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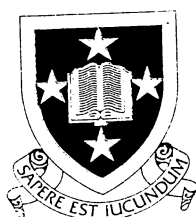
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RECEPTORS FOR GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTORS IN CARTILAGE USING ANTLER AS A MODEL FOR BONE GROWTH

**A thesis
submitted in fulfilment
of the requirements for the degree
of
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ABSTRACT

Bone growth in young mammals is a major determinant of eventual individual size, and ongoing bone metabolism is necessary to maintain a mineral balance for essential physiological processes. GH, IGF-I and IGF-II stimulate growth in many tissues and are implicated in cartilage and bone growth. Tissue sensitivity to a hormone may be gauged by receptor levels and the main objective of this thesis was to examine and characterise the receptors for GH and the IGFs in tissues of developing bone. Antler was used as a model for bone growth because it is similar in histochemistry to somatic long bones. It may be removed humanely, undergoes regeneration and grows rapidly, making it a useful tissue for research in growth.

Receptor localization in tissues of the growing antler tip was determined using autoradiographical studies and the receptors were characterised using competitive binding assays and affinity cross-linking studies. Production of IGFBPs was assessed and analysed for interference of hormonal binding to receptors. Ontogeny of receptor distribution patterns over the antler growth phase was also studied to assess changes in localization, or implied function, occurring with tissue maturation. Administration of testosterone to deer bucks during the antler growth phase to induce precocious tissue maturation was used as a tool to assess the direct effects of circulating androgens on cartilage development, and changes to receptor localization relative to this treatment.

No GH receptors were detected in antler tissues. IGF-I and IGF-II bound to receptors that had characteristics of type 1 and type 2 IGF receptors, respectively. Highest levels of IGF-I binding were in the maturation zone and IGF-II binding in the proliferative zone. IGFBPs were produced in higher levels in pre-cartilage than in cartilage tissues. Ontogenetic changes in IGF-I binding were observed by changes in distribution patterns at a late stage of antler growth. Distribution patterns of IGF-II binding did not change over the growth phase. Enhancement of antler mineralization and ossification was achieved to a limited degree by testosterone administration, suggesting that testosterone might not be as potent a stimulator of antler tissue maturation as its metabolites.

In conclusion, antler growth is independent of direct stimulation by GH. IGF-I and IGF-II each preferentially bind to distinct receptor populations in antler tissues, with different tissue distribution, hormonal specificity and ontogeneticity. Localization of IGF receptors in antler tissues implied a role for IGF-I in matrixogenesis and a role for IGF-II in cell proliferation. IGFBPs were implicated in the regulation of IGF-II actions *in vivo*. This evidence suggests that antler tissue provides a unique opportunity to study the endocrine effects of IGFs on cartilage and bone.

PREFACE

Some of the work in this thesis has been presented in several publications:

Refereed Publications (Appendix):

Elliott J. L., Oldham J. M., Ambler G. R., Bass J. J., Hodgkinson S. C., Breier B. H., Gluckman P. D., Spencer G. S. G. and Suttie J. M. (1992). Presence of insulin-like growth factor-I receptors and absence of growth hormone receptors in the antler tip. *Endocrinology* **130**, 2513-2520.

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Elliott J., Oldham J., Bass J., Spencer S., Hodgkinson S. and Suttie J. (1991). Histological localisation of insulin-like growth factor-I and II (IGF-I/II) receptors and no growth hormone receptors in the antler tip. *Proceedings of the 2nd Symposium on Insulin-like Growth Factors/Somatomedins*, San Francisco, Abstract B65, p. 200.

Elliott J. L., Oldham J. M. and Bass J. J. (1991). Further observations on IGF-1 and IGF-2 receptors in the antler tip. *Proceedings of the New Zealand Society of Endocrinology*, Palmerston North, Abstract 17.

Elliott J. L., Oldham J. M., Asher G. W., Molan P. C. and Bass J. J. (1992). Control of antler ossification and neck muscle hypertrophy by testosterone. Is insulin-like growth factor-I (IGF-I) involved? *Proceedings of the Endocrine Society of Australia*, Hamilton, Vol. 35 (Suppl.), Abstract NZ2.

Elliott J. L., Asher G. W., Molan P. C. and Bass J. J. (1993). The effect of testosterone on receptors for insulin-like growth factors in deer antlers. *75th Annual Meeting of the Endocrine Society*, Las Vegas, Abstract 1475.

Elliott J. L., Peterson A. J., Ledgard A. M., Asher G. W., Molan P. C. and Bass J. J. (1993). Production of insulin-like growth factor binding proteins by antler tissues in culture. *Proceedings of the Endocrine Society of Australia*, Dunedin, Vol. 36, Abstract 8.

Hodgkinson S. C., Napier J. R., Elliott J. L., Martyn J. A. K. and Spencer G. S. G. (1992). Studies on the production of anti-idiotypic antibodies to insulin-like growth factor-1 (IGF-1) in sheep. *Proceedings of the New Zealand Society of Endocrinology*, Hamilton, Abstract 49.

Suttie J. M., Fennessy P. F., Sadighi M., Elliott J. L., Corson I. D. and Lapwood K. R. (1991). Antler growth in deer. In: *Proceedings of a Deer Course for Veterinarians* Wilson P. R. (ed), pp. 155-168. Deer Branch of the New Zealand Veterinary Association, Sydney, Australia.

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ABBREVIATIONS

K _a	affinity constant
N-Met IGF-I	amino terminal methionyl recombinant human IGF-I
RGD	Arginine-Glycine-Asparagine
BSA	bovine serum albumin
CI	cation-independent
cpm	counts per minute
°C	degrees celsius
DNA	deoxyribonucleic acid
des IGF-I	desamino-(1-3) IGF-I
dpm	disintegrations per minute
K _d	dissociation constant
g	grams
x g	gravity
GH	growth hormone
GHRH	growth hormone releasing hormone
GHRH	growth hormone release inhibiting hormone
GTP	guanosine 5'-triphosphate
HCl	hydrochloric acid
IGFBP	insulin-like growth factor binding protein
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
kDa	kilodaltons
l/mol	litres per mole
M6P	mannose-6-phosphate
mRNA	messenger ribonucleic acid
µg/ml	micrograms per millilitre
µl	microlitres
µm	micrometres
mA	milliamperes
mg	milligrams
ml	millilitres
mmol/l	millimoles per litre
mol/l	moles per litre
M _r	molecular weight (from migration rate on polyacrylamide gels)
ng/ml	nanograms per millilitre
nmol/l	nanomoles per litre

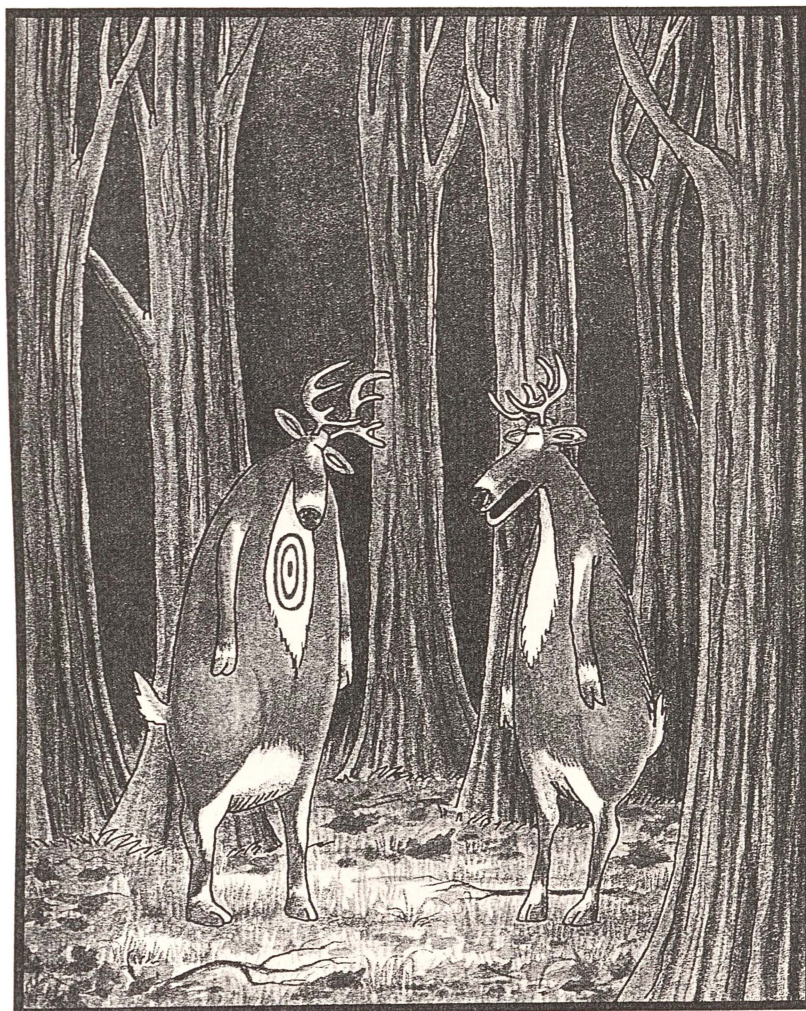
pH	negative log ₁₀ of the hydrogen ion concentration
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
¹²⁵ I-IGF-I	radioiodinated IGF-I
¹²⁵ I-IGF-II	radioiodinated IGF-II
SD	standard deviation
SED	standard error of the difference
SEM	standard error of the mean
V	volts
w/v	weight per volume
XAR	X-Omat AR5

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"Bummer of a birthmark, Hal."

(G. Larsen, 1986)

Dedicated to Wendy and Les Elliott

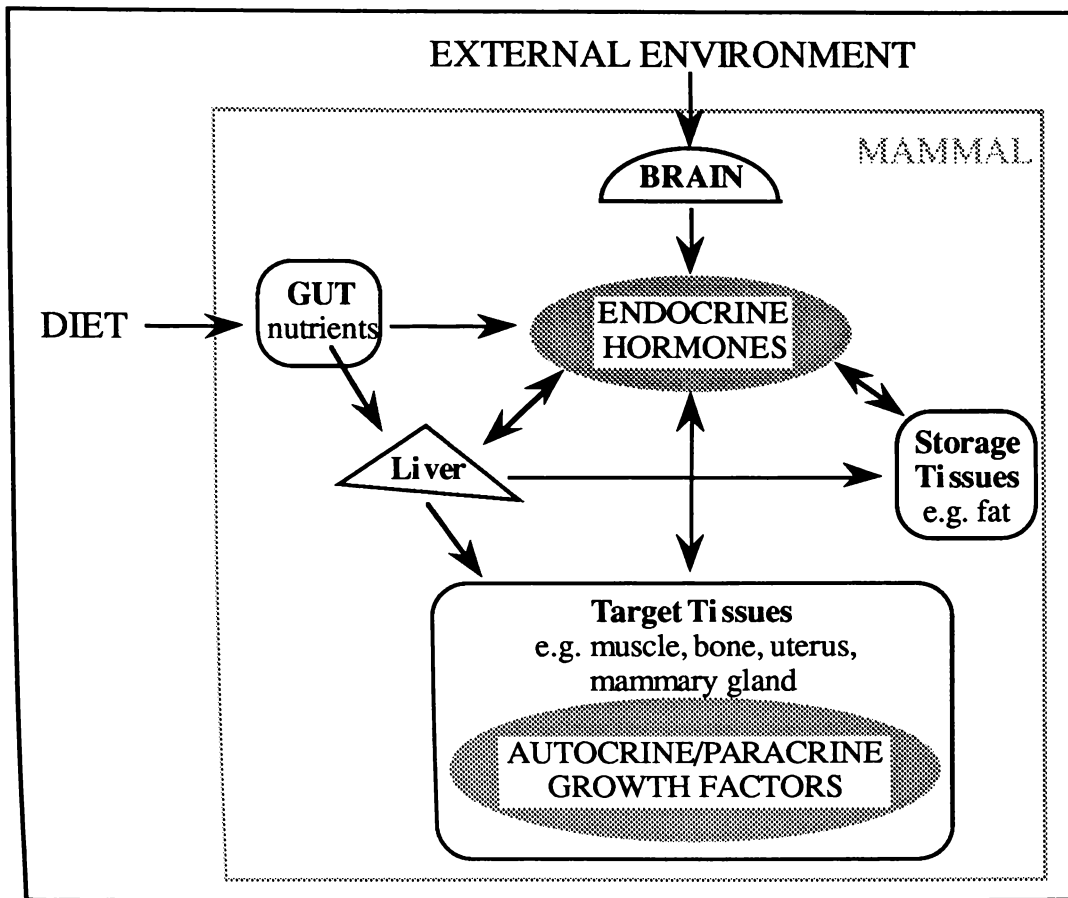
CHAPTER 1

INTRODUCTION

Mammals that grow eventually attain a predetermined body size that is programmed by the rate and duration of growth. This is mostly under genetic control, but may be controlled to some extent by nutritional and environmental conditions evoking physiological events in the gut and brain, which give rise to hormones and growth factors (Fig. 1.1). Growth constitutes an increase in size, and in developing mammalian tissues it occurs in two ways: hyperplasia (cell proliferation) and hypertrophy (cell enlargement). Coordination of cell and tissue growth is accomplished by the endocrine actions of hormones, combined with the autocrine/paracrine actions of local growth factors.

Growth hormone (GH) is implicated in growth processes by observations that normal somatic growth of mammals does not occur without it (Walker, Simpson, Asling & Evans, 1950; Frazer, Gavin, Daughaday *et al.*, 1982; Mathews, Hammer, Behringer *et al.*, 1987). Clinically, GH is used as a therapeutic tool in the treatment of patients with growth retardation disorders such as chronic short stature (Van Vliet, Styne, Kaplan & Grumbach, 1983), GH deficiency (Burns, Tanner, Preece & Cameron, 1981; Sandahl-Christiansen, Orskov, Binder & Kastrup, 1983; Albertsson-Wikland, Westphal & Wertgren, 1986) and hypopituitary dwarfism (Beck, McGarry, Dyrenfurth & Venning, 1957; Raben, 1958; Bierich, 1986). Insulin-like growth factor (IGF)-I is also used therapeutically as a growth promotant, in Laron dwarfism (Laron, Klinger, Blum *et al.*, 1992; Kanety, Karasik, Klinger *et al.*, 1993). Both IGF-I and IGF-II are implicated in fetal growth (Milner & Hill, 1987) and IGF-II is purported to have a role in tumour growth as it is highly expressed by certain tumour tissues (Wilkins, Molenaar, Ohlsson *et al.*, 1989).

Interplay between hormones and local factors in the growth physiology of a mammal (S. Gilmour, personal communication). External influences, such as nutritional and environmental conditions, can stimulate the production of hormones and growth factors, via sensitive organs. Hormones may act either directly, or indirectly by the stimulation of growth factors, to produce a growth response in the target tissues.

Figure 1.1 Physiology of mammalian growth.

The development of bones in fetal and pre-pubertal mammals is a major feature of longitudinal growth. Both GH and IGF-I influence bone growth by stimulating cartilage growth and development (Isaksson, Jansson & Gause, 1982; Russell & Spencer, 1985; Isgaard, Nilsson, Lindahl *et al.*, 1986; Hock, Centrella & Canalis, 1988). IGF-II is present in a large number of tissues, including bone, for which it is purported to have a role in growth (Mohan & Baylink, 1991).

1.1 BONE GROWTH

This thesis is concerned with aspects of the endocrine regulation of cartilage development as a component of bone growth. Deer antler tissue was selected as the growing cartilage

model for the current studies for a number of salient reasons. On compassionate grounds, antler tissue collection is a non-terminal exercise and the use of standard velvetting guidelines and animal husbandry practices, as spelt out by the NZ Game Industry Board, minimises the pain of, and risk to, the animals. The deer suffer no long term effects by antler removal as they are not prevented from being used as breeding stock and growing a normal set of antlers in the ensuing season, providing a renewable resource of material for research. Studies which engender an understanding of the mechanisms of growth in this tissue would have wide applicability to a broad range of other mammalian tissues. Antler is one of the fastest growing mammalian tissues and in large male deer can grow more than 2 cm/day in length (Goss, 1970) which makes it an exemplary model for growth. The histochemistry and cyto-architecture of the deer antler is similar to that of the epiphyseal growth plate in long bones (Goss, 1983a) and antler cartilage undergoes a form of endochondral ossification in the process of bone formation (Chapman, 1975; Banks & Newbrey, 1983a; Banks & Newbrey, 1983b; Goss, 1983a) as does somatic cartilage (Ham & Cormack, 1979b).

1.1.1 Bone growth and organisation of the growth plate

The first step in somatic long bone growth is the formation of a diminutive cartilage model which provides a template for development to the mature bone (Romer, 1955; Ham & Cormack, 1979a; Ham & Cormack, 1979b; Geneser, 1986; Wheeler, Burkitt & Daniels, 1987a; Rosen & Thies, 1992). The cartilage model is formed in the mammalian embryo by the condensation of mesenchymal cells which differentiate to form cartilage precursor cells, or chondroblasts. These chondroblasts then grow and proliferate, and secrete an extracellular matrix of fibres and ground substance, such as type II collagen and proteoglycans, respectively. As chondroblasts develop and undergo several mitotic divisions they differentiate into mature cartilage cells, or chondrocytes. The cell maturation sequence is most advanced towards the centre of the cartilage model and mature cartilage comprises chondrocytes embedded in large amounts of extracellular matrix. Chondrocytes grow in size (hypertrophy) but may still undergo mitotic division to

some extent, and can form groups of up to four cells which are held together in cavities in the extracellular matrix. A membrane forms at the periphery of the cartilage model and comprises a layer of fibroblasts, which secrete mainly collagen, and a layer of undifferentiated mesenchymal cells with cartilage-forming potential. The membrane creates a boundary between the developing cartilage and surrounding tissue and is called the perichondrium. This becomes infiltrated by capillaries in its outer surface (the fibroblastic layer), providing vascular support for continued growth of the cartilage model. As cartilage is avascular, chondrocytes depend on the diffusion of nutrients, gases and waste products to and from the capillaries outside the model, via the aqueous phase of the extracellular matrix. Extracellular matrix comprises 75 to 80% water which is bound up in the proteoglycan structure which itself comprises about 10% of the matrix, the remaining 10% being collagen (Ham & Cormack, 1979a; Ham & Cormack, 1979c; Geneser, 1986).

The physical properties of cartilage, such as turgor pressure and tensile strength, are imparted by the association between the collagen and proteoglycans of the extracellular matrix. The proteoglycans are sulphated glycosaminoglycans, of which chondroitin sulphate is the major component, that are covalently linked to a variety of non-collagenous proteins. These form large complex molecules containing 90 to 95% carbohydrates which are not flexible enough to form globular aggregates (Wheater, Burkitt & Daniels, 1987b) but instead exist in expanded amorphous forms which have a large surface area, occupying large volumes with small mass. These molecules have highly charged side groups, rendering them extremely hydrophilic and imparting a high water-holding capacity. The extensive hydration of the cartilage matrix means it is an efficient medium for diffusion of the solutes required for chondrocyte maintenance. Collagen fibrils form a reticular network that is interwoven throughout the ground substance.

As the chondrocytes in the middle of the cartilage model mature and undergo hypertrophy, resorbing some of the surrounding matrix, the remaining matrix becomes mineralized. Mineralization involves the precipitation of inorganic salts, predominantly hydroxyapatite

(calcium phosphate) crystals, until the cartilage is calcified (Geneser, 1986; Wheeler, Burkitt & Daniels, 1987a; Williams & Frolik, 1991). There are two proposed theories for the mechanism of initiation of calcification and both involve the extracellular matrix, within which calcification arises (Williams & Frolik, 1991). The *nucleation theory* espouses collagen fibrils providing a nucleation site for calcium phosphate crystallization, with crystal formation being controlled by noncollagenous proteins in the extracellular matrix. The *matrix vesicle theory* postulates that concentrations of calcium and phosphate, stored in matrix vesicles containing specific facilitatory enzymes, initiate calcification by depositing seed crystals at specific sites on collagen fibrils. With calcification the chondrocytes degenerate and die, probably due to the prevention of diffusion through the matrix (Ham & Cormack, 1979a; Geneser, 1986; Wheeler, Burkitt & Daniels, 1987a; Rosen & Thies, 1992). Simultaneously with calcification, the mesenchymal cells in the perichondrium surrounding the middle of the shaft (diaphysis) develop osteogenic potential and the perichondrium here becomes the periosteum. These cells differentiate into osteoblasts, possibly because of microenvironmental changes occurring involving improved oxygenation (Ham & Cormack, 1979b). Osteoblasts synthesize and secrete bone matrix and at this point they form a thin layer of bone around the middle of the shaft. Primitive vascular tissue grows through this bony layer by osteoclastic activity (osteoclasts are bone-resorbing cells that arise from the fusion of monocytes or macrophages), and invades the spaces left by dead chondrocytes in the cartilage matrix. The invading blood vessels carry with them mesenchymal cells that differentiate into osteoblasts and primitive bone marrow (hematopoietic cells). The osteoblasts use the calcified remnants of the cartilage matrix as a framework for the deposition of bone in the primary ossification step in the process of endochondral ossification (Hill & Logan, 1992).

The initial endochondral bone formation is at the centre of the diaphysis and this then spreads towards each end of the long bone, or epiphyses. At the same time, osteoclasts are resorbing the initial bone to make a marrow cavity, which also expands in the directions of the epiphyses. Each epiphysis is cartilaginous in nature and continues to grow by

proliferation and hypertrophy. The interface between the diaphysis and each epiphysis is an epiphyseal growth plate, which is spatially arranged into successive zones. From the epiphysis to the diaphysis the zonal arrangement is: 1) reserve cartilage, 2) proliferative zone, 3) zone of maturation and hypertrophy, 4) calcification zone, and 5) zone of cartilage removal and bone deposition, or metaphysis (Geneser, 1986). Bone increases in length at the epiphyseal growth plates which proliferate continuously, while at the metaphysis, cartilage is continuously being replaced by bone, pushing the epiphyseal growth plates apart (Hill & Logan, 1992). At birth, long bone shafts are ossified but the epiphyses are still cartilaginous.

Eventually the chondrocytes of the epiphyseal cartilage mature and calcify. Those at the centre of the epiphysis are the first to undergo calcification and die, and become replaced by bone, which forms from the osteogenic cells that invade it from the diaphysis. This process of secondary endochondral ossification spreads out from the epiphyseal centre as the chondrocytes surrounding it mature and die. Ossification of the epiphysis continues to completion except for the outermost layer which becomes articular cartilage. The epiphyseal growth plate remains cartilaginous in nature and continues to form cartilage, which is replaced by bone, and persists until postnatal longitudinal growth of the bone is completed.

Hormonal changes at sexual maturity prevent further cartilage proliferation and closure of the epiphysis occurs where the growth plates are replaced by bone from the diaphysis. Further modifications to bone involve remodelling where the first formed bone is resorbed by osteoclasts, in the proximity of blood vessels, and deposition of many layers of bone by osteoblasts occurs, filling in the spaces. This forms Haversian systems of concentric circles of lamellar bone, and this type of bone remodelling occurs throughout the life of the individual. Vascular components form an intricate series of channels throughout the bone, providing nutrients and allowing gas exchange. The final result of this highly complex

endochondral ossification process is a bone surrounded by periosteum and containing a central marrow cavity.

1.1.2 Antler as a model for bone growth

Antlers comprise deciduous bony protuberances from the frontal bones of the skull and are secondary sexual characters of male deer. Once antler growth has commenced, the growth rate is very rapid, and within approximately 16 weeks the deer effectively doubles the mineral deposits of the body (Putman, 1988). Antlers are voracious scavengers of circulating minerals and nutrients, so much so that if the individual animal's dietary intake is insufficient to meet the demands of the growing antlers, calcium stored in the skeleton is mobilised and transported to the antlers (Cowan, Hartsook & Whelan, 1968; Goss, 1970; Hillman, Davis & Abdelbaki, 1973; Goss, 1983c; Brown, 1990). For red deer stags it has been calculated that for 3 kg of hard antler produced, 536 g of calcium and 1.0 kg of other minerals will be deposited over the period of growth (Muir, Sykes & Barrell, 1985). Deer have been known to gnaw and nibble at the previous season's cast antlers, a valuable source of minerals (Putman, 1988), to minimise as much as possible the grievous mineral loss from the body that the casting entailed and to provide materials for the current season's growing antlers.

1.1.2.1 Morphology

Antlers grow from permanent bony pedicles and while they grow they are covered in velvet (Chapman & Chapman, 1975a), a special type of integument layer that is richly vascularized and innervated, and which is capable of enormous expansion (Goss, 1983a). It is well endowed with hairs and sebaceous glands which multiply profusely to keep pace with growth (Goss, 1983a). Once growth is complete, the velvet is shed leaving the bare antler, which is simply dead bone (Chapman & Chapman, 1975a). After the rutting season, during which behavioural changes are noticed - such as males using the hard antlers as weapons to establish social dominance - the antlers, but not the pedicles, are cast and the process is repeated each year. The morphological development of antlers has been

based on behavioural preferences where the fastest growing and largest antlers have been preferentially selected due to being improved weaponry and greater social status (Putman, 1988). An interesting observation about antlers is the increased propensity to growth during the life of the deer. Usually aging tissues do not grow as well as young tissue but in this case the size and morphological complexity of antlers increases with the age of the deer (Chapman & Chapman, 1975a; Putman, 1988).

Fallow deer are consigned to a different genus (*Dama*) from red deer (*Cervus*) because of the shape of their antlers which in fallow deer are palmate and in red deer are branched. Whilst velvet antlers from red deer are of economic value and are purported to have esoteric properties which are highly marketable (Goss, 1970), fallow deer antlers are not, and are generally removed and discarded in a farming situation (Asher, 1990). The studies reported in this thesis involve both fallow and red deer antlers, both of which have similar velvet growth phases (Goss, 1970).

1.1.2.2 *Histogenesis and Histology*

Soon after the casting of the previous season's hard antlers a scab of tissue forms on top of each pedicle stump which is quickly covered by velvet, migrating from the pedicle skin surrounding the wound (Goss, 1983b). A blastema of undifferentiated and hyperplastic cells forms beneath the velvet (whether this arises from the velvet or the periosteum of the pedicle, or both, is at present unknown) and this grows to form an antler (Goss, 1983b). Antler development occurs as a form of endochondral ossification (Chapman, 1975; Banks & Newbrey, 1983a; Banks & Newbrey, 1983b; Goss, 1983a). Initially a perichondrium is formed which comprises two layers (Speer, 1983), the outer layer consisting of proliferating fibroblasts embedded in a dense network of collagen fibres (Goss, Dinsmore, Grimes & Rosen, 1974), the inner being fibroblasts and mesenchymal cells. The mesenchymal cells persist at the apex throughout development (Banks & Newbrey, 1983a), the proliferation of which cause elongation. The descendants of these cells which are left behind differentiate into chondroblasts, then chondrocytes (Wislocki, 1942;

Wislocki, Aub & Waldo, 1947). Chondrocytes in antlers are organised differently from the longitudinal columns of somatic chondrocytes as they are arranged in polarised trabeculae, between which are numerous vascular channels (Goss, 1983a; Newbrey & Banks, 1983). Arteries entwine the growing antler, climbing distally in the velvet to deliver a copious supply of blood to the growing tip (Waldo, Wislocki & Fawcett, 1949) which is drained back through the veins running down to the pedicle through the developing cartilage (Goss, 1983a). The velvet is also endowed with sensory innervation (Adams, 1979; Geneser, 1986) so that the deer will not damage the soft growing tissue and impede the blood supply to the highly metabolising tissues.

The histochemistry of somatic and antler cartilage is similar (Goss, 1983a) and both tissues secrete large amounts of sulphated glycosaminoglycans into the extracellular matrix (Banks & Newbrey, 1983a; Banks & Newbrey, 1983b; Goss, 1983a), although there is a difference in the type of collagen produced by each: somatic chondrocytes secrete type II collagen and antler chondrocytes secrete type I and III collagens (Goss, 1983a). As in somatic cartilage, calcification occurs following chondrocyte hypertrophy of antler cartilage (Chapman, 1975; Goss, 1983a). Hydroxyapatite crystals form in a dense mass, surrounding the chondrocytes and isolating them from their nutrients. Death of the chondrocyte and subsequent bone deposition on the mineralised matrix occurs as in the epiphyses of long bones (Newbrey & Banks, 1983).

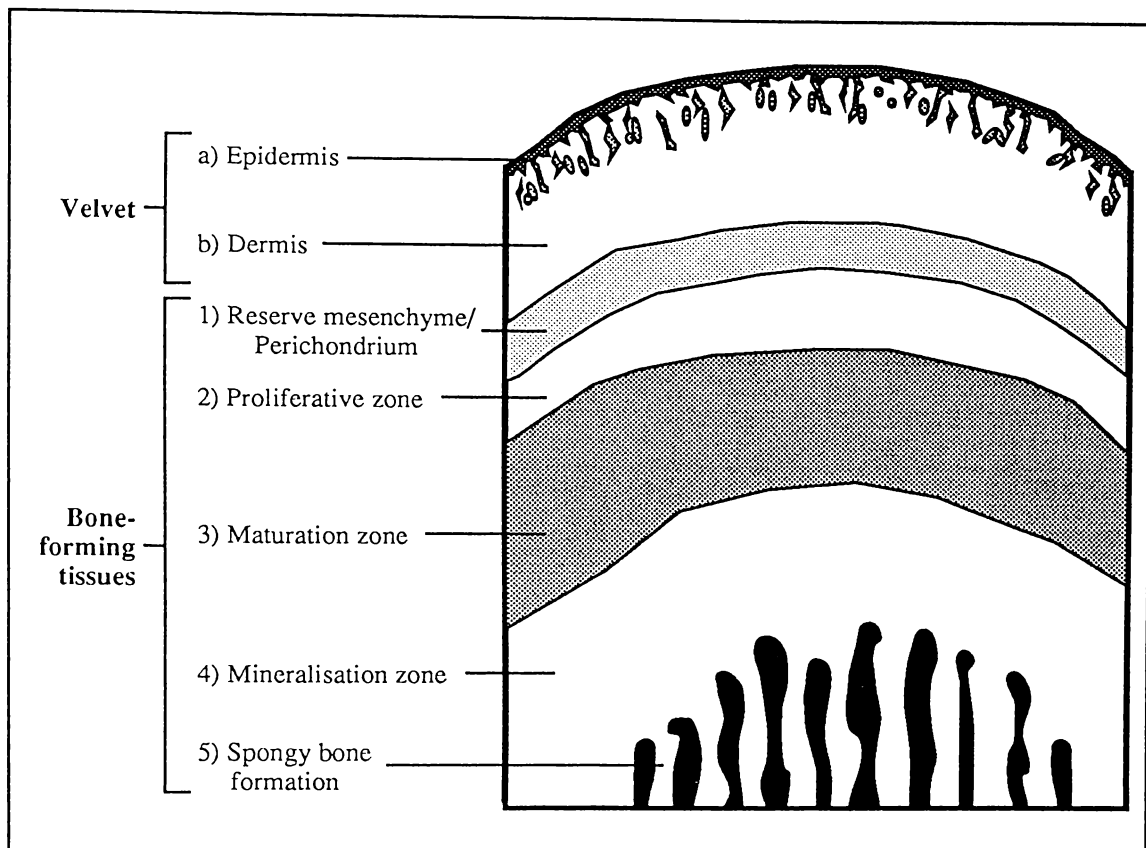
The spatial organisation of the growth zone of antlers is similar to that of the epiphyseal growth plate, but the zonation is less distinct (Newbrey & Banks, 1983) as antler cells and tissues undergo continuous, rather than intermittent, differentiation (Goss, 1983a). The zonal arrangement of the growing antler from the tip to the base (Fig. 1.2) is: a) epidermis and b) dermis, which comprise the velvet, and the developing bone tissues comprising: 1) reserve mesenchyme/perichondrium, 2) proliferative (chondroblastic) zone, 3) zone of chondrocyte maturation, 4) mineralisation zone, and 5) zone of cartilage removal and bone deposition (spongy bone). At certain stages during antler growth the growth zone widens

The growing antler tip comprises several histological zones, the epidermis and dermis are the velvet and the rest are cartilaginous. Of the cartilage zones, tissues that are proximal to the antler tip (top) are less differentiated than those distally.

and may bifurcate, as with red deer, or develop localised areas of growth, as with the fallow deer (Putman, 1988). It has been hypothesised that the rate of mitosis of cells at growth points distributed over the pedicle, the number of which is dependent on species and age, may be responsible for species-specific antler shapes (Putman, 1988).

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Figure 1.2 Diagrammatic representation of an antler tip.



Subsequent to chondrocyte death, the trabecular margins become lined with osteoblasts (typical of somatic tissues), and endochondral ossification occurs in a way indistinguishable from that seen in long bones (Banks & Newbrey, 1983a; Banks & Newbrey, 1983b). Chondroclasia occurs simultaneously with the laying down of bone matrix on the surfaces of the remnants of trabeculae, resulting in ossified trabeculae, or spongy bone (Goss, 1983a). The trabeculae thicken as the osteoblasts continue to lay down layers of bone matrix and the veins in between become progressively constricted. It

is this vascular constriction which is responsible for the death of the antler as the bone solidifies (Goss, Dinsmore, Grimes & Rosen, 1974; Goss, 1983a). Further growth is prevented as the ossification front catches up with the growth front, and the velvet dries and separates from the periosteum. The antler remains as spongy bone, no remodelling and Haversian system formation occurs and no bone marrow is formed (Chapman, 1975) as the antler is dead. Months later, the antler-pedicle junction is eroded by osteoclastic activity arising from the pedicle and the antler is cast (Chapman, 1975; Goss, 1983a; Goss, Van Praagh & Brewer, 1992), and a new antler is regenerated from the wound.

1.2 HORMONAL CONTROL OF BONE GROWTH

1.2.1 Somatic bones

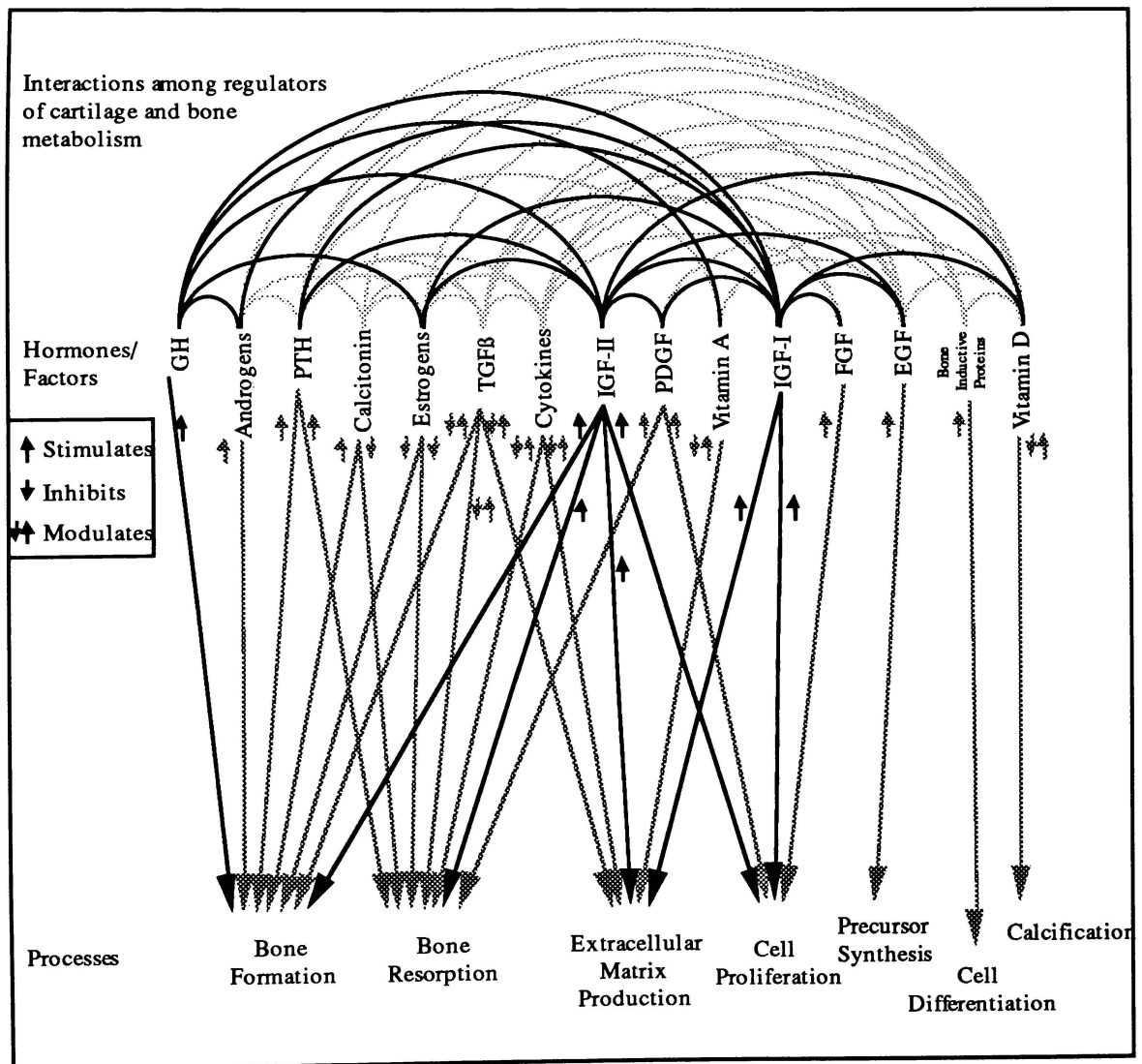
There are many factors affecting the growth, development and maintenance of bone tissue. Control involves an intricate interplay of inhibitory, stimulatory and modulatory effects which are regulated by interactions among endocrine and locally produced factors (Fig. 1.3). Whilst all of the processes listed in Figure 1.3 are essential in the growth and development of bone tissues, the processes occurring in mature bone are mainly resorption and formation. One important outcome of these two balanced processes are the mobilisation and binding of calcium, which aid in maintaining blood calcium homeostasis. The calcified skeleton is a major reservoir for ionic calcium, which is a key cation in the physiological regulation of many body functions, so its concentration in body fluids and tissues is carefully regulated.

GH and the androgens have endocrine roles which are primarily involved in bone formation directly (Williams & Frolik, 1991), and IGFs have autocrine/paracrine roles in the growth and development of tissue before bone formation (Williams & Frolik, 1991). Androgens inhibit the effects of parathyroid hormone, which stimulates bone resorption in response to low levels of calcium in the serum, in order to maintain normal blood calcium

Some of the known interactions of numerous hormonal and growth factor effects regulating cartilage and bone growth. Interactions of the GH/IGF axis are emphasised, to illustrate the complexity of the control of growth processes within a group of interrelated growth promoters. Information is from: Mohan & Baylink (1991), Williams & Frolik (1991), Hill & Logan (1992), Hill, Logan, McGarry & De Sousa (1992). Abbreviations: PTH, parathyroid hormone; TGF β , transforming growth factor- β ; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor.

homeostasis (Williams & Frolik, 1991). Parathyroid hormone also stimulates the release of IGF-I, IGF-II and other growth factors in neonatal mouse calvariae in culture by an independent mechanism to that of resorption (Linkhart & Mohan, 1989). This imparts, to some extent, a mechanism by which bone resorption is coupled to bone formation, in which IGF-II is purported to have a role (Mohan & Baylink, 1991).

Figure 1.3 Some aspects of the hormonal regulation of cartilage and bone growth.



1.2.2 Antlers

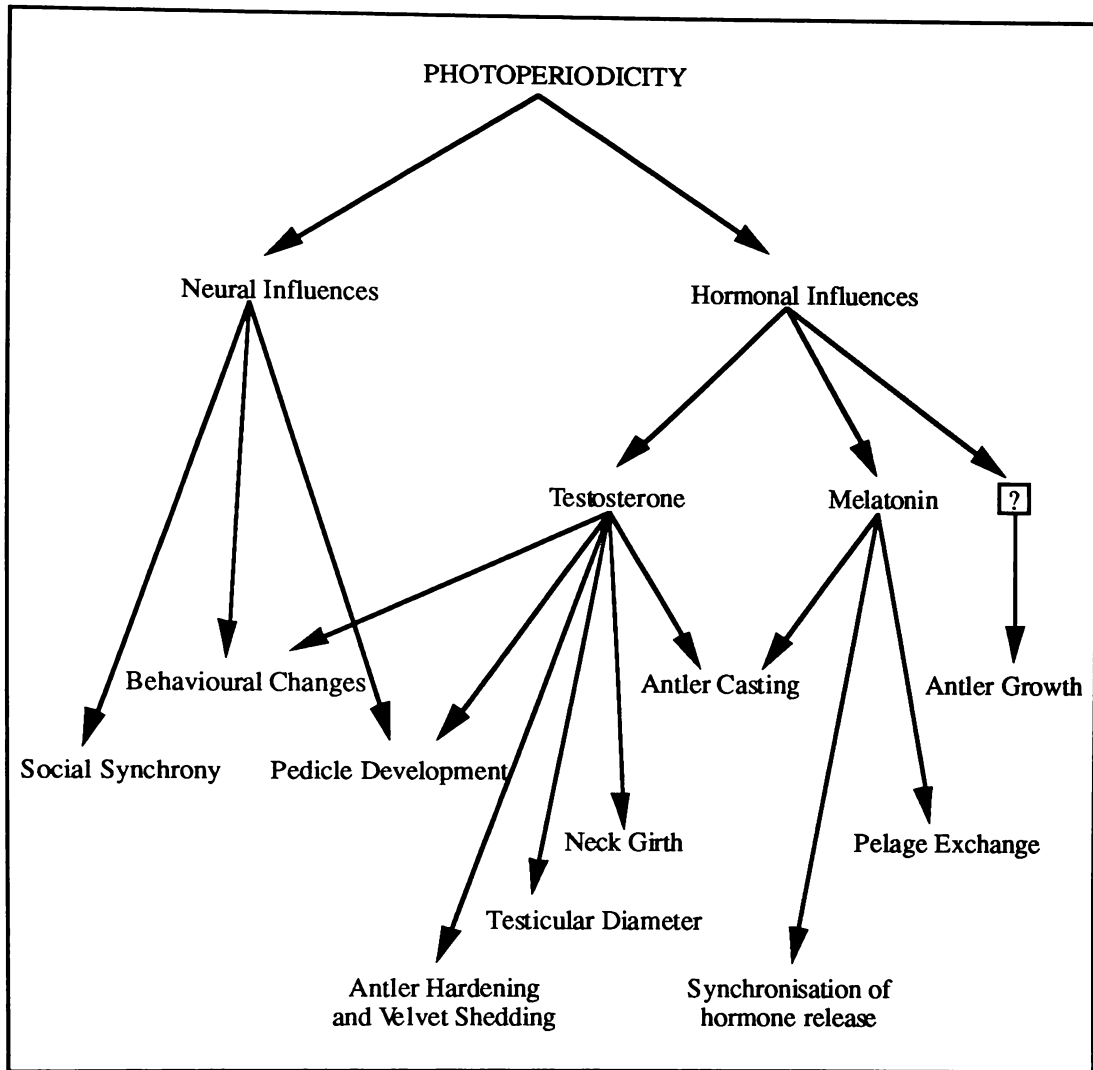
Antlers do not develop until after sexual maturation, as increasing testosterone secretion during puberty is required to complete the development of pedicles from which the antlers arise (Chapman, 1975; Chapman & Chapman, 1975b; Bubenik, 1983; Goss, 1983c; Putman, 1988). The seasonal development of antlers occurs in concert with other characteristics such as changes in testicular diameter, neck girth and behaviour (Bubenik, 1990). The regulation of these characteristics involves a combination of both neural and hormonal influences, with all changes being tightly controlled by photoperiodicity (Fig. 1.4) such that seasonal changes within a species are highly synchronous (Goss, 1969; Goss & Albach, 1974; Goss, 1983c; Snyder, Cowan, Hagen & Schanbacher, 1983; Bubenik, Schams & Coenen, 1987; Bubenik, 1990).

Factors affecting the growth of antler cartilage and bone are less well documented than those for somatic bones. Antler development is comparable to that of somatic long bones where endochondral ossification occurs (Banks & Newbrey, 1983b; Goss, 1983a; Sempere, Grimberg, Silve *et al.*, 1989), and various hormones and factors have been implicated in antler growth that are common to somatic bone growth, such as vitamin D (Sempere, Grimberg, Silve *et al.*, 1989), parathyroid hormone and calcitonin (Bubenik, 1983; Bubenik, 1990), androgens (Wislocki, Aub & Waldo, 1947; Goss, 1969; Chapman, 1975; Chapman & Chapman, 1975b; Brown, Cowan & Griel, 1978; Bubenik, 1983; Goss, 1983c; Morris & Bubenik, 1983; Bubenik, 1990), estrogens (Bubenik, 1983; Goss, 1983c), and IGF-I, IGF-II and GH (Suttie, Gluckman, Butler *et al.*, 1985; Suttie, Fennessy, Corson *et al.*, 1989; Bubenik, 1990; Suttie, Corson, Gluckman & Fennessy, 1991).

1.2.3 Importance of testosterone in bone

It has been known for many years that the antler cycles in deer are mainly under the control of the anterior pituitary gland and the testes (Wislocki, Aub & Waldo, 1947). More than 2000 years ago Aristotle wrote, "If stags be mutilated when, by reason of their age, they have as yet no horns, they never grow horns at all; if they be mutilated when they

Neural and hormonal influences responsible for the seasonal changes observed in the physiology and behaviour of male deer. Melatonin and testosterone were chosen as examples to illustrate the interplay of hormones with neural input and the known effects of these hormones on the antler cycle. At present it is not known which hormone(s) act to stimulate antler growth.

Figure 1.4 Photoperiodic control of seasonal changes in deer.

have horns, the horns remain unchanged in size and the animal does not lose them", (Goss, 1969; Chapman & Chapman, 1975b). It has been well known that castrated fawns fail to grow antlers (Gaskoin, 1856; Goss, 1969) and that following the castration of a deer in velvet antler, the antlers do not mineralise, shed their velvet or cast off compared with normal males (Gaskoin, 1856; Wislocki, Aub & Waldo, 1947; Chapman, 1975; Chapman & Chapman, 1975b; Goss, 1983c; Goss, 1983d; Bubenik, 1990). If deer are castrated in hard antler, the antlers are prematurely cast and new antlers are regenerated which remain in velvet and do not cast (Goss, 1969; Goss, Dinsmore, Grimes & Rosen, 1974). The changes in antler growth status are controlled by the fluctuations in sex hormone secretion

that occur naturally. The changes can be triggered prematurely by the administration of testosterone (Goss, 1969). If testosterone is administered while antlers are in velvet, further growth is aborted by inducing maturation of the partly grown antlers.

Administration of testosterone to castrates allows them to complete their antler development by turning cartilage to bone and shedding velvet. When the effects wear off, the old antlers drop off and new ones grow which remain in velvet. If testosterone injections are given before the old antlers are cast, casting and regrowth can be totally prevented (Goss, 1983c). Pedicle development is initiated by testosterone (Suttie, Lincoln & Kay, 1984). The onset of antler growth is correlated with a natural decline in testosterone output, along with cessation of spermatogenesis at that time of year (Goss, 1983c). Rising levels of testosterone bring about the maturation of antlers at the end of the growing season by causing solidification of the spongy bone, leading to constriction of vascular channels (Goss, Dinsmore, Grimes & Rosen, 1974; Muir, Sykes & Barrell, 1988). This causes death of the entire antler and the peeling off of the desiccated velvet (Goss, Dinsmore, Grimes & Rosen, 1974). High plasma levels of testosterone are then required to maintain the relationship between dead antler bone and the living pedicle (Bubenik, 1983; Goss, 1983c). A drop in testosterone levels occurs prior to osteoclasts appearing and degenerating the bone at the antler/pedicle junction (Wislocki, 1942; Goss, 1983a).

While it is well documented that testosterone and its metabolites (particularly dihydrotestosterone and estradiol) are involved in bone growth and mineralisation (Goss, 1969; Chapman, 1975; Goss, 1983c; Morris & Bubenik, 1983; Bubenik, 1990; Williams & Frolik, 1991; Correa, Leite, Borelli & Wajchenberg, 1992), the mechanism of action has not been defined as yet. Testosterone could have a direct role, via receptors, in increasing bone formation but this is difficult to assess due to its conversion to different metabolic forms (Morris & Bubenik, 1983). Testosterone can be converted to dihydrotestosterone in bone and cartilage (Williams & Frolik, 1991). The inhibition by testosterone of bone resorption (Williams & Frolik, 1991) may be through inhibition of parathyroid hormone

and enhancement of calcitonin effects. Some of the effects of testosterone may also be mediated through increases in serum levels of IGF-I (Williams & Frolik, 1991; Hobbs, Plymate, Rosen & Adler, 1993) which has matrixogenic and bone-forming effects (Williams & Frolik, 1991).

1.3 THE GH/IGF AXIS

In male mammals GH is released into the bloodstream from the pituitary gland. Secretion of GH is regulated by the neuroendocrine hormones: growth hormone releasing hormone (GHRH) and growth hormone release inhibiting hormone (GHRH) which are released from the hypothalamus (Frohman, Downs, Katakami & Jansson, 1987). GH circulates in plasma partly bound to a specific binding protein, and binds to cell surface receptors which are found in many target tissues throughout the body, including liver, muscle, fat and bone. One of the actions of GH is to stimulate the production of somatomedin (IGF-I), which can be released into the bloodstream to act in an endocrine manner, or can act locally in an autocrine or paracrine manner (Fig. 1.5) (Schalch, Heinrich, Draznin *et al.*, 1979; Zapf, Froesch & Humbel, 1981; Green, Morikawa & Nixon, 1985; Fant, Munro & Moses, 1986; Milner & Hill, 1987).

