hormone regulates the abundance of insulin-like growth factor I RNA in adult rat liver. *J. Biol. Chem.* **261**: 10025-8.
- 546 Murphy, L.J., Bell, G.I., Duckworth, L., Friesen, G. (1987) Identification, characterization, and regulation of a rat complementary deoxyribonucleic acid which encodes insulin-like growth factor-I. *Endocrinology* **121**: 684-91.
- 547 Matthews, L.S., Norstedt, G., Palmiter, R.D. (1986) Regulation of insulin-like growth factor I gene expression by growth hormone. *Proc. Natl. Acad. Sci. USA* **83**: 9343-7.
- 548 Phillips, L.S. and Unterman, T.G. (1984) Somatomedin activity in disorders of nutrition and metabolism. *Clin. Endocrinol. Metab.* **13**: 145-189.
- 549 Maes, M., Amand, Y., Underwood, L.E., Maiter, D. and Ketelslegers, J.-M. (1988) Decreased serum insulin-like growth factor I response to growth hormone in hypophysectomized rats fed a low protein diet: evidence for a post-receptor defect. *Acta Endocrinol.* **117**: 320-326.
- 550 Rotwein, P., Pollock, K.M., Didler, D.K. and Krivi, G.G. (1986) Organisation and sequence of the human insulin-like growth factor-I gene. Alternative RNA processing produces two insulin-

- like growth factor-I precursor peptides. *J. Biol. Chem.* **261**: 4828-4832.
- 551 Adamo, M.L., Gen-Hur, H., Roberts, C.T. Jr., and Le-Roith, D. (1991)
Regulation of start site usage in the leader exons of the rat insulin-like growth factor-I gene by development, fasting, and diabetes. *Mol. Endocrinol.* **5**: 1677-1686.
- 552 Lowe, W.L. Jr., Roberts, C.T. Jr., Laskey, S.R. and Leroith, D. (1987)
Differential expression of alternative 5' untranslated regions in mRNAs encoding rat insulin-like growth factor I. *Proc. Natl. Acad. Sci. USA* **84**: 8946-8950.
- 553 Roberts, C.T. Jr., Lasky, S.R., Lowe, W.L. Jr., Seaman, W.T. and Leroith, D. (1987a) Molecular cloning of rat insulin-like growth factor-I complementary deoxyribonucleic acids: differential messenger ribonucleic acid processing and regulation by growth hormone in extrahepatic tissues. *Mol. Endocrinol.* **1**: 243-248.
- 554 Adamo, M., Werner, H., Farnsworth, W., Roberts, C.T. Jr., Raizada, M. and Le-Roith, D. (1988) Dexamethasone reduces steady-state insulin-like growth factor I messenger ribonucleic acid levels in rat neuronal and ghial cells in primary culture. *Endocrinology* **123**: 2565-2570.
- 555 Holthuizen, E., Leroith, D., Lund, P.K., Roberts, C., Rotwein, P., Spencer, E.M., and Sussenbach, J.S. (1991) Revised nomenclature for the insulin-like growth factor gene and transcripts. In *Modern Concepts of Insulin-like Growth Factors*. E.M. Spencer, ed. (Elsevier Science Publishers, New York) pp. 733-736.
- 556 Saunders, J.C., Dickson, M.C., Pell, J.M. and Gilmour, R.S. (1991)
Expression of a growth hormone-responsive exon of the ovine insulin-like growth factor-I gene. *J. Molecular Endocrinology* **7**:

233-240.

- 557 Atkison, P.R., Weidman, E.R., Bhaumick, B., Bala, R.M. (1980) Release of somatomedin-like activity by cultured W1-33 human fibroblasts. *Endocrinology* **106**:2066-12.
- 558 Clemmons, D.R., Underwood, L.E., Van Wyk, J. (1981) Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. *J. Clin. Invest.* **67**: 10-9.
- 559 D'Ercole, A.J., Applewhite, G.T., Underwood, L.E. (1980) Evidence that somatomedin is synthesized by multiple tissues in the fetus. *Dev. Biol.* **75**: 315-28.
- 560 Clemmons, D.R., Van Wyk, J. (1985) Evidence for a functional role of endogenously produced somatomedin-like peptides in the regulation of DNA synthesis in cultured human fibroblasts and porcine smooth muscle cells. *J. Clin. Invest.* **75**: 1914-8.
- 561 Nilsson, A., Isgaard, J., Lindahl, A., Dahlström, A., Skottner, A., Isaksson OGP (1986) Regulation by growth hormone of number of chondrocytes containing IGF-I in rat growth plate. *Science* **233**: 571-4.
- 562 Wroblewski, J., Engström, M., Skottner, A., Madsen, K., Friberg, U. (1987) Subcellular location of IGF-I in chondrocytes from rat rib growth plate. *Acta Endocrinol. (Copenh)* **115**: 37-43.
- 563 Cook, J.J., Haynes, K.M., Werther, G.A. (1988) Mitogenic effects of growth hormone in cultured fibroblasts: Evidence for action via local insulin-like growth factor I production. *J. Clin. Invest.* **81**: 206-12.
- 564 Schlechter, N.L., Russell, S.M., Spencer, E.M., Nicoll, C.S. (1986) Evidence suggesting that the direct growth-promoting effect of growth hormone on cartilage *in vivo* is mediated by local

