



Effects of Insulin-like growth factor-I (IGF-I) peptides on the
growth and function of the gastrointestinal tract in adult and
suckling rats

Corinna-Britta Steeb (BSc. Hon.)

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LEAF AND STREAM

Find myself beside a stream of empty thought
like a leaf that's fallen to the ground,
and carried by the flow of water to my dreams
and woken only by your song.

Lord I've walked this path for many years
listened to the wind that calls my name.
The weeping trees of yesterday, they look so sad
await your breath of spring again.

Far beyond the hills where earth and sky will meet again
where shadows like an opening hand,
that hold the secrets that I've yet to find and wonder at
.....the light in which they stand.

(Wishbone Ash, Argus, 1972)



ABSTRACT

Growth and development of the gastrointestinal tract in mammals is regulated by the complex interaction of dietary, hormonal, neural and luminal factors. To investigate the effects of insulin-like-growth factor-I (IGF-I) peptide infusion on the growth and function of the gastrointestinal tract, adult and suckling rats (6 and 12 days old) have been treated with increasing doses of IGF-I or LongR³ IGF-I (LR³IGF-I), an IGF-I analog that shows greatly reduced binding affinity to several of the IGF binding proteins. Peptides were delivered via subcutaneously implanted mini-osmotic pumps. Control animals received vehicle carrier (0.1M acetic acid). Adult rats were maintained in metabolism cages throughout the treatment period for assessment of body weight gain, food and water intake and faecal and urinary output. Suckling rats were returned to their mother following pump implantation (9 pups/dam). At sacrifice, internal organs and the gastrointestinal tract were rapidly excised for subsequent histological, biochemical and autoradiographic analyses. Biological activities of Lactase-Phlorizin Hydrolase (LPH) and the alpha glucosidase sucrose-isomaltase were measured in tissue homogenates of suckling animals to assess IGF-I peptide effects on the maturation of GIT function. Distribution patterns of enzyme expression along the villus axis were determined in cryostat sectioned tissue samples.

In the adult rats, a dose-dependent increase in gut tissue weight and intestinal length was observed following peptide treatment for 14 days. Both mucosal and non-mucosal tissue components increased with proportional increments in proliferative cells within the crypt epithelium as indicated by proliferative cell nuclear antigen labelling (PCNA). Administration of IGF-I peptides rapidly induced proliferative activity as indicated by an increase in tritiated thymidine labelling following a 3 day peptide administration protocol to adult rats.

In 6-day old pups, treated for 6.5 days with LR³IGF-I but not IGF-I, increased body weight gain. However, both peptides increased the relative weights of organs, including the spleen and kidney. A selective action of IGF-I and the LR³IGF-I was indicated by the marked increase in gastrointestinal tissue components, so that total gut weight increased by up to 59% above control values following treatment with the highest dose of LR³IGF-I. Responses were particularly apparent in the small intestine and the stomach, and histological and biochemical analyses suggested that growth occurred through proportional increases of the mucosal and non-mucosal tissue mass. The thymidine labelling index increased in proportion to crypt population.

In the 12 day old pups, lactase activity, as measured in jejunal tissue homogenates, was significantly reduced following treatment with LR³IGF-I. Histochemical detection showed a significant reduction in surface staining for lactase along the entire length of duodenal villi. Conversely, in these animals, sucrase activity measured in either jejunal tissue homogenates or in cryostat sectioned tissue, was precociously induced.

The results of this study suggest that IGF-I peptides, in particular LR³IGF-I, significantly influence gastrointestinal growth in normal adult and suckling rats. IGF-I peptides stimulate intestinal proliferation and furthermore, influence the maturation and cytodifferentiation of enterocytes in the immature intestine. These findings indicate that IGF-I peptides may have therapeutic implications both in conditions of impaired gut function in the adult gastrointestinal tract and in the treatment of gut disease in the immature intestine.