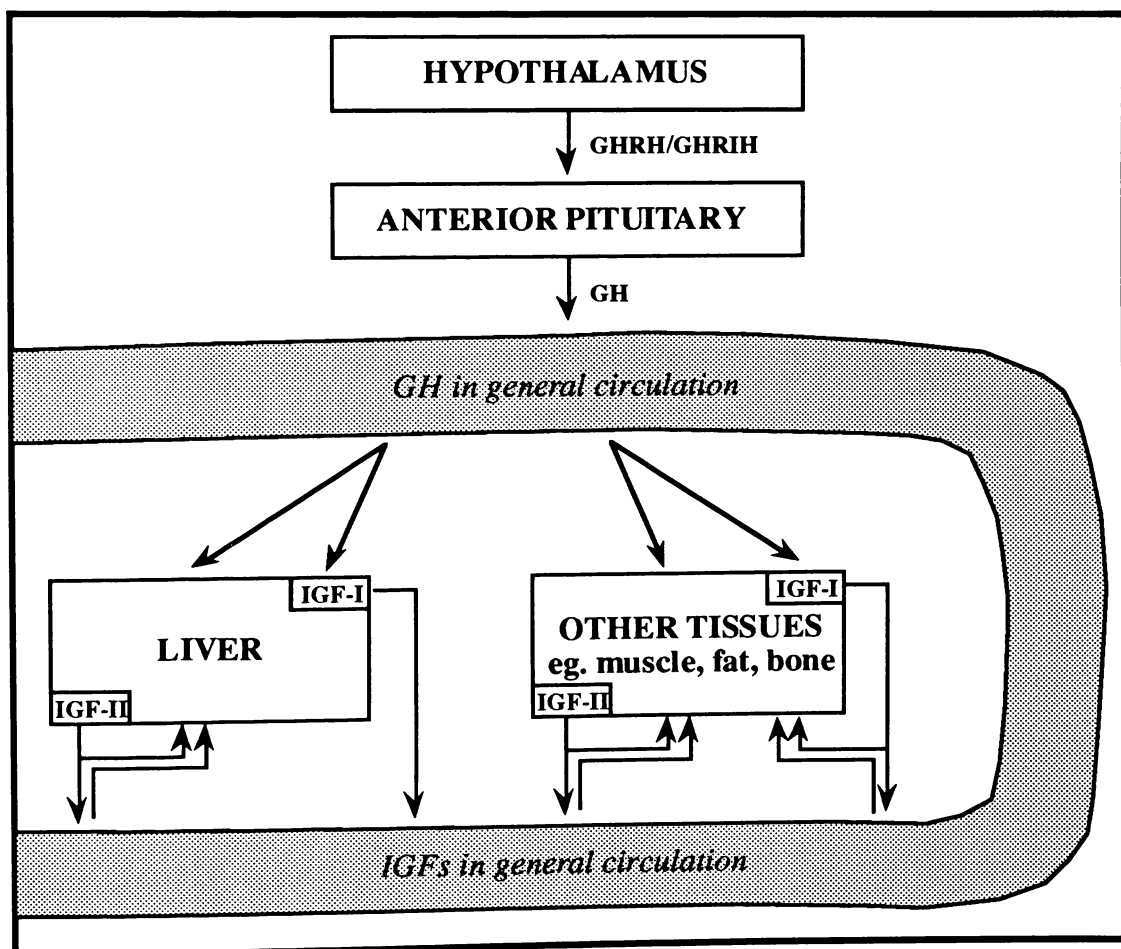
IGF-II is also produced in many tissues, but the production of IGF-II is not directly stimulated by GH (Richman, Benedict, Florini & Toly, 1985; Hammerman, 1989), although it has been tentatively suggested that GH may have some role in the regulation of IGF-II mRNA expression (Hynes, Van Wyk, Brooks *et al.*, 1987; Turner, Rotwein, Novakofski & Bechtel, 1988). The precise mechanisms governing IGF-II synthesis are as yet unclear but it seems that epidermal growth factor and dexamethasone are stimulatory (Richman, Benedict, Florini & Toly, 1985), dietary protein levels exert regulatory control to some extent (Straus & Takemoto, 1990) and expression is developmentally regulated as

Hypothalamic control of GH secretion into the general circulation is exerted by the neuroendocrine regulators GHRH and GHRH. GH has a dual effect on target tissues either by binding directly, or indirectly via IGF-I (Green, Morikawa & Nixon, 1985). IGF-II production is independent of GH. Both IGFs may exert endocrine or autocrine/paracrine effects on all target tissues, except for liver which has no receptors specific for IGF-I (Tandeski, Yemm & Hossner, 1990).

IGF-II mRNA abundance is highest during fetal development, and declines postnatally (Lund, Moats-Staats, Hynes *et al.*, 1986).

The liver is believed to be the major source of circulating IGFs (Schwander, Hauri, Zapf & Froesch, 1983), although many tissues have been shown to secrete IGFs into media (D'Ercole, Stiles & Underwood, 1984). Like GH, IGF-I and IGF-II bind specifically to circulating binding proteins, and to receptors in target tissues from which numerous biological responses are reported to arise (Froesch, Schmid, Schwander & Zapf, 1985).

Figure 1.5 Organisation of the mammalian GH/IGF axis in the male.



1.3.1 The GH, IGF-I and IGF-II peptides

GH mostly occurs as a polypeptide of 191 amino acids of M_r 22 kDa (Havel, Kauffman & Elzinga, 1988). The primary structure of ovine GH differs from bovine GH by only two amino acid residues (Wallis, 1973). Although most mammalian GH molecules are similar in structure, human GH differs from bovine GH by 35 to 40% of amino acid residues (Wallis, 1973; Hammerman, 1989). The heterogeneity in the primary structure of the GH molecules between species is not reflected in the tertiary structure which is a single polypeptide chain, folded and held in place by two intra-chain disulphide bridges (Havel, Kauffman & Elzinga, 1988). GH is structurally related to placental lactogen and prolactin (Wallis, 1988).

IGF-I and IGF-II are polypeptides that show a sequence homology with proinsulin (Rindernecht & Humbel, 1978a; Rindernecht & Humbel, 1978b; Froesch, Schmid, Schwander & Zapf, 1985) of 43% and 41% respectively (Zangger, Zapf & Froesch, 1987; Sara & Hall, 1990). The IGFs themselves have a sequence homology of 62% (Rindernecht & Humbel, 1978b). IGF-I is 70 amino acids long with a M_r of 7646 Da (Rindernecht & Humbel, 1978a) and IGF-II is 67 amino acids long with a M_r of 7471 Da (Rindernecht & Humbel, 1978b). Species differences in the primary structures of the mammalian IGFs are small. Bovine, porcine and human IGF-I are identical, while rat and mouse IGF-I differ from human IGF-I by three and four amino acid residues respectively (Sara & Hall, 1990). Cervine and human IGF-I differ by two amino acids (Moore, Jones, Lymburn *et al.*, 1993). Bovine IGF-II, which is identical to cervine IGF-II (Moore, Jones, Lymburn *et al.*, 1993), differs from human IGF-II by three amino acids, and rat and mouse IGF-II differ from human IGF-II by four and six amino acid residues respectively (Sara & Hall, 1990). Both IGFs are single-chain molecules with three intra-chain disulphide bridges in a tertiary structure that is similar to that of insulin (Sara & Hall, 1990). Shared conformations in specific regions of these molecules forms the basis of receptor cross-binding, enabling the functional interaction of insulin, IGF-I and IGF-II, to a certain extent, at the receptor level (Dafgård, Bajaj, Honegger *et al.*, 1985; Sara & Hall, 1990).

Whilst there is structural similarity in receptor binding regions of the molecules, regions of antibody binding are different, giving rise to a lack of immunological cross-reactivity (Froesch, Schmid, Schwander & Zapf, 1985).

1.3.2 Receptors for GH, IGF-I and IGF-II

Binding to specific cell surface receptors is a prerequisite for the physiological effects of peptide hormones such as GH and the IGFs. The GH receptor is a glycosylated polypeptide with a centrally located transmembrane region. As deduced from cloned complementary DNA sequences, the rabbit GH receptor is composed of 620 amino acids with a M_r of 110 to 140 kDa (Leung, Spencer, Cachianes *et al.*, 1987) and the human GH receptor is composed of 638 amino acids with a M_r of 130 kDa (Nilsson, 1989). GH receptors belong to a family of hematopoietin receptors (the type 1 cytokine receptor family) that include receptors for prolactin, erythropoietin, interleukins 2, 3, 4, 5, 6, 7 and 9, granulocyte-macrophage colony-stimulating factor, granulocyte colony stimulating factor, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor and a receptor-like proto-oncogene MPL (Dower, 1993; Ohlsson, Isgaard, Törnell *et al.*, 1993). GH receptors are structurally related to prolactin receptors, displaying extensive sequence homology in the extracellular domain and similar-sized cytoplasmic regions (Nilsson, 1989). Specific binding sites for GH have been recognised on numerous cells and tissue types of many organs, including adipocytes (Edén, Kostyo & Schwartz, 1982), lymphocytes, connective tissue, kidney, liver (Posner, 1976), skeletal muscle, ovary, fibroblasts and chondrocytes (Edén, Isaksson, Madsen & Friberg, 1983). GH has been implicated in many growth and metabolic activities, although the cellular events following GH receptor binding remain poorly characterised (Isaksson, Edén & Jansson, 1985; Hammerman, 1989).

The type 1 IGF receptor is a glycoprotein heterotetramer with a M_r of 300 kDa and is similar in structure to the insulin receptor. It consists of four disulphide-linked subunits: two α -subunits, each with a M_r of 130 kDa, and two β -subunits, each with a M_r of 90 kDa

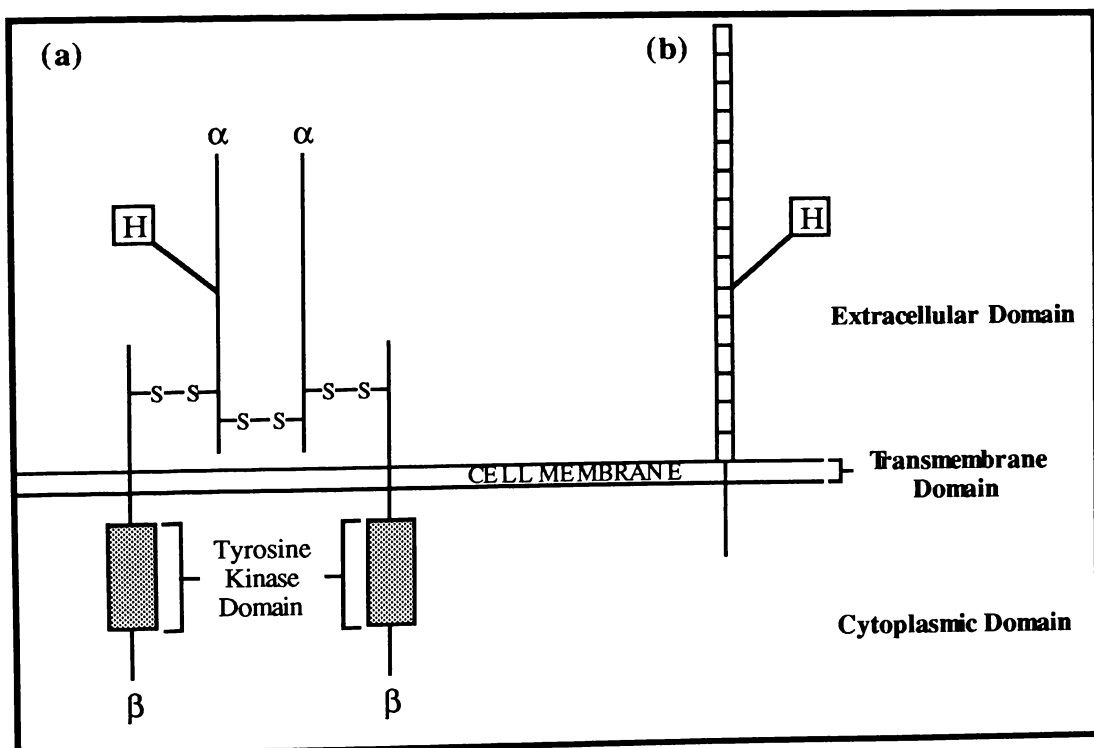
(Fig. 1.6) (Massagué & Czech, 1982; Rechler & Nissley, 1985; Underwood & Van Wyk, 1985). The amino acid sequence of the type 1 IGF receptor shares 50% homology with the primary sequence of the insulin receptor, which has the same $\alpha_2\beta_2$ subunit conformation and a high degree of similarity in the extracellular domain (Yarden & Ullrich, 1988), all of which, to a limited extent, contribute to a shared ligand specificity (Roth, Maddux, Wong *et al.*, 1983; Underwood & Van Wyk, 1985; Yarden & Ullrich, 1988). The insulin and type 1 IGF receptors belong to a superfamily of receptors that are identified as mediating their physiological processes by the activation of ligand-sensitive tyrosine kinase (Yarden & Ullrich, 1988; Bylund & Toews, 1993). Protein tyrosine kinases form the catalytic regions in the cytoplasmic domains of the transmembrane β -subunits. Ligand binding to the extracellular α -subunit activates the tyrosine kinase activity of the β -subunit (Yarden & Ullrich, 1988; Garofalo & Barenton, 1992), resulting in the autophosphorylation of the β -subunit, which is the first step in the transmission of the signal across the plasma membrane (Rechler & Nissley, 1985).

The type 2 IGF receptor is a monomer with of M_r 220 kDa, which appears to increase to 260 kDa under reducing conditions (Massagué & Czech, 1982; Underwood & Van Wyk, 1985). There is a single transmembrane region with an extracellular domain of 15 contiguous repeat sequences, comprising 93% of the receptor molecule, and a relatively small cytoplasmic domain (Fig. 1.6) (Hammerman, 1989). This receptor bears no resemblance to either the insulin receptor or the type 1 IGF receptor, but is identical to the cation-independent (CI) mannose-6-phosphate (M6P) receptor (Morgan, Edman, Standring *et al.*, 1987; MacDonald, Pfeffer, Coussens *et al.*, 1988; Roth, 1988; Tong, Tollefsen & Kornfeld, 1988). M6P receptors recognise and specifically bind M6P residues on lysosomal enzymes, and the CI M6P receptor (type 2 IGF receptor) binds ligands independently of divalent cations (von Figura & Hasilik, 1986). The type 2 IGF receptor displays multivalent binding and has two high affinity binding sites for M6P per molecule (Tong, Gregory & Kornfeld, 1989) and a single binding site per molecule for IGF-II (Kiess, Blichenstaff, Sklar *et al.*, 1988). As well as lysosomal enzymes, the M6P

Schematic structures of (a) the type 1 IGF receptor and (b) the type 2 IGF receptor. The type 1 IGF receptor is a disulphide-linked heterotetramer with a $\beta\alpha\alpha\beta$ subunit configuration, structurally similar to the insulin receptor. The type 2 IGF receptor is a monomer with most of the receptor molecule in the extracellular domain. The sites of binding of the IGFs are denoted by H (Sara & Hall, 1990).

recognition sites on the type 2 IGF receptor will also bind non-lysosomal ligands that contain M6P residues, such as transforming growth factor- β 1, proliferin and thyroglobulin (Nissley & Kiess, 1991). The binding sites for M6P and IGF-II are on the extracellular portion of the molecule, and are distinct entities which exhibit cooperativity (Kiess, Blichenstaff, Sklar *et al.*, 1988; MacDonald, Pfeffer, Coussens *et al.*, 1988). IGF-II and lysosomal enzymes, such as β -galactosidase, reciprocally inhibit the other ligand from binding to the same type 2 IGF receptor (Kiess, Thomas, Sklar & Nissley, 1990; Nissley & Kiess, 1991). Although free M6P has been reported to increase the binding of IGF-II to the type 2 IGF receptor (MacDonald, Pfeffer, Coussens *et al.*, 1988), it has been shown that free M6P competitively inhibits the binding of lysosomal enzymes to the receptor (Polychronakos, Guyda & Posner, 1988; Kiess, Thomas, Sklar & Nissley, 1990), thereby removing the source of inhibition of IGF-II binding, which results in an apparent increase (Nissley & Kiess, 1991).

Figure 1.6 Structural organisation of the IGF receptors.



The type 2 IGF receptor lacks the protein kinase activity of the insulin and type 1 IGF receptors (Nissley, Haskell, Sasaki *et al.*, 1985) and IGF-II signal transduction seems to occur via a G protein-coupled mechanism (Murayama, Okamoto, Ogata *et al.*, 1990; Okamoto, Katada, Murayama *et al.*, 1990; Okamoto, Nishimoto, Murayama *et al.*, 1990; Nishimoto, Ogata & Okamoto, 1991; Okamoto & Nishimoto, 1991). GTP binding proteins (G proteins) are membrane-associated regulatory proteins that transduce receptor-generated signals to effectors inside the plasma membrane (Gilman, 1987). In response to stimulation of type 2 IGF receptors by IGF-II, GTP binding proteins are activated by interaction with the cytoplasmic domain of the receptors (Okamoto & Nishimoto, 1991). Although the type 2 IGF receptor has two classes of ligand binding sites in its extracellular region, it seems that only IGF-II binding to the receptor is capable of activating GTP binding proteins, and not M6P (Okamoto, Nishimoto, Murayama *et al.*, 1990) which instead inhibits dose-dependently the G protein response by IGF-II (Murayama, Okamoto, Ogata *et al.*, 1990).

Homology among ligands and/or receptors can lead to cross-reactivity, where a receptor may bind more than one ligand. Because of GH receptor and prolactin receptor homology (Nilsson, 1989), GH may cross-bind to the prolactin receptor. As IGF-I and IGF-II share a certain degree of homology with insulin (Dafgård, Bajaj, Honegger *et al.*, 1985) and the insulin receptor and type 1 IGF receptor share structural homology (Yarden & Ullrich, 1988), a certain degree of receptor cross-binding can occur. This means that differential affinities exist, where the insulin receptor will preferentially bind insulin, but will bind the IGFs to a lesser extent; the type 1 IGF receptor has the greatest affinity for IGF-I but will also bind IGF-II and insulin; and the type 2 IGF receptor is specific for IGF-II, but will bind IGF-I to a small extent and insulin not at all (Underwood & Van Wyk, 1985). The purpose of receptor cross-binding may be of regulatory significance or could have a functional value, which will be discussed later in this chapter.

1.3.3 Binding Proteins

GH circulates throughout the body bound to a serum binding protein which is believed to be a proteolytically cleaved fragment of the GH receptor (Barnard & Waters, 1986; Leung, Spencer, Cachianes *et al.*, 1987; Baumann & Shaw, 1988; Spencer, Hammonds, Henzel *et al.*, 1988). Although serum levels of the GH binding protein are GH-dependent, they are not subject to sudden fluctuation, remaining stable in the face of acute GH changes (Carmignac, Wells, Carlsson *et al.*, 1992). The GH binding protein protects GH from degradation and prolongs its biological half-life (Baumann, Amburn & Buchanan, 1987), and it may modulate the ability of GH to bind to receptors (Nilsson, 1989).

IGFs, but not insulin, also bind to circulating binding proteins. The affinities of the insulin-like growth factor-binding proteins (IGFBPs) for IGF-I and IGF-II are comparable to, or greater than, the affinities of the type 1 and type 2 IGF receptors for the IGFs (McCusker, Camacho-Hubner, Bayne *et al.*, 1990; Sakano, Enjoh, Numata *et al.*, 1991; Kiefer, Schmid, Waldvogel *et al.*, 1992; Bach, Hsieh, Sakano *et al.*, 1993; Rechler, 1993). There are two classes of IGFBPs in serum or plasma, grouped according to their molecular size, and are designated 150 kDa IGFBPs or 30-40 kDa IGFBPs (Sara & Hall, 1990; Drop, Brinkman, Kortleve *et al.*, 1991). Binding of the IGFs to IGFBPs increases the biological half lives of the hormones: free IGFs have a half life of 10-12 minutes, IGFs bound to 30-40 kDa IGFBPs have half lives of 20-30 minutes and in the 150 kDa complex, can last for 6-15 hours in serum, depending on the species (Guler, Zapf, Schmid & Froesch, 1989; Hodgkinson, Moore, Napier *et al.*, 1989). Six IGFBPs have so far been identified and may be distinguished by differences in their amino-terminal amino acid sequences (Shimasaki, Shimonaka, Zhang & Ling, 1991).

IGFBP-1 was initially detected in human amniotic fluid and designated "amniotic fluid binding protein" (Drop, Kortleve & Guyda, 1984). Since then the protein has been found to be identical with placental protein 12, BP-28, IBP-1, α_2 -pregnancy associated endometrial globulin and growth hormone-independent binding protein, among others

(Rutanen & Pekonen, 1990; Drop, Brinkman, Kortleve *et al.*, 1991). The mature protein has a M_r of 28-30 kDa under non-reducing conditions, and binds both IGFs with a similar affinity (Rutanen & Pekonen, 1990). IGFBP-1 is present in low levels in serum, with serum levels being inversely dependent on insulin (Suikkari, Koivisto, Rutanen *et al.*, 1988) and rising dramatically during pregnancy. Synthesis of IGFBP-1 occurs in the endometrium, liver and luteal granulosa cells (Rutanen & Pekonen, 1990).

IGFBP-2 has a M_r of 33-35 kDa under non-reducing conditions (Hossenlopp, Segovia, Lassarre *et al.*, 1990) and preferentially binds IGF-II (Roghani, Hossenlopp, Lepage *et al.*, 1989; Rutanen & Pekonen, 1990). IGFBP-2 has been isolated from rat, bovine and human liver cell lines and bovine kidney cells (Rutanen & Pekonen, 1990; Drop, Brinkman, Kortleve *et al.*, 1991; Shimasaki, Shimonaka, Zhang & Ling, 1991), is found in human cerebrospinal fluid (Roghani, Hossenlopp, Lepage *et al.*, 1989), and is present in many fetal tissues (Brown, Chiarotti, Orlowski *et al.*, 1989; Delhanty & Han, 1993).

IGFBP-3 is the acid-stable IGF-binding subunit of the 150 kDa ternary complex which is the major form of IGFBP found in serum (Baxter & Martin, 1989b). The 150 kDa complex is comprised of an IGFBP-3 (β -subunit) bound to an IGF (γ -subunit) and an acid-labile protein of 80 kDa (α -subunit) which can only bind to IGFBP-3 in association with an IGF and under neutral conditions (Baxter & Martin, 1989b; Binoux, Roghani, Hossenlopp *et al.*, 1991; Rechler, 1993). Different glycosylated forms of IGFBP-3 migrate at different rates under SDS-PAGE, exhibiting a characteristic IGF-binding doublet at 41.5 and 38.5 kDa, from human serum (Sommer, Maack, Spratt *et al.*, 1991). These smaller forms of IGFBP-3 are capable of crossing the capillary barrier and are present in lymph and many tissues (Rechler, 1993), whereas the 150 kDa complex is restricted (Binoux & Hossenlopp, 1988). IGFBP-3 has also been known as BP-53, BP-29 and GH-dependent binding protein (Rutanen & Pekonen, 1990; Drop, Brinkman, Kortleve *et al.*, 1991) and binds IGF-I and IGF-II with relatively similar affinity (Rechler, 1993).

IGFBP-4 has been identified in culture media of human bone cells (Mohan, Bautista, Wergedal & Baylink, 1989), rat neuronal cells (Cheung, Smith, Shimasaki *et al.*, 1991) and human fibroblasts (Camacho-Hubner, Busby, McCusker *et al.*, 1992), and in rat serum (Shimonaka, Schroeder, Shimasaki & Ling, 1989). It exists in a 24 kDa form which can be glycosylated to a 28-29 kDa form (Bautista, Baylink & Mohan, 1991; Cheung, Smith, Shimasaki *et al.*, 1991; Camacho-Hubner, Busby, McCusker *et al.*, 1992), and has a slight preferential affinity for IGF-II over IGF-I (Rechler, 1993).

IGFBP-5 is present in fibroblast culture media (Camacho-Hubner, Busby, McCusker *et al.*, 1992), rat serum (Shimasaki, Shimonaka, Zhang & Ling, 1991) and human serum (Kiefer, Masiarz, Bauer & Zapf, 1991). It exists in a 31 kDa form (Camacho-Hubner, Busby, McCusker *et al.*, 1992) which shows a preferential affinity for IGF-II over IGF-I (Rechler, 1993).

IGFBP-6 is the most recently described IGFBP and exists in human serum in a 28-30 kDa form (¹Kiefer, Masiarz, Bauer & Zapf, 1991). IGFBP-6 has a 60 to 70-fold greater affinity for IGF-II than IGF-I (Rechler, 1993).

Recently, IGF binding proteins have been shown to form an integral part of some tissues by binding to extracellular matrices and cell surfaces (Clemmons, Elgin, Han *et al.*, 1986; Ooi, 1990; Clemmons, 1991; McCusker, Busby, DeHoff *et al.*, 1991; Jones, Gockerman, Busby *et al.*, 1993). A study by Jones, D'Ercole, Camacho-Hubner & Clemmons (1991) has shown that phosphorylated IGFBP-1 has increased affinity for IGF-I. They found that IGFBP-1 can be detected in the extracellular matrix, and contains the cell attachment sequence Arginine-Glycine-Asparagine (RGD), which is recognised by several cell surface receptor types. These authors went on to speculate that this could have important implications as phosphorylation of various RGD-containing bone matrix glycoproteins has

¹IGFBP-6 was termed IGFBP-4 in this reference, as noted by the Participants of the Second International IGF Symposium (1992). Report on the Nomenclature of the IGF binding proteins *Endocrinology* **130**, 125-126.

a role in altering cell-matrix interactions, and phosphorylation is known to be a step in multiple cellular metabolic and signalling pathways. IGFBP-2 also contains an RGD sequence near its carboxyl terminus, as with IGFBP-1, whereas IGFBP-3 does not contain such a sequence (Clemmons, 1991). Extracellular matrix has been shown to bind IGFBP-4 (Bautista, Baylink & Mohan, 1991) and IGFBP-5 (Jones, Gockerman, Busby *et al.*, 1993). The affinity of these surface-bound IGFBPs for IGFs is approximately 10-fold less than IGFBPs that are free in extracellular fluids (Conover, 1991; McCusker, Busby, DeHoff *et al.*, 1991), which have approximately the same or higher affinity for IGFs than IGF receptors (Rechler, 1993).

Binding of IGF-I to IGFBPs requires an intact amino acid sequence at the amino-terminus of the IGF-I molecule (Szabo, Mottershead, Ballard & Wallace, 1988). Analogues of IGF-I that have an altered amino-terminus show markedly affected abilities to binding to IGFBPs. In particular, desamino-(1-3)-IGF-I (des-IGF-I), which lacks the first three amino acid residues of the IGF-I amino-terminus, has a very low affinity for IGFBPs (Szabo, Mottershead, Ballard & Wallace, 1988; Ross, Francis, Szabo *et al.*, 1989; Canalis, McCarthy & Centrella, 1991). This truncated IGF-I occurs naturally in several tissues, leading to the hypothesis put forward by Sara and Hall (1990) that des-IGF-I is the locally acting autocrine/paracrine form of IGF-I and intact IGF-I, which binds to IGFBPs in plasma, is the endocrine form.

1.3.4 Roles of the GH/IGF axis

1.3.4.1 GH in General

GH and circulating IGFs exert a negative feedback control over secretion of GH from the pituitary gland (Hammerman, 1989). Other physiological controls operating on GH secretion include stimulation by physical exercise, hypoglycemia and high protein levels (Hammerman, 1989). These stimuli reflect the role of GH in carbohydrate metabolism where GH causes elevated glucose levels, which are antagonistic to the glucose-lowering action of insulin (Davidson, 1987). GH also has a role in lipid metabolism where it has

lipolytic effects (Goodman & Grichting, 1983; Davidson, 1987). Paradoxically, GH exhibits transient insulin-like effects in tissues of animals where plasma GH has been eliminated or suppressed (Isaksson, Edén & Jansson, 1985; Isgaard, 1989). These effects include increased glucose utilisation (Albertsson-Wikland & Isaksson, 1978; Davidson, 1987) and increased protein synthesis (Albertsson-Wikland & Isaksson, 1978). It is not known whether these insulin-like activities reported for GH are mediated by IGFs. GH also has an adipogenic effect which is active in myoblast differentiation (Nixon & Green, 1984), and facilitates the conversion of fibroblasts into adipocytes in a process that was shown not to involve IGF-I (Green, Zezulak & Djian, 1987).

Circulating GH acts both directly on sensitive cells in target tissues, and indirectly via stimulation of IGF production, which is the principle behind the dual effector theory of GH action (Green, Morikawa & Nixon, 1985). Whilst it is now generally accepted that the metabolic effects of GH are exerted directly, stimulation of somatic growth by GH seems to be indirect (Hammerman, 1989). This is supported by a number of studies showing that growth during fetal and early postnatal life in most species is independent of GH (Isaksson, Edén & Jansson, 1985) despite there being increased plasma levels of GH during this period relative to pre-pubertal GH plasma levels (Gluckman, Grumbach & Kaplan, 1981). Instead, GH-dependent growth is developmentally regulated and gradually increases to reach full dependence a few weeks after birth (Isaksson, Edén & Jansson, 1985; Isgaard, 1989). This is comprehensively demonstrated in a study where differential growth rates among GH-deficient mice, control mice and transgenic mice, with the GH gene inserted in the germ line to ensure continuous expression, were not achieved until three weeks post-partum (Mathews, Hammer, Behringer *et al.*, 1987). The progression to GH-dependent growth in lambs directly correlates with the number of GH receptors in ovine liver membranes, as binding site numbers are low during the fetal and neonatal period and increase postnatally (Gluckman, Butler & Elliott, 1983). Concomitant with the postnatal progression to GH-dependent growth in humans is an increase in circulating levels of GHBP (Daughaday, Trivedi & Andrews, 1987) which, in humans and rabbits,

are the proteolytically cleaved extracellular portions of the GH receptor (Trivedi & Daughaday, 1988). GH release is episodic in nature (Tannenbaum & Martin, 1976) and as the actions of GH are dependent on the total amount released and on its secretory pattern (Clark, Jansson, Isaksson & Robinson, 1985), circulating GHBP may affect the way in which GH exerts its hormonal action (Mannor, Winer, Shaw & Baumann, 1991).

In contrast to the findings with sheep, the presence of GH receptors with GH-binding capability have been demonstrated in human fetal tissues, including the liver (Hill, Riley, Bassett & Waters, 1992). These results tend to confound the existing explanations of GH-independent growth in the fetus and neonate. It is possible that relationships between elements of the GH/IGF axis are different in the fetus than they are postnatally. GH and IGF-I have been shown to exert independent effects on target cells in chondrogenesis (Hill & Logan, 1992), which could partly explain the GH-independent growth phenomenon. Further, IGF-I production is not an exclusive effect of GH as other hormones, such as placental lactogen (Hill, Crace & Milner, 1985), platelet-derived growth factor, fibroblast growth factor, adrenocorticotrophic hormone, thyrotropin, luteinizing hormone and follicle-stimulating hormone, may stimulate the synthesis of IGF-I from their target cells (reviewed by Sara & Hall, 1990). Local systems of growth control are implicated in many fetal tissues with the observation that the cellular localisation of IGFs and a 26 kDa IGFBP from amniotic fluid, probably IGFBP-1, show similar distribution patterns (Hill, Clemmons, Wilson *et al.*, 1989).

GH receptors have also been demonstrated on human growth plate chondrocytes at the fetal, perinatal and postnatal stages (Werther, Haynes, Edmondson *et al.*, 1993). Distribution of GH receptors in the growth plate changed ontogenetically with receptor occurrence depending on the degree of cellular maturation (Werther, Haynes, Edmondson *et al.*, 1993). This is also demonstrated in the rabbit tibial growth plate where GH receptors are limited to the hypertrophic chondrocytes in the neonate, but postnatally they are also found in the resting and proliferative zones, perichondrium and osteoblasts

(Barnard, Haynes, Werther & Waters, 1988). Suggested functional roles for GH on target tissues in the fetus and neonate include differentiation (Werther, Haynes, Edmondson *et al.*, 1993) and the stimulation of local IGF-I synthesis (Han, D'Ercole & Lund, 1987).

1.3.4.2 GH in Bones

There are numerous reports on the effect of GH on longitudinal bone growth in postnatal mammals (Salmon & Daughaday, 1957; Isaksson, Edén & Jansson, 1985; Russell & Spencer, 1985; Isgaard, Nilsson, Lindahl *et al.*, 1986; Nilsson, Isgaard, Lindahl *et al.*, 1986; Nilsson, Isgaard, Lindahl *et al.*, 1987; Nilsson, Lindahl, Edén & Isaksson, 1989). Local administration of GH or IGF-I directly to the tibia of hypophysectomised rats stimulates unilateral bone growth (Isaksson, Jansson & Gause, 1982; Russell & Spencer, 1985; Isgaard, Nilsson, Lindahl *et al.*, 1986; Nilsson, Isgaard, Lindahl *et al.*, 1987) as evidenced by increases in the width of the growth plate and proliferating zone (Loveridge & Farquharson, 1993). In the rabbit tibial growth plate, prechondrocytes and differentiating chondrocytes have a high number of GH receptors (Barnard, Haynes, Werther & Waters, 1988) and stimulatory effects of GH have been demonstrated on the proliferation of mouse cartilage progenitor cells (Maor, Hochberg, von der Mark *et al.*, 1989) and on the proliferation of rat chondrocytes (Lindahl, Nilsson & Isaksson, 1987). Both GH and IGF-I, directly and independently, bind to epiphyseal chondrocytes *in vivo*, depending on the stage of differentiation or maturation of the cells (Isaksson, Lindahl, Nilsson & Isgaard, 1987), and both stimulate chondrocyte mitogenesis *in vitro* (Ohlsson, Nilsson, Isaksson & Lindahl, 1992; Saggese, Federico & Cinquanta, 1993) and matrix production *in vitro* (Ohlsson, Nilsson, Isaksson & Lindahl, 1992). Although evidence ostensibly suggests that GH exerts direct growth effects on cartilaginous tissues (Isaksson, Jansson & Gause, 1982), there is no evidence in these studies to suggest that IGF-I expression is not occurring in these tissues. It has been hypothesised that GH may directly stimulate the differentiation of precursor cells and increase their responsiveness to IGF-I, thereby having indirect effects on the clonal expansion of chondrocytes (Isaksson, Lindahl, Nilsson & Isgaard, 1987). It is now generally regarded that the actions of GH in bone

growth are indeed by the stimulation of IGF-I production, either hepatically to increase levels of circulating IGF-I (Salmon & Daughaday, 1957), or locally (Isaksson, Lindahl, Nilsson & Isgaard, 1987; Isgaard, Möller, Isaksson *et al.*, 1988; Nilsson, Carlsson, Isgaard *et al.*, 1990) without changing circulating levels of IGF-I (Loveridge & Farquharson, 1993). This is supported by a study showing that the stimulatory effect on the tibial epiphyseal growth plate of GH, locally infused into the femoral artery, was abolished when antiserum to IGF-I was co-infused with GH (Schlechter, Russell, Spencer & Nicoll, 1986).

1.3.4.3 IGFs in General

In general, circulating levels of IGF-I are low in the fetus and rise postnatally to high levels at puberty, after which they decline with increasing age (Sara & Hall, 1990). Plasma levels of IGF-II are relatively high during fetal stages in all species, but postnatal levels vary. In man, IGF-II levels increase postnatally and remain high; in the sheep and guinea pig levels decline slightly but remain high; in the rat and mouse levels decrease to become negligible after weaning (Gluckman & Ambler, 1993).

IGFs circulate in the blood complexed to IGFBPs, with IGFBP-3 being the most prevalent species (Baxter & Martin, 1989a; Baxter & Martin, 1989b). The acid-labile subunit of the 150 kDa IGFBP-3 complex is induced by growth hormone (Baxter, 1991), whilst the IGFBP-3 subunit is induced by IGF-I (Clemmons, 1991). As more than 90% of circulating IGFs are bound in this IGFBP complex, it is a physiologically important molecule that is controlled by the coordination of GH and IGF-I (Clemmons, 1991). This relationship may confer some measure of control over IGF-II serum levels by GH as an increase in serum levels of IGF-I, induced by GH, could competitively inhibit binding of IGF-II to the IGFBP-3 complex, decreasing the half life and hence circulating levels of IGF-II (Owens, Johnson, Campbell & Ballard, 1990; Gluckman & Ambler, 1993).

IGFBPs are present in numerous tissues and fluids, and co-localization of IGFBPs and IGFs has been demonstrated in the rat kidney (Kobayashi, Clemmons & Venkatachalam,

1991) and in human fetal tissues except skin (Hill, Clemmons, Wilson *et al.*, 1989). Both groups of researchers suggested that the distribution of IGFBPs may define the sites of biological action of the IGFs. Suggested roles for the IGFBPs in tissues include regulation of the amount of IGF that can gain access to cellular receptors, mechanisms for tissue specificity, and control for the entry of IGFs into cells (Kobayashi, Clemmons & Venkatachalam, 1991). The IGFBPs may play a role in modulating or regulating the action of IGFs in bone (Baxter & Martin, 1989a; Sara & Hall, 1990) as bone cells have been shown to produce IGFBPs (Mohan, Linkhart, Rosenfeld & Baylink, 1989; Schmid, Zapf, Meier *et al.*, 1989). Effects of IGFBPs on IGF activity can be inhibitory (Baxter & Martin, 1989a; Cheung, Smith, Shimasaki *et al.*, 1991) or facilitatory (Elgin, Busby & Clemmons, 1987; Baxter & Martin, 1989a; Jones, Gockerman, Busby *et al.*, 1993). Local synthesis of IGFBPs has been hypothesised to provide local control of an IGF response in an autocrine or paracrine way, which seems to occur in the case of injuries where a localised proliferative response occurs and is confined to a small area (Elgin, Busby & Clemmons, 1987). Matrix-bound IGFBPs may act as local targeting agents for the IGFs (Clemmons, Elgin, Han *et al.*, 1986; De Vroede, Tseng, Katsoyannis *et al.*, 1986; Clemmons, 1991). IGF-II is present in human bone at levels 10 fold higher than that of IGF-I (Frolik, Ellis & Williams, 1988). A 29 kDa IGFBP (probably IGFBP-4) with a preferential affinity for IGF-II has also been isolated from human bone, and is postulated to be involved in the fixation of IGF-II in bone and in the potentiation of the proliferative actions of IGF-II (Bautista, Baylink & Mohan, 1991). IGFBPs may potentiate IGF receptor action, as expounded in a model by Conover (1992) in which soluble IGFBP-3 binds IGF, then binds to the cell surface and releases IGF into the vicinity of receptors via a progressive decrease in affinity, causing a local increase in IGF concentration. It has been suggested that the continuous release of IGF-I into the cellular environment produces an optimal biological response through maintaining high receptor occupancy and growth factor activity, and minimising receptor down-regulation (Blum, Jenne, Reppin *et al.*, 1989). Conversely the IGFBPs can inhibit action by sequestering IGFs and preventing receptor interaction (Conover & Powell, 1991). Release of IGFs from IGFBPs can occur

by the action of proteases or heparin (Clemmons, Underwood, Chatelain & Van Wyk, 1983; Hossenlopp, Segovia, Lassarre *et al.*, 1990; Frost, Macaulay, Wass & Holly, 1993), which implicates a new way of regulating IGFs at the tissue and cell levels (Clemmons & Van Wyk, 1985; Van Wyk & Trippel, 1987).

Messenger RNA for IGF-I has been detected in many tissues both from the fetus (Han, D'Ercole & Lund, 1987) and postnatally (Roberts, Lasky, Lowe & LeRoith, 1987). Immunohistochemical localization of IGF-I in many tissues of the rat indicate the widespread extrahepatic synthesis of this peptide (D'Ercole, Stiles & Underwood, 1984; Hansson, Nilsson, Isgaard *et al.*, 1988) and supports its role as an autocrine/paracrine growth factor. IGF-I gene expression in rat epiphyseal growth plate chondrocytes shows that IGF-I probably has a role in stimulating the clonal growth of differentiating chondrocytes via an autocrine/paracrine mechanism (Nilsson, Carlsson, Isgaard *et al.*, 1990). Another study showed IGF-II mRNA to be localized in cartilage and mesodermal structures, whereas IGF-I mRNA was associated with regions of osteogenic regions later in development, implying differential roles for the growth factors in this tissue (Shinar, Endo, Halperin *et al.*, 1993).

It has been postulated that differential regulation of IGF-I gene expression may give rise to either endocrine or autocrine/paracrine forms of IGF-I (LeRoith & Roberts, 1991), as opposed to post-translational modification by proteolysis to produce amino-terminal truncated IGF-I (Sara & Hall, 1990). Amino-terminal truncated forms of IGF-I (of which des-IGF-I is one) have very a low affinity for IGFBPs, which exempts them from IGFBP regulation and frees them for receptor binding. They are therefore invested with a higher biological potency than the endocrine form of IGF-I (Sara & Hall, 1990). In the rat, des-IGF-I enhances cell replication in calvarial cultures, as well as protein synthesis in calvariae and parietal bone, to a greater extent than IGF-I despite both peptides exhibiting similar receptor binding characteristics (Canalis, McCarthy & Centrella, 1991).

1.3.4.4 IGFs in Bone

The IGFs demonstrate myriad functions, both growth-promoting and metabolic, which appear to be specific to the maturational stage of the target cells: for instance, if cells are in a hyperplastic phase then the IGFs will stimulate proliferation (Sara & Hall, 1990). The actions of the IGFs that have so far been described mainly fall into three categories: insulin-like metabolic effects, mitogenic effects and effects caused in the mediation of GH action (Van Wyk & Trippel, 1987). In the epiphyseal growth plate in long bones, postnatal cartilage growth is under the control of IGFs (Daughaday & Rotwein, 1989). Roles for both IGFs in cartilage tissue have been demonstrated *in vitro* by mitogenesis (Hill, 1979; Sessions, Emler & Schalch, 1987; Mathieu, Rochefort, Barenton *et al.*, 1990), protein synthesis (McCarthy, Centrella & Canalis, 1989) and synthesis of matrix components in cartilage (Salmon & Daughaday, 1957; Hill, 1979; Ohlsson, Nilsson, Isaksson & Lindahl, 1992). These roles have been well reviewed (Froesch, Schmid, Schwander & Zapf, 1985; Sara & Hall, 1990).

In bovine growth plates, proliferative and maturing chondrocytes have a high IGF-I receptor concentration (Trippel, Van Wyk, Foster & Svoboda, 1983), and similar regions of the porcine epiphyseal growth plate, the chondrocytes of the proliferative and hypertrophic zones, are immunoreactive for IGF-I (Simon & Cooke, 1988), implying that IGF-I is active in these tissues. By contrast, IGF-II may be active in the growth and differentiation of mesenchymal cells (Moses, Nissley, Short *et al.*, 1980; Nagarajan, Anderson, Nissley *et al.*, 1985). Although IGF-I and IGF-II bind preferentially to the type 1 and type 2 IGF receptors respectively, because of receptor cross-binding among the IGF and insulin receptors, the specific effects of each receptor are difficult to assess (Rechler & Nissley, 1985; Underwood & Van Wyk, 1985). Mice carrying a disrupted gene for the type 1 IGF receptor are severely retarded in growth and achieve less than half of normal size at birth, as well as exhibiting effects such as organ hypoplasia and developmental delays in ossification (Liu, Baker, Perkins *et al.*, 1993). The use of antibodies to the type 1 IGF receptor has provided evidence that this receptor mediates the

actions of IGF-I, IGF-II and insulin in mitogenesis (Van Wyk, Graves, Casella & Jacobs, 1985; Conover, Misra, Hintz & Rosenfeld, 1986). This receptor is also implicated in the mediation of acute insulin-like effects by IGF-I and IGF-II in some tissues (Yu & Czech, 1984). Effects of IGF-I on bone matrix formation (Hock, Centrella & Canalis, 1988) are also probably mediated by the type 1 IGF receptor. A recent report has intimated that there are different binding sites on the type 1 IGF receptor for IGF-I and IGF-II (Germain-Lee, Janicot, Lammers *et al.*, 1992) which could give rise to differential responses of the same receptor to different ligands. The type 2 IGF receptor is a bifunctional receptor which binds both IGF-II and M6P-containing ligands and has a role in targeting lysosomal enzymes to lysosomes (von Figura & Hasilik, 1986). The question of whether this receptor mediates any biological responses of IGF-II has been thoroughly discussed (Roth, 1988) and reviews on this subject tend to be non-committal (Roth & Kiess, 1994). The type 2 IGF receptor has been propounded to mediate IGF degradation (Schalch, Sessions, Farley *et al.*, 1986), exhibit insulin-like biological response by the stimulation of glycogen synthesis (Hari, Pierce, Morgan *et al.*, 1987), and the effect of IGF-II in inhibiting lysosomal enzyme binding to the receptor may inhibit matrix digestion by the lysosomal action of osteoclasts, thereby having an active role in bone remodelling (Nissley & Kiess, 1991). In a recent study showing the developmental regulation of the type 2 IGF receptor during endochondral bone formation, the receptor was also implicated in bone resorption (Yu, Sklar, Nissley & Reddi, 1993). Both IGF-II and the receptor are highly expressed in fetal tissues (Brown, Graham, Nissley *et al.*, 1986; Sklar, Kiess, Thomas & Nissley, 1989), implying that they may have a role in growth. Several studies have tentatively suggested that IGF-II may stimulate mitogenesis through the type 2 IGF receptor (Tally, Li & Hall, 1987; Blanchard, Barenton, Sullivan *et al.*, 1988; Kojima, Nishimoto, Iiri *et al.*, 1988; Mohan, Linkhart, Rosenfeld & Baylink, 1989).

1.3.4.5 GH and IGFs in Antlers

Although there is a dearth of information regarding the GH/IGF axis in antlers, recent studies have begun to address this and have implicated a role for IGF-I in antler growth

(Suttie, Gluckman, Butler *et al.*, 1985). During antler growth, IGF-I concentrations in plasma are high (Suttie, Gluckman, Butler *et al.*, 1985) and positive correlations have been made between plasma IGF-I, liveweight gain and antler growth rate in red deer (Suttie, Fennessy, Corson *et al.*, 1989). IGF-I has been implicated in antler growth in other studies where surgical prevention of antler growth resulted in an increase in plasma concentrations of IGF-I, leading the authors to speculate that a target organ containing a large population of receptors had been removed (Suttie, Gluckman, Butler *et al.*, 1985; Suttie, Fennessy, Gluckman & Corson, 1988). GH concentrations also correlate with liveweight gain and antler growth rate, but with a delay of one month (Suttie, Fennessy, Corson *et al.*, 1989) so that during the time of peak antler growth, GH levels are relatively low (Bubenik, 1983; Suttie, Fennessy, Corson *et al.*, 1989). Circulating levels of IGF-II do not correlate positively with rates of antler growth (Suttie, Fennessy, Corson *et al.*, 1989). As IGFs are produced in many tissues, implying an autocrine/paracrine mode of action, circulating levels may not be as accurate an indicator of the physiological significance of these peptides as, for instance, tissue level measurements would be (D'Ercole, Stiles & Underwood, 1984; Hammerman, 1989). The majority of IGFs in blood are bound to the IGFBP-3 complex, which remains in the vascular compartment (Binoux & Hossenlopp, 1988; Clemmons, 1991) and the extent of availability of this IGF reservoir to the tissues is presently unknown.

Testosterone is responsible for marked changes in the behaviour and physiology of male deer (Wislocki, Aub & Waldo, 1947; Goss, 1969). Associated with increasing circulating levels of testosterone in deer is a dramatic maturation of antler tissues (Muir, Sykes & Barrell, 1988). As the actions of GH and the IGFs on tissues in developing cartilage and bones are dependent on the maturational stage of the target cells, testosterone may have marked effects on components of the GH/IGF axis.

1.4 AIMS OF THIS THESIS

Bones provide structural support in mammals and maintain a mineral balance for essential physiological processes, and the growth of long bones is a crucial determinant of the eventual size of an individual. GH and the IGFs have been implicated in cartilage and bone growth in numerous reports, and the main objective of this thesis was to investigate the nature and distribution of receptors for GH, IGF-I and IGF-II in cartilage. Studies were formulated to examine: the *in vitro* localisation of receptors *in situ*; the characteristics of the receptors, and; the possible extent of confounding of receptor binding by IGFBPs. A study of the ontogeny of receptors, and the effects of testosterone on tissue maturation and receptor binding, was included in order to look at the spatial and temporal arrangements of receptors. Antler was selected as the cartilage model because of the rapid growth of this tissue and it is intended that assessment of the data will impart information on the roles of the GH/IGF axis in bone development.

CHAPTER 2

MATERIALS AND METHODS

This chapter outlines the most commonly used methods in the experimental procedures referred to in this thesis, and includes method developments and validations where necessary.

2.1 ANTLER COLLECTION

2.1.1 Red Deer Antlers

Antler tips in the growth phase were collected from red deer (*Cervus elaphus*) stags under conditions approved by the Invermay (AgResearch, Invermay Agricultural Centre, Dunedin, New Zealand) animal ethics committee. The animals were restrained with an hydraulic crush. Local anaesthetic (Lignocaine, Astra Pharmaceuticals, Auckland, New Zealand) was administered as a ring block around the pedicle. The antler tips were sawn off and frozen in liquid nitrogen within one minute of removal, then stored at -20°C.

2.1.2 Fallow Deer Antlers

Antler tips in the growth phase were collected from fallow deer (*Dama dama*) bucks under conditions approved by the Ruakura (AgResearch, Ruakura Agricultural Centre, Hamilton, New Zealand) animal ethics committee. Adult bucks were restrained in a drop-floor cradle (Ruakura Engineering Development Group, Hamilton, New Zealand) and administered a local anaesthetic, Xylocaine (Lignocaine hydrochloride, Astra Pharmaceuticals Pty Ltd, N

Ryde, NSW, Australia) as a ring block around the pedicle. The antlers were sawn off and frozen in liquid nitrogen within one minute of removal and stored at -20°C.

2.2 HORMONES

Ovine IGF-I and IGF-II were kindly provided by Dr L. Moore (AgResearch, Wallaceville Research Station, Upper Hutt, New Zealand). IGF-I was purified from sheep plasma; it was homogeneous on reverse phase high pressure liquid chromatography and contained <0.01% ovine IGF-II (Moore & Mylek, 1993). Ovine IGF-II was also purified from sheep plasma and contained <0.01% IGF-I (Moore & Mylek, 1993). Bovine insulin and ovine prolactin were from Sigma Chemical Company (St. Louis, Missouri, USA). Ovine growth hormone was obtained from NIDDK (NIH, Bethesda, Maryland, USA). Recombinant amino-terminal methionyl human IGF-I (N-Met IGF-I) was kindly provided by Dr B D Burleigh (IMC/Pitman-Moore, Terre Haute, Indiana, USA). Desamino-(1-3)-IGF-I (des-IGF-I) was supplied by GroPep Ltd, Adelaide, Australia. Ovine IGF-I, IGF-II, insulin, GH, N-Met IGF-I and des-IGF-I were iodinated using the Iodogen method (Salacinski, McLean, Sykes *et al.*, 1981), as previously used for ovine IGF-I⁴ (Hodgkinson, Moore, Napier *et al.*, 1989) and ovine GH (Spencer, Bass, Hodgkinson *et al.*, 1991).