- production of somatomedin. *Proc. Natl. Acad. Sci. USA* **83**: 7932-4.
- 565 Isgaard, J. (1989) GH regulation of IGF-I expression in cartilage and skeletal muscle. PhD thesis. University of Göteborg, Göteborg.
- 566 Hoyt, E.C., Van Wyk, J.J., and Lund, P.K. (1988) Tissue and development specific regulation of a complex family of rat insulin-like growth factor I messenger ribonucleic acids. *Mol. Endocrinol.* **2**: 1077-1086.
- 567 Adamo, M., Lowe, W.L. Jr., Leroith, D. and Roberts, C.T. Jr. (1989) Insulin-like growth factor I messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed during development of the rat. *Endocrinology* **124**: 2737-2744.
- 568 Oldham, J.M. (1991) The regulation of post-natal growth in sheep through the growth hormone-insulin-like growth factor axis. PhD thesis, University of Waikato, NZ.
- 569 Samuelsson, E., Wadenstein, H., Hartmanis, M., Molis, T. and Uhlen, M. (1991) Facilitated in vitro refolding of human recombinant insulin-like growth factor-1 using a solubilizing fusion partner. *Biotech.* **9**: 363-366.
- 570 Francis, G.L., Read, L.C., Ballard, F.J., Bagley, C.J., Upton, F.M., Gravestock, P.M. and Wallace, J.C. (1986) Purification and partial sequence analysis of insulin-like growth factor-1 from bovine colostrum. *Biochem. J.* **233**: 207-213.
- 571 Yeoh, S.I. and Baxter, R.C. (1988) Metabolic regulation of the growth hormone-independent insulin-like growth factor binding protein in human plasma. *Acta. Endocrinol.* **119**: 465-473.
- 572 Prosser, C.G., Royle, C., Fleet, I.R. and Mephram, T.B. (1991) The galactopoietic effect of bovine growth hormone in goats is associated with increased concentrations of insulin-like growth

- factor-1 in milk and mammary tissue. *J. Endocrinol.* **128**: 457-443.
- 573 Prosser, C.G., Fleet, I.R., Corps, A.N., Froesch, E.R. and Heap, R.B.
(1990) Increase in milk secretion and mammary blood flow by
intra-arterial infusion of insulin-like growth factor-1 into the
mammary gland of the goat. *J. Endocrinol.* **126**: 437-443.
- 574 Han, V.K.M., D'Ercole, A.J. and Lund, P.K. (1987) Cellular localization
of somatomedin-c/insulin-like growth factor-1 messenger RNA in
the human fetus. *Science* **236**: 193-197.
- 575 Guler, H.P., Echardt, K.U., Zapf, J., Bauer, C. and Froesch, E.R. (1989)
Insulin-like growth factor-1 increases glomerular filtration rate and
renal plasma flow in man. *Acta Endocrinol. (Copenhagen)* **121**:
101-106.
- 576 Hodges, A.K. (1992) Developmental changes and histological localisation
of insulin-like growth factor I and II mRNA in sheep muscle and
liver. MSc Thesis, University of Waikato, NZ.
- 577 Collier, R.J., McNamara, J.P., Wallace, C.R., Dehoff, M.H. (1984) A
review of endocrine regulation of metabolism during lactation. *J.*
Anim. Sci. **59**: 498.
- 578 Asakawa-Yasumoto, K., Hizuka, N., Fukuda, I., Sakano, K., Shizume, K.
and Demura, H. (1992) Hypoglycemic effect of insulin-like
growth factor II in insulin resistant mice. Ninth International
Congress of Endocrinology, Nice, France. **Abstract P-09.03.007.**
- 579 Drop, S.L.S., Kortleve, D.J., Guyda, H.J. and Posner, B.I. (1984)
Immunoassay of a somatomedin-binding protein from human
amniotic fluid: levels in fetal, neonatal, and adult sera. *J. Clin.*
Endocrinol. Metab. **59**: 908

PUBLICATIONS ARISING FROM THIS THESIS

The work presented in this thesis has formed the basis of following publications:

K.M. Hua, J. Bass, R. Ord, S. Hodgkinson, S. Spencer and P. Molan (1991) Regulation of ovine plasma and tissue IGF-I by nutrition and GH. Proceedings of New Zealand Society of Endocrinology (Suppl.) Abstract 18.

J. Bass, K.M. Hua, R. Ord, S. Hodgkinson, S. Spencer and P. Molan (1991) Regulation of ovine plasma and tissue IGF-I by nutrition and GH. Proceedings of American Endocrine Society 73, Abstract 468.

K.M. Hua, S.C. Hodgkinson, P.C. Molan and J.J. Bass (1992) GH stimulates tissue insulin-like growth factor-I production in sheep. Proceedings of New Zealand Society of Endocrinology, Abstract 19.

K.M. Hua, S.C. Hodgkinson, P.C. Molan and J.J. Bass (1993) Effect of growth hormone administration on plasma levels of insulin-like growth factor binding protein three (IGFBP-3) in sheep. Proceedings of the Endocrine Society of Australia 36, A Combined Meeting With The New Zealand Society of Endocrinology, Abstract 145.

K.M. Hua, R. Ord, S. Kirk, Q.J. Li, S.C. Hodgkinson, G.S.G. Spencer, P.C. Molan and J.J. Bass (1993) Regulation of plasma and tissue levels of insulin-like growth factor-I by nutrition and treatment with growth hormone in sheep. Journal of Endocrinology 136, 217-224.