2.3 SECTIONING AND INCUBATION

For each antler, 8 µm - 10 µm thick sections were cut at -18°C to -20 °C and thaw-mounted in pairs onto gelatine chrome alum subbed microscope slides. Sections were stored at -20 °C and incubated within one week of being cut.

In vitro incubations were carried out using a modification of the method of Maurer & Reubi (1986). Slides were thawed and sections pre-incubated with 0.17 M Tris-HCl buffer (pH 7.4) for 10 minutes at room temperature. The pre-incubation buffer was aspirated and

¹Specific activity was 3.6556×10^{16} dpm/mole.

replaced with 50 μ l incubation buffer which comprised: 0.17 M Tris-HCl (pH 7.4), 1% bovine serum albumin (96-99% purified BSA; Sigma Chemical Company), 5 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride, together with iodinated hormone (20,000 cpm) with or without unlabelled competing hormones. After incubation for two hours at room temperature in an humidified slide chamber, the incubation buffer was aspirated and slides washed twice for five minutes in baths of cold 0.17 M Tris-HCl (pH 7.4), with 0.25% BSA and 0.01% Triton X-100. They were then fixed in neutral buffered formalin for 10 minutes, washed in distilled water for five minutes and air-dried. Slides were also incubated without radiolabelled hormones as controls for background grain counts, chemography and latent image fading of the autoradiographs (Rogers, 1973). For the incubations, IGF-I at 1 μ g/ml, IGF-II at 1 μ g/ml, insulin at 10 ng/ml and 10 μ g/ml or 250 μ g/ml were used to compete for ^{125}I -IGF-I binding sites. IGF-I, IGF-II and insulin at 1 μ g/ml were used to compete for ^{125}I -IGF-II binding sites. Unlabelled GH at 1 μ g/ml and unlabelled prolactin at 1 μ g/ml were used to compete for ^{125}I -GH binding sites. One section of each pair was used for total binding by incubation with radiolabelled hormone alone, while the other was incubated with the labelled hormone and a competing unlabelled hormone.

2.4 AUTORADIOGRAPHY

X-Omat AR5 (XAR) film (Eastman Kodak Company, Rochester, New York, USA) was apposed against the incubated slides under safelight conditions, then exposed in the dark for several days and developed, producing macroautoradiographs. These macroautoradiographs were used for the qualitative assessment of hormone binding and to estimate the exposure period for the sections while under photosensitive emulsion. LM1 emulsion (Amersham International, Amersham, Buckinghamshire, UK) or NTB-2 emulsion (Eastman Kodak Company) was applied to the sections on the slides under safelight conditions. The emulsion was dried overnight at room temperature with silica desiccant and exposed in the dark for two to five weeks at 4 °C. At the conclusion of the

exposure period the slides were equilibrated to room temperature and developed with D-19 developer (Eastman Kodak Company) then fixed with Fixaplug X-ray fixer (May and Baker). The processing times and temperatures were in accordance with the manufacturers' recommendations. The resulting microautoradiographs were washed in running tap water for 30 minutes, rinsed for five minutes in distilled deionised water, then stained with Ehrlich's haematoxylin and alcoholic eosin, or with toluidine blue, and mounted.

2.5 TIME EQUILIBRIUM

In order to gauge the effect of time on tracer binding, 48 frozen sections from one antler were cut, and mounted onto non-gelatinised coverslips which were inverted and glued to slides with Ados F2 adhesive (Ados Chemical Company, Lower Hutt, New Zealand), so that the sections were facing outwards. They were incubated as described above with ^{125}I -IGF-I in the presence or absence of unlabelled IGF-I for varying times with four pairs of sections being incubated for each nominated length of time. After exposure to X-Omat AR5 film the coverslips, with the incubated sections, were removed and gamma counted (1470 Wizard automatic gamma counter, Wallac Oy, Turku 10, Finland). Total binding reached equilibrium and was stable from 1/2 - 2 hours of incubation, as evidenced by macroautoradiograph intensity (data not shown), and background (non-specific) binding was negligible until four hours. In light of these trials a two hour incubation was considered optimal for use in subsequent incubations.

2.6 MICROSOMAL MEMBRANE PREPARATION

Crude microsomal fractions were prepared according to a modification of the method of Barenton, Patel, Khan, *et al.* (1988). Samples (4 g) of the cartilaginous portion of thawed antler tips (n=3) were homogenized (Ultra-Turrax) at high speed for three periods of

30 seconds each in 12 ml of ice-cold 0.25 M sucrose (pH 7.4) containing 4 mM imidazole and 1 mM of each of the inhibitors N-ethylmaleimide, p-hydroxymercuribenzoate and phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 1300 x g for 10 minutes to remove the incompletely homogenized material and connective tissue, and the supernatant was decanted. The pellet was re-suspended and re-centrifuged. The resulting pellet was discarded and the pooled supernatants were centrifuged at 3200 x g for 10 minutes, this time to remove intact nuclei and organelles. The resulting pellet was again re-suspended and re-centrifuged and the pooled supernatants were centrifuged at 200,000 x g for 30 minutes. The supernatant was discarded at this point and the pellet re-suspended and re-centrifuged. The final pellet, containing spun-down plasma, nuclear and intracellular membranes, was regarded as the crude microsomal fraction. This was re-suspended in Tris buffer consisting of 25 mM Tris-HCl with 10 mM MgCl₂ (pH 7.4) and centrifuged at 200,000 x g for 30 minutes as a washing step. The supernatant was discarded and the pellet stored at -20°C until use.

2.7 COMPETITIVE BINDING ASSAYS

2.7.1 Incubations of Antler Sections for Competitive Displacement

For one antler tip, cryogenically cut sections were mounted onto non-gelatinised coverslips glued to slides (as in Section 2.3). An *in vitro* incubation using ¹²⁵I-IGF-I was carried out with IGF-I (10 ng/ml to 100 µg/ml), IGF-II (10 ng/ml to 50 µg/ml) and insulin (10 ng/ml to 500 µg/ml) as competitors for binding sites (as described in Section 2.3). Two pairs of sections were incubated for each concentration of unlabelled hormone. After macroautoradiography (Section 2.4), the sections (on their coverslips) were removed from the slides and gamma counted. This method was later modified whereby cryogenically cut sections were mounted onto gelatinised slides, as described in Section 2.3, incubated, and wiped off the slide with a cotton bud soaked in 500 µl of a solution of 1% Decon 90 (Salmond Smith Biolab Ltd, Auckland, New Zealand) and 10% glycerol in a counting

tube. The cotton buds were broken off in their respective tubes and the tubes were then gamma counted.

Validation of this method was provided by competitive binding assays carried out using crude microsomal membrane preparations.

2.7.2 Competitive Binding Assays on Crude Microsomal Membranes

Microsomal membrane receptor activities and specificities were assayed in a competitive binding receptor assay. Tubes containing 100 μ l membrane sample (2 mg preparation/ml), 100 μ l standard (0, 3, 30, 300 or 3000 ng/ml unlabelled IGF-I or IGF-II, or 0, 10, 300, 3000 or 250,000 ng/ml unlabelled insulin) were incubated with 100 μ l tracer (either 125 I-IGF-I or 125 I-IGF-II at 50,000 cpm) for 16 hours at 4°C. Following incubation, 50 μ l of 12 mg/ml bovine serum gamma globulin (IgG; Sigma Chemical Company) in 0.064 M phosphate buffer (pH 7.4) and 750 μ l of 20% (w/v) polyethylene glycol (PEG, M_r 8000; Sigma Chemical Company) in the same phosphate buffer was added to all tubes except those with 0 ng/ml standard (totals). Tubes were vortex mixed, centrifuged at 2400 x g for 30 minutes, and the supernatants containing unbound tracer aspirated to waste. All tubes were gamma counted and the data analysed. Non-specific binding was taken to be the residual binding occurring in the presence of a large excess of unlabelled homologous hormone, as is the case in tubes with the highest concentration of homologous standard.

2.8 SCATCHARD ANALYSIS

Binding data from the competitive binding assays on crude microsomal membrane preparations (from Section 2.7.2) were analysed using the LIGAND-PC program (Biosoft, Cambridge, UK, adapted to be IBM-compatible). The LIGAND program was formulated to analyse the data from ligand binding experiments (Munson & Rodbard, 1980; Munson, 1983) and was used to fit the binding data from competitive displacement assays in this thesis to a Scatchard plot (Scatchard, 1949). The Scatchard equation: $B/F = -K_a B + K_a R^o$

was used to plot the ratio of bound hormone to free hormone, B/F (y variable) versus B (x variable) for different concentrations of total hormone present, including labelled and unlabelled hormone - the B/F observed for the labelled hormone is assumed to be equal to the B/F for total hormone present. From this Scatchard plot, which automatically allows for and assesses non-specific binding, estimates were able to be obtained for binding parameters: the negative slope of the plot being equal to the binding affinity constant (K_a) and the abscissa intercept being equal to the number of binding sites, called the receptor capacity (R_0). For homogeneous populations of independent receptors, the simplest Scatchard plot of a straight line can be obtained. These are ideal conditions though, and in reality a curvilinear plot is more often obtained as hormones can cross- bind to different receptors, or negative or positive cooperativity can occur where the hormone binding to a receptor affects the affinity of adjacent receptors for that hormone (Levey & Robinson, 1982). Because of this the LIGAND program can also assess the heterogeneity of binding sites, meaning that it can show if the labelled hormone is binding to more than one binding site, within statistical certainty, and can provide parameters for these. In this thesis, Scatchard analysis was used to calculate receptor capacity, binding affinity constant, binding dissociation constant (K_d , which equals $1/K_a$) and to assess homogeneity of binding sites for each of the radiolabelled hormones IGF-I and IGF-II.

2.9 SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out according to the method of Laemmli (1970). Briefly, stock solutions of 30% acrylamide (29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide, Bio-Rad Laboratories, Richmond, California, USA), 1.5 M Tris-HCl (pH 8.8) and 10% sodium dodecyl sulphate (SDS) were used to prepare separating gels with final concentrations of 7.5 or 15% acrylamide (as required), 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. Preparation of stacking gels involved using stock solutions of 30% acrylamide, 0.5 M Tris-HCl (pH 6.8) and 10% SDS to give final concentrations of 4% acrylamide, 0.124 M

Tris-HCl (pH 6.8) and 0.1% SDS. Gels were polymerized by the addition of 0.025% ammonium persulphate and 0.1% tetramethylethylenediamine (TEMED). Thickness of gels was 1.5 mm and the length of the separating gels was 5 cm, and stacking gels 1 cm. Of the 10 wells in each stacking gel, only the eight middle ones were used due to the uneven running of the wells on the edges.

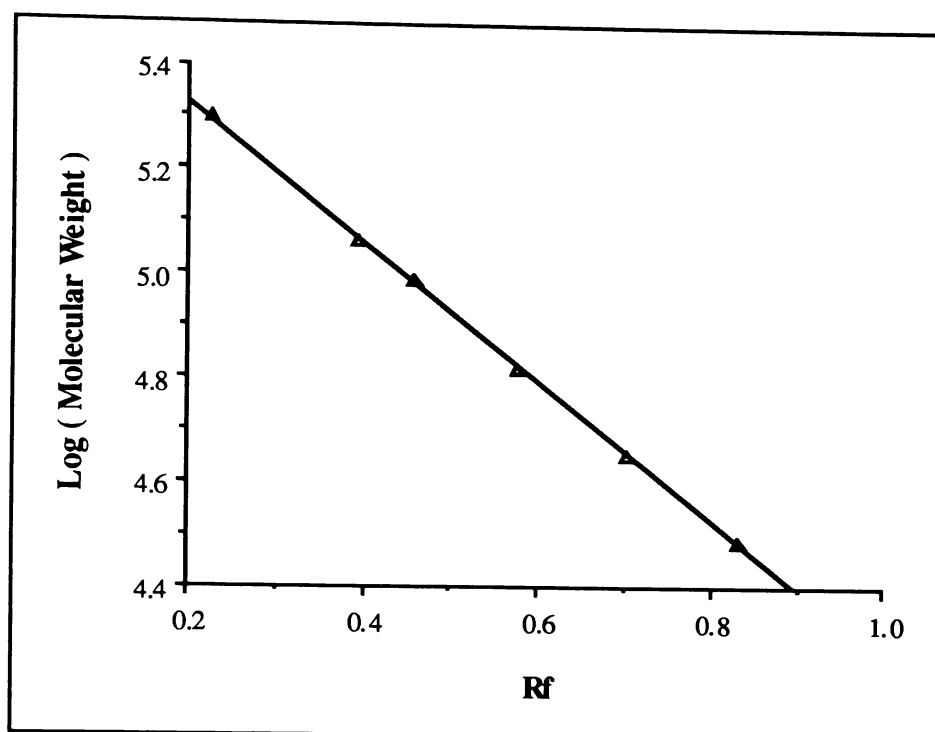
Samples were prepared as described in each section and loaded into the wells in the stacking gels using a Hamilton syringe (Hamilton Co., Reno, Nevada, USA). Not more than 50 μ l per well was loaded and the syringe was washed thoroughly between each well loading. Two gels were able to be run simultaneously on the Mini-Protean II Electrophoresis Cell system (Bio-Rad Laboratories, Hercules, California, USA) at a constant voltage of 150 V and variable current (80 mA average). Electrode buffer of 0.025 M Tris-HCl (pH 8.3), 0.192 M glycine and 0.1% SDS was used and electrophoresis was carried out at 4°C with running times ranging from 63 to 70 minutes, depending on the progress of the solvent front. For affinity cross-linking studies, a sample well containing 10 - 20 μ l of a molecular weight marker protein solution was run on each gel to enable an assessment of the molecular weights of the labelled protein bands to be made. The molecular weight marker proteins used were from a kit of high range SDS-PAGE standards (Bio-Rad Laboratories, Richmond, California, USA) and included myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (31 kDa). The distance migrated by each marker was used to calculate their relative mobility (Rf) using the equation:

$$Rf = \frac{\text{distance migrated by protein}}{\text{distance migrated by solvent front}}$$

The relative mobility of each unknown protein was compared with the relative mobility of the molecular weight standards on a standard curve (Fig. 2.1).

Marker proteins were subjected to SDS-PAGE using a 7.5% polyacrylamide gel and the curve was generated by plotting the log of the molecular weight of the broad range standards against the relative mobility (Rf). This particular plot was used for assessing molecular weights of IGF-I and IGF-II receptors in Chapters 3 and 4.

Figure 2.1 Example of a standard curve for SDS-PAGE molecular weight characterisations.



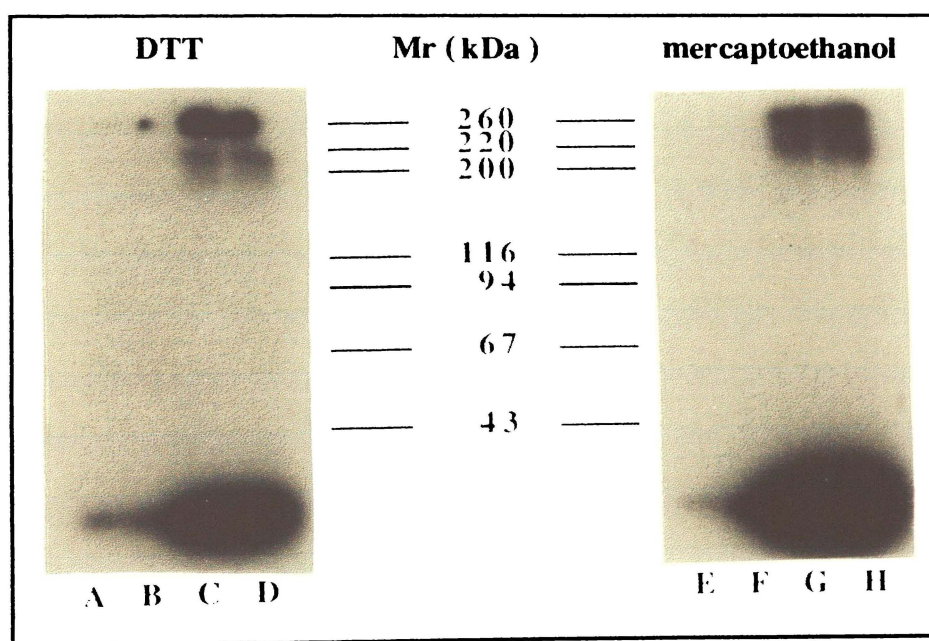
2.10 AFFINITY CROSS-LINKING STUDIES

Membrane samples (2 mg preparation/ml) were incubated overnight at 4°C with ^{125}I -IGF-I or ^{125}I -IGF-II (1×10^6 cpm) with or without excess unlabelled IGF-I or IGF-II ($1 \mu\text{g/ml}$), respectively, in a final volume of 0.5 ml Tris buffer (from Section 2.6) containing 0.1% (w/v) BSA. Samples then underwent PEG precipitation, affinity cross-linking with disuccinimidyl suberate and electrophoresis following the method of Barenton, Patel, Khan, *et al.* (1988). To elaborate, 1 ml of 0.125% rabbit IgG, then 1 ml of 25% PEG, both in Tris buffer, were added to the incubation tubes. They were vortex mixed and allowed to stand for 15 minutes at 4°C before centrifugation at $4000 \times g$ for 30 minutes. The supernatant was aspirated off and the pellets resuspended in 100 μl of 50 mM sodium phosphate buffer (pH 7.4). Disuccinimidyl suberate, freshly dissolved in dimethylsulphoxide, was added to the membrane suspension at a final concentration of

Macroautoradiographs of gels showing the molecular weights of the IGF-II hormone-receptor complex in antler cartilaginous tissues. Crude microsomal preparations of antler tip material (n=3) were incubated with ^{125}I -IGF-II in the presence (lanes A, B, E, F) and absence (lanes C, D, G, H) of excess unlabelled IGF-II, then affinity cross-linked and electrophoresed under reducing conditions with either DTT or mercaptoethanol as the reducing agent. Partial reduction of the receptor from 220 kDa to 260 kDa occurred in the presence of either agent, with DTT being more effective. In order to achieve a more complete reduction, the reducing agents were used together in subsequent experiments. The positions of molecular weight markers, which were run in adjacent lanes, are indicated.

0.35 mM, whereupon the tubes were vortex mixed and incubated on ice for 15 minutes. The reaction was quenched with the addition of 60 μ l of 1 M Tris buffer with 0.2 M EDTA (pH 6.8) to each tube and the tubes centrifuged as before. The supernatant was aspirated off and the pellet resuspended in 50 μ l of 10 mM sodium phosphate buffer (pH 7.0), to which were added 50 μ l of a solution containing 4% SDS, 20% glycerol, 0.001% bromophenol blue with or without the reducing agents 1 M mercaptoethanol and 0.2 M dithiothreitol (DTT) (Fig. 2.2).

Figure 2.2 Demonstration of the efficacy of the reducing agents.



A sample containing 50 μ l molecular weight marker proteins with 50 μ l of solution without reducing agents was also prepared. All of the samples were boiled for three minutes before application to 7.5% polyacrylamide slab gels and subjected to SDS-PAGE (Section 2.9). After electrophoresis the gels were fixed in solution containing 11.4% (w/v) trichloroacetic acid, 3.4% (w/v) sulphosalicylic acid and 30% methanol for 30 minutes, soaked in de-staining solution of 45% methanol and 10% acetic acid for 10 minutes, then stained with

Coomassie stain solution of 0.25% Coomassie brilliant blue R and 10% acetic acid for 30 minutes. The gels were de-stained overnight, dried for one hour in a solution of 45% methanol, 10% acetic acid and 5% glycerol, mounted on acetate and wrapped in cellophane. Gels were then exposed to X-Omat AR5 film with intensifying screens for three to seven days at -70°C, producing gel macroautoradiographs.

2.11 QUANTITATIVE SYSTEMS FOR EVALUATING RADIOGRAPHIC COUNTS

2.11.1 Manual Grain Counting

For each microautoradiograph (antler section coated with photosensitive emulsion and developed, as in Section 2.4), counts of radiographic grains were taken from four histological regions. Twelve sites were counted for each of the cartilaginous zone, reserve mesenchyme/perichondrium and dermis, and six sites for the epidermis. Fewer sites were taken from the epidermis to avoid site duplication as it was a much smaller component of the section. Hair follicles and sebaceous glands in the dermis (Plate 2.1) and blood vessels in the perichondrium were excluded from grain counting because of their heterogeneity in tissue structure and composition of receptors. Randomised co-ordinates of the microscope stage were used to select sites for counting. Grain counts were taken from areas corresponding to 20 x 20 μm^2 of tissue (Plate 2.2). Data were statistically analysed (Section 2.12) and comparisons were made between mean values for: total and non-specific binding to sections; specific binding for each animal; different tissue types within sections, and; the extent of binding with competing unlabelled hormones. The binding which remained after radiolabelled hormones were incubated with an excess of the unlabelled homologous hormone was called non-specific binding, and that remaining following incubation with heterologous hormones was termed residual binding.

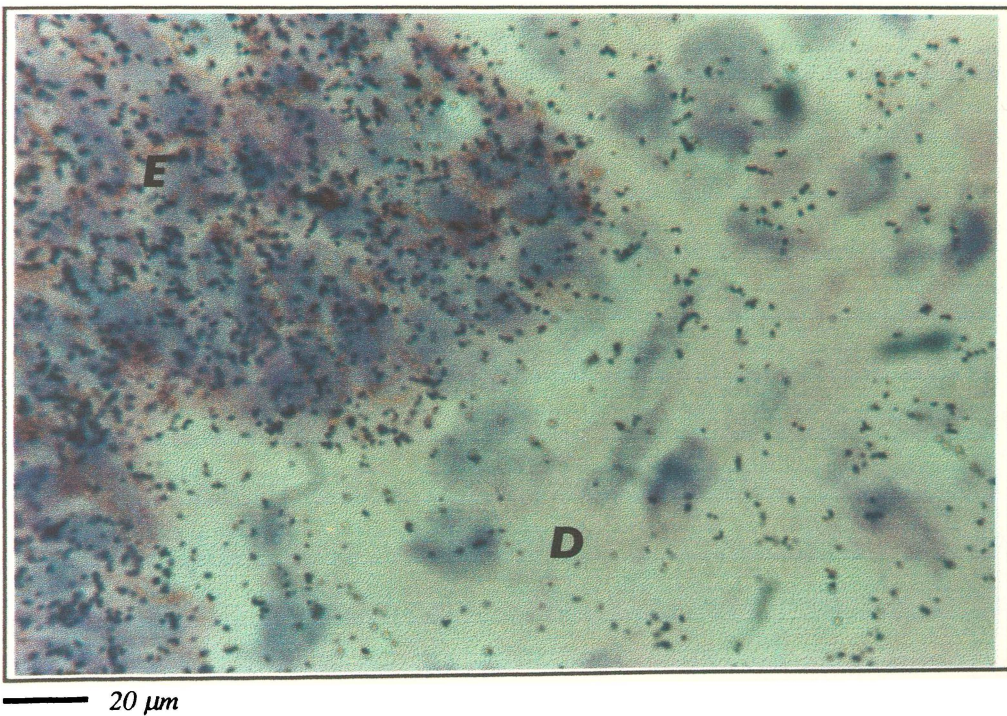
Photomicrograph of the velvet in a red deer antler section stained with haematoxylin and eosin. Salient cyto-architectonic structures are epidermis (E), dermis (D), hair follicles (F) and sebaceous glands (S).

Photomicrograph of a red deer antler section after incubation with ^{125}I -IGF-I, emulsion autoradiography and staining with haematoxylin and eosin. Counts of radiographic grains in each tissue - the epidermis (E) and dermis (D) are shown - were subsequently made in areas of $20 \times 20 \mu\text{m}^2$.

Plate 2.1 Histology of antler velvet.



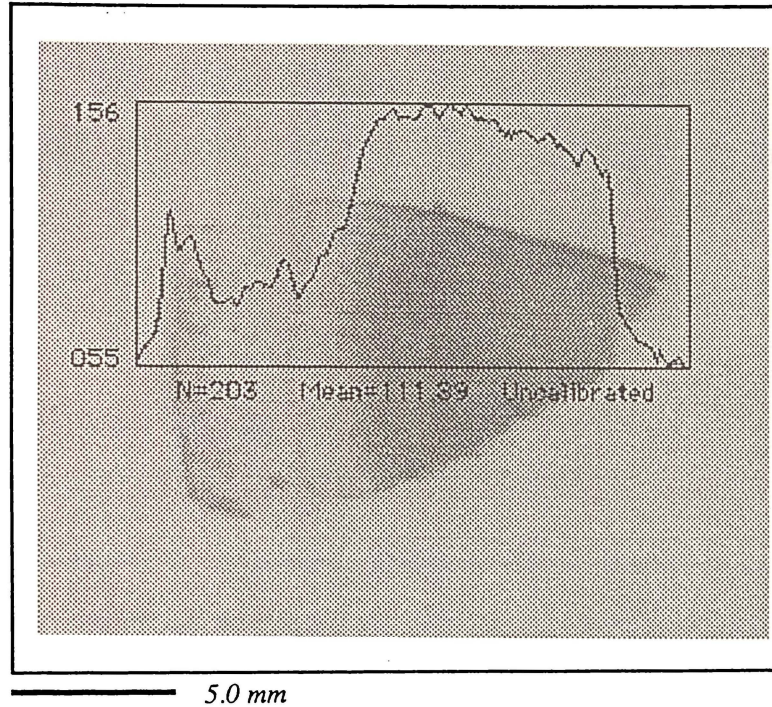
Plate 2.2 Total binding of ^{125}I -IGF-I showing radiographic grains.



Macroautoradiographical image of a red deer antler section after incubation with ^{125}I -IGF-I, then XAR autoradiography. The density of a line drawn across the image was analysed using IMAGE program and is depicted by the plot, showing that darker areas represent higher binding intensity.

Using the mouse a line was drawn across the image, transecting all histological zones. One line was drawn per section as initial investigation showed that lines drawn repeatedly in different places gave the same results. Mean plot values were calculated for each histological zone, which were determined from the appearance of the image.

Figure 2.3 Line plot of the density of binding on an antler macroautoradiograph.



and background density and was expressed in arbitrary greyscale units. The densities of each of the bands for each tissue were summed together and statistically analysed for differences among tissues (Section 2.12).

2.12 STATISTICAL ANALYSIS

Replicate data values were meaned and statistically analysed using analysis of variance (ANOVA, Genstat 5, Rothamsted Experimental Station, Harpenden, Hertfordshire, UK) enabling comparison among and between experimental parameters.

CHAPTER 3

GROWTH HORMONE AND IGF-I RECEPTORS IN ANTLE TISSUES

3.1 INTRODUCTION

Longitudinal bone growth in mammals involves the proliferation and maturation of chondrocytes in the epiphyseal growth plate. GH and IGF-I both stimulate DNA synthesis and matrix production in epiphyseal chondrocytes in culture (Ohlsson, Nilsson, Isaksson & Lindahl, 1992), although GH is believed to exert many of its stimulatory effects in cartilage via the production of IGF-I, rather than directly (Schlechter, Russell, Spencer & Nicoll, 1986). Synthesis of IGF-I is regulated by GH, but several other factors can regulate IGF-I secretion and some are involved in bone remodelling (Williams & Frolik, 1991), such as parathyroid hormone (Linkhart & Mohan, 1989) and 1,25-dihydroxyvitamin D₃ (Scharla, Strong, Mohan *et al.*, 1991). As well as having effects on bone formation, IGF-I is also implicated in bone resorption by facilitating osteoclast differentiation in the presence of 1,25-dihydroxyvitamin D₃ (Mochizuki, Hakeda, Wakatsuki *et al.*, 1992).

Receptors for GH are found on cartilage progenitor cells and early differentiating chondrocytes (Maor, Hochberg, von der Mark *et al.*, 1989) in the rabbit tibial growth plate (Barnard, Haynes, Werther & Waters, 1988; Ohlsson, Isgaard, Törnell *et al.*, 1993), and occur on hypertrophic chondrocytes, also, in the human growth plate (Werther, Haynes, Barnard & Waters, 1990). Proliferative and maturing chondrocytes have a high IGF-I receptor concentration in bovine growth plates (Trippel, Van Wyk, Foster & Svoboda,

1983), and the chondrocytes of the proliferative and hypertrophic regions in porcine epiphyseal growth plates are immunoreactive for IGF-I (Simon & Cooke, 1988). IGFBPs have been implicated in potentiation of the cellular response to IGF-I, particularly in cell replication (Elgin, Busby & Clemmons, 1987). This may occur by enhancing IGF-I binding to the type 1 IGF receptor (Clemmons, Elgin, Han *et al.*, 1986), an effect for which IGFBP-3 has been noted (Conover, 1992).

During the antler growth phase, circulating levels of IGF-I are high, and levels of GH decline (Suttie, Gluckman, Butler *et al.*, 1985; Suttie, Fennessy, Corson *et al.*, 1989), implying that IGF-I may have a role in antler growth. Immediately preceding the antler growth phase, IGF-I levels rise, and peak with the onset of growth.

GH and IGF-I are active promoters of somatic cartilage and bone growth, and IGF-I is implicated in the stimulation of antler growth. The objective of the study in this chapter was to examine and characterise the nature of GH and IGF-I binding to tissues in the growing tip of the antler in order to determine which tissues are responsive to these hormones, and to appraise their roles in cartilage growth.

3.2 MATERIALS AND METHODS

3.2.1 Binding Studies

3.2.1.1 Tissue Preparation

Antler tips in the growth phase were collected from seven adult red deer stags as described in Section 2.1.1. Collection occurred 60 days after casting of the previous hard antler. For each antler, 36 cryosections were cut and mounted in pairs onto 18 microscope slides. As a control for GH binding and hormone specificity, pieces of deer liver were obtained during a fallow deer slaughter at the Ruakura Agricultural Centre Abattoir. The tissues were

snap-frozen in liquid nitrogen within two minutes of removal and stored at -20°C until used. For the liver, 10 cryosections were cut and mounted onto five microscope slides.

3.2.1.2 *Incubations with ^{125}I -GH and ^{125}I -IGF-I*

IGF-I and GH were prepared and iodinated, as in Section 2.2, and the tissue sections underwent incubation using the method described in Section 2.3. For the incubations, IGF-I at 1 µg/ml, IGF-II at 1 µg/ml, insulin at 10 ng/ml and 10 µg/ml were used to compete for ^{125}I -IGF-I binding sites in antler tissues. Unlabelled GH at 1 µg/ml and prolactin at 1 µg/ml were used to compete for ^{125}I -GH binding sites in antler and liver tissues. Three pairs of antler sections from each animal and two pairs of liver sections were incubated for each combination of labelled hormone and unlabelled hormone.

3.2.1.3 *Autoradiography*

X-Omat AR5 film was exposed against the incubated slides for three days (Section 2.4), producing macroautoradiographs, after which LM1 emulsion was applied to the slides and exposed for 14 days, producing microautoradiographs, which were then stained with Ehrlich's haematoxylin and alcoholic eosin. In order to distinguish between the binding of IGF-I to prechondroblasts and chondroblasts, five more slides were prepared as described above and incubated with ^{125}I -IGF-I in the absence or presence of unlabelled IGF-I. After macroautoradiography, then microautoradiography using NTB-2 emulsion, the sections were stained with toluidine blue.

3.2.1.4 *Grain Counting and Statistical Analysis*

Microautoradiographs from two representative antlers were used for the counting of radiographic grains, which was carried out manually (Section 2.11.1). Data for histological zones for each competing hormone in each animal were pooled and the mean values analysed for difference using ANOVA (Section 2.12). Comparisons were made between total and non-specific binding to sections, between specific binding for each animal, among

different tissue types within sections and among binding with competing unlabelled hormones.

3.2.1.5 Incubations for Competitive Displacement of Tracer Binding

For one antler tip, 68 sections were cut and mounted onto non-gelatinised coverslips which were glued to slides. An incubation using ^{125}I -IGF-I was carried out as described in Section 2.7.1. The incubation was repeated twice more with two more animals ($n=3$), then all of the slides underwent macroautoradiography (Section 2.4), after which the slides were removed and gamma counted. Binding data were analysed for competitive displacement.

3.2.2 Receptor Analysis

3.2.2.1 Competitive Binding Assays

Antler tissues from three animals were used in the preparation of a microsomal membrane preparation as outlined in Section 2.6. This preparation was stored at -20°C until radioreceptor assays were performed.

Competitive binding assays were performed for ^{125}I -IGF-I receptor binding. The receptor preparation was incubated with ^{125}I -IGF-I and various concentrations of unlabelled IGF-I, IGF-II and insulin (Section 2.7.2), after which the incubation tubes were gamma counted.

3.2.2.2 Scatchard Analysis

The binding data were analysed for competitive binding and a Scatchard analysis was carried out (as described in Section 2.8), where estimates were obtained for affinity constants and receptor capacity.

3.2.2.3 Affinity Cross-Linking Studies

Membrane samples were incubated, affinity cross-linked and subjected to SDS-PAGE, using ^{125}I -IGF-I as the tracer, with or without excess unlabelled IGF-I (Section 2.10).

Sections of antler and liver tissue were incubated with ^{125}I -GH, in the presence or absence excess unlabelled GH. After incubation, the sections were fixed, subjected to macroautoradiography for seven days, then developed. A, total binding of GH; B, non-specific binding.

X-Omat AR5 film was exposed against the gels for seven days, producing macroautoradiographs.

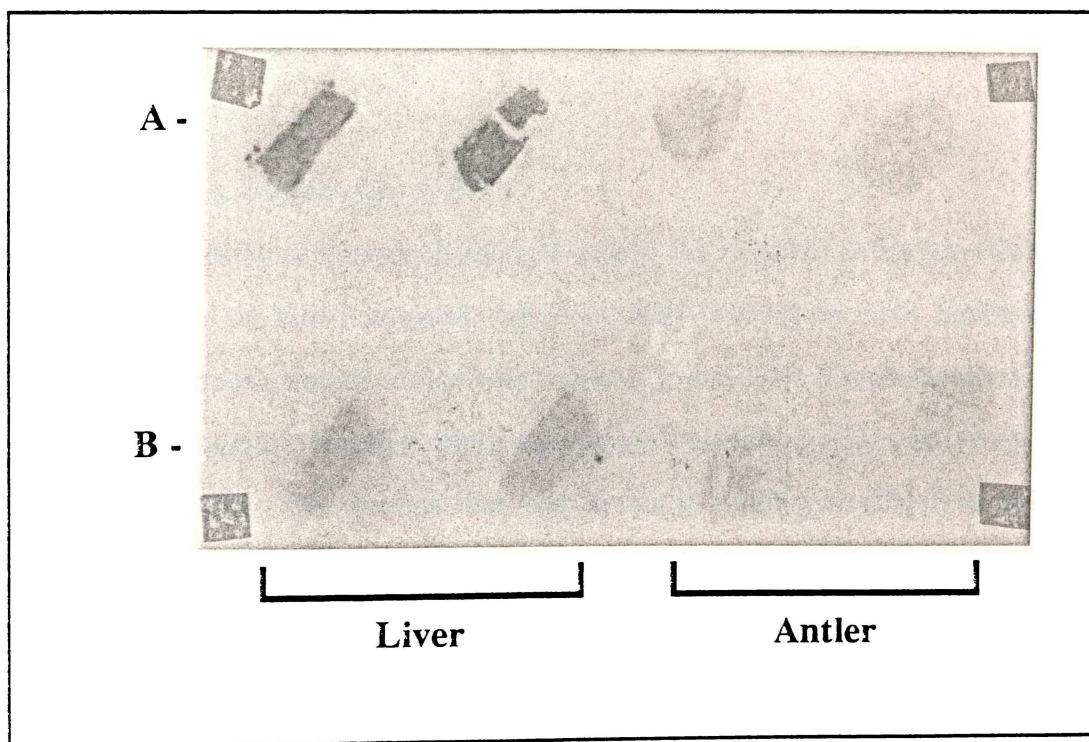
3.3 RESULTS

3.3.1 Growth Hormone

3.3.1.1 GH Binding

Qualitative assessment of the macroautoradiographs from all seven antler tips that underwent incubation with ^{125}I -GH showed negligible binding of GH to antler tissues. In contrast, macroautoradiographs of liver sections, which provided a negative control for the specificity of the hormone, showed that there was GH binding in this tissue¹ (Fig. 3.1).

Figure 3.1 Macroautoradiograph of GH binding to deer liver and antler tissues.



¹The control used to demonstrate the technique of GH binding was liver from *Dama dama* whilst antler tissue was from *Cervus elaphus*. It is possible that there may be a difference between species in binding ovine GH but identical results were achieved when the experiments were repeated using antler material from *Dama dama*.

*Radiographic grain counts of ^{125}I -GH binding to receptors in different tissues of the deer antler tip (n=7) and in the deer liver (n=2). Counts were made from microautoradiographs of tissue sections, in areas of $20 \times 20 \mu\text{m}^2$, under a light microscope. Specific binding was calculated as total binding minus non-specific binding. *, $p < 0.05$; **, $p < 0.01$; NS, no significant binding. SEM, standard error of the mean.*

3.3.1.2 Receptors for GH

Following microautoradiography, radiographic grain counts of the antler sections confirmed the lack of specific binding of GH to any of the tissues of the antler, in contrast to the liver to which specific binding was observed (Table 3.1).

Table 3.1 Binding of GH to tissues of the deer antler and liver.

HISTOLOGICAL ZONE	TOTAL BINDING	NON-SPECIFIC BINDING	SPECIFIC BINDING TO PROLACTIN RECEPTORS	SPECIFIC BINDING TO GH RECEPTORS	SEM
Epidermis	16	18	-	-2 NS	4.9
Dermis	7	9	-	-2 NS	1.8
Reserve Mesenchyme and Perichondrium	7	7	-	0 NS	1.6
Cartilaginous Zone	8	8	-	0 NS	2.0
Liver	45	11	22*	12**	5.2

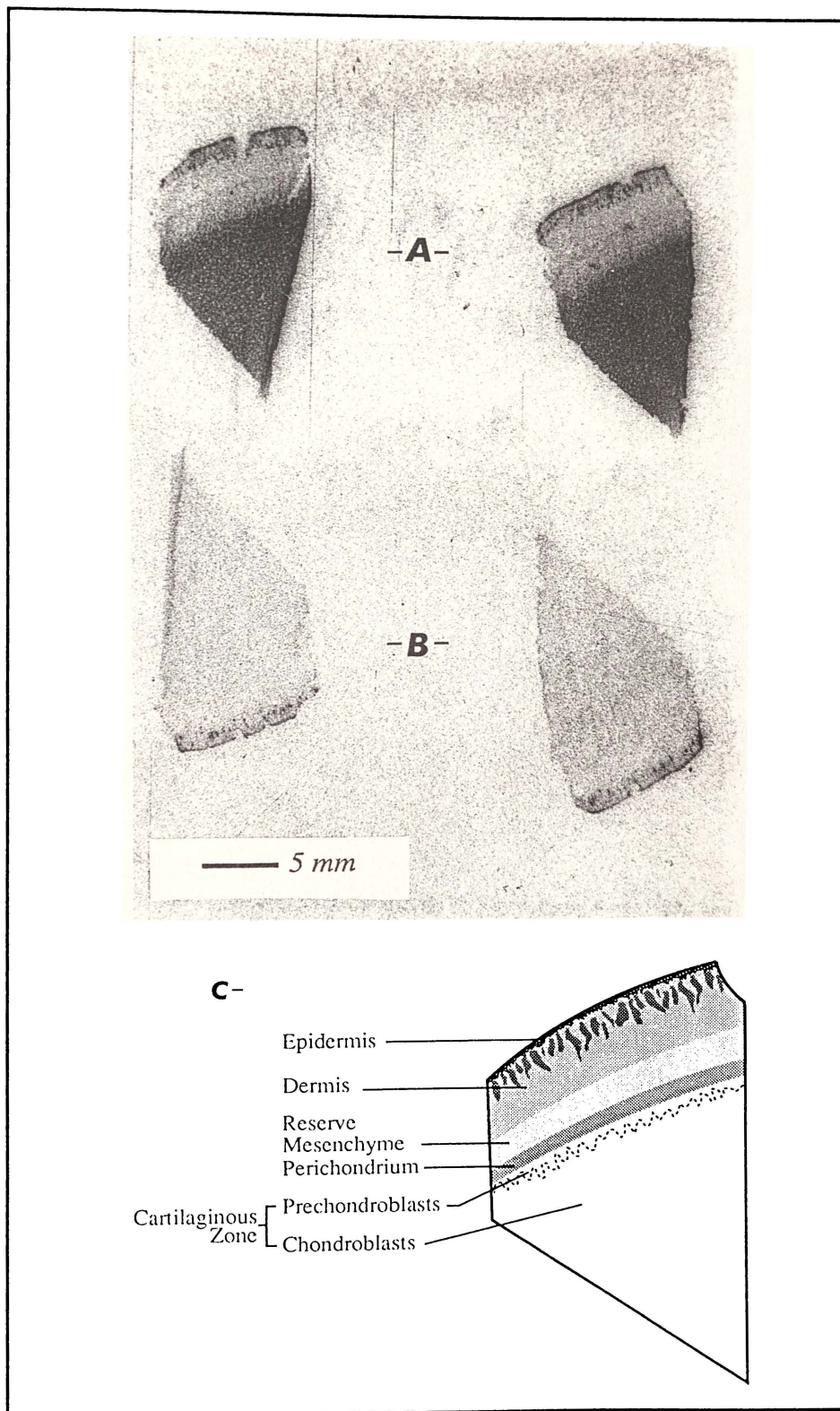
3.3.2 IGF-I

3.3.2.1 Binding Distribution of IGF-I

Qualitative assessment of the macroautoradiographs for all seven antler tips that underwent incubation with ^{125}I -IGF-I showed that there was binding in each histological zone (Fig. 3.2). On sections treated to show total binding, the epidermis, cartilaginous zone, and dermis were all heavily labelled while the perichondrium and the reserve mesenchyme showed faint signals. The dermis had high levels of binding localised to hair follicles (Plate 3.1), and the perichondrium and reserve mesenchyme had high levels of binding localised to blood vessel walls (Plate 3.2). These heterogeneous structures were not included in radiographic grain counting. Non-specific binding was greatest in the epidermis than in the other regions.

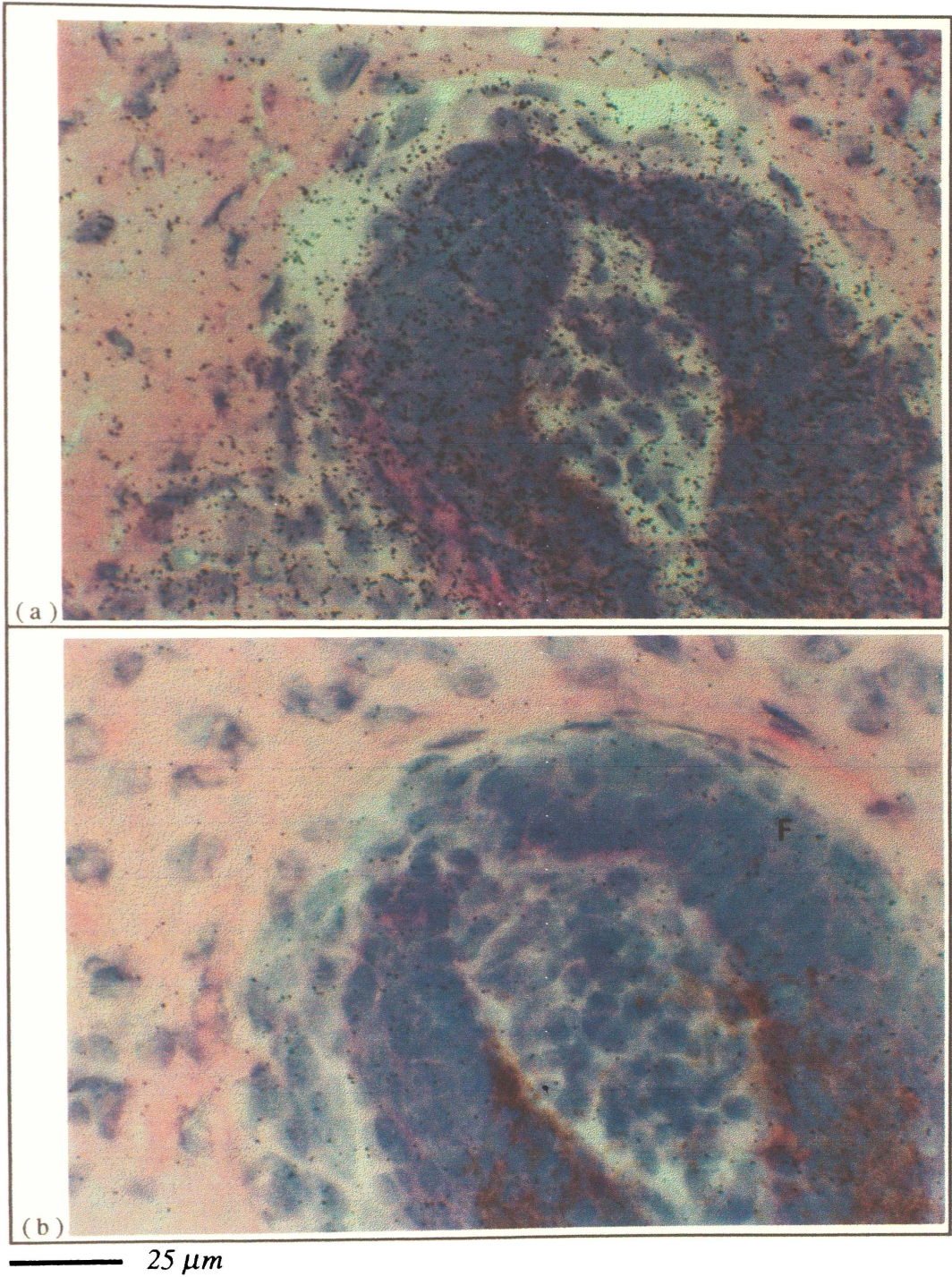
Macroautoradiographs from one representative antler tip (n=7), showing qualitatively the extent of binding of IGF-I to each histological region. After incubation with ^{125}I -IGF-I with or without excess unlabelled IGF-I, the sections were fixed and exposed against XAR film for four days, then developed. A, total binding of IGF-I; B, non-specific binding; C, diagram of an antler tip section showing the positions of the histological regions.

Figure 3.2 Binding of IGF-I to antler tissues.



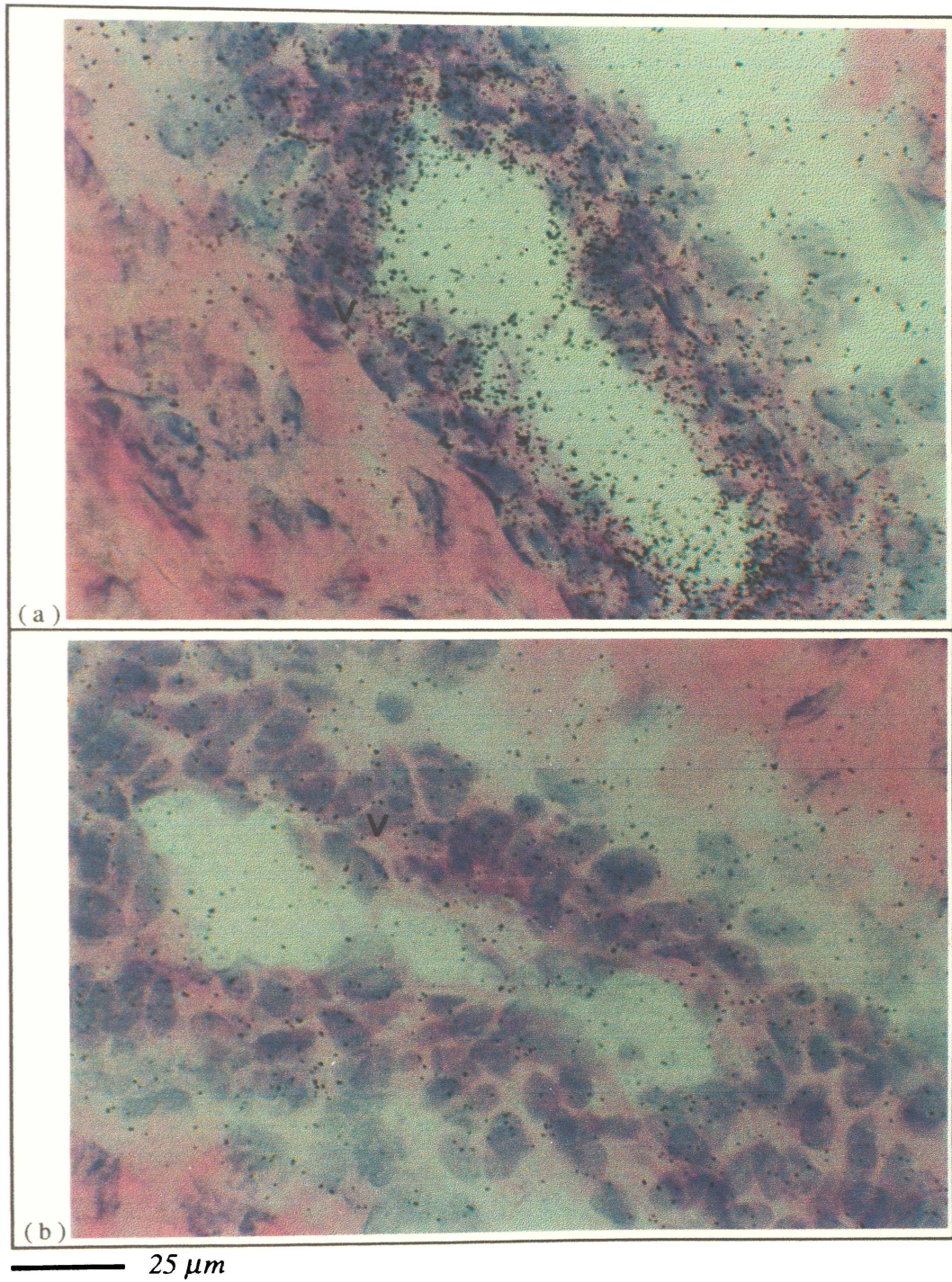
Microautoradiographs showing radiographic grains representing (a) total binding of ^{125}I -IGF-I, and (b) non-specific binding to hair follicles (F) in the dermis of an antler. Following autoradiography, sections were stained with haematoxylin and eosin.

Plate 3.1 Binding of IGF-I to hair follicles.



Microautoradiographs showing radiographic grains representing (a) total binding of ^{125}I -IGF-I, and (b) non-specific binding to blood vessel walls (V) in the reserve mesenchyme and perichondrium zone of an antler. Following autoradiography, sections were stained with haematoxylin and eosin.

Plate 3.2 Binding of IGF-I to blood vessel walls



*Counts of radiographic grains were made from microautoradiographs of antler tip sections (n=2). Specific binding was calculated as differences between total binding and non-specific or residual binding after incubation in the presence of various unlabelled hormones in excess; SEM, standard error of the mean. Values shown are means; values in parentheses are percentages denoting sensitivity of ^{125}I -IGF-I to competitive displacement by heterologous hormones compared with specific binding (100%). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NS, no significant difference.*

Data from each histological zone, comprising counts from 12 counting sites per section for each zone except the epidermis (six), for three sections per animal, were pooled for each treatment. Initial analysis showed no significant difference between the two animals so the data from the two animals were pooled. Standard errors were calculated on the pooled means.

3.3.2.2 Specific Binding

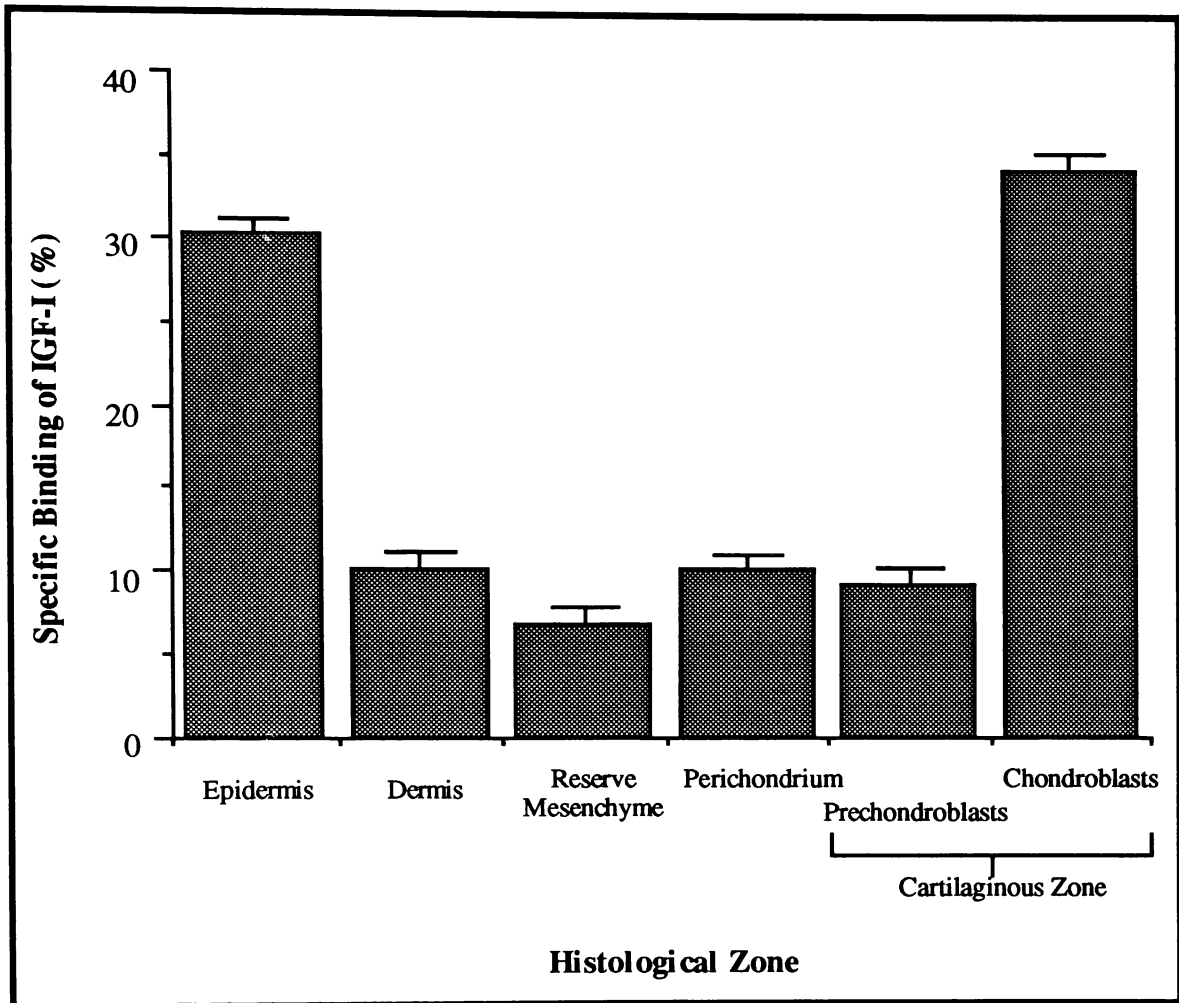
Radiographic grain counts of ^{125}I -IGF-I binding in each histological zone were not significantly different between animals ($p>0.05$). Differences in binding were significant between corresponding zones in the total binding and non-specific binding sections ($p<0.001$), among the different treatments with unlabelled hormones ($p<0.001$), and among the histological zones within a section ($p<0.05$) (Table 3.2).

Table 3.2 Specific binding of ^{125}I -IGF-I in different tissues of the antler.

HISTOLOGICAL ZONE	SPECIFIC BINDING	Binding displaceable by treatment with:			SEM
		1 $\mu\text{g/ml}$ IGF-II	10 $\mu\text{g/ml}$ insulin	10 ng/ml insulin	
Epidermis	58 ***	30 ** (52)	3 (NS)	12 (NS)	9.2
Dermis	20 ***	17 *** (85)	9 * (45)	2 (NS)	3.5
Reserve Mesenchyme	14 ***	15 *** (100)	4 * (29)	4 (NS)	2.1
Perichondrium	20 ***	17 *** (85)	6 (NS)	0 (NS)	3.5
Cartilaginous Zone	32 ***	26 *** (81)	15 ** (47)	-5 (NS)	4.2

Most of the specific binding of IGF-I tracer occurred in the epidermis and cartilaginous zone. Differential binding of ^{125}I -IGF-I to bands within the cartilaginous zone, which were morphologically indistinguishable in sections stained with haematoxylin and eosin, was resolved by staining with toluidine blue. Binding in the cartilaginous zone was then re-analysed and the greatest level of binding was found to be to chondroblasts (Fig. 3.3).

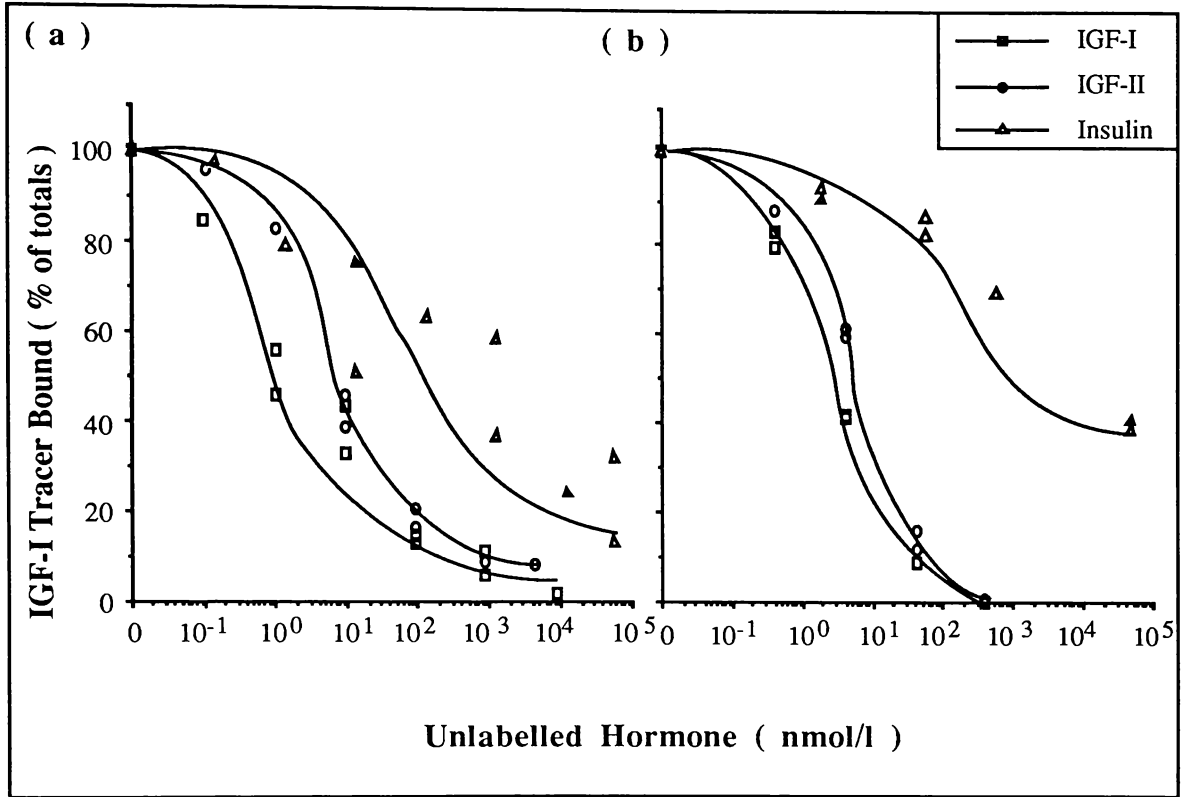
Antler sections were subjected to an in vitro incubation with ^{125}I -IGF-I, then macroautoradiography, microautoradiography, and staining with toluidine blue. After grain counting, specific binding was calculated as the difference between mean total and mean non-specific radiographic grain counts, for each histological zone. Values are expressed as percentages of the total specific binding from all zones; errors shown are standard error of the mean.

Figure 3.3 Specific binding of IGF-I to each histological zone of the antler.

3.3.2.3 Receptor Characterization

The cross-binding of hormones to receptors has been used to identify type 1 and type 2 receptors for IGFs based on their relative affinities for IGF-I, IGF-II and insulin (Underwood & Van Wyk, 1985). Receptors for IGF-I were characterized from displacement curves of IGF-I binding to antler tissue. Results from the competitive displacement of labelled hormone using whole antler sections showed that IGF-I bound to sites with properties typical of type 1 IGF receptors, with a high affinity for IGF-I, less affinity for IGF-II and less again for insulin (Fig. 3.4a). These results agreed with the displacement curve obtained by using microsomal membrane preparations (Fig. 3.4b), which is more commonly used in competitive binding assays.

Competitive displacement of IGF-I binding to (a) antler sections (n=3), and (b) microsomal membrane preparations of antler tissue (n=3), after incubation with ^{125}I -IGF-I and various concentrations of unlabelled IGF-I, IGF-II and insulin. Values are expressed as percentages of total binding, which are the counts obtained after incubation with tracer alone.

Figure 3.4 Competitive displacement curves for IGF-I.

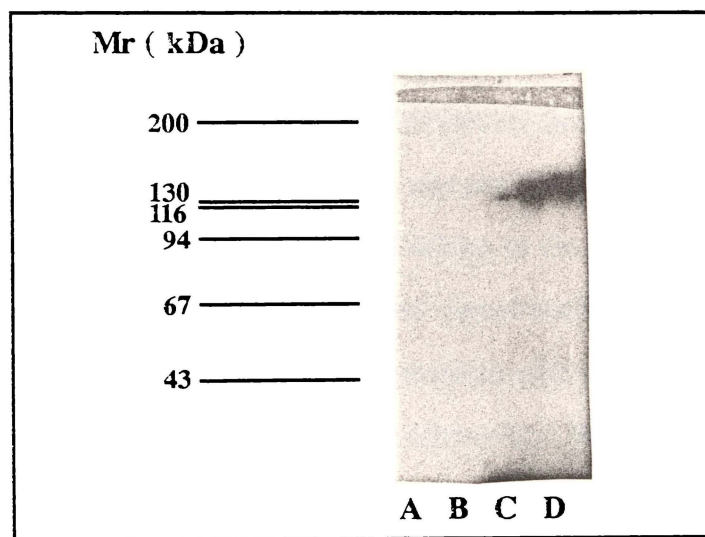
Scatchard analysis of the counts obtained from the competitive binding assay revealed an affinity constant (K_a) of $4.48 \times 10^8 \pm 1.52 \times 10^8$ l/mol (which is equivalent to a dissociation constant, K_d , of 2.23 ± 0.76 nmol/l) for the interaction of IGF-I with the IGF-I receptor and a receptor capacity of 1.31 ± 0.17 pmol/mg of initial tissue weight. These values are similar to those obtained for the K_d for ^{125}I -IGF-I binding to receptors in bovine adrenals (Penhoat, Chatelain, Jaillard & Saez, 1988) and the ovine ovary (Monget, Monniaux & Durand, 1989), and for the K_d and receptor capacity obtained for ^{125}I -IGF-I binding to receptors in the rat uterus (Yallampalli, Osuamkpe & Nagamani, 1992). A tendency to curvilinearity was observed in the Scatchard plot, but LIGAND-PC analysis showed no improvement in using a two-site rather than a one-site model, indicating that the tracer was binding to a single class of receptor.

Macroautoradiograph of a gel showing the molecular weight of the IGF-I tracer binding constituent in membranes of antler cartilaginous tissue. Membrane preparations of antler tip material (n=3) were incubated with ^{125}I -IGF-I in the presence (lanes A, B) and absence (lanes C, D) of excess unlabelled IGF-I, then affinity cross-linked and subjected to SDS-PAGE. The positions of molecular weight markers, which were run in an adjacent lane, are indicated.

3.3.2.4 Molecular Weight Characterization

The molecular weight of the receptor for IGF-I was assessed by affinity cross-linking studies carried out on crude antler membranes. Following affinity labelling with ^{125}I -IGF-I, SDS-PAGE and autoradiography, a single band of radioactivity was detected at 130 kDa which was absent in the presence of excess unlabelled IGF-I (Fig. 3.5). These findings are indicative of IGF-I binding to the α -subunit of the type 1 IGF receptor (Massagué & Czech, 1982; Rechler & Nissley, 1985; Underwood & Van Wyk, 1985; Jansen, van Buul-Offers, Hoogerbrugge *et al.*, 1989).

Figure 3.5 IGF-I affinity cross-linked to binding sites in antler tissue.



3.4 DISCUSSION

This study has demonstrated the presence of receptors for IGF-I and the absence of receptors for GH in antler tip tissues. From competitive displacement studies, Scatchard analysis and affinity cross-linking studies it was apparent that the IGF-I tracer bound to a single class of binding site with the properties of a type 1 receptor in antler tissues. As IGF-I binding to IGFBPs cannot be displaced by high concentrations of insulin (Monget,

Monniaux & Durand, 1989) the ability of insulin to completely inhibit IGF-I tracer binding at high concentrations in these zones suggests that the binding sites are actual receptors with minimal participation of IGFBPs. The IGF-I binding sites in the epidermis showed less sensitivity to IGF-II than any of the other zones and no sensitivity to insulin. Reasons for this could include the tracer binding to a receptor with different affinity, or a heterogeneous receptor population, or to IGFBPs. These would not have been detected in the competitive displacement analysis using autoradiography of entire antler sections as the epidermis comprised only a minute fraction of the whole.

Autoradiography showed the number of available receptors for IGF-I in each tissue type of the antler to be different, with large numbers in the epidermis and cartilaginous zone. In contrast, the lowest levels of specific binding of ^{125}I -IGF-I were measured in the dermis, reserve mesenchyme and perichondrium. However, because of their heterogeneous composition, the dermis, reserve mesenchyme and perichondrium contained isolated areas of high binding. Sebaceous glands and hair follicles in the dermis showed high total and non-specific IGF-I tracer binding. The blood vessel walls in the perichondrium were densely labelled with ^{125}I -IGF-I, which is in agreement with the specific binding of IGF-I to blood vessel endothelial cells demonstrated by others (Bar, Boes & Yorek, 1986; Bornfeldt *et al.*, 1988; Oldham *et al.*, 1989).

The reserve mesenchyme is characterised by high mitotic activity (Banks & Newbrey, 1983a) and both the reserve mesenchyme and the perichondrium contain areas of hyperplasia (Banks & Newbrey, 1983a; Banks & Newbrey, 1983b). Although these zones are the sites of the most vigorous proliferation in the antler, the current results show that the reserve mesenchyme and perichondrium (the most undifferentiated tissue and the most rapidly differentiating tissue, respectively) have the lowest specific binding of ^{125}I -IGF-I.

The highest binding of IGF-I to receptors was to the chondroblasts of the cartilaginous zone. This binding correlates directly with the presence of a highly concentrated extracellular

substance of the nature of a sulphated glycosaminoglycan, probably chondroitin sulphate (B. J. Young, *personal communication*), and increases abruptly, concomitantly with the maturation of chondroblasts. Chondroblast maturation and increased matrix production through proteoglycan formation involves the sulphation of glycosaminoglycans and accumulation of collagen fibrils. The stimulatory effect of IGF-I on sulphation is well known and forms the historical basis for IGF research on sulphate uptake by chondroblasts (Salmon & Daughaday, 1957). In ruminants, IGF-I has been shown to increase proteoglycan synthesis in articular cartilage (McQuillan, Handley, Campbell *et al.*, 1986). This evidence suggests that IGF-I is probably instrumental in the development of cartilaginous matrix in the antler, exhibiting its sulphation factor activity, rather than having a role in mitosis. Thus the stimulating effects of IGF-I on antler growth, suggested from correlations between antler growth rate and plasma levels of IGF-I (Suttie, Fennessy, Corson *et al.*, 1989), may have a major role in extracellular matrix production rather than stimulating cell division.

From the results of an experiment in which red deer stags were surgically prevented from growing antlers, it was observed that peripheral plasma IGF-I concentrations during velvet antler growth were elevated by up to 1.5 times those of normal antlered stags (Suttie, Fennessy, Gluckman & Corson, 1988). It was suggested that IGF-I was not locally produced in the antler, but was under endocrine control, which is supported by the lack of GH receptors found in this study. Furthermore, *in situ* hybridization studies have so far failed to detect significant levels of mRNA for IGF-I in the antler (A. K. Hodges, *unpublished observation*).

These results permit the interesting speculation that the type 1 receptor may have a function in mediating the effects of IGF-I in matrixogenesis in the cartilage of the antler. However, the role of the IGF-I binding site in the essentially non-cartilaginous tissue of the antler (epidermis) is not clear. IGF-I binding in this tissue displayed a different competitive

displacement characteristics, compared with cartilaginous tissues, which presumably reflects the different function of the epidermis in antler growth.

This study has demonstrated the binding pattern of IGF-I in different histological zones of the antler growing tip, and defined the nature of the binding site. The specific binding pattern of IGF-I, which showed the majority of the tracer binding to chondroblasts, concomitant with maturation of the extracellular matrix, suggests that IGF-I has a more important role in chondroblast matrixogenesis than in hyperplasia. The binding seemed to be to a receptor demonstrating type 1 IGF receptor characteristics rather than to IGF binding proteins and this will be studied and discussed in a more definitive way later in this thesis. The absence of GH receptors in the antler tip suggests that IGF-I may have an endocrine role in antler growth.

CHAPTER 4

IGF-II RECEPTORS IN ANTLER TISSUES

4.1 INTRODUCTION

There are indications from a number of studies that IGF-II is involved in bone growth. It is stored in the bone matrix of several species (Frolik, Ellis & Williams, 1988; Mohan, Jennings, Linkhart & Baylink, 1988; Yu, Sklar, Nissley & Reddi, 1993) and is proposed, upon release, to have a paracrine role in stimulating bone formation at its storage site. This has the effect of counteracting bone resorption and maintaining calcium levels in the bone mineral reservoir (Mohan & Baylink, 1991). IGF-II mRNA is highly expressed in the germinal layer of the long bones of early postnatal rats, implicating a role for IGF-II in early bone development and the longitudinal growth of the epiphyseal growth plate (Shinar, Endo, Halperin *et al.*, 1993). Mitogenic and developmental effects of IGF-II have been reported in rat chondrocytes (Sessions, Emler & Schalch, 1987), mouse osteoblasts (Slootweg, Hoogerbrugge, de Poorter *et al.*, 1990) and fetal rat bone calvariae (Centrella, McCarthy & Canalis, 1990). Bone cells have also been shown to produce IGFBPs (Mohan, Linkhart, Rosenfeld & Baylink, 1989; Schmid, Zapf, Meier *et al.*, 1989), which may play a role in modulating or regulating the action of IGF-II in this tissue (Baxter & Martin, 1989; Sara & Hall, 1990; Bautista, Baylink & Mohan, 1991).

Receptors for IGF-II have been characterized in skeletal tissues in a variety of species, such as growth plate chondrocytes in cattle (Trippel, Chernausek, Van Wyk *et al.*, 1988), rabbit chondrocytes (Jansen, van Buul-Offers, Hoogerbrugge *et al.*, 1989), mouse osteoblasts

(Slootweg, Hoogerbrugge, de Poorter *et al.*, 1990), chick and mouse bone cells (Mohan, Linkhart, Rosenfeld & Baylink, 1989), and fetal rat osteoblasts (Centrella, McCarthy & Canalis, 1990). IGF-II binds preferentially to the type 2 IGF receptor, but because of receptor cross-binding it is possible that interaction of IGF-II with the type 1 or insulin receptors may account for many of the actions of IGF-II (Rechler & Nissley, 1985; Underwood & Van Wyk, 1985; Trippel, Chernauek, Van Wyk *et al.*, 1988; Van Wyk, Casella, Hynes & Lund, 1988). Some researchers postulate that there is a common IGF receptor, a type 3 IGF receptor, that is also sensitive to insulin (Hintz, Thorsson, Enberg & Hall, 1984). Recently, IGF-II has been shown to recognise another type of receptor (XR) with which it interacts to serve placental growth in mice (Baker, Liu, Robertson & Efstratiadis, 1993).

IGF-II appears to have direct growth-promoting effects on cartilage and bone cells. The aim of this chapter was to examine and characterise IGF-II binding in antler tip tissues in order to assess whether the antler is responsive to IGF-II, and which could implicate a role for IGF-II in rapid tissue growth.

4.2 MATERIALS AND METHODS

4.2.1 Binding Studies

4.2.1.1 Tissue Preparation

Antler tips (the distal 2.5 cm where rapid growth was occurring) were collected from nine adult red deer stags as described in Section 2.1.1. Collection occurred 60 days after casting of the previous hard antler. For each antler, 18 cryosections were cut and mounted in pairs onto nine microscope slides.

4.2.1.2 Incubations with ^{125}I -IGF-II

IGF-II was iodinated (Section 2.2) and antler sections were subjected to an incubation using

^{125}I -IGF-II as the tracer (Section 2.3). For the incubations, IGF-II at 1 $\mu\text{g/ml}$, IGF-I at 1 $\mu\text{g/ml}$, and insulin at 10 $\mu\text{g/ml}$ were used for competitive inhibition of tracer binding. Three pairs of sections from each animal were incubated for each combination of labelled hormone and unlabelled hormone.

4.2.1.3 *Authoradiography*

After incubation, XAR film was exposed against the slides for two days, then developed (Section 2.4), producing macroautoradiographs. NTB-2 emulsion was applied to the sections on the slides for three animals. These were dried overnight at room temperature with silica desiccant and exposed for five weeks at 4 °C. The microautoradiographs were then developed, fixed and stained with Ehrlich's haematoxylin and alcoholic eosin (Section 2.4).

4.2.1.4 *Grain Counting and Statistical Analysis*

Microautoradiographs from the three antlers underwent manual radiographic grain counting (Section 2.11.1). Data were pooled as in Section 3.2.1.4 and means were statistically analysed using ANOVA (Section 2.12). Comparisons were made between total and non-specific binding to sections, between total and residual binding to sections, between specific binding for each animal, and among different tissue types within sections.

4.2.1.5 *Incubations for Competitive Displacement of Tracer Binding*

For one antler tip, 68 sections were cut and mounted onto non-gelatinised coverslips which were glued to slides. An *in vitro* incubation using ^{125}I -IGF-II as the tracer, using a series of unlabelled hormones to compete for tracer binding to sections, was carried out as described in Section 2.7.1. The experiment was repeated with an antler tip from another deer, but with the sections mounted directly onto gelatinised slides. All slides underwent macroautoradiography (Section 2.4), then the sections were removed and gamma counted.

In order to nullify the involvement of a putative insulin-sensitive IGF-II receptor (Hintz, Thorsson, Enberg & Hall, 1984), antler sections were incubated with unlabelled insulin before the addition of tracer IGF-II. A further 14 sections from each animal were cut and mounted onto coverslips, which were mounted two per slide. These sections were subjected to an incubation protocol where they were pre-incubated as described in Section 2.3, then incubated for two hours in 50 μ l of incubation buffer with unlabelled insulin (10 μ g/ml) in place of the tracer. Following this unlabelled incubation, the sections were included with the first series of competitive displacement incubations, described above, and incubated with IGF-II tracer in the presence of a series of concentrations of insulin (10 ng/ml to 500 μ g/ml). After macroautoradiography, the sections were removed and gamma counted.

Counts obtained from sections that underwent identical treatment were meaned and analysed for competitive displacement. Mean total binding was assigned as 100% and other binding data were calculated relative to it.

4.2.2 Receptor Analysis

4.2.2.1 Microsomal Membrane Preparation

Microsomal membrane preparations of antler tissue from three animals were prepared as described in Section 2.6. The preparations were stored at -20 °C until radioreceptor assays were performed.

4.2.2.2 Competitive Binding Assays

Radioreceptor assays were performed for the type 2 IGF receptor in antler. A series of competitive binding assays were performed for binding of 125 I-IGF-II. The membrane preparation was incubated with 125 I-IGF-II and various concentrations of unlabelled IGF-II, IGF-I and insulin. Assay conditions were as described in Section 2.7.2.

4.2.2.3 *Scatchard Analysis*

The binding data were analysed as described in Section 2.8. From Scatchard analysis estimates were obtained for receptor capacity and dissociation constants.

4.2.2.4 *Affinity Cross-Linking Studies*

Membrane samples were incubated, affinity cross-linked and run on SDS-PAGE, as described in Section 2.10, using ^{125}I -IGF-II (1×10^6 cpm) as the tracer, with or without excess unlabelled IGF-II. XAR film was exposed against the electrophoresis gels for three days and developed, producing macroautoradiographs.

4.3 RESULTS

4.3.1 IGF-II Binding

4.3.1.1 *Binding Distribution*

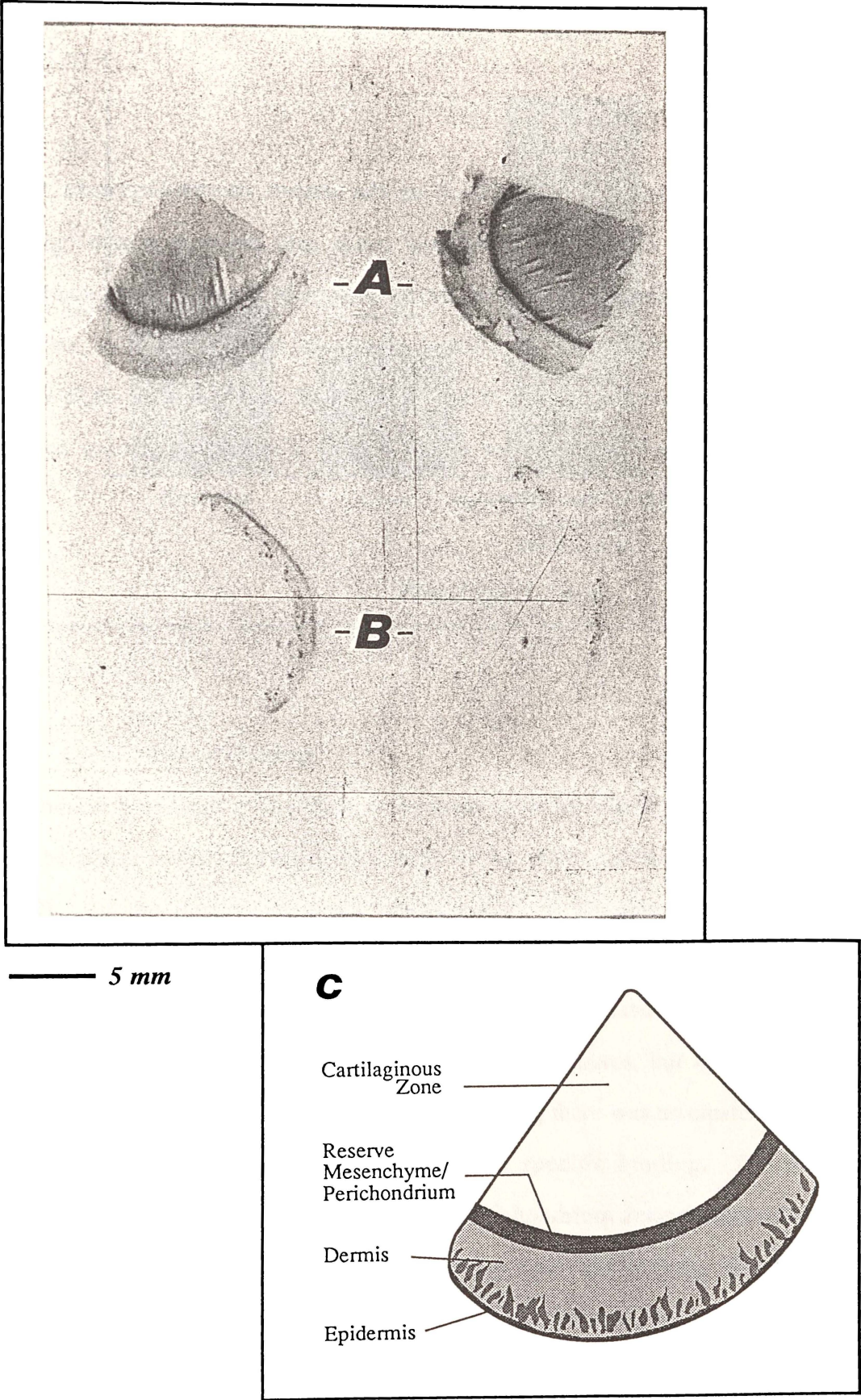
The macroautoradiographs of the incubated sections for all nine antler tips showed IGF-II binding in each histological zone (Plate 4.1). On sections incubated with tracer alone, the perichondrium and reserve mesenchyme zone was heavily labelled whilst the cartilaginous zone, epidermis and dermis were moderately labelled. The very faint signal from the sections incubated to show non-specific binding indicated that virtually all of the total binding was specific binding.

4.3.1.2 *Specific Binding*

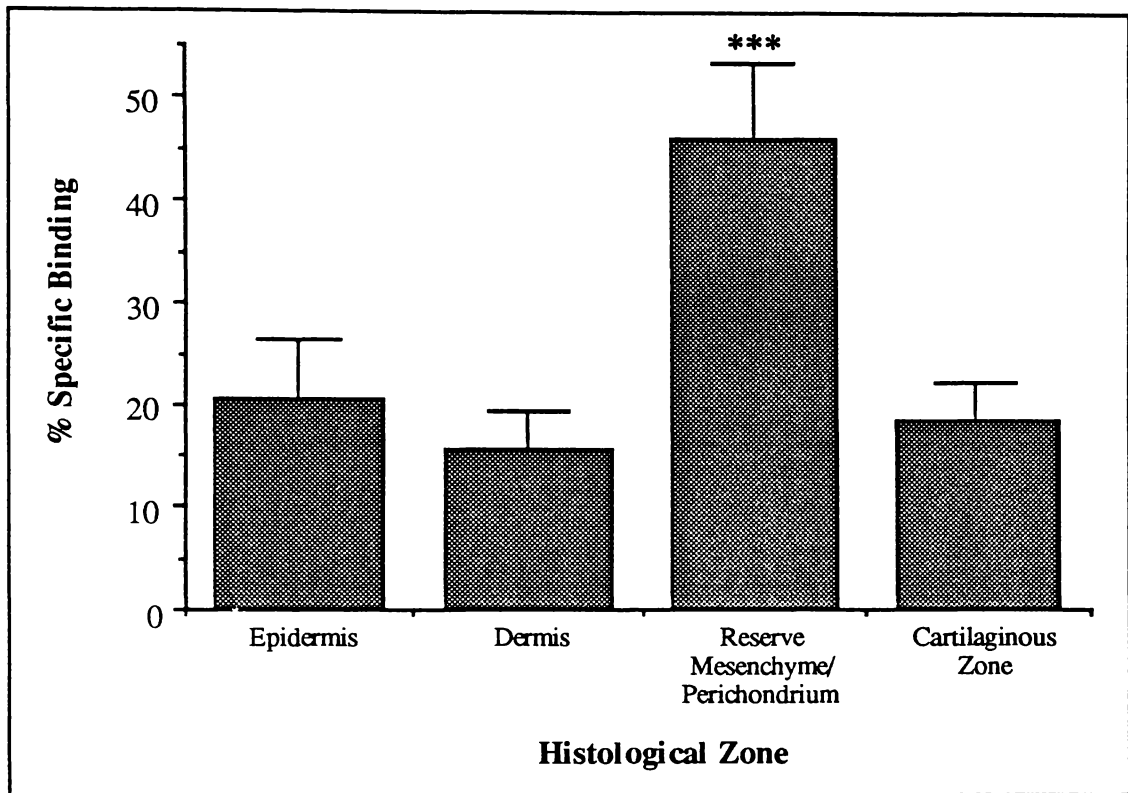
Grain counts of ^{125}I -IGF-II binding per unit area of tissue on the microautoradiographs were not significantly different among animals. There was significant specific binding ($p < 0.001$) in each histological zone, with significantly higher binding ($p < 0.001$) in the reserve mesenchyme and perichondrium zone (Fig. 4.1). IGF-I and insulin did not displace binding of the IGF-II tracer in any of the zones.

Macroautoradiographs from one representative antler tip (n=9), showing qualitatively the extent of binding of ^{125}I -IGF-II to each histological region. After incubation with tracer, with or without excess unlabelled IGF-II, the sections were fixed and XAR film was exposed against them for four days, then developed. (A) Total binding of IGF-II; (B) non-specific binding; (C) diagram of an antler tip section showing the histological regions.

Plate 4.1 Binding of IGF-II to antler tip sections.



*After in vitro incubation with ^{125}I -IGF-II as the tracer, autoradiographs from three animals were stained with haematoxylin and eosin, and the radiographic grains were counted. Specific binding was calculated as differences in means of grain counts between total and non-specific binding for each histological zone, and expressed as percentages of total specific binding over all zones. There was significant binding in each histological zone; error bars show standard error of the mean. Comparison among zones: ***, $p < 0.001$, otherwise no significant difference.*

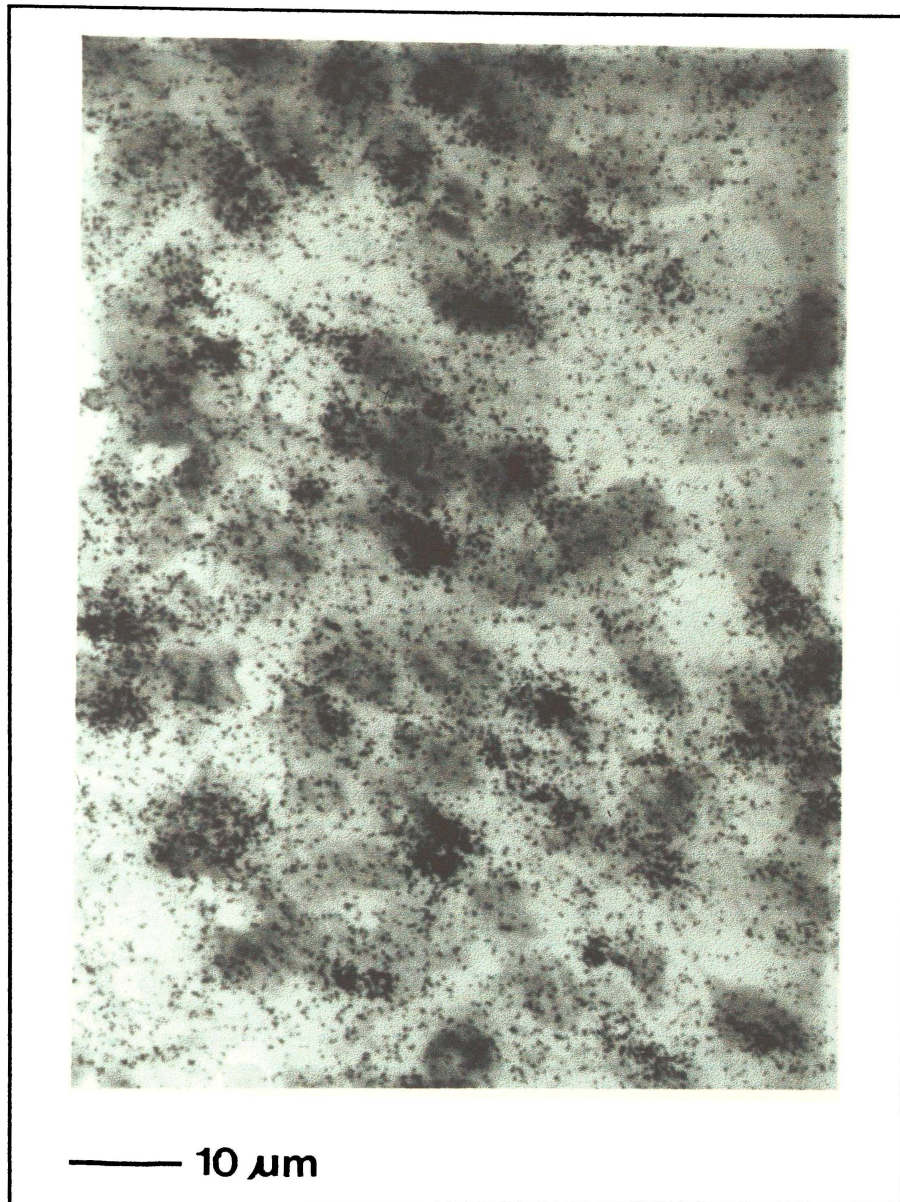
Figure 4.1 Specific binding of IGF-II to histological zones of the antler.

4.3.1.3 Localization of Binding

The distribution of radiographic grains on sections showing total binding was not uniform. In all of the zones except the epidermis most of the grains occurred clustered in groups (Plate 4.2). In the dermis, clusters were sparse; in the reserve mesenchyme and perichondrium zone, clusters were dense, and in the cartilaginous zone, clusters occurred in spaces in the extracellular matrix. The clusters were not associated with structures such as vascular endothelial tissue, hair follicles and sebaceous glands, but was associated with various nuclei. In sections showing non-specific binding there was no clustering, indicating that the clusters of ^{125}I -IGF-II grains represented specific binding. The increase in clustering seen in the reserve mesenchyme and perichondrium zone corresponded with an increase in cell number and nuclear mass. Grains were more evenly distributed in the epidermis and did not show the localized clusters of binding seen in the other tissues of the antler tip.

Microautoradiograph showing total binding of ^{125}I -IGF-II in the reserve mesenchyme and perichondrium zone. After incubation with tracer the sections were fixed, subjected to macroautoradiography, then exposed to photosensitive emulsion for five weeks and developed. Radiographic grains are observed to be clustered to some but not all nuclei.

Plate 4.2 Clustering of the radiographic grains of IGF-II tracer.



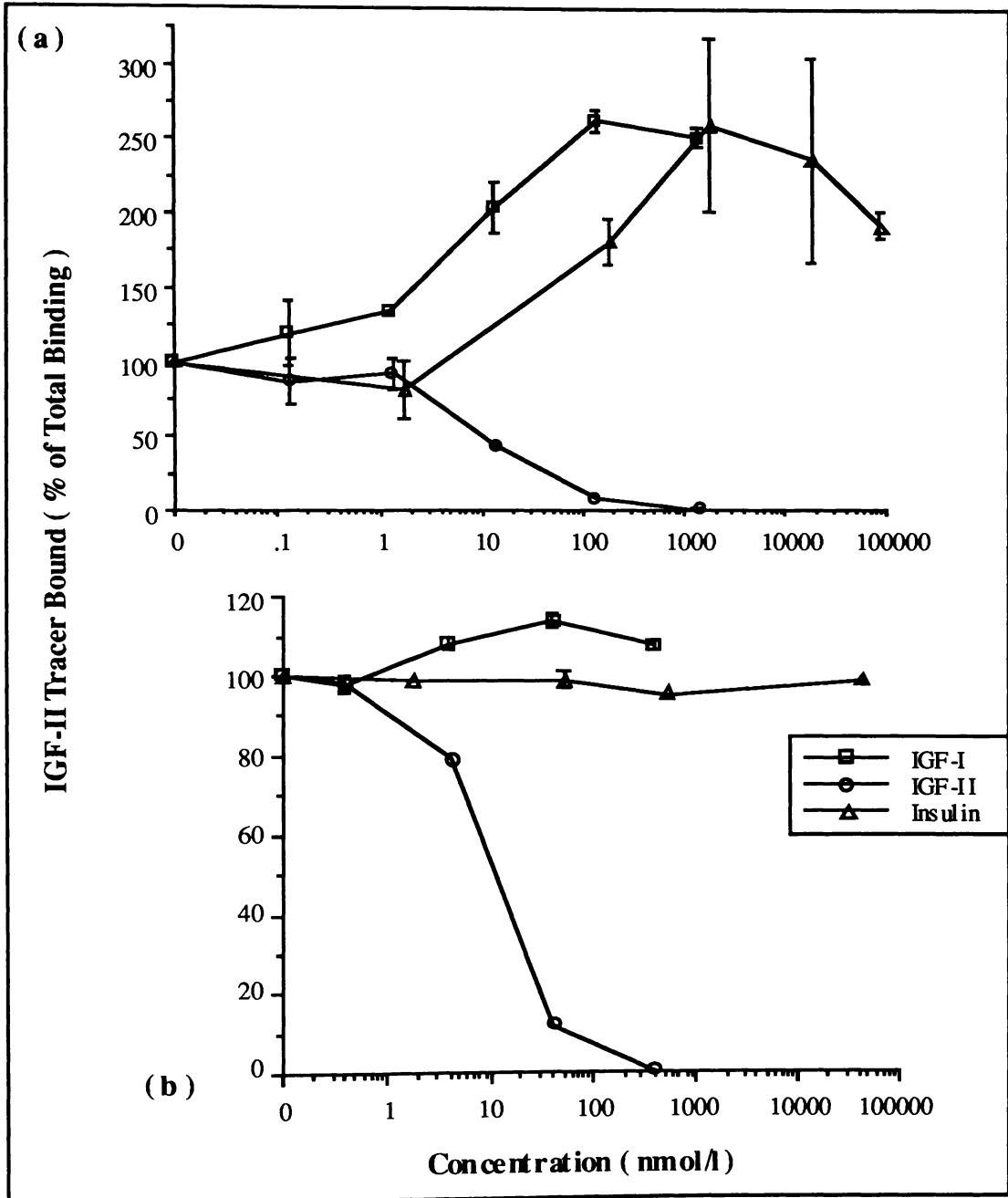
4.3.2 Receptors for IGF-II

4.3.2.1 Characterization by Competitive Binding

Displacement curves for IGF-II were obtained from the competitive displacement of ^{125}I -IGF-II by unlabelled hormones in whole antler sections (Fig. 4.2a) and microsomal membrane preparations of antler tissue (Fig. 4.2b). Binding of tracer to binding sites was readily displaceable by unlabelled IGF-II, but IGF-I and insulin both enhanced tracer binding to antler sections in a concentration-dependent manner. The sections which were initially incubated with 10 $\mu\text{g/ml}$ insulin showed a dramatic and unexpected increase in tracer binding compared with sections which had not undergone the prior incubation with insulin (Fig. 4.3). The enhancement of IGF-II tracer binding to antler sections by IGF-I and insulin, which was shown to be specific binding, was prominent in the macroautoradiographs (Fig. 4.4). In the microsomal membrane preparations, a slight enhancement of tracer binding with increasing concentrations of IGF-I, but not insulin, was observed. These binding characteristics for IGF-II are not generally considered typical for a type 2 IGF receptor, as determined by competitive binding methods. However, Scatchard analysis revealed a receptor capacity of 79.89 nmol/mg of membrane preparation and a dissociation constant (K_d) of 2.33 nmol/l for the interaction of IGF-II with its receptor. This value is in agreement with previously reported K_d values for low-affinity type 2 IGF receptors in crude microsomal membranes of human erythroleukemia cells (Blanchard, Barenton, Sullivan *et al.*, 1988), osteoblast-enriched cultures from fetal rat bone (Centrella, McCarthy & Canalis, 1990) and affinity-purified rat placental receptors (MacDonald, Pfeffer, Coussens *et al.*, 1988). Despite the presence of other receptors in the membrane preparations, eg. type 1 IGF receptors (Chapter 3), the LIGAND-PC programme (Section 2.8) fitted the competitive binding data to a one-site rather than a two-site model, implying that the tracer was binding to a relatively homogeneous receptor population.

Competitive displacement curves for IGF-II binding to (a) antler sections (n=2), and (b) microsomal membrane preparations of antler tissue (n=3), after incubation with ^{125}I -IGF-II and various concentrations of unlabelled IGF-I, IGF-II and insulin. Data are expressed as percentages of total binding, which are the counts obtained after incubation with tracer alone. Each data point is the average of two replicate samples from each animal, ie. the mean of four values for antler sections and the mean of six values for microsomal membrane preparations. Error bars are standard error of the mean and are shown when they are larger than the plot symbol.

Figure 4.2 Hormonal specificity of receptors for IGF-II in antler tissues.



Competitive displacement graph of IGF-II binding, as depicted in Figure 4.2a, but including data from an extra series of insulin concentrations from antler sections that had been previously incubated with 10 µg/ml of unlabelled insulin (Insulin2). All sections underwent an incubation with ^{125}I -IGF-II, with or without unlabelled IGF-I, IGF-II or insulin in various concentrations. After macroautoradiography, sections were removed from slides and gamma counted. Data are expressed as percentages of total binding, which is the mean of counts obtained after incubation with tracer alone. Each data point is the average of two replicate samples from both animals, ie. the mean of four values. Error bars are standard error of the mean and are shown when they are larger than the plot symbol.

Sections from one representative antler (n=9), which were incubated with ^{125}I -IGF-II tracer in the absence (Total Binding) or presence of excess unlabelled competing hormones IGF-II, IGF-I at 1 µg/ml, or insulin at 10 µg/ml. Further sections from two of the animals were incubated with tracer and 10 µg/ml insulin after a previous incubation with 10 µg/ml of unlabelled insulin only (insulin2). The sections were fixed and autoradiographed with XAR film for three days, producing macroautoradiographs.

Figure 4.3 Enhancement effect of insulin on IGF-II binding to antler sections.

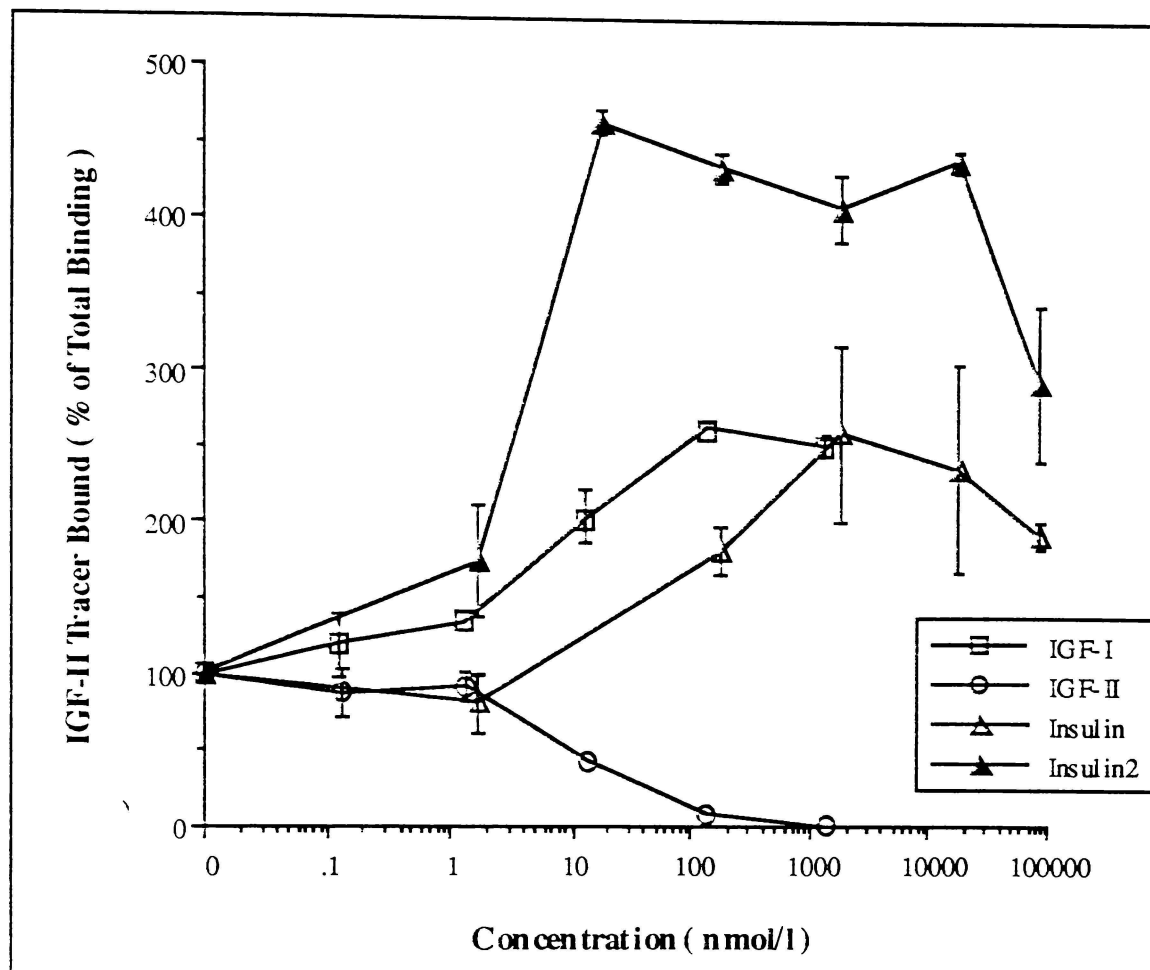
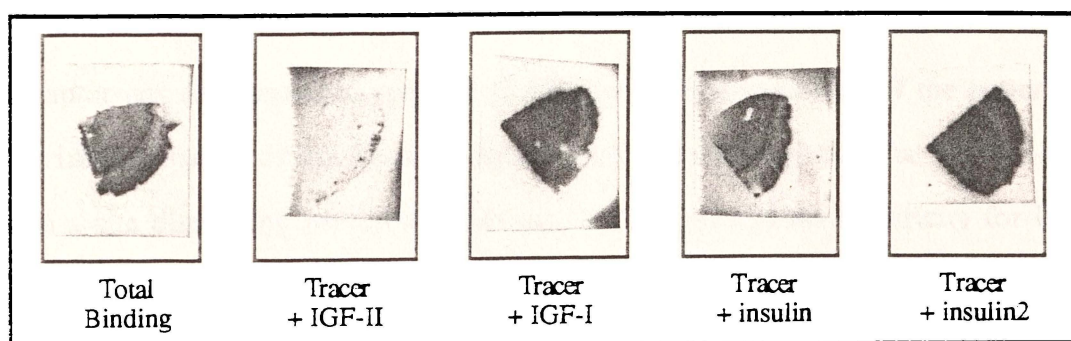


Figure 4.4 Binding of IGF-II to antler sections.

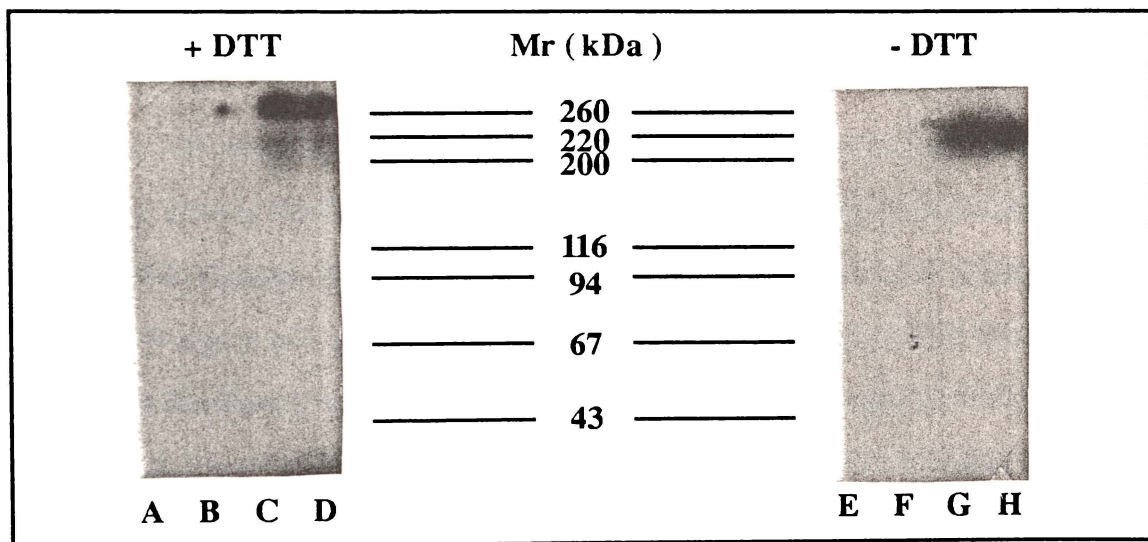


Macroautoradiographs of SDS-PAGE gels showing the molecular weights of the IGF-II hormone-receptor complex in antler cartilaginous tissues. Membrane preparations of antler tip material (n=3), which did not include the epidermis and dermis, were incubated with ^{125}I -IGF-II in the presence (lanes A, B, E, F) and absence (lanes C, D, G, H) of excess unlabelled IGF-II, then affinity cross-linked and subjected to SDS-PAGE under reducing (+DTT) and non-reducing (-DTT) conditions. The positions of molecular weight markers, which were run in adjacent lanes, are indicated.

4.3.2.2 Characterization by Molecular Weight

After affinity labelling of antler microsomal membranes with ^{125}I -IGF-II, followed by electrophoresis and autoradiography, a single band of radioactivity was detected on the macroautoradiographs, which was localized at 220 kDa and was absent in the presence of excess unlabelled IGF-II (Fig. 4.5). Under reducing conditions the radioactive band was at a M_r of 260 kDa. These findings are indicative of the tracer binding to a type 2 IGF receptor (Kasuga, Van Obberghen, Nissley & Rechler, 1981; Barenton, Patel, Khan *et al.*, 1988).

Figure 4.5 IGF-II affinity cross-linked to binding sites in antler tissue.



4.4 DISCUSSION

This Chapter has demonstrated specific binding of IGF-II in tissues of the antler tip and characterized the nature of this binding. Radioreceptor assays showed that the IGF-II tracer bound to a site displaying preferential affinity for IGF-II and no specificity for IGF-I or insulin. The heterogeneous nature of the membrane preparation used does not rule out the possibility that the tracer may have been binding to more than one binding site, although the Scatchard analyses suggest that the tracer was binding to a relatively homogeneous

population of receptors. The finding of a major band migrating at 220 kDa under non-reducing conditions in the affinity cross-linking study, together with the high specificity for IGF-II displayed by the specific binding, suggests that tracer binding was predominantly to a site with the properties of the type 2 IGF receptor. There was little evidence that the tracer was binding to a type 1 IGF receptor as displacement of the tracer was not apparent with unlabelled IGF-I. There was also no evidence for the tracer binding to insulin receptors as insulin, having a greater affinity for the insulin receptor than IGF-II (Underwood & Van Wyk, 1985), would have shown a displacement of the tracer. Likewise there was no evidence for tracer binding to the putative type 3 IGF receptor described by Hintz, Thorsson, Enberg & Hall (1984).

The type 2 IGF receptor has high affinity for IGF-II, does not recognise insulin and has a low, if any, affinity for IGF-I, depending on the tissue or cell type under study (Massagué & Czech, 1982; August, Nissley, Kasuga *et al.*, 1983; Oppenheimer & Czech, 1983; Roth, Maddux, Wong *et al.*, 1983; Beguinot, Kahn, Moses & Smith, 1985; Mohan, Jennings, Linkhart & Baylink, 1988; Jansen, van Buul-Offers, Hoogerbrugge *et al.*, 1989; Centrella, McCarthy & Canalis, 1990; Sara & Hall, 1990; Bautista, Baylink & Mohan, 1991; Nissley & Kiess, 1991; Fournier, Ferralli, Price & Schlaeppli, 1993). At higher concentrations (above 1 nmol/l), both unlabelled IGF-I and insulin caused a dose-dependent and parallel increase in IGF-II tracer binding which reached a plateau at 130 nmol/l for IGF-I and 1308 nmol/l for insulin. Pre-incubation of sections with unlabelled insulin before competitive binding studies were carried out had the effect of priming the system, so that the enhancement of binding effect by insulin was increased in these sections. Ovine kidney tissue displays a similar enhancement effect, and in an experiment where membrane preparations of kidney tissue were subjected to affinity cross-linking and SDS-PAGE, following co-incubation with ¹²⁵I-IGF-II and unlabelled IGF-I, results showed that the increase in tracer binding was due to an increase in binding to type 2 IGF receptors (J. Martyn, *personal communication*). The enhancement of IGF-II tracer binding by IGF-I and insulin seen in antler sections, and by IGF-I but not insulin in crude microsomal

membrane preparations, does not agree with the properties usually ascribed to the type 2 IGF receptor.

The phenomenon of enhanced tracer binding has been described in the literature as paradoxical binding, and seems to occur when an IGF tracer is co-incubated with low concentrations of competing unlabelled hormones. Paradoxical binding (or the 'hook' effect) occurs in several cell types such as myoblasts (Ballard, Ross, Upton & Francis, 1988), fibroblasts (Clemmons, Elgin, Han *et al.*, 1986; De Vroede, Tseng, Katsoyannis *et al.*, 1986; Ballard, Ross, Upton & Francis, 1988; McCusker, Camacho-Hubner, Bayne *et al.*, 1990; Conover, 1992), several muscle cell types (McCusker & Clemmons, 1988), erythroleukemia cells (Blanchard, Barenton, Sullivan *et al.*, 1988), mesangial cells (Aron, Rosenzweig & Abboud, 1989), neural cells (Orlowski, Chernauek & Akeson, 1989), and tissues in the liver (Barenton, Patel, Khan *et al.*, 1988), brain (Lesniak, Hill, Kiess *et al.*, 1988) and ovary (Teissier, Monget, Monniaux & Durand, 1994). A mechanism that has been suggested for paradoxical binding involves the secretion, or detachment from the cell surface, of IGFBPs in the presence of free IGFs or insulin. These would preferentially bind IGFs in the extracellular medium (Funk, Kessler, Eisenmenger *et al.*, 1992), as they have higher affinity for the IGFs than cell surface receptors, which have higher affinity again than IGFBPs bound to the cell surface (McCusker, Camacho-Hubner, Bayne *et al.*, 1990; Conover, 1992). IGFBPs released into the extracellular medium have the ability to modulate the availability of IGFs to cell surface binding sites and give rise to paradoxical binding (De Vroede, Tseng, Katsoyannis *et al.*, 1986; McCusker, Busby, DeHoff *et al.*, 1991). This involves IGFBPs re-associating with the cell surface after binding free hormones in the medium, then releasing the hormone in the vicinity of cell surface receptors through a series of affinity-decreasing steps (McCusker & Clemmons, 1988; Conover, 1992), resulting in a localised high concentration of IGF. An IGFBP from human bone is postulated to fix IGF-II for storage in bone tissue, and to potentiate some of the actions of IGF-II on bone cells (Bautista, Baylink & Mohan, 1991). The possible involvement of IGFBPs in IGF regulation in antler tissues will be investigated in Chapter 5.

Insulin increases the number of cell surface type 2 IGF receptors in cell culture systems of adipocytes through the redistribution of type 2 IGF receptors from an intracellular compartment to the cell surface (Oppenheimer, Pessin, Massagué *et al.*, 1983; Oka, Mottola, Oppenheimer & Czech, 1984; Wardzala, Simpson, Rechler & Cushman, 1984; Corvera & Czech, 1985; Tanner & Lienhard, 1989). The redistribution of type 2 IGF receptors is linked with the redistribution of transferrin receptors to the plasma membrane, which is also stimulated by insulin (Davis, Corvera & Czech, 1986; Tanner & Lienhard, 1987), as both receptors occur in the same intracellular compartments (Tanner & Lienhard, 1989). IGF-I can also stimulate transferrin receptor redistribution to a limited extent, and decrease internalization (Davis, Faucher, Racaniello *et al.*, 1987). Insulin also increases the steady state cell surface concentration of type 2 IGF receptors in adipocytes (Corvera & Czech, 1985) and increases the apparent affinity of type 2 IGF receptors for IGF-II (Oppenheimer, Pessin, Massagué *et al.*, 1983). These observations implicate insulin in the facilitation of IGF-II activity via the type 2 IGF receptor *in vivo*, but cannot explain the binding characteristics of IGF-II to antler sections, which were from tissue that had been frozen, and cell membranes would not be intact.

It has been suggested that IGF-II is an important factor in the growth and differentiation of mesenchymal cells (Moses, Nissley, Short *et al.*, 1980; Nagarajan, Anderson, Nissley *et al.*, 1985), and has been implicated in mitogenesis (Sessions, Emler & Schalch, 1987; Mathieu, Rochefort, Barenton *et al.*, 1990), and protein synthesis (McCarthy, Centrella & Canalis, 1989). Mohan & Baylink (1991) showed that IGF-II can stimulate bone cell proliferation, and surmised that this action was mediated by type 2 IGF receptors. In the antler, the histological region with the highest specific binding of IGF-II was the reserve mesenchyme and perichondrium zone. This is the zone which undergoes the most rapid differentiation and mitosis in the antler (Banks & Newbrey, 1983a; Banks & Newbrey, 1983b) and where binding of IGF-I is relatively low (Chapter 3). Thus, the growth effects in the reserve mesenchyme and perichondrium zone could be due to IGF-II. IGF-II has been reported to

have mitogenic effects either alone (Adams, Nissley, Kasuga *et al.*, 1983; van Dijk, Tanswell & Challis, 1988) or in the presence of other growth factors (Slootweg, Hoogerbrugge, de Poorter *et al.*, 1990).

Clustering of IGF-II binding to nuclei observed in the dermis, reserve mesenchyme and perichondrium and cartilaginous zones suggests a role for IGF-II in cell replication in the antler. A similar phenomenon is seen in skeletal muscle where ^{125}I -IGF-II binding is localized to fibroblast nuclei in some regions of connective tissue (Oldham, Hodges, Schaare *et al.*, 1993). The expression of IGF-II mRNA in rat tibial growth plates, at presumed sites of local action of IGF-II, has also been shown not to be evenly distributed (Shinar, Endo, Halperin *et al.*, 1993). In the dermis, clustering was associated with nuclear-stained material amongst relatively large areas of sparsely labelled connective tissue and, in the reserve mesenchyme and perichondrium zone and cartilaginous zone, clusters were observed to occur associated with some, but not all, nuclei, suggesting that there may be sites available for binding IGF-II on cells at a specific stage of the cell cycle. Superficially, the present observations support those of Adams, Nissley, Kasuga *et al.* (1983), who used cell multiplication experiments where variations in response to IGF-II were explained as being due to differences in the mitogenic phase of fibroblast cells in culture. However, the heterogeneous nature of the antler tissue sections meant that a variety of cyto-architectonic structures, including components of the intracellular milieu, would be exposed to the tracer and other added hormones. Type 2 IGF receptors in nuclear membranes (Payne, Kendall & Hodgkinson, 1991) of nuclei which are exposed to the external environment through damage due to freezing or sectioning could well bind the tracer and give rise to the clustering phenomenon. Nuclei which were shielded from hormonal contact by a diffusion barrier would not be seen to bind the tracer.

The pattern of binding of IGF-II in the antler is different from the pattern of binding of IGF-I (Chapter 3). In contrast to IGF-I, which displays highest specific binding to cartilage tissue, which is differentiated, most of the specific binding of IGF-II was to the reserve

mesenchyme and perichondrium zone, in which tissues are undergoing rapid differentiation and proliferation. This indicates that the receptor for IGF-II in the antler differs from the IGF-I receptor in function, as well as in properties and distribution. Therefore, IGF-II probably has an effect on proliferating tissue, as is the case in human erythroid precursors where proliferation is mediated via the type 2 IGF receptor (Blanchard, Barenton, Sullivan *et al.*, 1988).

In conclusion, this Chapter showed that, in antler tissues, IGF-II binds mainly to sites displaying preferential affinity for IGF-II and with the properties of a type 2 IGF receptor. This receptor population showed enhanced binding of IGF-II tracer in the presence of unlabelled IGF-I and insulin, suggesting that IGFBPs may be involved in regulating IGF-II binding and possibly actions. From distribution patterns of specific binding, which showed highest binding to reserve mesenchyme and perichondrium zones, and nuclear localisation, it is postulated that the main role of IGF-II in growing antler tissues is in proliferation. These results indicate that IGF-II binds to a distinct population of receptors, with a different role, to that of IGF-I (Chapter 3).

CHAPTER 5

IGF BINDING PROTEINS IN ANTLEER TISSUES

5.1 INTRODUCTION

Specific binding of IGFs comprises binding to receptors and IGFBPs. Both of these aspects must be taken into consideration in order to fully comprehend the biological actions of the IGFs. IGFBPs play a pivotal, although incompletely defined, role in regulating IGF action in many tissues, as suggested by similar distribution patterns in tissues of IGFBPs and IGFs demonstrated in human and ovine fetuses (Hill, Clemmons, Wilson *et al.*, 1989; Delhanty & Han, 1993), human and rat placentas (Zhou & Bondy, 1992) and rat kidneys (Kobayashi, Clemmons & Venkatachalam, 1991).

Putative roles for the IGFBPs in tissues include: regulation of the amount of IGF that can gain access to receptors; regulation of IGF actions; providing a mechanism for tissue specificity for the IGFs; and control for the transport of IGFs into tissues (Binoux, Hossenlopp, Hardouin *et al.*, 1986; Kobayashi, Clemmons & Venkatachalam, 1991). Regulation of IGF availability to cellular receptors can be inhibitory (Baxter & Martin, 1989a; Cheung, Smith, Shimasaki *et al.*, 1991) or may enhance the cellular response to growth factors (Elgin, Busby & Clemmons, 1987; Baxter & Martin, 1989a; Jones, Gockerman, Busby *et al.*, 1993). Local synthesis of IGFBPs could control an IGF response in an autocrine or paracrine way, which is postulated to occur in the case of injuries where a localised proliferative response occurs, possibly augmented by IGFBPs, which is confined to a small area (Elgin, Busby & Clemmons, 1987).

IGFBPs containing RGD sequences are able to bind to some types of cell surface receptors, such as integrin receptors (Ruoslahti & Pierschbacher, 1987), so may potentially activate a cellular response (Jones, D'Ercole, Camacho-Hubner & Clemmons, 1991). IGFBPs also bind to the extracellular matrix by associating with glycosaminoglycans. Of these, heparin is a commonly occurring glycosaminoglycan (Jackson, Busch & Cardin, 1991), and all IGFBPs except IGFBP-4 contain consensus sequences for binding to heparin (S. Hodgkinson, *unpublished data*). IGFBP-5 binds to bone cells (Jones, Gockerman, Busby *et al.*, 1993) and may regulate the activity of IGF-II in coupling bone formation to bone resorption (Mohan, Strong, Linkhart & Baylink, 1994) or osteoblast mitogenesis (Andress & Kiefer, 1994). Extracellular matrix-bound IGFBPs have a lower affinity for IGFs than the IGF receptors do (Rechler, 1993), and free heparin dissociates IGFs from their IGFBPs (Clemmons, Underwood, Chatelain & Van Wyk, 1983). These premises form the basis of a proposed mechanism of IGFBP action in enhancing IGF binding and biological response in many cells (Elgin, Busby & Clemmons, 1987; Conover, 1991) where IGFBPs in the extracellular medium bind IGFs, followed by the association of the IGFBPs with heparin in the extracellular matrix, after which dissociation of IGFs from IGFBPs occur in the proximity of receptors at the cell surface (McCusker, Camacho-Hubner, Bayne *et al.*, 1990; Conover, 1991). In this way, extracellular matrix-binding IGFBPs may act as local targeting agents that enhance the concentration of IGFs in the local tissue microenvironment (Clemmons, 1991).

Receptors for the IGFs have a lower affinity for IGFs than do IGFBPs free in extracellular fluids (Conover, 1991; McCusker, Busby, DeHoff *et al.*, 1991) and formation of the IGF-IGFBP complex can inhibit the binding of IGFs to receptors (Orlowski, Chernausek & Akeson, 1989; Ross, Francis, Szabo *et al.*, 1989; Kiefer, Schmid, Waldvogel *et al.*, 1992). In blood, most of the IGF present is associated with the biologically inactive 150 kDa IGFBP-3 complex (Baxter & Martin, 1989a; Baxter & Martin, 1989b; Rutanen and Pekonen, 1990; Clemmons, 1991) which does not cross the capillary barrier (Binoux & Hossenlopp, 1988; Clemmons, 1991). Release of sequestered IGFs can occur by

proteolytic degradation of the IGFBPs, increasing the bioavailability of the IGFs to cells and tissues (Clemmons, Underwood, Chatelain & Van Wyk, 1983; Van Wyk & Trippel, 1987; Hossenlopp, Segovia, Lassarre *et al.*, 1990; Binoux, Roghani, Hossenlopp *et al.*, 1991; Frost, Macaulay, Wass & Holly, 1993; Kanzaki, Hilliker, Baylink & Mohan, 1994), implying a local form of regulatory control (Clemmons & Van Wyk, 1985; Van Wyk & Trippel, 1987).

IGFBPs are present in numerous tissues and fluids, and because of the importance of the local production and distribution of IGFBPs in tissues, the aim of the studies in this chapter was to characterise the nature of the IGFBPs produced by cartilage tissues of the antler. These studies also undertook to determine the extent to which IGFs specifically bind to extracellular matrix-associated IGFBPs in antler tissues.

5.2 MATERIALS AND METHODS

5.2.1 IGFBP Production

5.2.1.1 Tissue Collection and Culturing

The right-side antler tips from three fallow deer bucks were collected during the mid-growth phase under ethics committee-approved conditions (Section 2.1.2). Pre-cartilaginous and cartilaginous tissues were dissected out and weighed. Each tissue was chopped finely, washed twice in saline and cultured in 10 ml of serum-free Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, Maryland, USA) under aseptic conditions. Samples of media were taken from each tissue culture at 18 and 42 hours. The samples were analysed for IGFBPs using SDS-PAGE and Western-ligand blotting with either ^{125}I -IGF-I or ^{125}I -IGF-II as the tracer, then subjected to autoradiography (Section 2.10) and quantified by densitometry.

5.2.1.2 *³⁵S-Methionine Uptake Experiment*

To establish that protein synthesis was occurring in the antler tissue, samples of pre-cartilage and cartilage tissues were collected, dissected out and cultured in methionine-free Dulbecco's modified Eagle medium (GIBCO BRL) using the method described above, but with the addition of ³⁵S-methionine (Amersham International) to the medium. Samples of media were collected after three hours, dialysed to remove unincorporated ³⁵S-methionine and run on a 15% polyacrylamide gel. After electrophoresis the gel was stained with Coomassie stain solution and destained overnight (Section 2.10), then subjected to autoradiography (Section 2.4).

5.2.1.3 *Protein Assay*

To load even amounts of proteins onto the polyacrylamide gels in SDS-PAGE, the protein concentration of each sample was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, California, USA), using BSA as the protein standard.

5.2.1.4 *SDS-PAGE*

Gels were prepared by the method described in Section 2.9 with the separating gel having a final concentration of 15% acrylamide. Preparation of samples involved diluting each sample of antler culture media, the dilution depending on protein concentrations assessed in Section 5.2.1.3, in a treatment buffer of 2.5% SDS and 0.156 mol/l Tris-HCl (pH 6.8), giving final concentrations of 1% SDS, 62.5 mmol/l Tris-HCl (pH 6.8) and 1 mg/ml protein. A sample of fallow deer serum was prepared similarly, with a final dilution of 20%. To each sample was added 5 µl of 0.3% bromophenol blue in 50% ethanol, after which tubes were vortex mixed and 40 µl of each culture media sample and 10 µl of serum sample were loaded into wells in the stacking gels. For each electrophoresis run, two gels were run simultaneously, one loaded with 18 hour samples and the other with 42 hour samples.

5.2.1.5 *Western-Ligand Blotting and Autoradiography*

Following electrophoresis, gels were soaked in transfer buffer of 48 mmol/l Tris/39 mmol/l glycine (pH 9.2) with 20% methanol for 20 minutes. Chromatography paper pieces and 0.2 μ m-thick nitrocellulose immobilizing membranes (Trans-Blot transfer medium, Bio-Rad Laboratories, Hercules, California, USA) were also soaked in transfer buffer. Protein transfer from SDS polyacrylamide gels to nitrocellulose membranes was carried out using a semi-dry horizontal electrophoretic transfer method (Towbin, Staehelin & Gordon, 1979) on a LKB Multiphor II NovaBlot system (LKB-Produkter AB, Bromma, Sweden). Briefly, transfer units of the continuous buffer system, comprising: six pieces of soaked chromatography paper; three pieces of soaked chromatography paper; soaked nitrocellulose membrane; soaked post-electrophoresis SDS polyacrylamide gel; three pieces of soaked chromatography paper; and six pieces of soaked chromatography paper, were stacked on top of the saturated graphite plate of the anode electrode, with bubbles being excluded at every layer. The saturated graphite plate of the cathode electrode was placed on top, the current pins connected and the whole unit was connected by anode and cathode pins to a Multiphor II power supply (Bio-Rad Laboratories, Hercules, California, USA). A current 0.8 mA/cm² was applied to the unit and transfer took place over 1.5 hours. After transfer, the nitrocellulose membranes were removed for autoradiography and the SDS polyacrylamide gels were stained with Coomassie stain solution and destained overnight, as described in Section 2.10.

Following Western blotting the nitrocellulose membranes were subjected to a detection method which involved iodinated IGFs binding to transferred IGFBPs. All stages of the method were carried out on an IKA-VIBRAX-VXR electronic vibrating platform (Janke and Kunkel, Staufen, Germany) at room temperature. The membranes were first soaked for 30 minutes in a solution of Tris-saline (0.01 mol/l Tris with 0.15 mol/l NaCl, pH 7.5) and 3% Nonidet P40 (BDH Laboratory Supplies, Poole, England), which was replaced with a solution of Tris-saline and 1% BSA for two hours. The membranes were then soaked in Tris-saline and 0.1% Tween 20 (Aldrich Chemical Company Inc., Milwaukee, Wisconsin, USA) for 10 minutes before being incubated overnight in a solution of

Tris-saline with 1% BSA and 0.1% Tween 20 to which had been added iodinated tracer (either IGF-I or IGF-II) at approximately 25,000 cpm/ml. Following this, the membranes were passed through a series of five post-incubation baths of 15 minutes each, the first two being Tris-saline and 0.1% Tween 20, and the final three being just Tris-saline. The membranes were then removed from the vibrating platform, dried and subjected to autoradiography for four days at -70°C, as described in Section 2.10, producing macroautoradiographs.

5.2.1.6 *Densitometry (ImageQuant)*

Bands on the macroautoradiographs, representing IGF tracer binding to IGFBPs, were measured by densitometry using the ImageQuant programme (Section 2.11.3.2) and statistically analysed using ANOVA (Section 2.12). The molecular weights of the IGFBPs were estimated by comparison with the migration of proteins of known molecular weight which were run on the same gel.

5.2.1.7 *Immunodetection of IGFBP-2 and IGFBP-4*

Nitrocellulose membranes were immunoblotted using antibodies to bovine IGFBP-2 (Upstate Biotechnology Inc., Lake Placid, New York, USA) and human IGFBP-4 (Upstate Biotechnology Inc., Lake Placid, New York, USA), and based on the method of Ventling & Hurley (1988). Briefly, the membranes were immersed in 5% BSA in a buffer of 0.1 mol/l Tris buffered saline (pH 7.6) with 0.05% w/v Nonidet P40 (TBS-NP40 buffer) for 1-2 hours at room temperature, to block non-specific binding sites. The blocked membranes were incubated in the primary antibody (either IGFBP-2 or IGFBP-4) diluted in TBS-NP40/1% BSA for 1-2 hours at room temperature, with agitation. At the conclusion of the incubation, the membranes were washed (3 x 15 minutes) with TBS-NP40. They were then incubated with 1:200 diluted horseradish peroxidase conjugated anti-rabbit immunoglobulin G (Sigma Chemical Co., St Louis, Missouri, USA) for 4 hours at 25°C. After washing (3 x 15 minutes) with TBS-NP40, the chromogenic substrate 3,3'-diaminobenzidine tetrahydrochloride (Gibco BRL, Auckland, New Zealand) in TBS-NP40/0.03% H₂O₂ was added to each membrane for five minutes at room

temperature. The reaction was stopped by rinsing in TBS-NP40 and the membranes were air-dried.

5.2.2 Binding to IGFBPs *In Vitro*

5.2.2.1 *Des-IGF-I Incubation*

Desamino-(1-3)-IGF-I (des-IGF-I) has a very low affinity for IGFBPs (Szabo, Mottershead, Ballard & Wallace, 1988; Ross, Francis, Szabo *et al.*, 1989; Canalis, McCarthy & Centrella, 1991). To gauge the extent to which IGF-I bound to extracellular matrix-bound IGFBPs *in vitro* in antler tissues, competitive displacement studies using des-IGF-I as a tracer and as a competitive inhibitor and as a tracer were carried out. Two fallow deer antlers were collected from two bucks and immediately frozen (Section 2.1.2). From each antler, 20 sections were cryogenically cut, mounted onto 10 microscope slides and subjected to an *in vitro* incubation using ¹²⁵I-IGF-I and ¹²⁵I-des-IGF-I as tracers. For the incubation, IGF-I, des-IGF-I, and IGF-II, all at 1 µg/ml, and insulin at 10 ng/ml and 250 µg/ml were used for displacement studies. After thawing, the sections were pre-incubated, then incubated with either tracer at 20,000 cpm, with or without unlabelled competing hormones, and passed through a series of post-incubation washes as described in Section 2.3. The incubated slides were air-dried, then subjected to autoradiography for three days and developed (as in Section 2.4), producing macroautoradiographs. They were then exposed to NTB-2 emulsion for 3 weeks at 4°C and developed, producing microautoradiographs (Section 2.4), and then stained with haematoxylin and eosin.

5.2.2.2 *Immunohistochemical Localisation of IGFBP-2*

Further sections from each antler collected as in Section 5.2.2.1 were cryogenically cut and mounted onto slides. The sections were fixed for 10 minutes in 10% formalin and washed for five minutes in distilled water and air-dried. They were then stained using an immunoperoxidase method modified from the method of Seppälä, Wahlström, Koskimies *et al.* (1984). Half of the sections were digested with trypsin for 30 minutes then washed in PBS. All of the sections were then incubated with PBS with 0.5% Tween 20 and 1% BSA containing a 1:100 dilution of an antibody to bovine IGFBP-2 and left in an

Macroautoradiograph of ^{35}S -methionine-labelled proteins. ^{35}S -methionine was added to the culture media of pre-cartilage and cartilage tissues from two antlers, (A) and (B). The tracer was taken up by the tissues and used in the synthesis of new proteins, which were detected when samples of media from each tissue culture were subjected to SDS-PAGE and autoradiography.

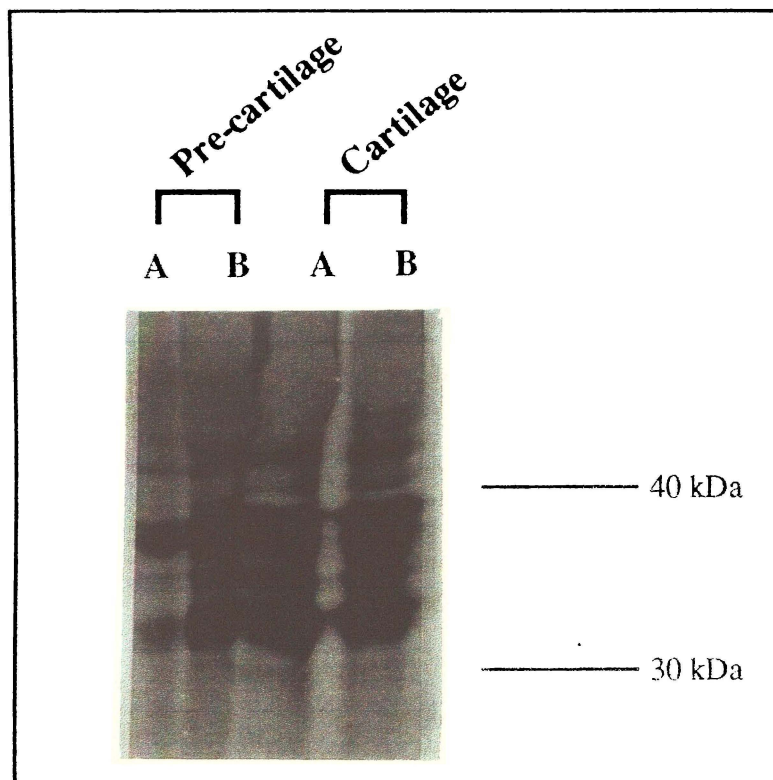
humidified slide chamber overnight at 4°C. Control sections incubated without the antibody were also processed. The sections were then washed with PBS and incubated with 1:200 diluted horseradish peroxidase-conjugated anti-rabbit immunoglobulin G for 4 hours at 25°C. After washing in PBS, 3,3'-diaminobenzidine tetrahydrochloride in 0.05 mol/l Tris/0.03% H₂O₂ was added for 11 minutes at room temperature. The reaction was stopped by washing in PBS and the sections were counterstained with light green (Sigma Chemical Company) and examined microscopically.

5.3 RESULTS

5.3.1 IGFBP Production

De novo synthesis of protein by the cultured antler tissues was confirmed by the uptake of ³⁵S-methionine into proteins secreted into the media, some of which were within the molecular weight range of the IGFBPs (Fig. 5.1).

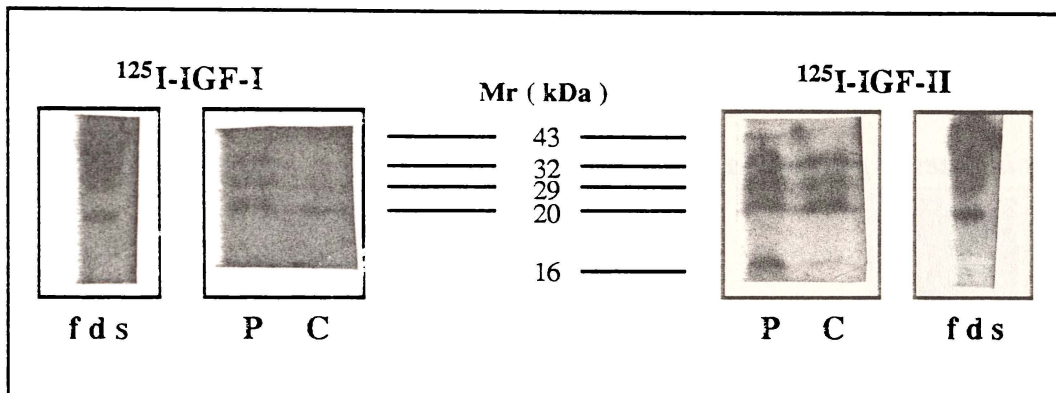
Figure 5.1 Protein synthesis by antler cartilaginous tissues in culture.



Molecular weights (M_r) of bands, which represent IGF tracers binding to IGFBPs in lanes of pre-cartilage (P) and cartilage (C) tissue culture media and to samples of fallow deer serum (fds), were obtained by comparison with the migration rates of marker proteins.

The identities of the IGFBPs produced in the array of bands that bound ^{125}I -IGF-I and ^{125}I -IGF-II were presumed by comparing with the molecular weights of marker proteins and fallow deer serum that were electrophoresed simultaneously (Fig. 5.2). IGFBPs were at 43 kDa, 32 kDa, 29 kDa and 20 kDa. In addition, the pre-cartilaginous tissue secreted a lower molecular weight binding protein of 16 kDa which was specific for IGF-II.

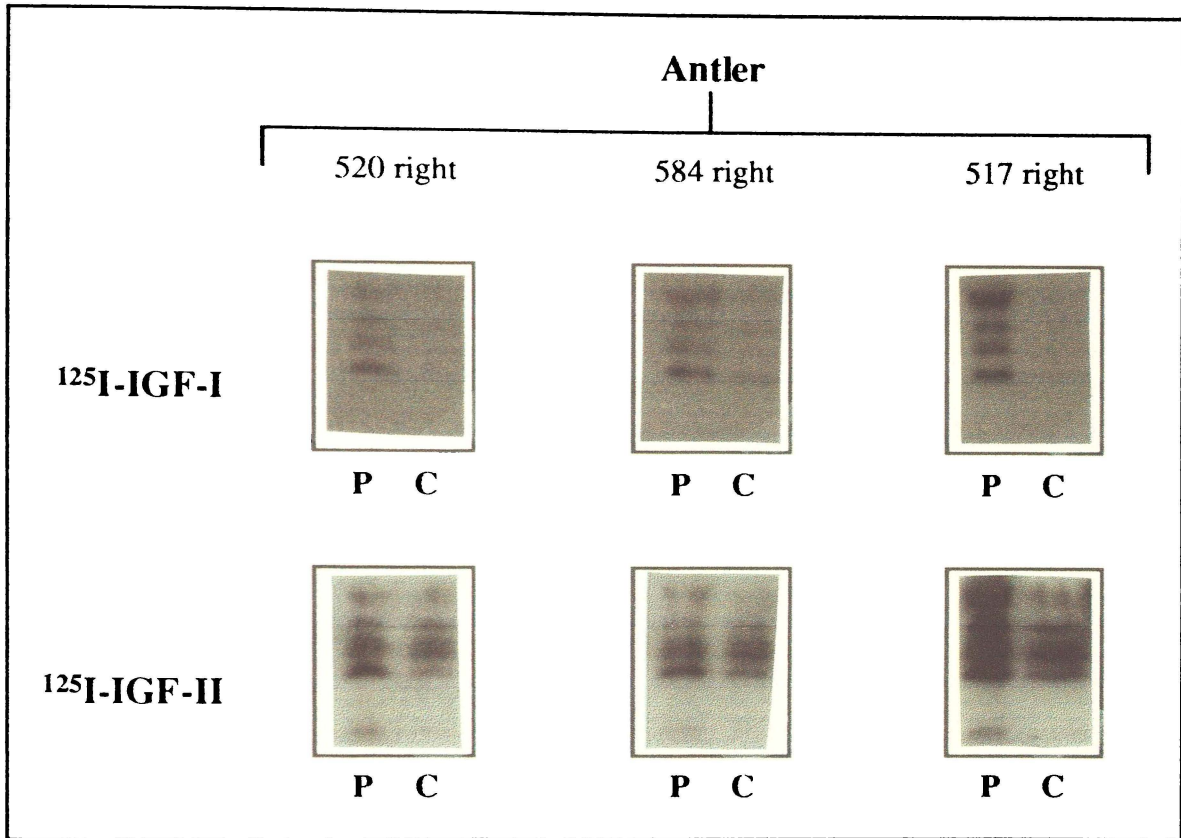
Figure 5.2 Molecular weights of IGFBPs produced in antler tissues.



The 43 kDa IGFBP seen in both antler tissue media and fallow deer serum was presumably IGFBP-3, the most common IGFBP in the serum of most species. Immunodetection of IGFBP-2 on nitrocellulose membranes using an antibody to IGFBP-2 confirmed that bands corresponding to 32 kDa and 20 kDa were IGFBP-2-related proteins (data not shown). No bands were labelled using an antibody to IGFBP-4, but subsequent immunoassays showed that this antibody was species specific and would not cross-react with any ovine, bovine or cervine IGFBP-4 (A. J. Peterson, *personal communication*), so the identity of the band at 29 kDa was unable to be verified. The band at 16 kDa, which was specific for IGF-II, was also unable to be identified because stocks of antibodies to IGFBPs other than IGFBP-2 were not readily available.

The macroautoradiographs of ^{125}I -IGF-I and ^{125}I -IGF-II binding to IGFBPs in the culture media of tissues from all three antlers (Fig. 5.3) were qualitatively assessed. Pre-cartilage tissues were shown to produce proportionately greater levels of IGFBPs than cartilage tissues.

Macroautoradiographs of IGF-I and IGF-II binding to IGFBPs produced by antler tissues in culture. Each sample of culture media was subjected to SDS-PAGE and Western-ligand blotting, using either ^{125}I -IGF-I or ^{125}I -IGF-II as the tracer, and the resulting nitrocellulose membranes were autoradiographed. Bands represent tracer binding to IGFBPs in lanes of pre-cartilage (P) and cartilage (C) tissue culture media.

Figure 5.3 IGFBPs produced by antler tissues in culture.

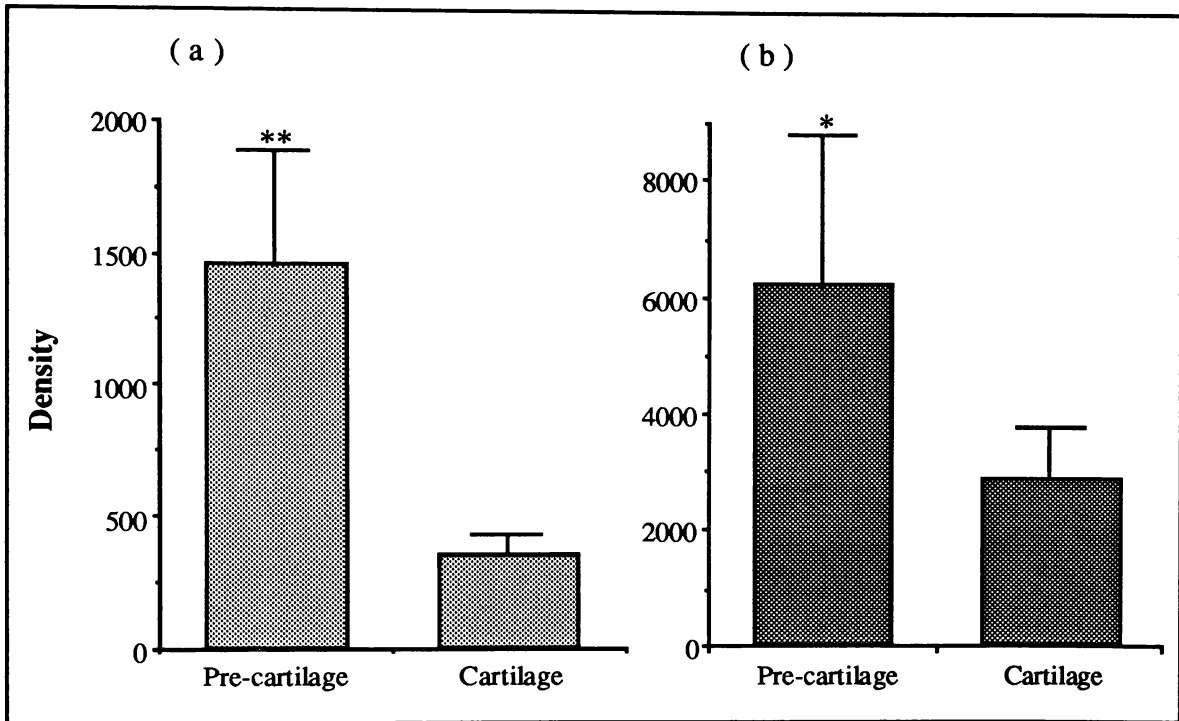
Results from the analysis of the macroautoradiographs of IGF-I binding to IGFBPs by densitometry showed that total secretion of IGFBPs by cartilage tissue was significantly less ($p < 0.001$) than secretion of IGFBPs by pre-cartilage tissue (Fig. 5.4a). A similar result was obtained for IGF-II binding to IGFBPs produced by antler tissues (Fig. 5.4b).

5.3.2 Binding of IGFs to IGFBPs *in vitro*

Qualitative assessments of the macroautoradiographs from the *in vitro* incubation using ^{125}I -IGF-I and ^{125}I -des-IGF-I as tracers showed that des-IGF-I was able to displace ^{125}I -IGF-I to the same extent as IGF-I (Fig. 5.5). ^{125}I -des-IGF-I was also seen to bind equally as well to antler tissues in each histological zone as ^{125}I -IGF-I. In addition, the extent of displacement of each of the tracers with heterologous hormones appeared to be similar.

*Graph of densitometry results from macroautoradiographs of radiolabelled IGFs binding to IGFBPs. Samples of culture media from pre-cartilage and cartilage tissues of antlers were subjected to SDS-PAGE, Western-ligand blotting using either ^{125}I -IGF-I (a) or ^{125}I -IGF-II (b) as the tracer, and autoradiography. The bands on the resulting macroautoradiographs were quantified by densitometry using the ImageQuant programme so that the total production of IGFBPs for each tissue was able to be calculated. Errors shown are SEM, and significant differences result from comparisons between tissues for each hormone; **, $p < 0.01$; *, $p < 0.05$.*

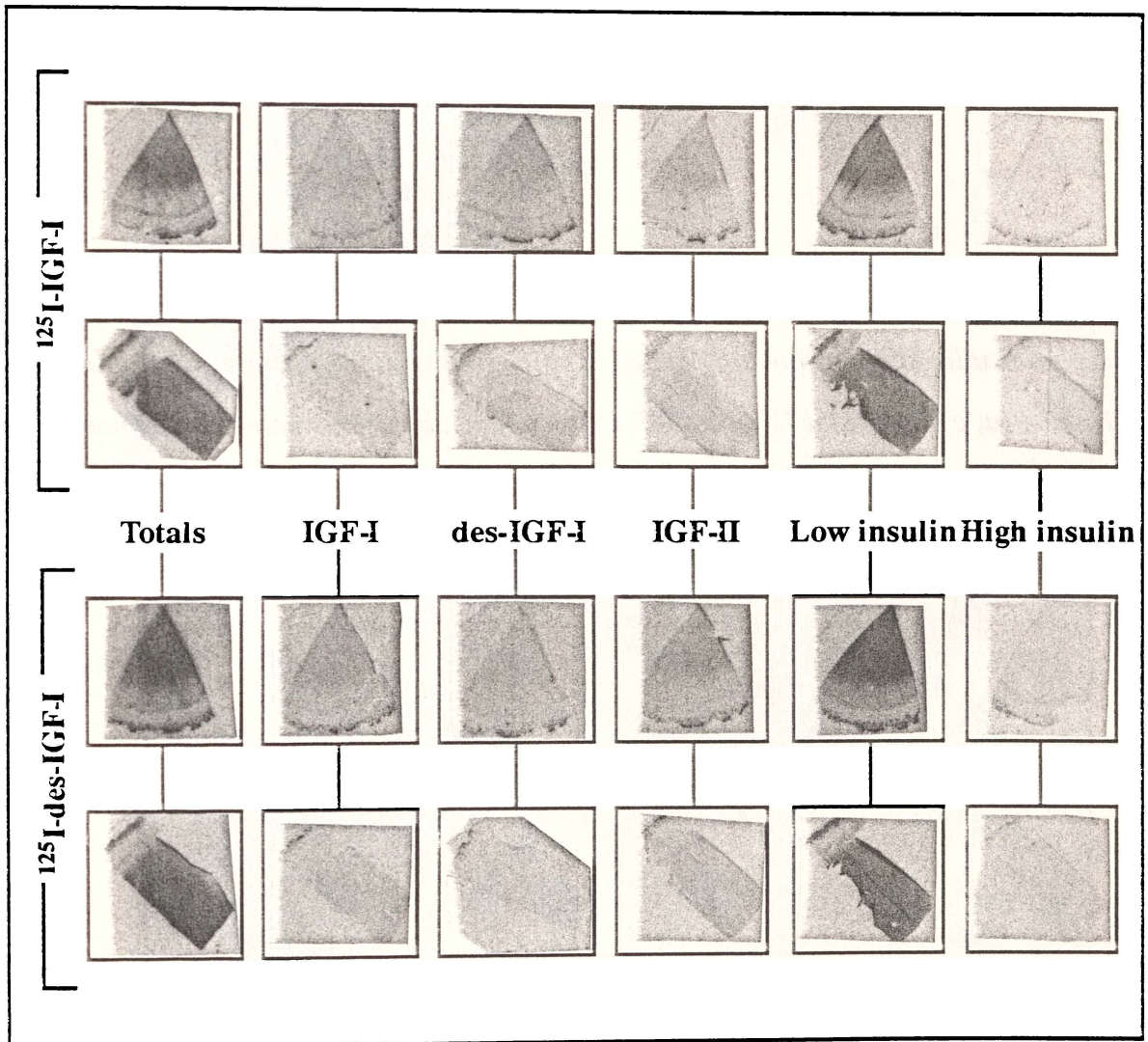
Figure 5.4 Pre-cartilage produces proportionately more IGFBPs than cartilage.



An interesting observation that arose from these results was that, in contrast to the cartilaginous tissues, binding of ^{125}I -IGF-I to the epidermis, hair follicles and to vascular tissue throughout antler sections was not competitively inhibited by des-IGF-I, and ^{125}I -des-IGF-I did not bind as highly to these tissues as ^{125}I -IGF-I (Plate 5.1). The presence of IGFBPs in these tissues was confirmed by results from the immunohistochemical detection of IGFBP-2 (Plate 5.2) which showed staining of the epidermis, hair follicles and blood vessel walls.

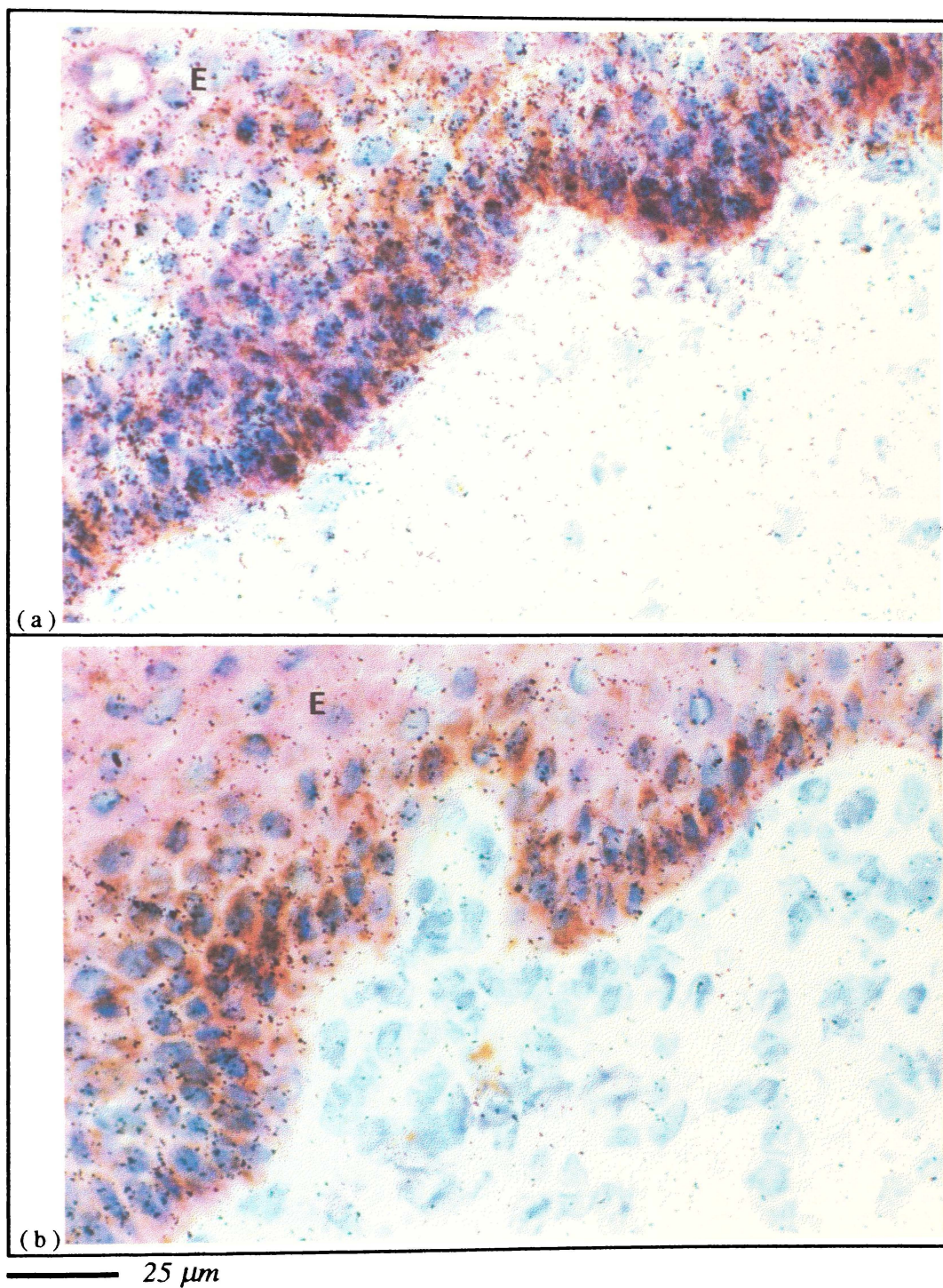
Antler sections from two animals were incubated with either tracer in the absence (Totals) or presence of excess unlabelled competing hormones IGF-I, des-IGF-I, IGF-II at 1 µg/ml, low insulin at 10 ng/ml, or high insulin at 250 µg/ml. After fixing, XAR film was exposed against the sections for three days, producing macroautoradiographs.

Figure 5.5 Comparison of ^{125}I -IGF-I and ^{125}I -des-IGF-I binding to antler sections.

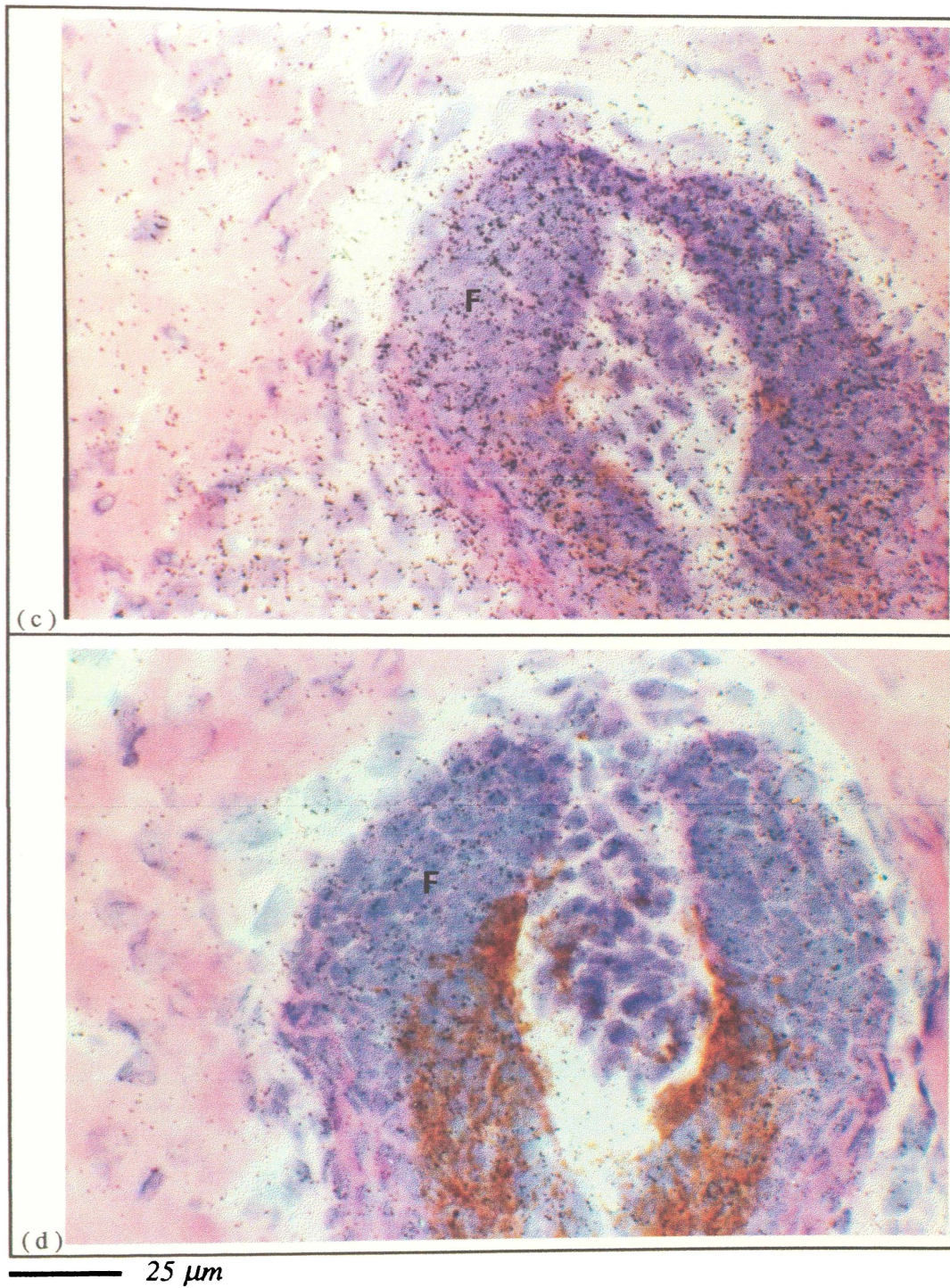


Microautoradiographs showing radiographic grains representing total binding of ^{125}I -IGF-I (a, c and e) and ^{125}I -des-IGF-I (b, d and f) to various non-cartilaginous tissues in antler sections. Sections were incubated with either tracer, fixed and subjected to autoradiography. The resulting microautoradiographs were stained with haematoxylin and eosin.

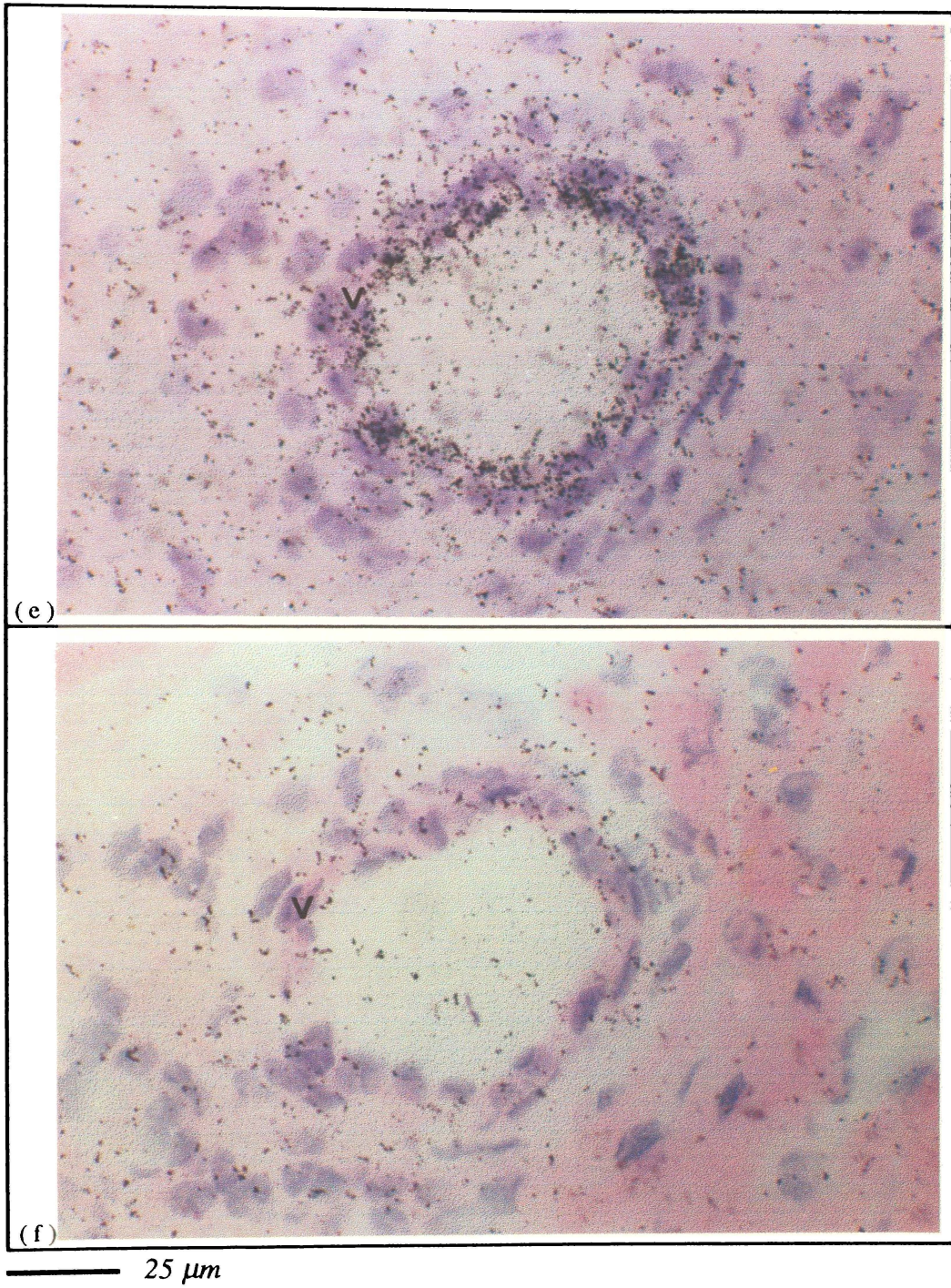
Plate 5.1 Comparison of IGF-I and des-IGF-I tracer binding to non-cartilaginous antler tissues.



(a) and (b) Epidermis (E).



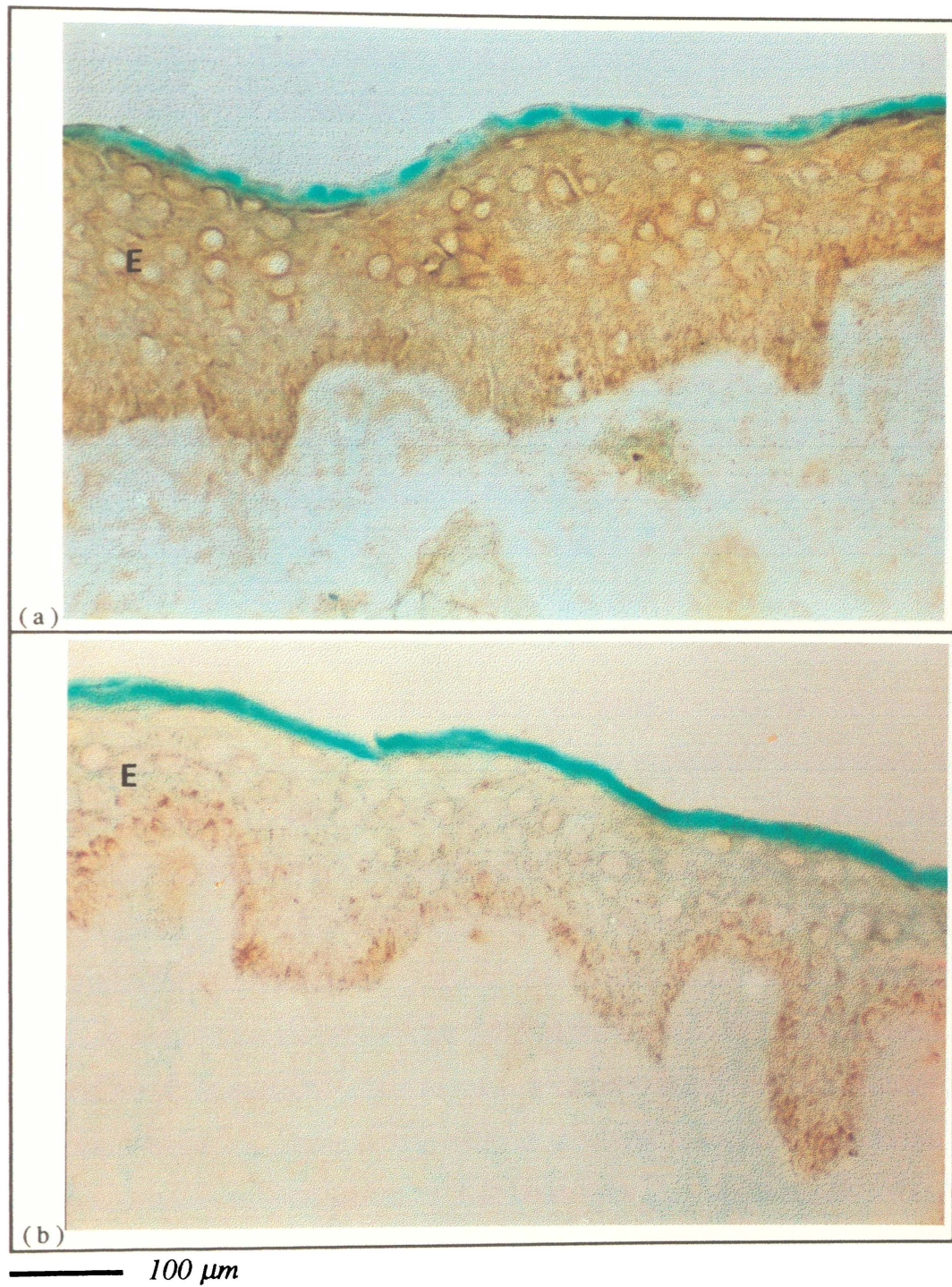
(c) and (d) Hair follicles (F).



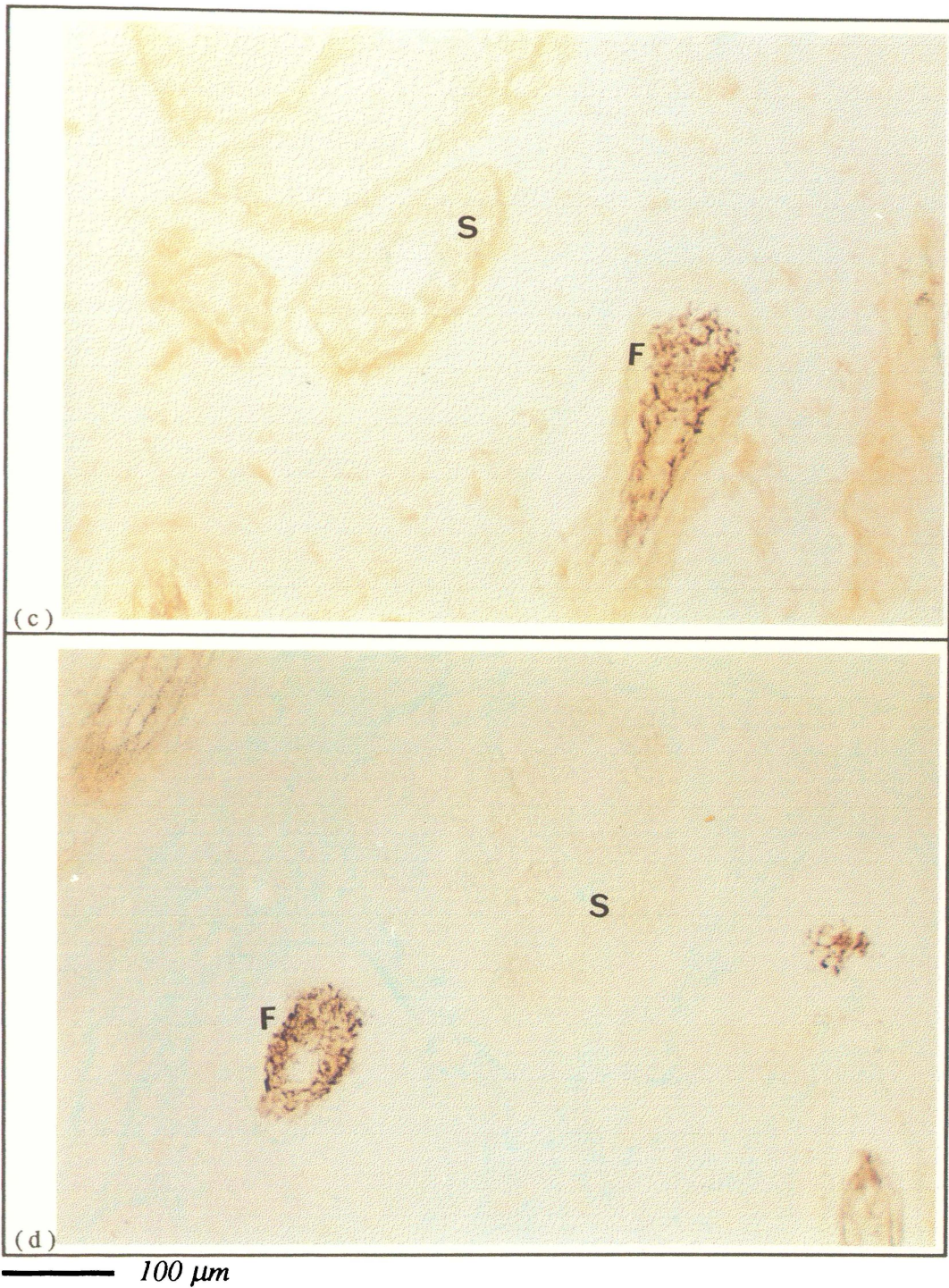
(e) and (f) Blood vessel walls (V).

Localization of IGFBP-2 in antler tissues was detected by immunohistochemistry. Antler sections were subjected to immunohistochemistry using an antibody to IGFBP-2, and counterstained with light green. Tissues in which IGFBP-2 was localized were stained brown (a, c and e) compared with control tissues (b, d and f) from sections incubated without the antibody.

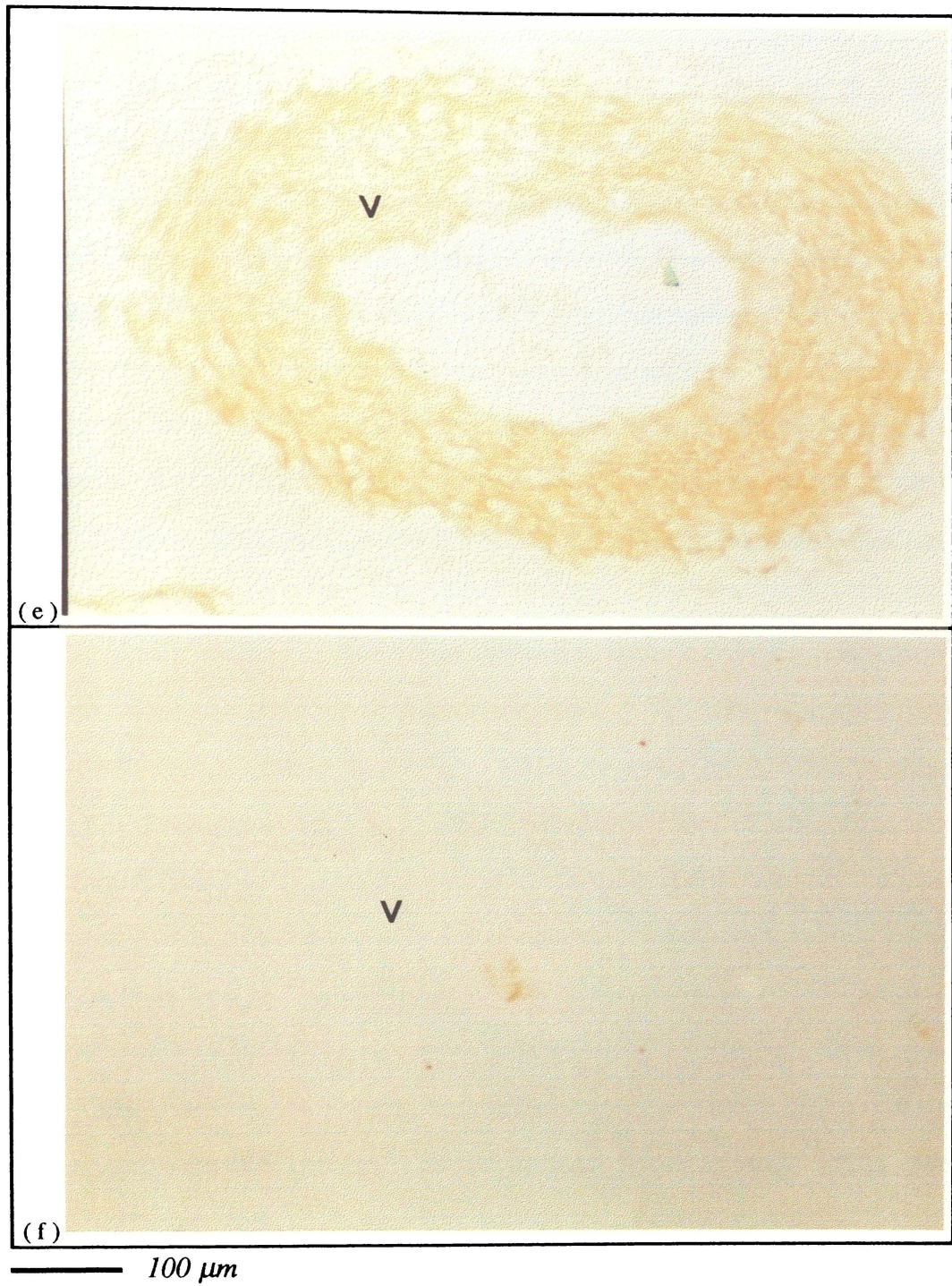
Plate 5.2 Localization of IGFBP-2 in antler tissues.



(a) and (b) Epidermis (E).



(c) and (d) Hair follicles (F) and sebaceous glands (S). The presence of the natural hair pigment, melanin (M), did not interfere with the immunodetection method used.



(e) and (f) Blood vessel walls (V).

5.4 DISCUSSION

This chapter has demonstrated the production of IGFBPs by antler tissues in culture and has attempted to characterise these IGFBPs with a view to assessing their biological relevance. The IGFBP composition of tissues confers specificity for the IGFs and can modulate IGF availability to, and actions on, the tissues (Binoux, Hossenlopp, Hardouin *et al.*, 1986; Kobayashi, Clemmons & Venkatachalam, 1991), which depends on the nature and proportions of the IGFBPs present. Partial characterisation of IGFBP components produced by antler cartilaginous tissues was achieved by the identification by immunoblotting of IGFBP-2. IGFBP-3 was probably also present by molecular weight comparisons. All of the IGFBPs produced had a higher specificity for IGF-II than IGF-I, suggesting that there is a higher level of regulation for this peptide in antler cartilage.

The intense specific binding of IGF-I demonstrated in antler cartilage tissue (Chapter 3) is not reflected by corresponding excessive secretion of IGFBPs, indicating very little interference to IGF-I binding to the type 1 IGF receptor by IGFBPs in this tissue. Truncated IGF-I analogues, such as des-IGF-I, display reduced affinity of binding to the IGFBPs (Sara & Hall, 1990). Results from IGF-I and des-IGF-I tracer incubations in this study showed that unlabelled des-IGF-I was able to competitively inhibit IGF-I tracer binding equally as well as unlabelled IGF-I. This indicates that no IGFBPs bound to the extracellular matrix of antler cartilage were detected using this method, which could mean that either there were very few present, or they had a non-competitive affinity for the IGF-I tracer compared with IGF receptors, as has been reported (Conover, 1991; McCusker, Busby, DeHoff *et al.*, 1991). Presumably, soluble or heparin-bound IGFBPs would have been washed off the sections before tissue fixation, which occurred during the post-incubation phase of the procedure.

Results from affinity cross-linking studies carried out on antler microsomal membrane preparations revealed that IGF-I tracer was bound to a single band migrating at 130 kDa (Section 3.3.2.2), and IGF-II tracer bound to a single band migrating at 220 kDa, and at

260 kDa under reducing conditions (Section 4.3.2.2). These results reflect tracer binding to the type 1 and type 2 IGF receptors, respectively, and there were no bands of IGF tracer binding apparent in the molecular weight range of the IGFBPs, implying that IGFBPs in antler cartilaginous tissues are not membrane-bound.

IGF-II binds highly and specifically to pre-cartilage tissues in the antler (Chapter 4). Results of the current studies showed that antler pre-cartilage tissues produced proportionately greater levels of IGFBPs than did antler cartilage tissues. This implies that the IGFBPs could act as targeting agents for the tissue localization of IGFs (Clemmons, 1991), especially IGF-II in the case of antler, in close proximity to type 2 IGF receptors in the pre-cartilage zone. IGFBP secretion correlates with the capacity of cells to proliferate *in vitro* (McCusker & Clemmons, 1988), which is a rapidly occurring event in antler pre-cartilage tissue (Banks & Newbrey, 1983a). A likely role for IGFBPs in this zone in particular that is suggested by this is in the facilitation of IGF-II binding to the type 2 IGF receptor, and modulation of the biological response arising from this, resulting in an enhancement of cell proliferation.

Several non-cartilaginous types of antler tissues were shown to contain bound IGFBPs. Results from des-IGF-I tracer incubations, along with results from immunohistochemistry using an antibody to IGFBP-2, indicated that there were IGFBPs bound in the epidermis, to hair follicles, and in blood vessel walls. These would not have been detected by affinity cross-linking studies using microsomal membranes or SDS-PAGE of tissue culture media as these sample preparations were of cartilaginous tissues only and did not include the velvet tissues of epidermis and dermis. There would have been insufficient vascular endothelial tissue in these sample preparations for appreciable levels of IGFBPs to have been detected. Soluble low molecular weight IGFBPs are able to permeate the vasculature (Bar, Booth, Boes & Dake, 1989; Ooi, 1990), have been found in extracellular environments of many cell types (Clemmons, 1991). Vascular endothelial cells produce IGFBPs (Bar, Harrison, Baxter *et al.*, 1987) that have properties peculiar to that tissue, part of which may involve the transport of IGFs between the circulation and subendothelial

tissues, and altering tissue IGF activity (Bar, Booth, Boes & Dake, 1989). Finding that IGFBP-2 is a constituent of the epidermis, hair follicles and vascular tissues in the antler has implications for the regulation of IGF-II actions in these tissues. A close correlation between IGF-II mRNA and IGFBP-2 mRNA in placental and fetal tissues has led researchers to suggest that IGFBP-2 may be an important modulator of IGF-II actions (Zhou & Bondy, 1992; Delhanty & Han, 1993).

The studies reported in this chapter showed that IGFBPs are produced in antler cartilaginous tissues *in vitro* and tend to be free rather than membrane-bound or bound to the extracellular matrix. Bound forms of IGFBPs were present in several non-cartilaginous structures of the antler. IGFBPs produced by antler tissues in culture had a higher specificity for IGF-II than IGF-I, and were present in proportionately greater amounts in culture media from pre-cartilage than from cartilage tissues. The substantially higher level of specific binding of IGF-II that is seen in pre-cartilage tissue (Chapter 4) leads to the speculation that the main role of IGFBPs in antler tissues is to target IGF-II for localization to areas of where proliferation is occurring, and to enhance IGF-II binding and actions.

CHAPTER 6

THE EFFECT OF TESTOSTERONE ON RECEPTORS FOR IGF-I AND IGF-II IN GROWING ANTLERS

6.1 INTRODUCTION

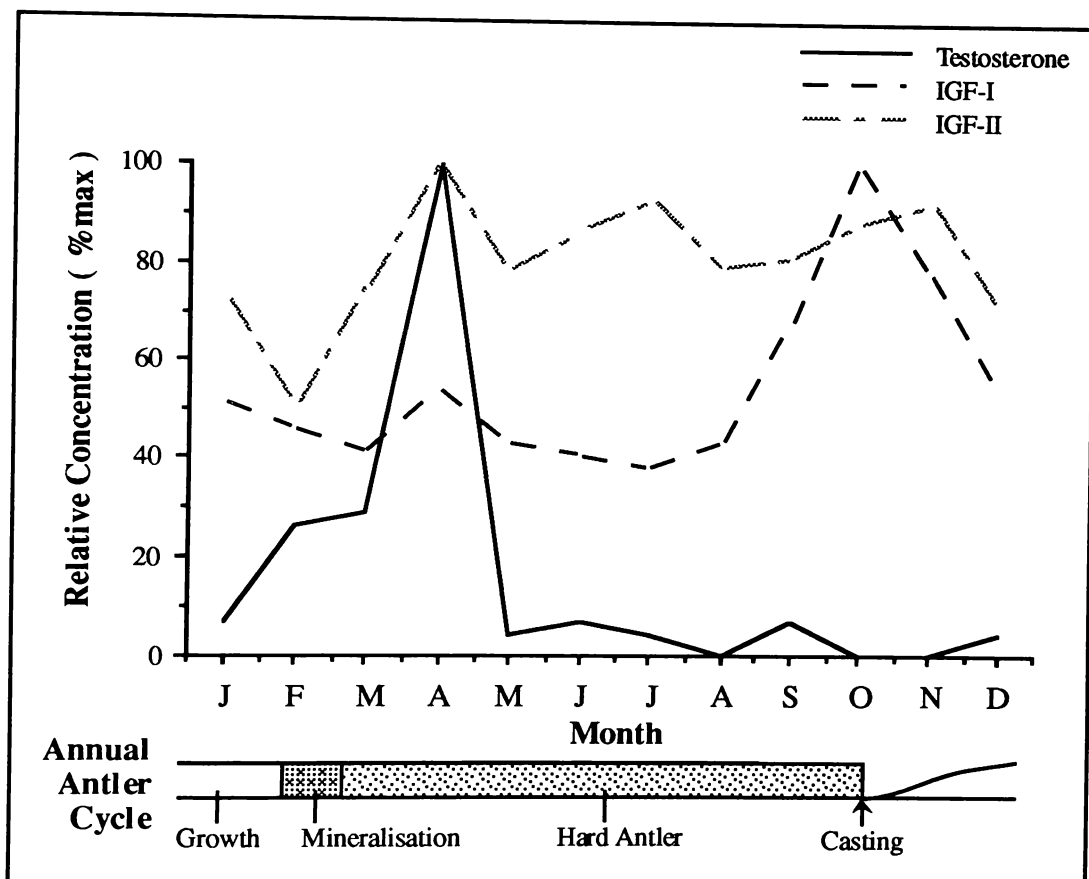
Antlers are secondary sexual characteristics of male deer which show dramatic changes in association with seasonal levels of plasma testosterone (Goss, 1969; Asher, Day & Barrell, 1987), and following the castration of a deer in velvet antler, antlers fail to mature or cast off (Wislocki, Aub & Waldo, 1947; Goss, 1969; Chapman, 1975; Chapman & Chapman, 1975b; Goss, 1983a; Goss, 1983b; Bubenik, 1987). Circulating testosterone concentrations are low and sometimes unmeasurable during initial antler growth in adult male fallow deer, but in later growth stages, and during mineralisation and hard antler stages, testosterone rises and can reach peak levels of 100 nmol/l (Asher & Peterson, 1991), concurrent with bone matrix deposition in antler tissues (Suttie, Lincoln & Kay, 1984; Muir, Sykes & Barrell, 1988; Asher, Peterson & Bass, 1989) as illustrated in Figure 6.1. Testosterone is metabolised to different derivatives in the peripheral circulation (Morris & Bubenik, 1983), which causes difficulties in interpreting modes of testosterone action.

Testosterone administration *in vivo* stimulates an increase in circulating levels of IGF-I (Hobbs, Plymate, Rosen & Adler, 1993; Weissberger & Ho, 1993), an effect which is possibly secondary to the increase in hepatic GH receptor capacity that occurs with testosterone treatment over time (Breier, Gluckman & Bass, 1988). Testosterone

Relative hormonal concentrations were calculated from data presented by Asher, Peterson & Bass (1989) for testosterone and Suttie, Fennessy, Corson et al. (1989) for IGF-I and IGF-II. A diagrammatic representation of antler development beneath the graph enables comparison of circulating hormonal levels with physiological changes of antler tissues.

administration to osteoblastic cells *in vitro* enhances the mitogenic effects of IGF-II (Kasperk, Fitzsimmons, Strong *et al.*, 1990). During the annual antler cycle, circulating levels of IGF-I rise and peak during the early stages of growth, and IGF-II levels are high throughout the year (Suttie, Fennessy, Corson *et al.*, 1989), as shown in Figure 6.1.

Figure 6.1 Temporal relationships between plasma levels of various hormones during one annual antler cycle of southern hemisphere male deer.



Testosterone induces maturational changes in antler tissues and may also have effects on cell replication. Results from the previous studies in this thesis have implicated IGF-I in antler tissue maturation and IGF-II in cell replication and/or differentiation. This study was undertaken to examine the changes in IGF receptor populations that occur during normal antler growth, and to compare these with receptor populations from antlers of animals

where testosterone was exogenously administered during the peak growth phase, to see if the effects of testosterone are reflected in the ontogeny of the IGF receptor populations.

6.2 MATERIALS AND METHODS

6.2.1 Antler Collection

Eleven antlers from six fallow deer bucks were collected singly at 20 day intervals between 20 days and 100 days after casting of the previous hard antlers. Collection involved the removal of a single antler from each of two animals at the 20, 40, 80 and 100 days, and each of three animals at 60 days. Each animal underwent two collections at different random times, except one which underwent one collection. On day 50, nine additional bucks were each administered with 4 ml of a 5% (w/v) solution of a mixture of testosterone esters (Durateston, Chemavet Division, Pharmaco (NZ) Ltd, Auckland, New Zealand) by intramuscular injection, a dosage designed to cause complete mineralisation, ossification and velvet shedding in 14 days following injection. Antlers from these bucks were removed singly on days 1, 2, 4, 6, 8, 9, 10 and 13 following injection, under the conditions described in Section 2.1.2. One of the antlers, collected four days after testosterone injection, had to be discarded because of damage incurred in the paddock.

6.2.2 Binding Studies

6.2.2.1 Sectioning

From each antler, 16 longitudinal sections (8 μm to 10 μm thick) of the antler tip were cryogenically cut at -18°C to -20°C and thaw-mounted in pairs onto eight gelatine-coated microscope slides. Sections were stored at -20°C and incubated within one week of being cut.

6.2.2.2 Incubations with ^{125}I -IGF-I and ^{125}I -IGF-II

IGF-I and IGF-II were iodinated as previously described (Section 2.2) and used for *in vitro* incubations (Section 2.3). Briefly, sections were pre-incubated, then incubated with in the presence of either ^{125}I -IGF-I or ^{125}I -IGF-II at 20,000 cpm, with or without competing unlabelled hormones. Competing unlabelled hormones used were IGF-I at 1 $\mu\text{g}/\text{ml}$, IGF-II at 1 $\mu\text{g}/\text{ml}$, and insulin at 1 $\mu\text{g}/\text{ml}$ and 250 $\mu\text{g}/\text{ml}$. Following incubation, sections were washed, fixed and air-dried. Control sections were also subjected to the incubation protocol, but without tracer. For each animal, one section of each pair was incubated with ^{125}I -IGF-I or ^{125}I -IGF-II alone (total binding), and the other was incubated with one of the tracers and a competing unlabelled hormone.

6.2.2.3 Autoradiography

XAR film was exposed against the incubated slides for four days and developed, producing macroautoradiographs (Section 2.4). NTB-2 emulsion was applied to the sections on the slides, dried overnight at room temperature with silica desiccant and exposed for three weeks at 4°C. The microautoradiographs were developed and fixed, then stained with toluidine blue and mounted (Section 2.4).

6.2.2.4 Microautoradiographical Analysis and Statistical Analysis

IGF-II macroautoradiographs were analysed using IMAGE densitometry (Section 2.11.3.1) where binding for a section was calculated as the intensity of the image underneath a line drawn across a macroautoradiograph. The intensity was expressed as plot values and mean values were calculated so as to represent the level of binding in each of five histological zones - chondrocytes, chondroblasts, reserve mesenchyme and perichondrium zone, dermis, and epidermis. Means for total binding and non-specific binding for each ontogenetic age and treatment group were used to calculate mean specific binding. IGF-I macroautoradiographs were not suitable for analysis by densitometry as the control and treated antlers were mounted on separate sheets of film which had different background densities, so it was not feasible to make comparisons between them. Instead, IGF-I

microautoradiographs underwent radiographic grain counting using an automated counting system. For each section, counts were made from five sites in each histological zone. Hair follicles and sebaceous glands in the dermis and blood vessels in the reserve mesenchyme and perichondrium zone were excluded from counting. Sites were selected from random microscope stage co-ordinates and tissue counts taken from areas of uniform light intensity using the automated counting system VISILOG (Section 2.11.2). Mean specific binding was calculated as the difference between means of total and non-specific binding. All data were statistically analysed using ANOVA (Section 2.12) and were used to compare specific binding in each histological zone between different ages and treatments. To aid clarity of presentation, treatment groups that showed no significant differences in magnitude or distribution of binding from a preliminary analysis, where all data were analysed separately, were pooled, and the pooled SEDs used in the calculation of t-values.

6.3 RESULTS

6.3.1 Ontogenetic Changes in Antler Histology

Over the growth period in this study, several ontogenetic changes in antler tip histology were observed. After 20 days of growth, the reserve mesenchyme and perichondrium zone was relatively wide and difficult to distinguish from other zones, and mineralisation of the cartilage was absent. Tissues progressively matured up to 100 days where the reserve mesenchyme and perichondrium zone had compacted, before forming the periosteum, and comprised a small zone close to the tip (Plate 6.1). Ossification of the cartilage was occurring at this late stage of development, forming spicules of spongy bone.

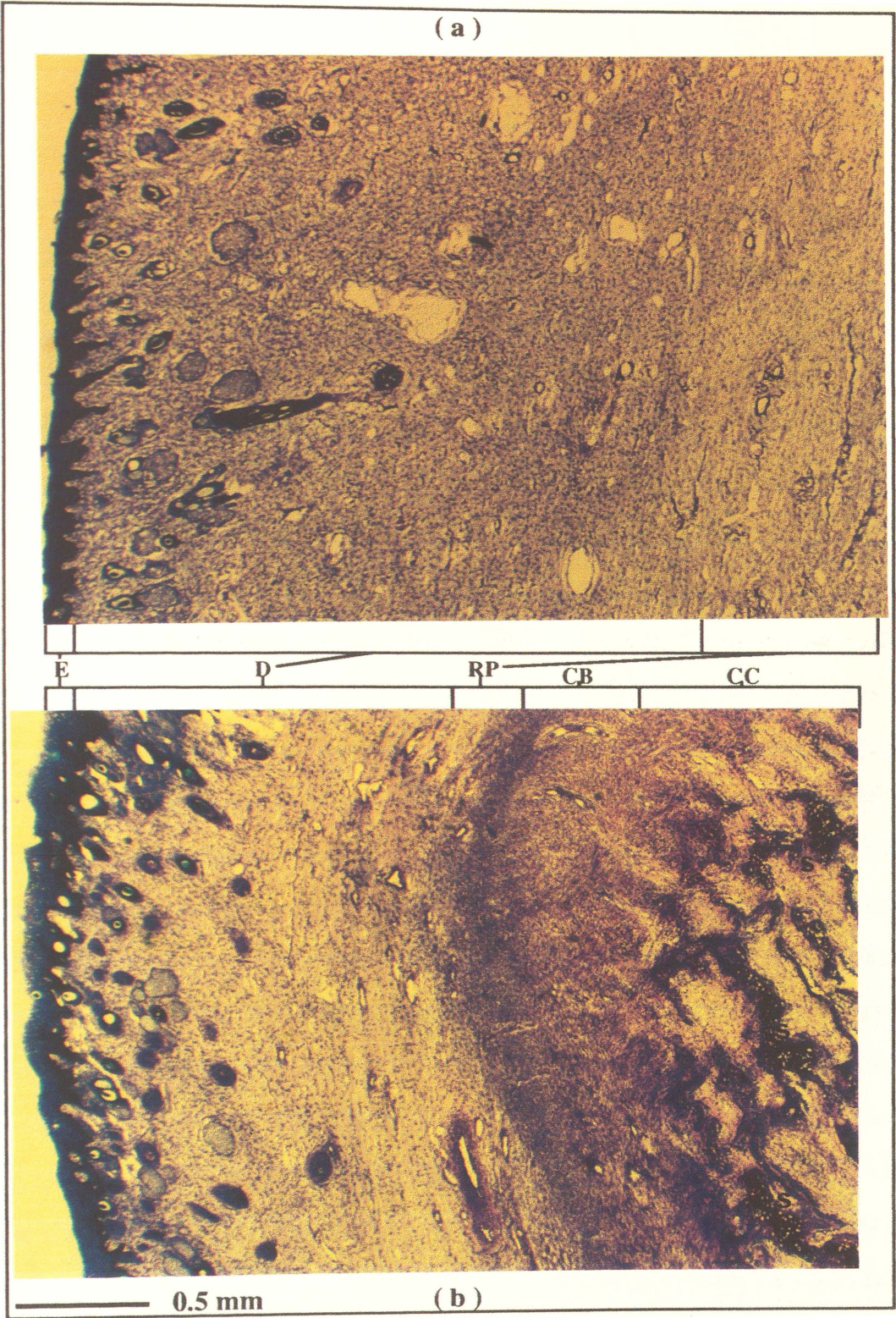
6.3.2 Receptor Binding

6.3.2.1 *Ontogenetic changes in ^{125}I -IGF-I and ^{125}I -IGF-II binding*

Specific binding patterns of IGF-I in antler tip tissues were not significantly different in magnitude or distribution throughout the growth period from 20 days to 80 days, but

Photomicrographs of longitudinal antler sections showing the changes in ontogeny that occurred from (a) 20 days to (b) 100 days of the growth period under study. Antler sections were cryogenically cut at 8 μ m thickness, then thaw-mounted onto slides, fixed in 10% formalin and stained with toluidine blue. E, epidermis; D, dermis; RP, reserve mesenchyme and perichondrium zone; CB, chondroblast zone; CC, chondrocyte zone; S, spicules of newly formed bone.

Plate 6.1 Ontogenetical changes in antler histology.



changed in distribution at 100 days, at a time when the antler tip was in the process of ossification. Distribution of binding from 20 to 80 days was in a pattern consistent with that obtained previously for IGF-I (Chapter 3) where highest binding occurred in the chondrocyte zone. Binding to the epidermis was variable within and among each age, but this was not statistically significant. At 100 days the binding pattern changed markedly with a significant decrease in binding to the chondrocyte zone ($p < 0.01$) and a significant increase in binding in the reserve mesenchyme and perichondrium zone ($p < 0.001$) from a mean of 2.34×10^{-2} grain counts/ μm^2 at 20 days, to 26.1×10^{-2} grain counts/ μm^2 at 100 days (Fig. 6.2a).

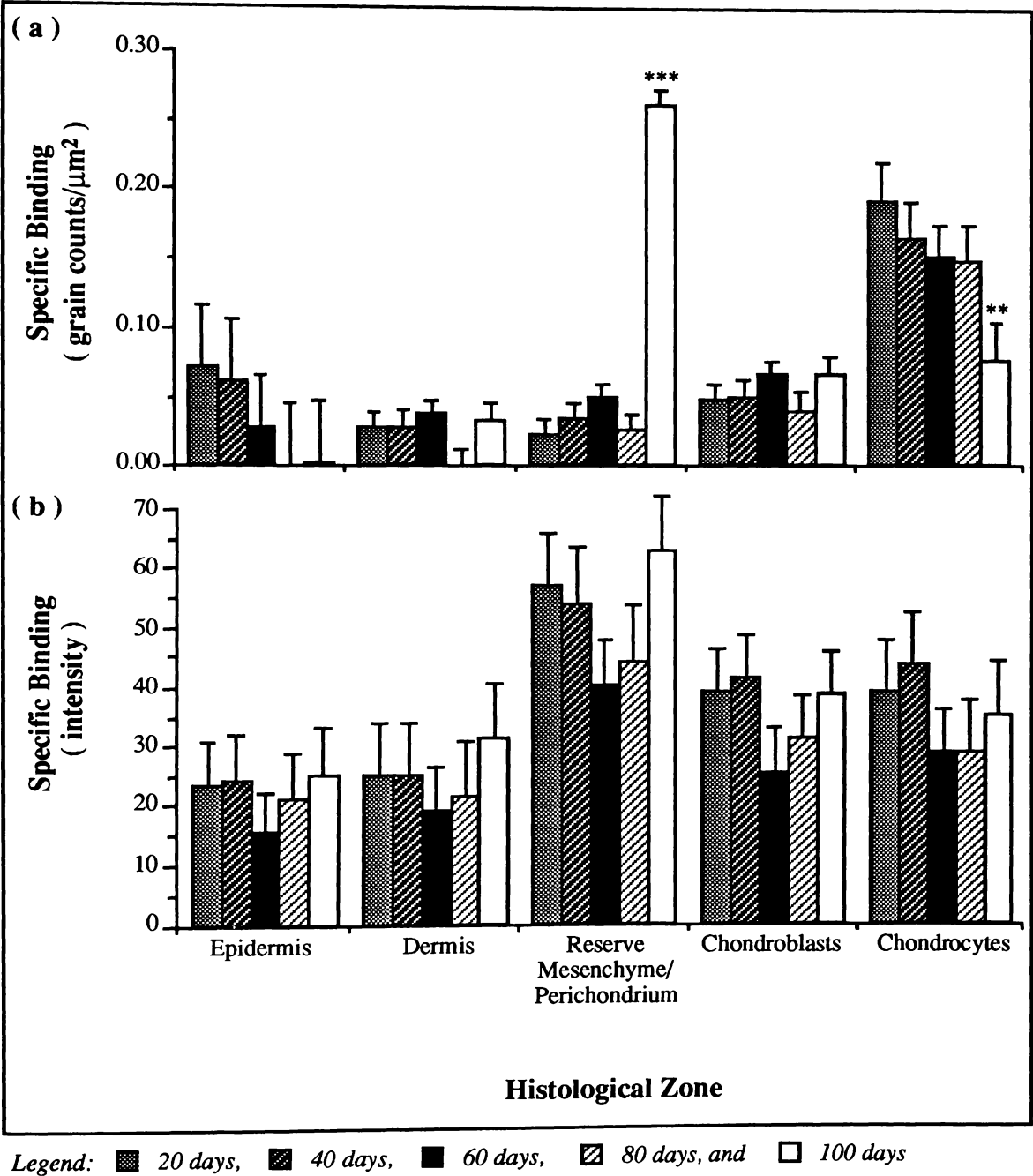
Specific binding patterns of IGF-II in antler tip tissues were consistent with those obtained previously (Chapter 4) and were constant throughout the growth period with no significant changes in magnitude or distribution (Fig. 6.2b).

6.3.2.2 Competitive Displacement of Tracer Binding

The cross-binding of hormones to receptors has been used to identify type 1 and type 2 receptors for IGFs based on their relative affinities for IGF-I, IGF-II and insulin (Underwood & Van Wyk, 1985). Displacement of ^{125}I -IGF-I or ^{125}I -IGF-II by competing unlabelled hormones varied depending on the nature of the unlabelled hormone used. Qualitative assessments of all of the macroautoradiographs produced showed that unlabelled IGF-I displaced the homologous tracer to a greater extent than the same concentration of IGF-II, and much greater again than insulin (Fig. 6.3a). Very high concentrations of insulin were able to displace IGF-I tracer completely. Unlabelled IGF-II was the only hormone that was able to successfully compete with the IGF-II tracer for binding sites in this study, with no competitive displacement observed with unlabelled IGF-I or insulin (Fig. 6.3b). These observations were constant for all of the antler tissues used in this study, from both the untreated ($n=6$) and testosterone-treated ($n=9$) series of bucks.

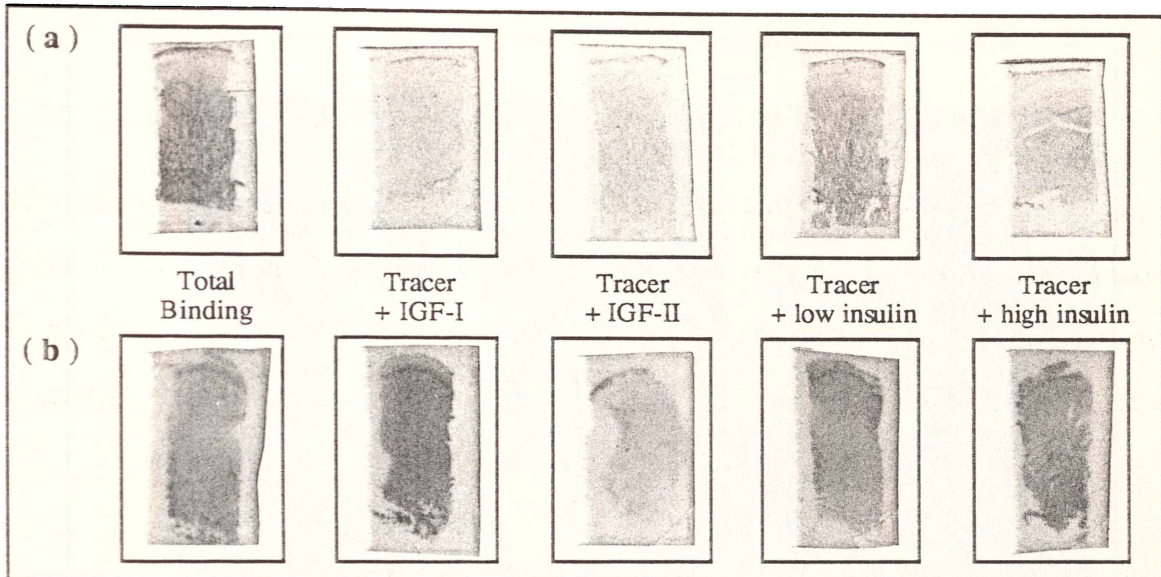
*Antler sections were subjected to an in vitro incubation using either (a) ^{125}I -IGF-I or (b) ^{125}I -IGF-II as the tracer. Antler sections incubated with ^{125}I -IGF-I underwent macroautoradiography, microautoradiography, and staining with toluidine blue. Counts of radiographic grains were made from the microautoradiographs using a light microscope and specific binding was calculated as the difference between mean total and mean non-specific counts. Sections incubated with ^{125}I -IGF-II underwent macroautoradiography and densitometry. Specific binding was calculated as the difference between means of total and non-specific binding densities. All values are means from two animals for each growth stage except for 60 days where $n=3$. Errors shown are standard error of the mean among ages within each zone; **, $p<0.01$; ***, $p<0.001$; otherwise not significantly different from 20 days growth.*

Figure 6.2 Specific binding to tissues of the antler tip throughout growth.



Antler sections were incubated with (a) ^{125}I -IGF-I or (b) ^{125}I -IGF-II in the absence (Total Binding) or presence of excess competing unlabelled hormones IGF-I, IGF-II and low insulin at 1 $\mu\text{g/ml}$, or high insulin at 250 $\mu\text{g/ml}$. After fixing, the sections were autoradiographed against XAR film for four days, producing macroautoradiographs.

Figure 6.3 Macroautoradiographs of IGFs binding to antler tip tissues.



6.3.3 Effects of Testosterone

6.3.3.1 Testosterone-induced changes in antler histology

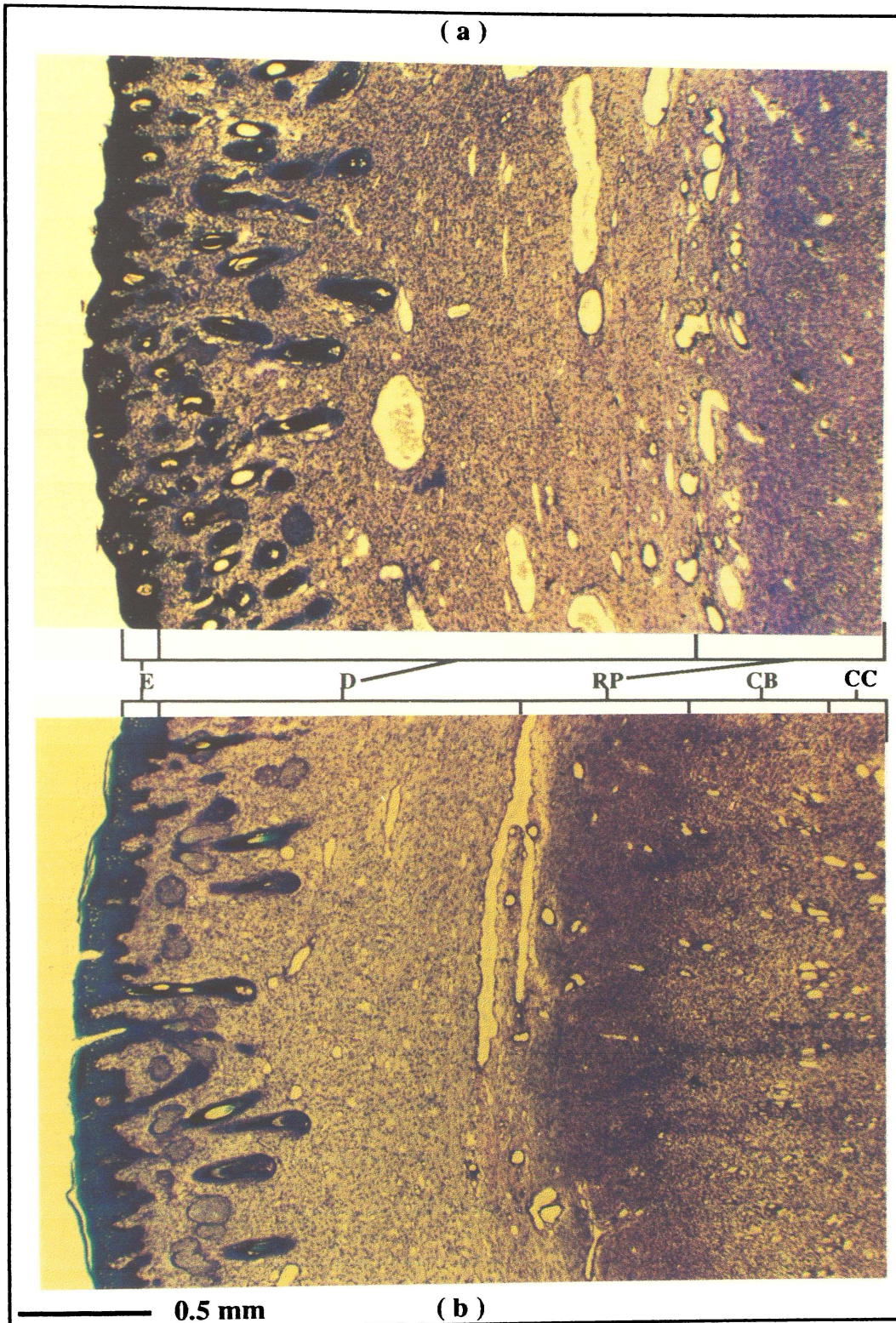
Testosterone treatment enhanced the maturation of the antler cartilage, as seen by histological changes (Plate 6.2). When compared with antler tissues of a control buck at 60 days of growth, the antler tissues of a treated buck at 60 days (10 days after testosterone administration) were relatively advanced. The reserve mesenchyme and perichondrium zone was closer to the tip, as were cartilaginous trabeculae, and ossification of the antler base had occurred.

6.3.3.2 Testosterone-induced changes in receptor binding

Testosterone treatment appeared to decrease specific binding of IGF-I in all antler tissues except for the epidermis, when compared with control antler tissues at a similar stage of growth (60 days), although any control group from 20 to 80 days could have been used as a comparison. In the treated series of tissues, specific binding of IGF-I in the epidermis was highly variable, but insignificant. The chondroblast zone seemed to respond most rapidly to

Photomicrographs of longitudinal antler sections demonstrating the advanced tissue maturation that occurred with testosterone treatment. Antler sections from (a) a control buck at 60 days and (b) a treated buck also at 60 days were cryogenically cut at 8 μ m thickness, then thaw-mounted onto slides, fixed in 10% formalin and stained with toluidine blue. E, epidermis; D, dermis; RP, reserve mesenchyme and perichondrium zone; CB, chondroblast zone; CC, chondrocyte zone.

Plate 6.2 Changes in antler histology induced by testosterone treatment.

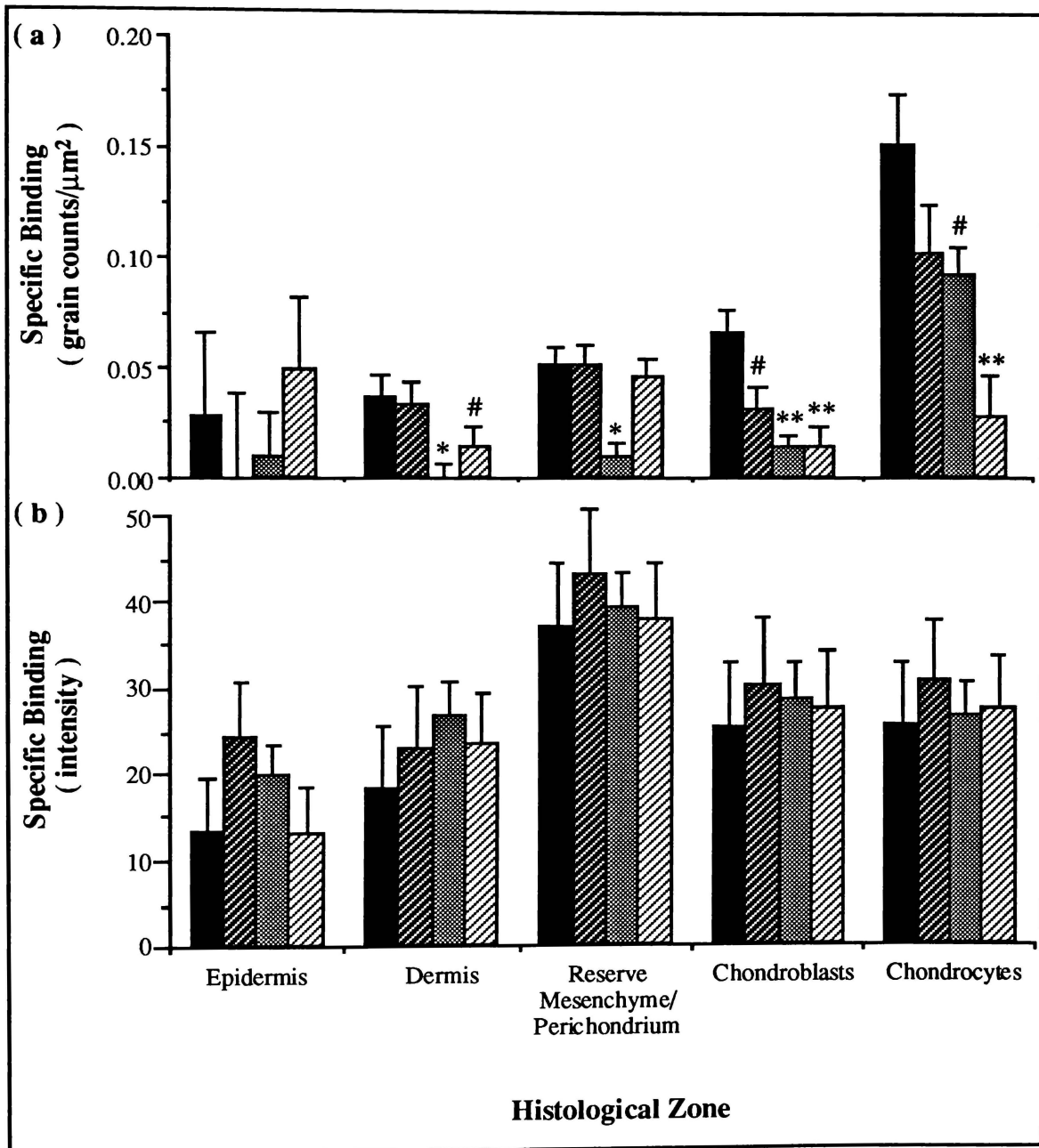


the effect of testosterone as specific binding had decreased ($p < 0.10$) after one day of treatment (51 days) but had not in any other tissue. By 52 days, specific binding of IGF-I had decreased significantly in the dermis and reserve mesenchyme and perichondrium zones ($p < 0.05$), chondroblast zone ($p < 0.01$) and chondrocyte zone ($p < 0.10$) and remained low until 58 days. This enabled the data from the 52-58 days treatment groups to be pooled as binding was not seen to change significantly during this period. From 59 to 63 days the specific binding decreased further or remained low in all of these zones, except for the reserve mesenchyme and perichondrium zone where the binding increased relatively, to become not significantly different from the 60 day control tissue (Fig. 6.4a). These data were pooled to form a 59-63 days group. Data for the specific binding of IGF-II in the same tissues were grouped in the same way as the IGF-I data, although in this case there were no significant differences in binding in each tissue among the treatment groups (Fig. 6.4b).

6.4 DISCUSSION

The effects of testosterone in this study were to enhance antler cartilage maturation, which was associated with changes in receptor binding patterns of IGF-I. Testosterone administration had no effect on specific binding of IGF-II in tissues of the growing antler tip. Previous reports have shown that plasma levels of testosterone show a rapid increase from 89 days to 104 days after casting, between which times there is a decrease in depth of cartilage tissue in the antler tip and an increase in bone matrix deposition and degree of mineralisation (Muir, Sykes & Barrell, 1988). These differences were noticed in the control tissues from the present study where there were minimal changes in antler tip histology from 20 days to 80 days after casting and a marked tissue maturity by 100 days. Changes in magnitude of the specific binding of IGF-I correlate with these maturational changes of tissues as binding was higher in developing and maturing cartilage (20 days to 80 days after casting) than in ossifying tissues (100 days). Specific binding of IGF-I was significantly

*Antler sections from testosterone-treated bucks were subjected to in vitro incubations using either (a) ^{125}I -IGF-I or (b) ^{125}I -IGF-II as the tracer. Sections incubated with ^{125}I -IGF-I underwent macroautoradiography, microautoradiography, and staining with toluidine blue. Counts of radiographic grains were made from the microautoradiographs using an automated system, and specific binding was calculated as the difference between mean total and mean non-specific counts. Sections incubated with ^{125}I -IGF-II underwent macroautoradiography and densitometry. Specific binding was calculated as the difference between means of total and non-specific binding intensities. Means of specific binding for the treated groups at 51 days ($n=3$), 52-58 days ($n=10$) and 59-63 days ($n=4$) were compared with the mean of a control group at 60 days ($n=3$). Errors shown are standard error of the mean; #, $p<0.10$; *, $p<0.05$; **, $p<0.01$; otherwise not significantly different from 60 days control.*

Figure 6.4 Specific binding to tissues of antler tips from treated bucks.

Legend: ■ 60 days, control, ▨ 51 days, treated, ▩ 52-58 days, treated, and ▪ 59-63 days, treated.

lower ($p < 0.01$) in the cartilage tissue of mineralised antlers at 100 days of growth in the control series of antlers, at a stage when endogenous circulating levels of testosterone would have been elevated compared with earlier growth stages (Suttie, Lincoln & Kay, 1984; Muir, Sykes & Barrell, 1988; Asher, Peterson & Bass, 1989). No changes in specific binding of IGF-II occurred during, or as a result of, maturational changes of the antler tissues. With testosterone treatment an overall decrease in specific binding of IGF-I was observed to occur, but there were no differences observed in the specific binding of IGF-II.

Competitive displacement data in this study reproduced in part the results obtained from earlier, more comprehensive and quantitative competitive displacement studies using antler tissues (Sections 3.3.2 and 4.3.2), which showed that IGF-I and IGF-II bound predominantly to characteristic type 1 and type 2 IGF receptors, respectively, in these tissues. Competitive displacement of IGF-I tracer by high concentrations of insulin in this study indicated minimal IGF-I tracer binding to IGF binding proteins (Monget, Monniaux & Durand, 1989).

Testosterone treatment evoked a significant decrease in specific binding of IGF-I in all tissues except for the epidermis and temporarily in the reserve mesenchyme/perichondrium zone. No significant levels of messenger RNA for IGF-I have been detected in antler tissues (G. Ambler, *unpublished observation*; A. Hodges, *unpublished observation*), testosterone does not directly stimulate IGF-I or IGF-I receptor gene expression in the rat epiphyseal growth plate (Phillip, Palese, Hernandez *et al.*, 1992) and androgens have no effect on IGF-I binding to type 1 IGF receptors (Kasperk, Fitzsimmons, Strong *et al.*, 1990), so the effect of testosterone on IGF-I binding in antler tissues is most likely to be indirect. This is supported by a study in which androgens had no effect on IGF-I activity in bone cell cultures (Kasperk, Fitzsimmons, Strong *et al.*, 1990), implying that systemic input was required to achieve an effect. A recent study on hypogonadal men has shown that testosterone administration has the effect of increasing IGF-I serum levels and is involved in the modulation of the GH/IGF axis in adult men (Weissberger & Ho, 1993). Androgens

may mediate some effects on bone through increases in serum levels of IGF-I (Williams & Frolik, 1991), which has implications for the present study as elevated levels of IGF-I, arising through the systemic effects of administered testosterone, could cause down-regulation of type 1 IGF receptors.

In contrast to IGF-I, specific binding patterns of IGF-II did not change throughout the growth period or under the experimental treatment conditions in this study, indicating that type 2 IGF receptors are not affected by testosterone. This is not in agreement with results from studies carried out on bone cells *in vitro* where androgens enhanced the binding and mitogenic actions of IGF-II (Kasperk, Fitzsimmons, Strong *et al.*, 1990). However, these authors went on to say that an autocrine mechanism of TGF β , induced by the androgens, may mediate the increased responsiveness to IGF-II that was observed and that further studies were required. The results of the current study indicate that IGF-I and IGF-II bind to distinct receptor populations which respond to different regulatory controls and also suggest that IGF-II may have little to do with the tissue maturation activated by testosterone. Despite the ability of IGF-II to cross-bind to the type 1 IGF receptor, binding of IGF-II did not increase significantly in the reserve mesenchyme and perichondrium zone, indicating that IGF-II was binding to a distinct receptor population which was not affected by testosterone either directly or indirectly.

Although testosterone had an overall effect of decreasing IGF-I binding to type 1 IGF receptors in antler tissues, within the tissues there was an uneven response of IGF-I binding with testosterone administration. At 9-13 days after injection, binding in the reserve mesenchyme and perichondrium zone had increased relative to the other treated tissues, which had a tendency to decrease, perhaps indicating that testosterone had a slightly enhancing effect on IGF-I binding in this tissue. In the control group this was demonstrated at the 100 day stage when IGF-I binding in this zone showed a marked increase. Administration of a higher dose of testosterone to bucks would be required to examine this observation in antlers under the conditions of the current trial.

Actions of IGF-I and IGF-II appear to be specific to the maturational stage of the target cells (Sara & Hall, 1990). In the context of the current study, the receptor ontogeny of both of the IGFs is a probable reflection of their respective functions in which case the results indicate that IGF-I has a major role in late cartilage development and/or growth, but not in pre-cartilage or bone, and that IGF-II has a major role in pre-cartilage growth and/or differentiation. There is no evidence that these roles changed throughout antler growth, but the temporal spatial arrangement of the receptor population for IGF-I did change which probably reflected the change in temporal spatial arrangement of the target cells that occurred during tissue maturation. No differences in the spatial arrangement of the IGF-II receptor population were observed, probably because the pre-cartilage cells are located in the same place proximal to the antler tip throughout the growth period.

This study showed that testosterone enhanced deer antler maturation and ossification which would have the effect of inhibiting growth by arresting cell growth activities through advancing the mineralisation of the cell matrix. The dosage of testosterone which was administered, however, failed to cause mineralisation of cartilage tissue in the antler to the degree expected. This may be because testosterone is readily metabolised to different derivatives, each of which exert specific and distinct effects on target tissues, particularly the antler (Morris & Bubenik, 1983). The authors of a study in which different metabolites of testosterone were administered to mature castrated white-tailed deer bucks during antler growth surmised that the normal development and maturation of antler bone may require the participation of at least two different metabolites (Morris & Bubenik, 1983). Results from other studies support this as receptors for estradiol, which is effective at mineralising growing antlers when administered systemically with testosterone (Wislocki, Aub & Waldo, 1947), have been shown to be localised in antler tissues (Lewis & Barrell, 1991). In a recent report, the aromatization of testosterone to estradiol was shown to be partially responsible for modulating the GH/IGF axis in adult males (Weissberger & Ho, 1993) and estradiol modulation of GH receptors in steers has been implicated in somatic growth in

ruminants (Breier, Gluckman & Bass, 1987) and rats (Chen, Liu, Bates & Hintz, 1991). Estradiol also stimulates cell proliferation in cultures of human osteoblast-like cells and has a biphasic effect on human chondrocyte mitogenesis, where it is stimulatory at low concentrations (0.1 nmol/l) and inhibitory at a supraphysiological dose of 10 nmol/l (Saggese, Federico & Cinquanta, 1993). In adult male deer estradiol will effectively cause antler mineralisation (Morris & Bubenik, 1983; Barrell & Lewis, 1994) faster than testosterone (Morris & Bubenik, 1983). Durateston is a mixture of four testosterone esters which are designed to be sequentially degraded so as to provide elevated levels of circulating, biologically active, levels of testosterone for up to six weeks (Chemavet Division, Pharmaco (NZ) Ltd). The ability of testosterone to be metabolised into other forms is limited by esterification and, therefore, would have been restricted to some extent in this trial. In light of the current findings it seems that the metabolisation of testosterone to other forms may be required for full effectiveness and that testosterone alone does not stimulate a complete mineralisation and ossification response in antlers. This suggests that the elevated endogenous levels of testosterone in male deer during the late stages of antler growth, mineralisation and hardening, may form a reservoir of metabolisable testosterone rather than acting *per se*.

This study showed that IGF-I and IGF-II each bound to distinct receptor populations in the antler throughout growth with different tissue distribution patterns and regulatory controls. Increased endogenous levels of testosterone in control bucks during the late stages of antler growth may have given rise to the maturation of tissues that changed the temporal spatial arrangement of the type 1 IGF receptor population. Decreases in intensity of IGF-I receptor binding, caused by the administration of testosterone, could have arisen from systemic effects of testosterone on the GH/IGF axis. Testosterone had no effect on either the intensity or distribution of IGF-II receptor binding in antler tissues in the growth phase. The effect of enhanced cartilage maturation that was expected with testosterone treatment was only seen to a limited extent in this study as assessed by histological changes. These results have implications regarding distinct roles for the IGFs in developing cartilage and bone

tissues and also suggest that metabolites of testosterone, rather than testosterone itself, are more potent stimulators of antler tissue maturation.

CHAPTER 7

GENERAL DISCUSSION

7.1 GH/IGF AXIS IN CARTILAGE

In the epiphyseal growth plate, receptors for GH are localised to chondroprogenitor cells (cartilage stem cells) from early infancy to puberty, and are not detected after closure of the epiphyseal growth plate following puberty (Barnard, Haynes, Werther & Waters, 1988). Although GH has been shown to bind directly to receptors in epiphyseal growth plate chondrocytes in somatic cartilage (Isaksson, Jansson & Gause, 1982; Edén, Isaksson, Madsen & Friberg, 1983), no GH receptors were detected in tissues of the antler (Chapter 3). There is no role for GH in hematopoiesis as bone marrow is not formed in antlers (Chapman, 1975).

Receptors for IGF-I in the growing antler tip were relatively homogeneous with the properties typical of the type 1 IGF receptor (Chapter 3). This receptor population was present in all tissues of the antler and in very high concentrations in cartilaginous tissue, from which it is proposed that the role of IGF-I in antler cartilage is mediated by type 1 IGF receptors, and involved in the matrix development of differentiated chondrocytes. This hypothesis agrees with findings reported in the literature for somatic chondrocytic and osteoblastic cells which are shown to stimulate matrix synthesis in response to exogenous IGF-I (Hock, Centrella & Canalis, 1988; McCarthy, Centrella & Canalis, 1989; Williams & Frolik, 1991; Hill, Logan, McGarry & De Sousa, 1992; Ohlsson, Nilsson, Isaksson & Lindahl, 1992).

The production of extracellular matrix by cartilage is crucial in providing the foundation for mineralisation and endochondral ossification to occur. This is illustrated in an experiment where demineralised, mainly collagenous, rat long bone matrix was transplanted in powder form to subcutaneous sites in the thorax and abdomen of rats (Reddi & Anderson, 1976), causing condensation of apposing fibroblasts and differentiation to chondroblasts, then chondrocytes. The chondrocytes produced excessive matrix, involving considerable incorporation of ^{35}S into chondroitin sulphate, indicating that sulphation factor activity, which is a characteristic of IGF-I (Salmon & Daughaday, 1957), was occurring. Subsequently the chondrocytes hypertrophied, the matrix mineralised, and vascular invasion and endochondral ossification ensued, suggesting that extracellular matrix has an important role in cellular development and maturation, particularly for chondrocytes. The extracellular matrix also contains glycosaminoglycans (Banks & Newbrey, 1983b; Jackson, Busch & Cardin, 1991) which provide binding sites for the IGFBPs that may occur in the regulation of IGF action (McCusker, Camacho-Hubner, Bayne *et al.*, 1990; Clemmons, 1991; Conover, 1992).

The conclusion that the major role of IGF-I in antler cartilage is in matrixogenesis is based on the spatial arrangement of type 1 IGF receptors in this tissue, their coincidence with high levels of sulphated extracellular matrix components and the reported sulphation and matrixogenic effects of IGF-I (Salmon & Daughaday, 1957; Hill, 1979; Ohlsson, Nilsson, Isaksson & Lindahl, 1992). There is no evidence to exclude IGF-I also having a mitogenic role in the growth of this tissue as DNA synthesis is a well documented effect of IGF-I (Sara & Hall, 1990; Han & Hill, 1992) on chondrocytes (Hock, Centrella & Canalis, 1988; Ohlsson, Nilsson, Isaksson & Lindahl, 1989) and operates via mechanisms distinctly different from that of matrix synthesis (Hock, Centrella & Canalis, 1988).

A population of receptors with highest specificity for IGF-II were also detected and characterised in the antler. Receptors for IGF-II were present in all tissues but were distributed differently from those for IGF-I, and analysis showed that these receptors were

typical of type 2 IGF receptors (Chapter 4). The tissue region with the highest concentration of receptors for IGF-II was the reserve mesenchyme and perichondrium zone. This tissue is where the most rapid proliferation and differentiation occurs in the antler (Banks & Newbrey, 1983a; Banks & Newbrey, 1983b), therefore the major role of IGF-II in cartilage is probably stem cell proliferation and possibly also differentiation, mediated via the type 2 IGF receptor. Proliferation and differentiation often occur in conjunction with each other, making it difficult to distinguish between these processes in tissues comprising progenitor cells where they occur concurrently. Radiographic grains from ^{125}I -IGF-II microautoradiographs showed a distinctive pattern of clustering where some, but not all nuclei exhibited an intense localisation of receptors (Chapter 4). This observation favours IGF-II having mainly a proliferative role via type 2 IGF receptors in antler tissues. In various cell systems many reported actions of IGF-II, for instance cell replication (Sessions, Emler & Schalch, 1987), collagen synthesis (McCarthy, Centrella & Canalis, 1989) and anabolism (Beguinot, Kahn, Moses & Smith, 1985), are mediated via type 1 IGF receptors. However, Mohan, Linkhart, Rosenfeld & Baylink (1989) showed that cell proliferation in mouse bone cells and in rat hepatoma cells is inhibited in the presence of a type 2 IGF receptor antibody, and concede that at least part of the mitogenic action of IGF-II is mediated through type 2 rather than type 1 IGF receptors.

7.2 ENDOCRINE VERSUS AUTOCRINE/PARACRINE

IGF-I has been suggested as having a paracrine role in pre-pubertal bone growth and an endocrine role thereafter (Daughaday & Rotwein, 1989). Several studies have reported the expression of IGF-I and IGF-II mRNA in longitudinal bones (Isgaard, Möller, Isaksson *et al.*, 1988; Nilsson, Carlsson, Isgaard *et al.*, 1990; Shinar, Endo, Halperin *et al.*, 1993) where IGF-I mRNA was localised to regions of mineralisation and ossification and IGF-II mRNA was localised to growth zones. A study on the production of IGFs and their binding proteins by rabbit articular chondrocytes suggested that the cells stimulated their

own proliferation by the secretion of IGF-I (Froger-Gaillard, Hossenlopp, Adolphe & Binoux, 1989). In antlers, however, no mRNA for IGF-I or IGF-II have so far been detected (G. R. Ambler, *unpublished observation*; A. K. Hodges, *unpublished observation*), indicating an endocrine role for both IGFs in antler tissues. The absence of GH receptors in antler tissues (Chapter 3) supports the case for endocrine actions of IGF-I, and if GH exerts any direct effects on somatic cartilage growth other than stimulating the production of IGF-I, they would appear to be nonessential, as cartilage seems to be able to grow rapidly and normally in their absence, which appears to be the case with the antler. The endocrine actions of IGF-I on antler growth are probably regulated by GH action on the liver, the main source of circulating IGF-I (Schwander, Hauri, Zapf & Froesch, 1983) which does have receptors for GH (Chapter 3).

IGF-I and IGF-II exert differential effects on tissues, depending on the receptor constitution of the tissues and on the specificity of IGFBPs produced by the tissues for the IGFs (Binoux, Hossenlopp, Hardouin *et al.*, 1986; Kobayashi, Clemmons & Venkatachalam, 1991). IGFBPs are produced by many tissues and cell types (McCusker & Clemmons, 1988; Bar, Booth, Boes & Dake, 1989; Cheung, Smith, Shimasaki *et al.*, 1991; Camacho-Hubner, Busby, McCusker *et al.*, 1992) including antler tissues (Chapter 5). Results from competitive displacement experiments where unlabelled des-IGF-I, which has very low affinity for IGFBPs, successfully inhibited binding of radiolabelled IGF-I (Chapter 5) show that IGF-I does not bind to matrix-bound IGFBPs to a significant degree in antler cartilage tissue. In contrast, excess unlabelled des-IGF-I failed to completely competitively inhibit binding of IGF-I tracer in vascular tissue, hair follicles and epidermal tissue in the antler (Chapter 5), implicating the presence of matrix-bound IGFBPs in these locations. Although IGF binding was not quantified in these tissues, IGF receptors were also present in these structures, revealed by the partial inhibition of IGF-I tracer binding by des-IGF-I, and by the binding of des-IGF-I tracer exceeding the non-specific binding levels in these tissues. The role of the matrix-bound IGFBPs in these structures is a source of speculation at present, but may perhaps impart a

competitive advantage over neighbouring tissues in scavenging IGFs from extracellular fluids.

Type 2 IGF receptors exhibited unusual binding traits whereby enhancement of IGF-II tracer binding to antler sections occurred in the presence of high concentrations of unlabelled IGF-I and insulin. This 'paradoxical binding' (or 'hook') effect was probably attributable to IGFBPs released into the incubation media in the presence of peptide hormones having a facilitatory effect on receptor binding of IGF tracer. The presence of IGFBPs was demonstrated in antler tissue (Chapter 5) and could have the effect of increasing the concentration of IGFs, especially IGF-II, in the local tissue microenvironment, thereby increasing hormonal availability to receptors and eventuating enhanced receptor binding (Ooi, 1990; Conover, 1992) in a tissue specific manner (Ballard, Ross, Upton & Francis, 1988). IGFBPs are released from cells undergoing proliferation and differentiation (McCusker & Clemmons, 1988) and local tissue targeting of IGFs by IGFBPs can lead to the stimulation of DNA synthesis (Clemmons, 1991). In the reserve mesenchyme and perichondrium zone, IGFBP production is proportionately greater than in cartilage tissue (Chapter 5), and this zone has the highest concentration of type 2 IGF receptors than any other tissue (Chapter 4). IGF-II has a higher affinity for IGFBPs than IGF-I (Rechler, 1993) and recognises them to a far greater extent in antler tissue culture media (Chapter 5), implying that major functions of IGFBPs in antler tissues are in targeting IGF-II from the extracellular medium and/or facilitating the actions of IGF-II. Although IGF-I was seen to bind to some of the IGFBPs produced by antler tissues *in vitro*, especially in the reserve mesenchyme and perichondrium zone (Chapter 5), IGF-I binding to the IGFBPs is likely to be competitively inhibited *in vivo* by IGF-II which is present in greater concentrations.

In deer, circulating levels of IGF-II during antler growth are approximately 10 fold higher than the corresponding levels of IGF-I (Suttie, Fennessy, Corson *et al.*, 1989). If the antler is essentially under endocrine control it seems reasonable to assume that tissue levels of

the IGFs in the antler may reflect the concomitant plasma levels. Unlike antler cartilage, somatic cartilage is avascular (Barnard, Haynes & Werther, 1988), in which case access of nutrients and growth components to cells comprising cartilage relies largely upon diffusion processes. The antler, on the other hand, is extensively vascularised throughout growth with unrestricted cellular access to ready supplies of hormones occurring at all stages of growth, and the need to produce a local supply is obviated. In this way the energy that would have been expended in protein synthesis could be channelled into other areas of tissue growth, maximising the efficiency of energy use by the rapidly developing antler.

7.3 TESTOSTERONE AND IGF RECEPTOR ONTOGENY

Androgens stimulate bone formation and inhibit bone resorption (Williams & Frolik, 1991). Increased circulating levels of testosterone in male deer are associated with antler cartilage maturation and bone formation (Goss, 1969; Goss, Dinsmore, Grimes & Rosen, 1974; Chapman, 1975; Bubenik, 1983; Goss, 1983c; Morris & Bubenik, 1983; Muir, Sykes & Barrell, 1988; Bubenik, 1990; Williams & Frolik, 1991; Correa, Leite, Borelli & Wajchenberg, 1992). Testosterone is able to be converted to various different metabolites in the peripheral circulation and bone cell cultures, and the aromatization of testosterone to estradiol may be at least partly responsible for the mineralisation and ossification effects on antler tissues that are attributed to testosterone (Morris & Bubenik, 1983), and which estradiol has been shown to directly stimulate (Barrell & Lewis, 1994). Administration of testosterone esters, which are not able to be metabolised readily, yielded a limited maturation response in growing tissues of the antler (Chapter 6), implying that metabolites of testosterone, rather than testosterone itself, may be responsible for many of the perceived actions of testosterone in these tissues.

Aromatization of testosterone to estradiol may also be partially responsible for modulating the GH/IGF axis in males, as is suggested by results from a number of studies (Breier,

Gluckman & Bass, 1987; Weissberger & Ho, 1993). Testosterone administration increases serum levels of IGF-I (Williams & Frolik, 1991; Hobbs, Plymate, Rosen & Adler, 1993; Weissberger & Ho, 1993), which may have given rise to an indirect tissue maturation effect in antlers in response to exogenous testosterone administration (Chapter 6). Testosterone treatment had a net effect of significantly decreasing specific binding of IGF-I in antler cartilage and dermal tissues (Chapter 6). This may have been a systemic effect from increased serum levels of IGF-I arising from testosterone stimulation. Specific binding of IGF-II did not appear to be affected by testosterone, notwithstanding the ability of IGF-II to cross-bind to the type 1 IGF receptor.

Receptor ontogeny studies revealed that IGF-I and IGF-II bound to distinct receptor populations in antler tissues throughout the growth phase (Chapter 6). Type 1 IGF receptors underwent a distribution change in the mineralised and ossifying antler tip, whereas the type 2 IGF receptor population remained constant in distribution and intensity (Chapter 6). These results are consistent with the evolving functions of IGF-I and IGF-II in bone growth, and indicate that the actions of IGFs are specific to the maturational stage of the target cells (Sara & Hall, 1990). In the rat it is postulated that GH has a role in prechondrocyte differentiation, which is an initial stage of cellular maturation, whilst interacting with IGF-I at a later developmental stage (Nilsson, Isgaard, Lindahl *et al.*, 1986; Lindahl, Nilsson & Isaksson, 1987). Postnatal serum levels of IGF-II seem to vary according to species, and are negligible in the rat (Gluckman & Ambler, 1993). However, serum levels of IGF-II are comparatively higher than serum levels of IGF-I in male deer (Suttie, Fennessy, Corson *et al.*, 1989). Type 2 IGF receptors are present in relatively high concentrations in the prechondrocyte regions (reserve mesenchyme/perichondrium zone) of the antler (Chapter 4), whilst GH receptors are not (Chapter 3), which could possibly imply that in the prechondrocytic regions of the antler, IGF-II actions replace the actions of GH seen in the rat growth plate.

7.4 BONE GROWTH

The development, growth and metabolism of longitudinal bones are regulated by a complex system of interactions among endocrine hormones and autocrine/paracrine growth factors (Williams & Frolik, 1991), some of which are represented in Figure 1.3 (Chapter 1). Cartilage grows by clonal expansion initiated in cartilage precursor cells of perichondrial/mesenchymal origin (Ham & Cormack, 1979a; Geneser, 1986; Wheeler, Burkitt & Daniels, 1987a; Hill & Logan, 1992; Rosen & Thies, 1992), which interact with a progression of mutually regulating hormones and growth factors as the cells progress to maturity (Hill & Logan, 1992). Several growth factors, such as insulin-like growth factors, basic fibroblast growth factor, transforming growth factor- β , epidermal growth factor, platelet-derived growth factor and acidic fibroblast growth factor, can regulate the proliferation and/or differentiation of cartilage and/or bone cells *in vitro* (Hill & Milner, 1984; Rodan, Wesolowski, Thomas & Rodan, 1987; Hill & Han, 1991; Han & Hill, 1992; Hill & Logan, 1992; Jiang, Savona, Chambaz & Feige, 1992; Ohlsson, Isgaard, Törnell *et al.*, 1993). Platelet-derived growth factor and IGFs, acting in combination, are potent growth stimulators (Stiles, Capone, Sher *et al.*, 1979), and fibroblast growth factor, the IGFs and transforming growth factor- β interact to regulate the synthesis of extracellular matrix components in chondrocytes (Han & Hill, 1992; Hill & Logan, 1992). Endocrine factors, such as GH, androgens, estrogens, parathyroid hormone, calcitonin, vitamin A and vitamin D, are also implicated in the regulation of cartilage and bone metabolism (Williams & Frolik, 1991). Components of the GH/IGF axis have known effects on cartilage and bone growth and development (Isaksson, Jansson & Gause, 1982; Russell & Spencer, 1985; Isgaard, Nilsson, Lindahl *et al.*, 1986; Hock, Centrella & Canalis, 1988; Mohan & Baylink, 1991).

The results from the studies in this thesis provide evidence that mammalian cartilage and bone growth can occur without the direct involvement of GH, and suggest that growing antlers utilize IGFs in circulation rather than manufacture their own. Growth hormone in

this case would appear to be a misnomer as GH only has peripheral effects on antler growth, being a growth regulator in this tissue rather than having a direct role in antler growth. This has also been suggested in the case of somatic long bone cartilage where, although GH receptors and mRNA for IGF-I are present in the growth plate, the growth effects of GH are purported to be mediated exclusively by IGF-I (Schlechter, Russell, Spencer & Nicoll, 1986; Nilsson, Carlsson, Isgaard *et al.*, 1990). IGF-I and IGF-II bind to distinct receptor populations, localised to tissues in different maturational stages in the antler tip, which have different regulatory controls and ontogeneticity. The production of IGFBPs *in vitro* by antler tissues has implications for the modulation of IGF receptor binding and activity, especially for IGF-II, at the local tissue level. Maturation of antler tissues evoked a change in IGF-I receptor distribution, which was only partially simulated by the administration of exogenous testosterone at the dosage used, implying that testosterone metabolites may be the main potentiators of maturation effects. Extrapolation of the findings in the antler growth model to other bony tissues, such as the epiphyseal growth plate, is applicable as both share a similar histochemistry (Banks & Newbrey, 1983a), although there seem to be some differences in cellular organisation and receptor constitution. Antler tissue affords a unique opportunity to look at the purely endocrine effects of the IGFs on cartilage and bone growth and development.

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APPENDIX

PUBLICATIONS ARISING FROM THIS THESIS

Paper I

Presence of Insulin-Like Growth Factor-I Receptors and Absence of Growth Hormone Receptors in the Antler Tip

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ABSTRACT. Red deer antler tips in the growing phase were removed 60 days after the recommencement of growth for autoradiographical studies and RRAs. Sections were incubated with radiolabeled GH or insulin-like growth factor-I (IGF-I), with or without excess competing unlabeled hormones, and were analyzed autoradiographically. There was negligible binding of [¹²⁵I]GH in any histological zone of antler sections. [¹²⁵I]IGF-I showed highest specific binding in the chondroblast zone to a receptor demonstrating binding characteristics of the type 1 IGF receptor. The lowest specific binding of [¹²⁵I]IGF-I was to pre-

chondroblasts. RRAs on antler microsomal membrane preparations confirmed the absence of GH receptors and the presence of type 1 IGF receptors found by autoradiography. These findings suggest that IGF-I may act in an endocrine manner in antler growth through a receptor resembling the type 1 IGF receptor. The presence of type 1 receptors in the chondroblast zone implicates IGF-I involvement in cartilage formation through matrixogenesis. There is no support for IGF-I having a major role in mitosis in the antler. (*Endocrinology* 130: 2513-2520, 1992)

LOCAL administration of GH or insulin-like growth factor-I (IGF-I) directly to the tibia of hypophysectomized rats has been reported to stimulate unilateral bone growth (1-4). Both GH and IGF-I, directly and independently, bind to epiphyseal chondrocytes *in vivo*, depending on the stage of differentiation/maturation of the cells (5). Maor *et al.* (6) have demonstrated a stimulatory effect of GH on cartilage progenitor cell proliferation, and Barnard *et al.* (7) showed that prechondrocytes and differentiating chondrocytes have a high number of GH receptors in the rabbit tibial growth plate.

In bovine growth plates, proliferative and maturing chondrocytes have a high IGF-I receptor concentration (8), whereas chondrocytes of the proliferative and hypertrophic regions in porcine epiphyseal growth plates are immunoreactive for IGF-I (9). IGF-I stimulates cartilage proteoglycan synthesis *in vitro* (10), and transplanted collagenous matrix components stimulate the formation of new bone (11), suggesting that extracellular matrix can have an important role in cellular development and maturation, particularly for chondroblasts.

Antler growth is very rapid, and the growing tip is composed of fibroblasts which divide and differentiate into chondroblasts and eventually osteoblasts (12-14).

During antler growth, IGF-I concentrations in plasma are high (15), and positive correlations have been found between plasma IGF-I, live weight gain, and antler growth rate in red deer (16).

Administration of GH *in vivo* stimulates the production of IGF-I in various tissues of hypophysectomized rats (17, 18). There is also evidence that IGF-I may be produced in the epiphyseal growth plate of rats (19, 20). Messenger RNA for IGF-I has been detected in chondrocytes of the proliferative, hypertrophic, and early degenerative zones and was GH dependent.

It has been speculated that IGF-I is not locally produced in antler tissue but acts in an endocrine way on antler growth. The objective of the present study was to examine GH and IGF-I binding to receptors in the growing tip of the antler.

Materials and Methods

Autoradiographical studies

Materials. Antler tips in the growth phase were collected from seven adult red deer (*Cervus elaphus*) stags under conditions approved by the Invermay Animal Ethics Committee. The animals were restrained with a hydraulic crush, and local anaesthetic (Lignocaine, Astra Pharmaceuticals, Auckland, New Zealand) was administered as a ring block around the pedicle. Collection occurred 60 days after casting of the previous hard antler. The antler tips were frozen in liquid nitrogen

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within 1 min of removal and stored at -70°C .

IGF-I was purified from sheep plasma [ovine IGF-I (oIGF-I)]; it was homogeneous on reverse-phase HPLC and contained less than 0.01% oIGF-II (21). Ovine IGF-II was also purified from sheep plasma and contained less than 0.01% IGF-I (21). Ovine GH was obtained from NIDDK (Baltimore, MD); bovine insulin and oPRL were from Sigma Chemical Co. (St. Louis, MO). Ovine IGF-I and oGH were iodinated using the Iodogen method (22), as previously used for oIGF-I (23) and oGH (24).

Time equilibrium. In order to gauge the effect of time on tracer binding, 48 frozen sections from one antler were cut and mounted onto nongelatinized coverslips which were glued to slides (Ados F2 adhesive, Ados Chemical Co., Petone, New Zealand). They were incubated as described below with [^{125}I] IGF-I in the presence or absence of unlabeled IGF-I for varying times. Four pairs of sections were incubated for each nominated length of time. After exposure to X-Omat AR5 film (Eastman Kodak, Rochester, NY), the coverslips were removed and γ -counted. Equilibrium of binding was stable from 0.5–2 h of incubation, and nonspecific binding was negligible until 4 h. In light of these trials a 2-h incubation was considered optimal for use in the subsequent incubations.

Incubations. For each animal, 36 cryosections ($8\text{ }\mu\text{m}$ thick) were cut at -18 to -20°C and thaw-mounted in pairs onto 18 gelatine-coated microscope slides. Sections were stored at -20°C and incubated within 1 week of being cut.

In vitro incubations were carried out using a modification of the method of Maurer and Reubi (25). Slides were thawed and sections preincubated with 0.17 M Tris-HCl buffer (pH 7.4) for 10 min at room temperature. The preincubation buffer was replaced with 50 μl incubation buffer which comprised 0.17 M Tris-HCl (pH 7.4), 1% BSA (Sigma Chemical Co.), 5 mM MgCl_2 , and 1 mM phenylmethylsulfonyl fluoride, together with iodinated hormone (20,000 cpm) and unlabeled peptide where appropriate. The incubation proceeded at room temperature in a humidified slide chamber, after which the incubation buffer was aspirated and slides washed twice for 5 min in baths of cold 0.17 M Tris-HCl (pH 7.4), with 0.25% BSA and 0.01% Triton X-100. They were then fixed in neutral buffered formalin for 10 min, washed in distilled water for 5 min and air-dried. Slides were also incubated without iodinated ligand as controls for background grain counts, chemography, and latent image fading of the autoradiographs (26). For the incubations, oIGF-I at 1 $\mu\text{g/ml}$, oIGF-II at 1 $\mu\text{g/ml}$, and bovine insulin at 10 ng/ml and 10 $\mu\text{g/ml}$ were used to compete for [^{125}I]oIGF-I binding sites. Unlabeled oGH at 1 $\mu\text{g/ml}$ and unlabeled oPRL at 1 $\mu\text{g/ml}$ were used to compete for [^{125}I]oGH binding sites. One section of each pair was used for total binding by incubation with radiolabeled hormone alone, whereas the other was incubated with the labeled hormone and a competing unlabeled hormone. Three pairs of sections from each animal were incubated for each combination of labeled hormone and unlabeled hormone.

Autoradiography. The incubated slides were exposed against X-Omat AR5 film for 3 days and the developed film used to estimate the exposure period for the sections while under emulsion. LM1 emulsion (Amersham International, Buckingham-

shire, UK) was applied to the sections under safelight conditions, and these were dried overnight with silica dessicant at room temperature before being exposed for 14 days at 4°C . At the conclusion of the exposure period the slides were equilibrated to room temperature and developed with D-19 developer (Eastman Kodak Company) then fixed with Fixaplast x-ray fixer (May and Baker, Lower Hutt, New Zealand). The processing times and temperatures were in accordance with the manufacturer's recommendations. The resulting autoradiographs were washed in running tap water for 30 min, rinsed for 5 min in distilled deionized water, then stained with Ehrlich's hematoxylin and alcoholic eosin and mounted. In order to distinguish between the binding of IGF-I to prechondroblasts and chondroblasts, five slides were prepared as described above and subjected to an *in vitro* incubation of [^{125}I]IGF-I in the absence or presence of unlabeled IGF-I. After exposure to X-Omat AR5 film and NTB-2 emulsion (Eastman Kodak Company) and development, the sections were stained with toluidine blue, and the radiographic grains counted and statistically analyzed.

Grain counting and statistical analysis. Sections from two representative animals were used for the counting of radiographic grains. For each section, grain counts were taken from five histological regions. Twelve sites were counted for the cartilaginous zone and the reserve mesenchyme, and six sites each for the epidermis, dermis, and perichondrium. Fewer sites were taken from the latter tissues to avoid site duplication as they were much smaller components of the section. Hair follicles and sebaceous glands in the dermis and blood vessels in the perichondrium were excluded from grain counting. Randomized coordinates of the microscope stage were used to select sites for counting. Grain counts were taken from areas corresponding to $20 \times 20\text{ }\mu\text{m}^2$ of tissue. Data were statistically analyzed using analysis of variance. Comparisons were then made between total and nonspecific binding to sections, specific binding for each animal, different tissue types within sections, and the extent of binding with competing unlabeled hormones. The binding which remained after radiolabeled hormones were incubated with an excess of the unlabeled homologous hormone was called nonspecific binding, and that remaining after incubation with heterologous hormones was termed residual binding.

Incubations for competitive displacement. For one antler tip, 68 sections were cut and mounted onto nongelatinized coverslips glued to slides. An *in vitro* incubation using [^{125}I]IGF-I was carried out but with oIGF-I (10 ng/ml to 100 $\mu\text{g/ml}$), oIGF-II (10 ng/ml to 50 $\mu\text{g/ml}$), and insulin (10 ng/ml to 500 $\mu\text{g/ml}$) as competitors for binding sites. Two pairs of sections were incubated for each concentration of unlabeled hormone. After exposure to X-Omat AR5 film, the coverslips were removed from the slides and γ -counted. The whole incubation was repeated twice more ($n = 3$) and the counts analyzed using the LIGAND-PC program (Biosoft, Cambridge, UK; adapted to be IBM-compatible).

Receptor analysis

Microsomal membrane preparation. Antler tissue from three animals was thawed at 4°C , the skin and fur layer removed,

and the remaining cartilaginous portion cut into small pieces (0.5–1 g) and washed in cold (4 °C) 0.3 M sucrose. The tissue was then weighed and homogenized (1:3 wt/vol) in 0.3 M sucrose containing 1000 Kallikrein inhibitor units (KIU)/ml Trasylol (Bayer Pharmaceuticals, Botany, New South Wales, Australia). Homogenization was performed with an Ultra-Turrax homogenizer (Janke and Kunkal, Staufen, Germany) for 4 min at high speed. The flask was kept in an ice bath or at 4 °C during all steps. The homogenate was centrifuged at $1,500 \times g$ for 20 min and the supernatant obtained was recentrifuged at $15,000 \times g$ for 20 min and this supernatant centrifuged at $100,000 \times g$ for 90 min. The resulting pellet was incubated with 3.5 M $MgCl_2$ (mixed by gentle homogenization) at a ratio of 1:2 initial antler wt/vol for 20 min. The mixture was then centrifuged at $100,000 \times g$ for a further 90 min, the pellet washed with cold buffer containing 0.025 M Tris (pH 7.6) and 0.01 M $CaCl_2$, and centrifuged at $40,000 \times g$ for 60 min. The final pellet was re-suspended in buffer containing 0.025 M Tris (pH 7.6), 0.01 M $CaCl_2$, and 1000 KIU/ml Trasylol at a ratio of 1 ml/g initial tissue wt. These microsomal membranes were stored at $-20^\circ C$ until RRAs were performed.

RRAs. RRAs were performed for the GH receptor in antler [using bovine GH (bGH) as the labeled and unlabeled hormone], and for the IGF type 1 receptor [using recombinant human IGF-I (rhIGF-I)] in antler. Recombinant bGH was obtained from American Cyanamid Co. (Princeton, NJ) and rhIGF-I from Ciba Geigy Ltd. (Basel, Switzerland).

Bovine GH was iodinated using lactoperoxidase (27) and the radiolabeled hormone separated by Sephadex G-100 chromatography using 0.05 M barbitone (pH 8.6) as the eluent. Recombinant hIGF-I was iodinated with chloramine-T (28) and the radiolabeled hormone separated by Sephadex G-50 chromatography using 0.01 M PBS (pH 6.2) containing 0.1% (wt/vol) gelatin as the eluent. The iodinated hormones were used in RRAs within 4 days of iodination.

Initially, specific binding of [^{125}I]bGH to a range of concentrations of microsomal membrane was assessed. Assays were performed in triplicate using membrane volumes ranging from 5–300 μ l incubated with [^{125}I]bGH (~30,000 cpm) in a total incubation volume of 0.5 ml. The buffer used for the IGF-I RRAs was 0.025 M Tris (pH 7.6), 0.2% BSA, 0.02% sodium azide and 1000 KIU/ml Trasylol. For the GH RRAs this buffer also contained 0.01 M $CaCl_2$. Nonspecific binding was assessed using tubes containing excess unlabeled hormone (1000 ng/tube for bGH and 20 ng/tube for rhIGF-I). Incubation was for 20 h at room temperature and was terminated by the addition of 2 ml ice-cold buffer. Bound and free hormone were separated by centrifugation at $3000 \times g$ for 45 min at 4 °C.

The time course of [^{125}I]rhIGF-I binding to the membranes was also assessed for binding in antler (data not shown). The binding was shown to be reversible by the addition of excess unlabeled hormone (20 ng/tube rhIGF-I).

A series of competitive binding assays were performed for [^{125}I]IGF-I binding. The receptor preparation (40 μ l/tube) was incubated with [^{125}I]rhIGF-I and various concentrations of unlabeled rhIGF-I (0–500 ng/tube), oIGF-II (0–500 ng/tube), and insulin (0–1000 ng/tube). Assay conditions were otherwise as described above.

The binding data were analyzed using the LIGAND program (Version 3.0, Biosoft). As for Scatchard analysis (29), this uses weighted nonlinear iterative curve-fitting procedures and allows statistical comparison of a one- vs. two-site model. Estimates were obtained for receptor capacity and affinity constants.

Results

Autoradiography

GH binding. Both the XAR and emulsion autoradiographs indicated negligible binding of GH tracer. Grain counting confirmed the lack of specific binding of GH to any of the tissues of the antler (Table 1).

IGF-I binding. The XAR autoradiographs for all seven antler tips were qualitatively assessed and showed IGF-I binding in each histological zone (Fig. 1). On total binding sections, the epidermis, cartilaginous zone, and dermis were all heavily labeled, whereas the perichondrium and the reserve mesenchyme showed faint signals. The dermis had high levels of localized binding to sebaceous glands and hair follicles, and the perichondrium and reserve mesenchyme had high levels of binding to blood vessel walls. These structures were not included in grain counting. Nonspecific binding was greater to the epidermis than the other regions.

Most of the binding of IGF-I tracer occurred in the epidermis and cartilaginous zone. Differential binding of [^{125}I]IGF-I to bands within the cartilaginous zone, which were morphologically indistinguishable in hematoxylin- and eosin-stained sections, was resolved by staining with toluidine blue. Binding in the cartilaginous zone was then reanalyzed, and the greatest level of binding was found to be to chondroblasts (Fig. 2).

Grain counts of [^{125}I]IGF-I binding were not significantly different between animals. Differences in binding did occur between the total binding and nonspecific binding sections ($P < 0.001$), between the different treatments with unlabeled hormones ($P < 0.001$), and between the histological zones within a section ($P < 0.05$) (Table 2).

TABLE 1. Grain counts of [^{125}I]GH binding to receptors in different tissues of the antler tip

Histological zone	Total binding	Nonspecific binding	Pooled SED	Specific binding
Epidermis	18	23	1.2	0 (NS)
Dermis	9	9	1.2	0 (NS)
Reserve mesenchyme	6	10	1.2	0 (NS)
Perichondrium	5	6	1.2	0 (NS)
Cartilaginous zone	8	7	1.2	1 (NS)

Counts were made from autoradiographs of antler tip sections from two animals ($n = 2$), in areas of $20 \times 20 \mu m^2$, under a light microscope. Specific binding was calculated as total binding minus nonspecific. NS, No significant binding; SED, standard error of the difference.

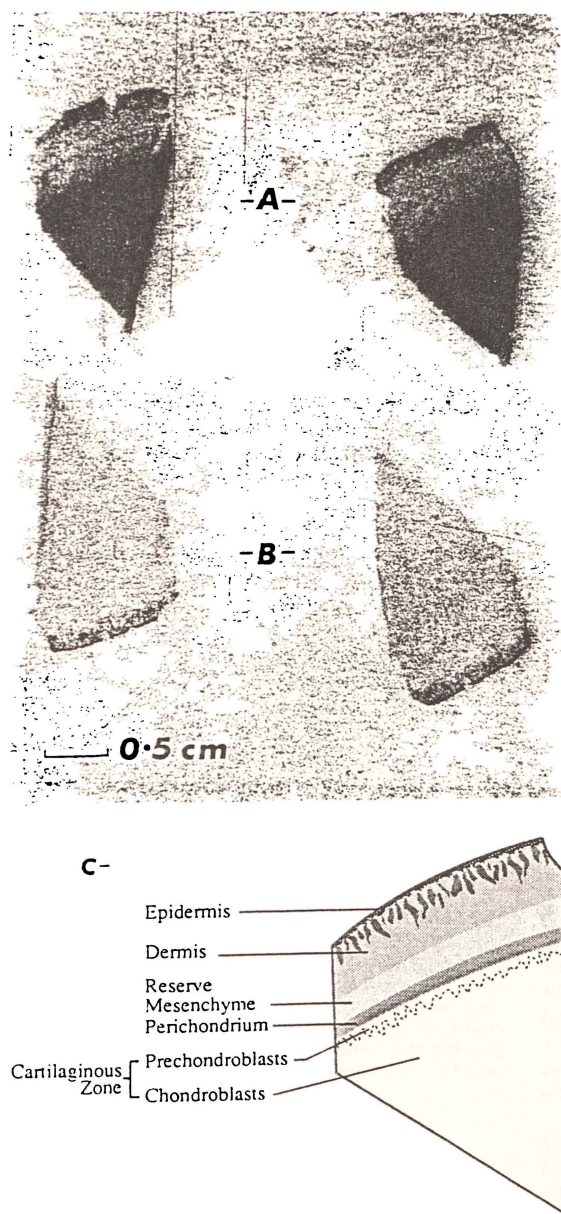


FIG. 1. XAR autoradiographs from one representative antler tip ($n = 7$), showing qualitatively the extent of binding of [^{125}I]IGF-I to each histological region. After incubation with tracer $\pm$ excess unlabeled IGF-I, the sections were fixed and exposed against XAR film for four days, then developed. A, Total binding of IGF-I; B, nonspecific binding; C, diagram of an antler tip section showing the positions of the histological regions.

The cross-binding of hormones to receptors has been used to identify type 1 and type 2 receptors for IGFs based on their relative affinities for IGF-I, IGF-II, and insulin (30). The affinity curve for IGF-I receptors, obtained from the competitive displacement of labeled hormone in whole antler sections, showed the IGF-I binding sites to be typical of type 1 IGF receptors with a high affinity for IGF-I, less affinity for IGF-II, and less again for insulin (Fig. 3). These results agree with those obtained by receptor analysis using microsomal membrane preparations. Analysis of the grain counts obtained from each histological zone of sections also agrees with this trend (Table 2), however, the binding sites of the epidermis display a pattern of sensitivity to competitive displacement by unlabeled hormones, which is different to the other zones. These zones exhibit statistically similar patterns of competitive displacement.

Membrane receptor analysis

GH receptors. GH RRA revealed specific binding to antler microsomal membranes of less than 1% at all membrane concentrations examined (Fig. 4). Deer liver was used as a control for the assay system with specific binding of 30% being observed at 100 μl /tube of membrane preparation.

IGF-I receptors. Specific binding of [^{125}I]IGF-I plateaued at a membrane volume of approximately 100 μl /tube (Fig. 4) and was observed to be 17.6% at 100 μl /tube of membrane preparation. Cross-reactivity studies demonstrated the classical properties of the type 1 IGF receptor. IGF-I bound with 18 times the affinity of oIGF-II and approximately 1400 times the affinity of insulin (Fig. 5).

Scatchard analysis (LIGAND) revealed an affinity constant of 2.24×10^{10} liters/mol for the interaction of IGF-I with the IGF-I receptor, a value similar to the affinity constant previously obtained for IGF-I receptors and IGF-binding proteins (31, 32), and a receptor capacity of 15.8 pmol/100 mg initial tissue wt (Fig. 6). A tendency to curvilinearity was observed in the Scatchard plot, but LIGAND analysis showed no improvement using a two-site rather than a one-site model.

Discussion

This study has demonstrated the presence of IGF-I receptors in the antler tip, and the absence of GH receptors. From competitive displacement studies, RRAs, and Scatchard analysis it was apparent that the IGF-I tracer was bound to a relatively homogeneous binding site with the properties of a type 1 receptor in the dermis, reserve mesenchyme, perichondrium, and cartilaginous zone. As IGF-I binding to IGF binding proteins cannot be displaced by high concentrations of insulin (33) the ability of insulin to completely inhibit IGF-I tracer binding at

FIG. 2. Specific binding of IGF-I tracer to each histological zone of the antler. After *in vitro* incubation with [125 I]IGF-I as the tracer and autoradiography, sections were stained with toluidine blue. After grain counting, specific binding was calculated as the difference between total and nonspecific binding. The values were calculated as percentages of the differences in means of grain counts; errors shown are SEM.

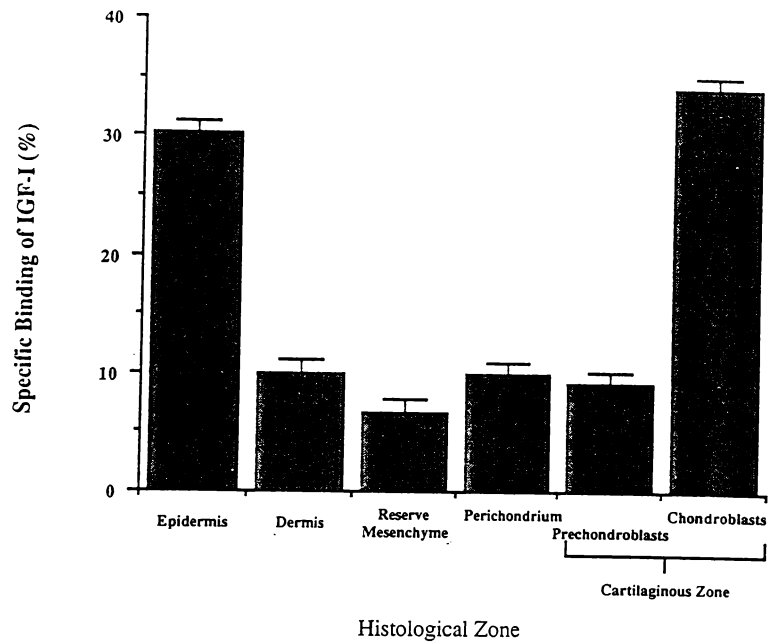


TABLE 2. Specific binding of [125 I]IGF-I in different tissues of the antler tip

Histological zone	Specific binding	Specific binding after treatment			
		IGF-II (1 μ g/ml)	Insulin (10 μ g/ml)	Insulin (10 ng/ml)	SEM
Epidermis	58 ^a	30 ^b (52)	3 (NS)	12 (NS)	9.2
Dermis	20 ^a	17 ^a (85)	9 (45)	2 (NS)	3.5
Reserve mesenchyme	14 ^a	15 ^a (100)	4 ^c (29)	4 (NS)	2.1
Perichondrium	20 ^a	17 ^a (85)	6 (NS)	0 (NS)	3.5
Cartilaginous zone	32 ^a	26 ^a (81)	15 ^a (47)	-5 (NS)	4.2

Counts from autoradiographs of antler tip sections from two animals ($n = 2$), in areas of $20 \times 20 \mu\text{m}^2$ under a light microscope. Specific binding was calculated as differences (NS, No significant difference) between total binding and nonspecific or residual binding after incubation in the presence of various unlabeled hormones in excess. Values shown are means; values in parentheses are percentages denoting sensitivity of [125 I]IGF-I to competitive displacement of heterologous hormones compared with specific binding (100%).

^a $P < 0.001$.

^b $P < 0.01$.

^c $P < 0.05$.

high concentrations in these zones suggested that the binding sites were actual receptors with minimal IGF binding protein participation. The IGF-I binding sites in the epidermis showed less sensitivity to IGF-II than any of the other zones and no sensitivity to insulin. Reasons for this could include the tracer binding to a receptor with different affinity, or a heterogeneous receptor population, or even to IGF binding proteins. These would

not have been detected in the affinity analysis using autoradiography of entire antler sections, as the epidermis comprised only a minute fraction of the whole.

Autoradiography has shown the number of available receptors in each tissue type of the antler to be different, with large numbers in the epidermis and cartilaginous zone. In contrast, the lowest levels of specific binding of [125 I]IGF-I were measured in the dermis, reserve mesenchyme, and perichondrium. However, because of their heterogeneous composition, the dermis, reserve mesenchyme, and perichondrium contained isolated areas of high binding. Sebaceous glands and hair follicles in the dermis showed high total and nonspecific IGF-I tracer binding. The blood vessel walls in the perichondrium were densely labeled with [125 I]IGF-I, which is in agreement with the specific binding of IGF-I to blood vessel endothelial cells demonstrated by others (34-36).

The reserve mesenchyme is characterized by high mitotic activity (13), and both the reserve mesenchyme and the perichondrium contain areas of hyperplasia (13, 14). Although these zones are the sites of the most vigorous proliferation in the antler, our results show that the reserve mesenchyme and perichondrium (the most undifferentiated tissue and the most rapidly differentiating tissue, respectively) have the lowest specific binding of [125 I]IGF-I.

The highest level of specific binding of IGF-I was found to be to the chondroblasts of the cartilaginous zone. This

FIG. 3. Competitive displacement of IGF-I binding to whole antler sections ($n = 3$) after incubation with [125 I]IGF-I and various concentrations of unlabeled IGF-I, IGF-II, and insulin.

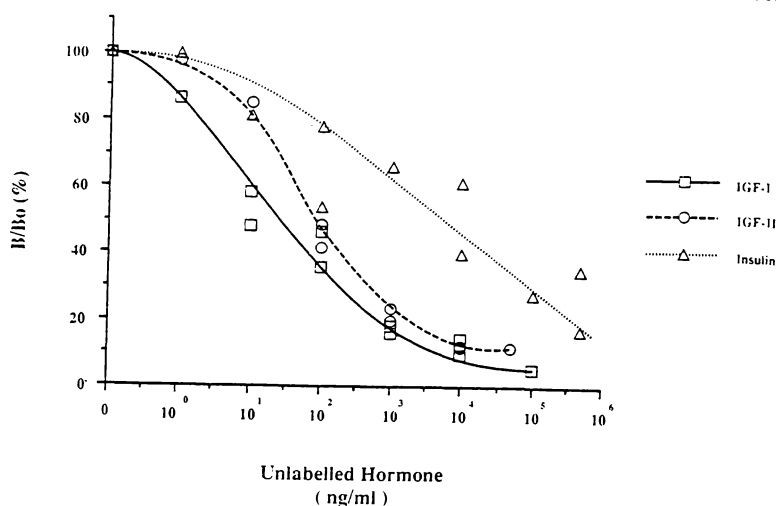
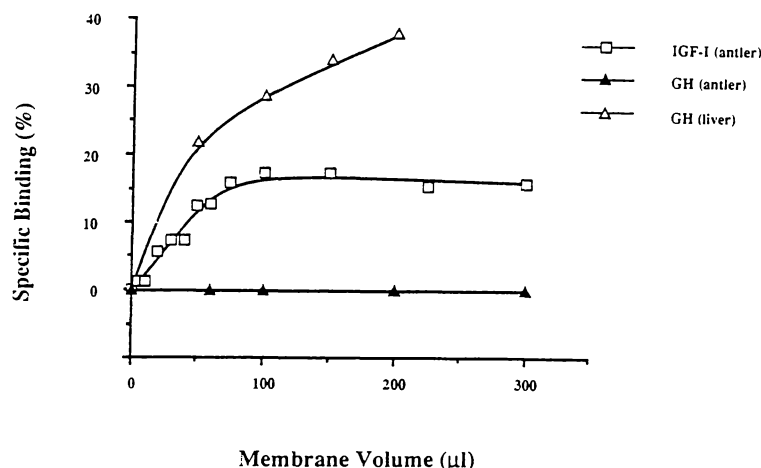


FIG. 4. Specific binding of radiolabeled IGF-I or GH to a range of volumes of microsomal membrane preparation of antler ($n = 3$) in the incubation mixture. [125 I]GH binding to a microsomal membrane preparation of normal deer liver was carried out as a control for [125 I]GH binding to the antler.



binding correlates directly with the presence of a highly concentrated extracellular substance of the nature of a sulfated glycosaminoglycan, probably chondroitin sulfate (Young, B., personal communication), and occurs as an abrupt increase with the maturation of prechondroblasts to chondroblasts. This chondroblast maturation and increased matrix production through proteoglycan formation involves the sulfation of glycosaminoglycans and collagen fibril accumulation. The sulfation stimulatory effect of IGF-I is well known and forms the historical basis for IGF research (10), and IGF-I has been shown to increase proteoglycan synthesis in bovine articular cartilage (37). This evidence suggests that IGF-I is probably instrumental in the development of cartilaginous matrix in the antler, exhibiting its sulfation factor activ-

ity, rather than having a role in mitosis. Thus the antler growth-stimulating effects of IGF-I suggested from correlations between antler growth rate and plasma levels of IGF-I would seem to operate by increasing the collagenous matrix rather than stimulating cell division.

From the results of an experiment in which red deer stags were surgically prevented from growing antlers, it was observed that peripheral plasma IGF-I concentrations during velvet antler growth were elevated by up to 1.5 times those of normal antlered stags (38). It was suggested that IGF-I was not locally produced in the antler, and this is supported by the lack of GH receptors found in this study, both by autoradiography and RRA. Furthermore, *in situ* hybridization studies have so far failed to detect significant levels of messenger RNA for

FIG. 5. Displacement of [125 I] IGF-I binding to microsomal membrane preparations by various concentrations of unlabeled IGF-I, IGF-II, and insulin. The membrane preparations were of antler tip material ($n = 3$) and did not include the epidermis and dermis.

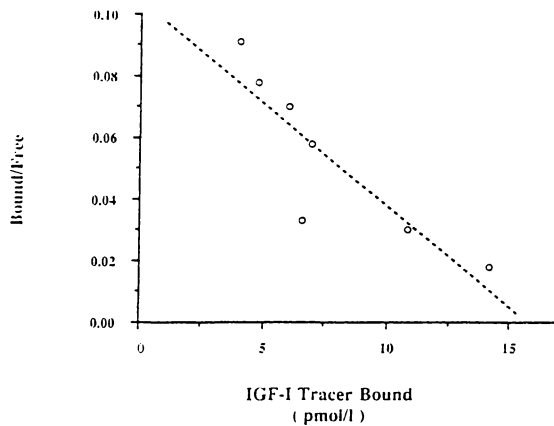
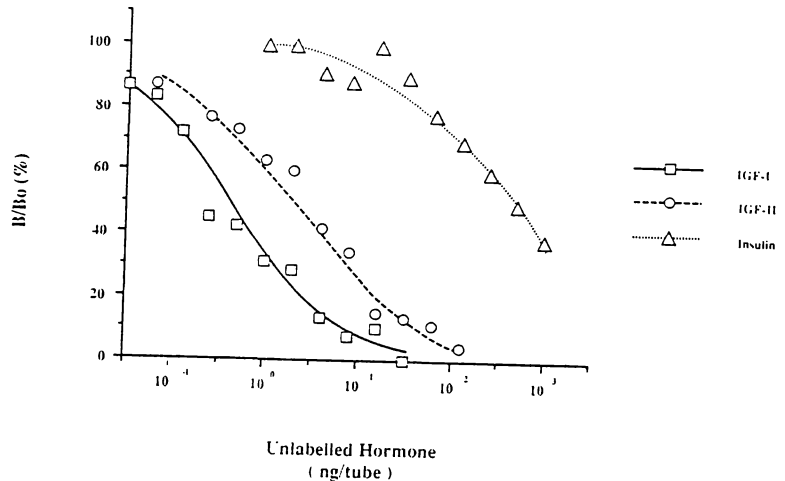


FIG. 6. Scatchard plot of the interaction of [125 I]IGF-I with the type 1 IGF receptor in antler microsomal membrane preparation.

IGF-I in the antler (Hodges, A., personal communication).

These results permit the interesting speculation that the putative type 1 receptor may have a function in mediating the effects of IGF-I in matrixogenesis in the cartilage of the antler. However, the role of the IGF-I binding site in the essentially noncartilaginous tissue of the antler (epidermis) is not clear; this site displays a different affinity pattern to the cartilaginous tissue and presumably has a different functional role.

This study has demonstrated the binding pattern of [125 I]IGF-I in different histological zones of the antler growing tip, and defined the nature of the binding site. The specific binding pattern of [125 I]IGF-I, which showed

the majority of the tracer binding to chondroblasts, suggests that IGF-I has a more important role in chondroblast matrixogenesis than in hyperplasia. The binding was to a receptor demonstrating type 1 IGF receptor characteristics rather than to IGF binding proteins. The absence of GH receptors in the antler tip suggests that IGF-I may have an endocrine role in antler growth.

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Paper II

Receptors for insulin-like growth factor-II in the growing tip of the deer antler

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ABSTRACT

Insulin-like growth factor-II (IGF-II) binding in the growing tip of the deer antler was examined using autoradiographical studies, radioreceptor assays and affinity cross-linking studies. Antler tips from red deer stags were removed 60 days after the commencement of growth, and cryogenically cut into sections. Sections were incubated with radiolabelled IGF-II, with or without an excess of competing unlabelled IGF-II and analysed autoradiographically. Radiolabelled IGF-II showed high specific binding in the reserve mesenchyme and perichondrium zones, which are tissues undergoing rapid differentiation and cell division in the antler. Binding to all other structural zones was low and significantly ($P < 0.001$) less than binding to the reserve mesenchyme/perichondrium zones. Radioreceptor assays on antler microsomal membrane preparations revealed that the IGF-II binding

was to a relatively homogeneous receptor population ($K_d = 1.3 \times 10^{-10}$ mol/l) with characteristics that were not entirely consistent with those normally attributed to the type 2 IGF receptor. Tracer binding was partly displaceable by IGF-I and insulin at concentrations above 10 nmol/l. However, affinity cross-linking studies revealed a single band migrating at 220 kDa under non-reducing conditions, indicative of the type 2 IGF receptor. These results indicate that, in antler tip tissues, IGF-II binds to sites which have different binding patterns and properties from receptors binding IGF-I. This may have functional significance as it appears that, whilst IGF-I has a role in matrix development of cartilage, IGF-II may have a role in the most rapidly differentiating and proliferating tissues of the antler.

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INTRODUCTION

The histochemistry of the deer antler is similar to that of the epiphyseal growth plate in long bones (Goss, 1983). The growing antler tip is composed of fibroblasts which divide and differentiate into chondroblasts and eventually osteoblasts (Wislocki, 1942; Banks & Newbrey, 1983a,b). In the growth plate, cartilage growth is under the control of insulin-like growth factors (IGFs) (Daughaday & Rotwein, 1989). IGF-I has also been implicated in the growth of the antler (Suttie *et al.* 1985; Elliott *et al.* 1992).

Cell surface receptors, which are required to mediate the physiological actions of IGFs, have been characterized and are reviewed elsewhere (Sara & Hall, 1990). IGF-I and IGF-II bind preferentially to the type 1 and type 2 IGF receptors respectively, but because of receptor cross-binding it is possible that interaction of IGF-II with the type 1 or insulin receptors may account for many of the actions of IGF-II (Rechler & Nissley, 1985; Underwood & Van Wyk, 1985; Trippel *et al.* 1988). Binding competition studies on bovine (Trippel *et al.* 1988) and rabbit (Jansen *et al.* 1989) chondrocyte membranes have

shown that there are separate receptor sites for both IGF-I and IGF-II. IGFs also bind to a range of carrier proteins but the physiological role of these IGF-binding proteins (IGFBPs) is not clear (Baxter, 1991). The IGFBPs may play a role in modulating or regulating the action of IGFs in bone (Baxter & Martin, 1989; Sara & Hall, 1990) as bone cells have been shown to produce IGFBPs (Mohan *et al.* 1989a; Schmid *et al.* 1989).

The actions of IGF-I and IGF-II appear to be many and varied, and in many cases seem to overlap. It has been suggested that IGF-II is an important factor in the growth and differentiation of mesenchymal cells (Moses *et al.* 1980; Nagarajan *et al.* 1985), and both IGFs have been implicated in mitogenesis (Mathieu *et al.* 1990), DNA synthesis (Sessions *et al.* 1987) and protein synthesis (McCarthy *et al.* 1989). These roles have been well reviewed (Froesch *et al.* 1985; Sara & Hall, 1990).

We have previously demonstrated the presence of high concentrations of receptors for IGF-I in the chondroblast zone of the antler tip but relatively few receptors in tissues where excessive proliferation and rapid differentiation are occurring (Elliott *et al.* 1992). As this IGF-I binding was seen to be localized chiefly to the extracellular matrix of cells in the chondroblast zone we surmised that IGF-I was more likely to have a role in matrixogenesis than in controlling cell division. The aims of this study were to examine IGF-II binding to receptors in the growing trip of the antler and to compare their spatial relationship to receptors binding IGF-I.

MATERIALS AND METHODS

Autoradiographical studies

Materials

Antler tips (the distal 2.5 cm where rapid growth was occurring) were collected from nine adult red deer (*Cervus elaphus*) stags under conditions approved by the Invermay Animal Ethics Committee. The animals were restrained with a hydraulic crush, and local anaesthetic (Lignocaine, Astra Pharmaceuticals, Auckland, N.Z.) was administered as a ring block around the pedicle. Collection occurred 60 days after casting of the previous hard antler. The antler tips were frozen in liquid nitrogen within 1 min of removal and stored at -70°C .

IGF-II was purified from sheep plasma: it was homogeneous on reverse-phase high-pressure liquid chromatography and contained <0.01% ovine IGF-I (Moore *et al.* 1989). Ovine IGF-I was also purified from sheep plasma and contained <0.01% IGF-II (Moore *et al.* 1989), and bovine insulin was from Sigma Chemical Co. (St Louis, MO, U.S.A.). Ovine

IGF-II was iodinated using the Iodogen method (Salacinski *et al.* 1981), as previously used for ovine IGF-I (Hodgkinson *et al.* 1989) and ovine growth hormone (Spencer *et al.* 1991).

Incubations

For each antler, 18 sections (8 μm thick) were cut at -18 to -20°C and thaw-mounted in pairs onto nine gelatin chrome-alum subbed microscope slides. Sections were stored at -20°C and incubated within 1 week of being cut.

The slides were incubated under the conditions described previously for ^{125}I -labelled IGF-I and ^{125}I -labelled growth hormone (Elliott *et al.* 1992) using ^{125}I -labelled IGF-II as the tracer. Briefly, slides were thawed and sections preincubated with Tris-HCl buffer (0.17 mol/l; pH 7.4) for 10 min at room temperature. The preincubation buffer was aspirated before sections were incubated with 50 μl buffer of Tris-HCl (0.17 mol/l, pH 7.4), 1% bovine serum albumin (BSA; Sigma Chemical Co.), MgCl_2 (5 mmol/l), phenylmethylsulphonyl fluoride (1 mmol/l, and ^{125}I -labelled IGF-II (20 000 c.p.m.) with and without unlabelled IGF-II. After incubation for 2 h at room temperature in a humidified slide chamber, the incubation buffer was aspirated and slides washed twice for 5 min in baths of cold Tris-HCl (0.17 mol/l, pH 7.4), with 0.25% BSA and 0.01% Triton X-100. They were then fixed in neutral buffered formalin for 10 min, washed in distilled water for 5 min and air-dried. Slides were also incubated without iodinated hormone as controls for background grain counts, chemography and latent image fading of the autoradiographs (Rogers, 1973). For the incubations, ovine IGF-II at 1 $\mu\text{g}/\text{ml}$, ovine IGF-I at 1 $\mu\text{g}/\text{ml}$, and bovine insulin at 10 $\mu\text{g}/\text{ml}$ were used for competitive inhibition of tracer binding. One section of each pair was used for total binding by incubation with ^{125}I -labelled IGF-II alone while the other was incubated with ^{125}I -labelled IGF-II and a competing unlabelled hormone. Three pairs of sections from each animal were incubated for each combination of labelled hormone and unlabelled hormone.

Autoradiography

After incubation, the slides were exposed against X-Omat AR5 film (Eastman Kodak Company, Rochester, NY, U.S.A.) for 2 days, to produce macroautoradiographs. NTB-2 emulsion (Eastman Kodak Company) was applied to the sections on the slides for three animals. These were dried overnight at room temperature with silica desiccant and exposed for 5 weeks at 4°C . The microautoradiographs were then developed, fixed and stained with Ehrlich's haematoxylin and alcoholic eosin.

Grain counting and statistical analysis

The autoradiographs from the three antlers underwent radiographic grain counting and statistical analysis. Sites from four histological zones were counted for each section – twelve sites from the cartilaginous zone, reserve mesenchyme/perichondrium and dermis, and six sites from the epidermis. Hair follicles and sebaceous glands in the dermis and blood vessels in the perichondrium were excluded from grain counting. Sites were selected from random microscope stage co-ordinates and tissue counts taken from areas of $20 \times 20 \mu\text{m}^2$. Data were statistically analysed using analysis of variance and comparisons made between total and non-specific binding to sections, total and residual binding to sections, specific binding for each animal, and different tissue types within sections. Significance was assessed using Student's *t*-test.

Receptor analysis

Microsomal membrane preparation

Microsomal membrane preparations of antler tissue from three animals were prepared as described previously (Elliott *et al.* 1992). Briefly, the antler tips were thawed at 4°C , the cartilaginous portion was removed, fragmented and washed in sucrose (0.3 mol/l). The tissue was then weighed and homogenized (1:3, w/v) in sucrose (0.3 mol/l) and 1000 KIU Trasylol/ml (Bayer Pharmaceuticals, Botany, NSW, Australia) for 4 min at high speed with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany) at 4°C . The homogenate was centrifuged at 1500 *g* for 20 min, the supernatant recentrifuged at 15 000 *g* for 20 min and then again at 100 000 *g* for 90 min. The resulting pellet was incubated with MgCl_2 (3.5 mol/l) at a ratio of 1:2 initial tissue weight/volume for 20 min. The mixture was then centrifuged at 100 000 *g* for a further 90 min, the pellet washed with cold buffer containing Tris (0.025 mol/l, pH 7.6) and CaCl_2 (0.01 mol/l) centrifuged at 40 000 *g* for 60 min. The final pellet was resuspended in buffer containing Tris (0.025 mol/l, pH 7.6), CaCl_2 (0.01 mol/l) and 1000 KIU Trasylol/ml at a ratio of 1 ml/g of initial tissue weight. These microsomal membranes were stored at -20°C until radioreceptor assays were performed.

Radioreceptor assays

Radioreceptor assays were performed for the type 2 IGF receptor (using ovine IGF-II) in antler. Ovine IGF-II purified from sheep serum was iodinated with chloramine-T (Greenwood *et al.* 1963) and the radiolabelled hormone separated by Sephadex G-50 chromatography using phosphate buffered saline (0.01 mol/l; pH 6.2) containing 0.1% (w/v) gelatin as

the eluent. The iodinated hormone was used in radioreceptor assays within 4 days of iodination.

Initially, specific binding of ^{125}I -labelled IGF-II to a range of concentrations of microsomal membrane was assessed. Assays were performed in triplicate using membrane volumes ranging from 5 to 300 μl incubated with ^{125}I -labelled IGF-II (approximately 30 000 c.p.m.) in a total incubation volume of 0.5 ml. The buffer used for the radioreceptor assays was Tris (0.025 mol/l, pH 7.6), 0.2% BSA, 0.02% sodium azide and 1000 KIU Trasylol/ml. Non-specific binding was assessed using tubes containing excess unlabelled hormone of 50 ng/tube. Incubation was for 20 h at room temperature and was terminated by the addition of 2 ml ice-cold buffer. Preliminary experiments confirmed that equilibrium conditions were achieved after incubation for 20 h at room temperature, and reversibility of binding was observed with addition of excess unlabelled ligand. Bound and free hormones were separated by centrifugation at 3000 *g* for 45 min at 4°C .

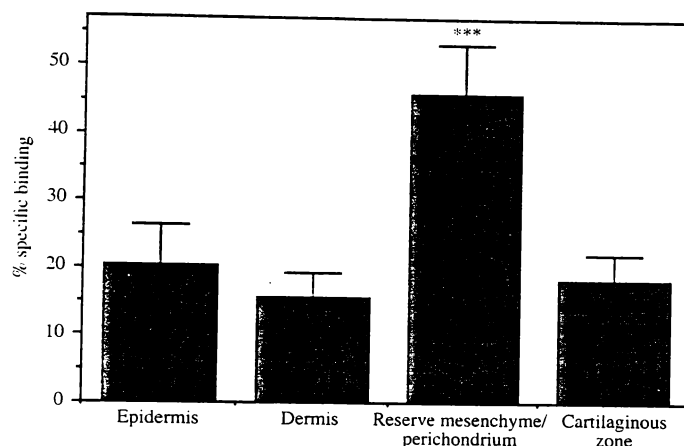
A series of competitive binding assays was performed for ^{125}I -labelled IGF-II binding. The receptor preparation (5 μl /tube) was incubated with ^{125}I -labelled IGF-II and various concentrations of unlabelled ovine IGF-II (0–500 ng/tube), recombinant human IGF-I (Ciba Geigy Ltd, Basel, Switzerland) (0–500 ng/tube) and recombinant human insulin (Eli Lilly, Indianapolis, IN, U.S.A.) (0–1000 ng/tube). Assay conditions were otherwise as described above.

The binding data were analysed using the LIGAND program (Version 3.0, Biosoft, Cambridge, Cambs, U.K.). From Scatchard analysis (Scatchard, 1949), estimates were obtained for receptor capacity and affinity constants.

Affinity cross-linking studies

Microsomal membrane preparation

Crude microsomal membrane fractions were prepared according to a modified method of Barenton *et al.* (1988). Briefly, 4 g of the cartilaginous portion of a thawed antler tip was homogenized (Ultra-Turrax) at high speed for three periods of 30 s in 12 ml ice-cold sucrose (0.25 mol/l, pH 7.4) containing imidazole (4 mmol/l) and the inhibitors N-ethylmaleimide, *p*-hydroxymercuribenzoate and phenylmethylsulphonyl fluoride (all at 1 mmol/l). The homogenate was centrifuged at 1300 *g* for 10 min, and the supernatant decanted. The pellet was resuspended and recentrifuged after which it was discarded and the pooled supernatants were centrifuged at 3200 *g* for 10 min. The resulting pellet was again resuspended and recentrifuged and the pooled supernatants were centrifuged at 200 000 *g* for 30 min. The supernatant



TEXT-FIGURE 1. Specific binding of ^{125}I -labelled IGF-II to each histological zone of the antler. After *in vitro* incubation with ^{125}I -labelled IGF-II, autoradiographs from three animals ($n=3$) were stained with haematoxylin and eosin, and the radiographic grains were counted. Specific binding was calculated as differences in means of grain counts between total and non-specific binding for each histological zone, and expressed as percentages. There was significant binding in each histological zone; errors shown are standard error of the mean (d.f. = 12). *** $P < 0.001$ compared with other zones.

was discarded at this point and the pellet resuspended and recentrifuged. The final pellet was regarded as the crude microsomal fraction. This was resuspended in Tris buffer consisting of Tris-HCl (25 mmol/l) with MgCl_2 (10 mmol/l; pH 7.4) and centrifuged at 200 000 *g* for 30 min. The supernatant was discarded and the pellet stored at -20°C until use.

Cross-linking, gel electrophoresis and autoradiography
Membrane samples (2 mg preparation/ml) were incubated overnight at 4°C with ^{125}I -labelled IGF-II (1×10^6 c.p.m.) with or without excess unlabelled IGF-II (1 $\mu\text{g}/\text{ml}$) in Tris buffer containing 0.1% BSA (0.5 ml). Samples then underwent polyethylene glycol precipitation, affinity cross-linking with disuccinimidyl suberate and electrophoresis following the method of Barenton *et al.* (1988). After electrophoresis the gels were fixed in solution containing 11.4% (w/v) trichloroacetic acid, 3.4% (w/v) sulphosalicylic acid and 30% methanol for 30 min, soaked in destaining solution of 45% methanol and 10% acetic acid for 10 min, then stained with Coomassie stain solution of 0.25% Coomassie brilliant blue R and 10% acetic acid for 30 min. The gels were destained overnight, dried for 1 h in a solution of 45% methanol, 10% acetic acid and 5% glycerol and preserved in cellophane. Gels were then exposed to X-Omat AR5 film with

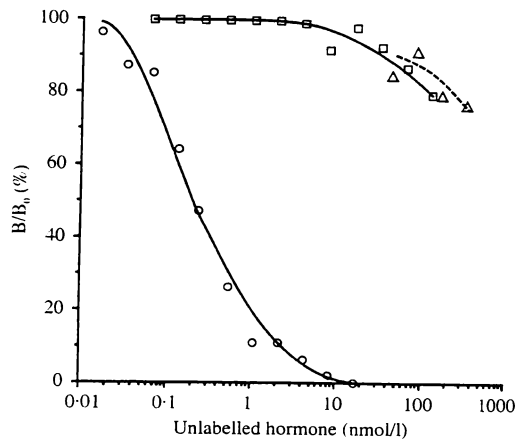
intensifying screens for 3 days at -70°C . Marker proteins used were: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa).

RESULTS

IGF-II binding

The macroautoradiographs for all nine antler tips were qualitatively assessed and showed IGF-II binding in each histological zone (Pl. 1). On total binding sections, the perichondrium and reserve mesenchyme were heavily labelled whilst the cartilaginous zone, epidermis and dermis were moderately labelled. The very faint signal of the non-specific binding sections indicated that virtually all of the total binding was specific binding.

Grain counts of ^{125}I -labelled IGF-II binding per unit area of tissue on the microautoradiographs were not significantly different between animals. There was significant ($P < 0.001$) specific binding in each histological zone, with significantly ($P < 0.001$) higher binding in the reserve mesenchyme/perichondrium zone (Text-fig. 1). IGF-I and insulin did not displace binding of the IGF-II tracer in any of the zones.

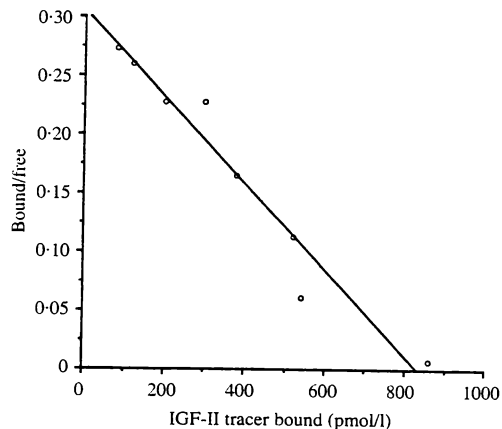


TEXT-FIGURE 2. Displacement of ^{125}I -labelled IGF-II binding to microsome preparations by various concentrations of unlabelled IGF-II ($\circ$), IGF-I ($\square$) and insulin ($\triangle$). The membrane preparations were of antler tip material ($n=3$) which did not include the epidermis and dermis.

The distribution of radiographic grains on sections of total binding was not uniform. In all zones except the epidermis most of the grains occurred clustered in groups (Pl. 2). In the dermis, clusters were sparse, in the reserve mesenchyme/perichondrium zone, clusters were dense and in the cartilaginous zone, clusters occurred in spaces in the extracellular matrix. The clusters were not associated with heterogeneous structures, such as vascular endothelial tissue, hair follicles and sebaceous glands, but did seem to occur in conjunction with various nuclei. Sections of non-specific binding revealed no clustering, indicating that the clusters of ^{125}I -labelled IGF-II grains represented specific binding. The increase in clustering seen in the reserve mesenchyme/perichondrium zone corresponded with an increase in cell number and nuclear mass. Grains were more evenly distributed in the epidermis and did not show the localized clusters of binding seen in the other antler tip tissues.

IGF-II receptors

Specific binding of ^{125}I -labelled IGF-II to membrane proteins was found to be concentration-dependent and plateaued (69.8%) at a membrane volume of approximately 100 μl /tube (data not shown). Cross-reactivity studies (Text-fig. 2) demonstrated that the binding of ^{125}I -labelled IGF-II to the antler membranes was to binding sites collectively displaying a preferential affinity for IGF-II and a slight affinity for



TEXT-FIGURE 3. Scatchard plot of the interaction of ^{125}I -labelled IGF-II with its receptor in antler microsome preparations.

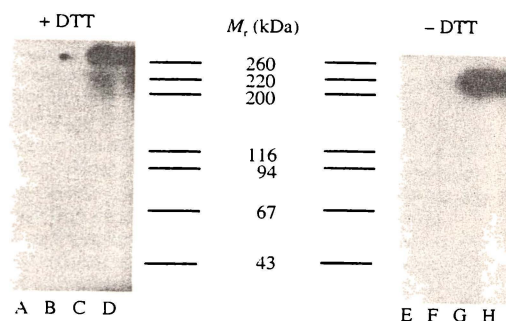
IGF-I and insulin. Scatchard analysis revealed a straight line, showing a receptor capacity of 803 pmol/100 mg initial tissue weight and a K_d of 1.3×10^{-10} mol/l for the interaction of IGF-II with its receptor (Text-fig. 3). This value is in agreement with K_d values from other studies for the type 2 IGF receptor in the rat (August *et al.* 1983) and cow (Tong *et al.* 1988).

Affinity cross-linking of IGF-II

After affinity labelling of crude antler membranes with ^{125}I -labelled IGF-II, electrophoresis and autoradiography, a single band of radioactivity was detected, which was localized at 220 kDa and disappeared in the presence of excess unlabelled IGF-II (Text-fig. 4). Under reducing conditions the radioactive band migrated at M_r 260 kDa. These findings seem indicative of the tracer binding to a type 2 IGF receptor (Kasuga *et al.* 1981; Barenton *et al.* 1988).

DISCUSSION

This study has demonstrated the presence of IGF-II specific binding in the antler tip and characterized the nature of this binding. Results from radioreceptor assays showed that the IGF-II tracer bound to a binding site displaying preferential affinity for IGF-II and low specificity for IGF-I and insulin. The heterogeneous nature of the membrane preparation used does not rule out the possibility that the tracer may have been binding to more than one binding site, although the results of the Scatchard analysis would



TEXT-FIGURE 4. Autoradiographs of gels showing the molecular weights of the IGF-II hormone-receptor complex in antler cartilaginous tissues. Antler membrane preparations were incubated with ^{125}I -labelled IGF-II in the presence (lanes A,B,E,F) and absence (lanes C,D,G,H) of excess unlabelled IGF-II, then affinity cross-linked and electrophoresed under reducing (+DTT) and non-reducing (- DTT) conditions, as described by Barenton *et al.* (1988). The positions of molecular weight markers in adjacent lanes are indicated.

suggest that the tracer is binding to a relatively homogeneous population of receptors. The finding of a major band migrating at 220 kDa under non-reducing conditions in the affinity cross-linking study, together with the high specificity for IGF-II displayed by the specific binding, would suggest that tracer binding was predominantly to a site with the properties of the type 2 IGF receptor (Kasuga *et al.* 1981; Underwood & Van Wyk, 1985; Nissley & Kiess, 1991). The displacement of IGF-II tracer binding by IGF-I and insulin seen in this study, however, does not agree with the properties traditionally ascribed to the type 2 receptor. There was little evidence that the tracer was binding to a type 1 IGF receptor as displacement with high concentrations of unlabelled IGF-I (10 nmol/l). Likewise, there was no evidence for the IGF-II tracer binding to insulin receptors as insulin, having a greater affinity for the insulin receptor than IGF-II (Underwood & Van Wyk, 1985), would have shown a displacement of the tracer at low concentrations, and this was not apparent.

Results also gave very little evidence for tracer IGF-II binding to IGFBPs. Tracer binding to IGFBP-1 or IGFBP-3, which do not display preferential affinity for either of the IGFs (Baxter *et al.* 1987; Forbes *et al.* 1988), would have been competitively displaced by unlabelled IGF-I at low concentrations. IGFBP-2, IGFBP-4 and IGFBP-6, which display preferential binding for IGF-II (Forbes *et al.* 1988; Mohan *et al.* 1989b; Roghani *et al.* 1989;

Martin *et al.* 1990), show a lower affinity for IGF-I. However, in this study, IGF-I and insulin displaced IGF-II tracer at relatively similar concentrations, implying that tracer binding to the established IGFBPs was minimal, as IGFBPs have no affinity for insulin (Monget *et al.* 1989).

There are indications from a number of studies (Frolik *et al.* 1988; Mohan *et al.* 1988) that IGF-II is involved in bone growth. It has been extracted from the bone matrix of several species, and characterization of receptors for IGF-II in skeletal tissues in a variety of species has been reported, e.g. for bovine growth plate chondrocytes (Trippel *et al.* 1988), rabbit chondrocytes (Jansen *et al.* 1989), and mouse osteoblasts (Slootweg *et al.* 1990). IGF-II has been shown to stimulate bone cell proliferation by Mohan & Baylink (1991) and these authors surmised that this action was mediated by type 2 IGF receptors. In the antler, the histological region with the highest specific binding of IGF-II was the reserve mesenchyme/perichondrium. This is the zone which undergoes the most rapid differentiation and mitosis in the antler (Banks & Newbrey, 1983a,b) and where binding of IGF-I is low (Elliott *et al.* 1992). From this it may be speculated that the growth effects in the reserve mesenchyme/perichondrium zone could be due to IGF-II. Mitogenic effects of IGF-II have been reported elsewhere, either alone (Adams *et al.* 1983; van Dijk *et al.* 1988) or in the presence of other growth factors (Slootweg *et al.* 1990), so it is possible that one of the main roles of the receptor for IGF-II in the antler is that of mediating hyperplasia.

The clustering of IGF-II binding to nuclei observed in the dermis, reserve mesenchyme/perichondrium and cartilaginous zones would seem to endorse this. A similar phenomenon is seen in skeletal muscle where localization of ^{125}I -labelled IGF-II binding to fibroblast nuclei in some regions of connective tissue has been observed (Oldham *et al.* 1993). In the dermis, clustering was associated with nuclear-stained material amongst relatively large areas of sparsely labelled connective tissue and, in the reserve mesenchyme/perichondrium and cartilaginous zones, clusters were observed to occur associated with some, but not all, nuclei, suggesting that there may be sites available for binding IGF-II on cells at a specific stage of the cell cycle. Our observations appear to support those of another study (Adams *et al.* 1983), which used cell multiplication experiments where variations in response to IGF-II were explained as being due to differences in the mitogenic phase of fibroblast cells in culture.

The pattern of binding of IGF-II in the antler is different from the pattern of binding of IGF-I (Elliott *et al.* 1992). In contrast to IGF-I, which displays highest specific binding to cartilage tissue, which is

differentiated (Elliott *et al.* 1992), most of the specific binding of IGF-II was to the reserve mesenchyme and perichondrium, which are tissues undergoing rapid differentiation and proliferation. From this it appears probable that the receptor for IGF-II in the antler differs from the IGF-I receptor in function, as well as in properties and distribution. Therefore, IGF-II may have an effect on rapidly differentiating and proliferating tissue whilst IGF-I may have a role in the maturation of cartilaginous tissues of the antler.

In conclusion, IGF-I and IGF-II binding in the antler exhibit different properties; IGF-I binding displays classical affinity properties of the type 1 IGF receptor (Elliott *et al.* 1992) whilst the data in this study showed that labelled IGF-II binding was to binding sites displaying preferential affinity for IGF-II. Although the membrane preparations used in this study were of a heterogeneous nature, and there is a possibility that all or part of the tracer binding may have been to other binding sites, the evidence supports the majority of IGF-II binding being to a type 2 IGF receptor in the growing tip of the deer antler.

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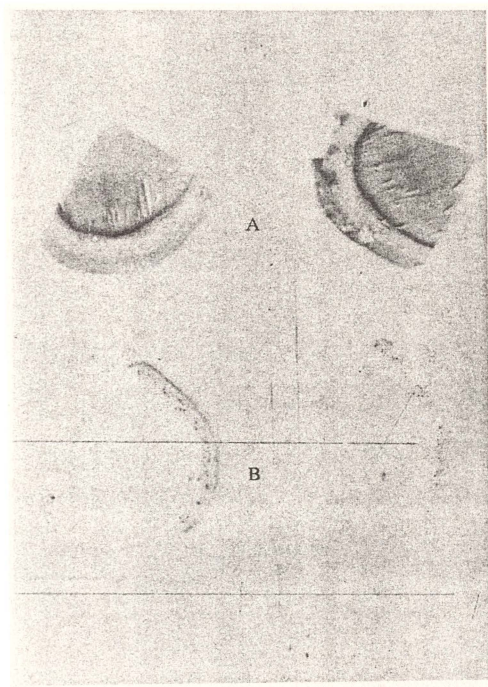
DESCRIPTION OF PLATES

Plate 1

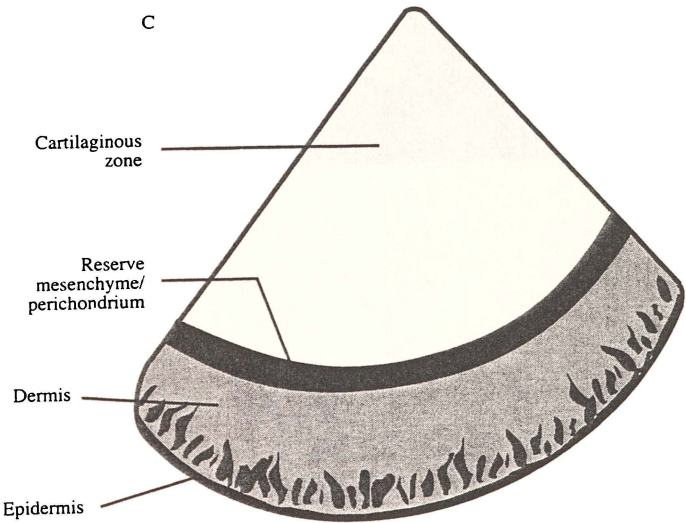
Macroautoradiographs from one representative antler tip ($n=9$), showing qualitatively the extent of binding of ^{125}I -labelled IGF-II to each histological region. After incubation with tracer $\pm$ excess unlabelled IGF-II, the sections were fixed and exposed against X-Omat AR-5 film for 4 days, then developed. (A) Total binding of IGF-II, (B) non-specific binding and (C) diagram of an antler tip section showing the histological regions.

Plate 2

Microautoradiograph showing total binding of ^{125}I -labelled IGF-II in the reserve mesenchyme/perichondrium zone. After incubation with tracer the sections were fixed, subjected to macroautoradiography, then exposed to photosensitive emulsion for 5 weeks and developed. Radiographic grains are observed to be clustered to some but not all nuclei.



0.5 cm





— 10 µm