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STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any other University or other tertiary institution and, to the best of my belief and knowledge, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Date: May 5, 1995

Signed: _____

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Publications arising from thesis research

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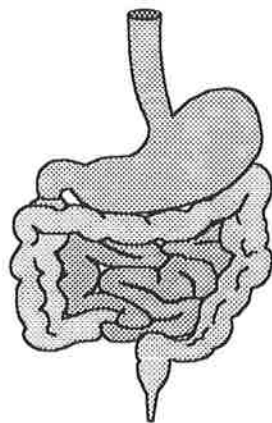
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COMMONLY USED ABBREVIATIONS

³ [H]TdR	tritiated thymidine
ANOVA	analysis of variance
CGF	crypt growth fraction
CGF	crypt growth fraction
DNA	deoxyribonucleic acid
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
GH	growth hormone
GIT	gastrointestinal tract
i.p.	intraperitoneal
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFBP	insulin-like growth factor binding proteins
LPH	lactose-phlorizin hydrolase
LR ³ IGF-I	LongR ³ insulin-like growth factor-I
mRNA	messenger ribonucleic acid
N-terminus	amino terminus
P	probability
PCNA	proliferating cell nuclear antigen
rIgG	rat immunoglobulin G
s.c.	subcutaneous
SEM	standard error of the mean
SI	sucrase-isomaltase
T4	thyroxine
TPN	total parenteral nutrition

CHAPTER 1:

GASTROINTESTINAL GROWTH AND DEVELOPMENT IN ADULT AND SUCKLING RATS AND ITS REGULATION INSULIN-LIKE GROWTH FACTOR PEPTIDES





CHAPTER 1

Gastrointestinal growth and development in adult and suckling rats and its regulation by insulin-like growth factor-I peptides.

Throughout life, all organs and tissues of the body grow in a highly ordered fashion in relation to the body as a whole; however, many organs including the heart, lungs, kidneys skeletal muscles and the gastrointestinal tract are capable of particularly rapid growth in response to a stimulus, diseases or additional demand even when the body has ceased to grow (Widdowson, 1985). The main objective of this thesis is to examine the effect of insulin-like growth factor-I peptide administration on the growth and function of the gastrointestinal tract in adult and suckling rats.

The first part of this literature review examines: **1)** general aspects of gastrointestinal development and maturation; **2)** the development of the gastrointestinal tract during foetal and the immediate perinatal period; **3)** the postnatal maturation of the intestinal epithelium and patterns of enzyme activities; and **4)** aspects of intestinal proliferation in the mature gastrointestinal tract. The factors that control and govern gastrointestinal development and function during neonatal, postnatal and adult life will be discussed in each of these sections. Considering the complexity of the topic, this review, whilst not attempting to be comprehensive, will focus on the major issues related to the work presented in this thesis. Because aspects of gastrointestinal growth and function have been studied most extensively in rodents, the majority of the work cited in this historical literature review relates to studies in rats (mice); however, where appropriate, comparisons to other mammals have been included.

The second part of the literature review aims to elucidate some characteristics of: 1) the structure and chemistry of insulin-like growth factors (IGFs), their production and localisation; 2) the distribution and proposed function of the IGF-binding proteins; 3) the IGF receptors; 4) the biological activities of IGFs and their binding proteins; and 5) IGFs and the gastrointestinal tract. The biological significance of IGF physiology and function will be related to the growth and function of the mature and immature gastrointestinal tract in rats.

This review generally cites work published prior to January 1991, the commencement of my PhD candidature. Review of the literature revealed that little was known about the *in vivo* action of IGF peptides on the gastrointestinal tract. Especially, very little was known of the effects of IGF action in the adult or developing gastrointestinal tract in normal animals. Furthermore, the *in vivo* potencies of the IGF analogues were not well established. Because the field of IGF research has been expanding at an accelerating rate in the last 4 years, more recent literature has been included appropriately in some parts of the literature review, in particular in the section "IGFs and the gastrointestinal tract". Information relevant to this thesis published since 1991 is included in the appropriate discussion sections of each Chapter.

Part 1: Growth and development of the mature and immature gastrointestinal tract

1.1. General aspects of gastrointestinal development and maturation

The development of the gastrointestinal tract (GIT) is determined by the complex interaction between four major determinants (Lebenthal, 1989). Firstly, the inherent genetic endowment, which is contained in the base sequence of DNA (deoxyribonucleic acid) molecules, provides the ultimate control for the differentiation of tissues and cells. Secondly, a species-specific intrinsic developmental clock determines the temporal sequence of developmental processes. Thirdly, endogenous regulatory mechanisms such as pancreatico-biliary secretions, hormonal and endocrine factors as well as growth factors and tissue interactions regulate the expression of the genetic endowment at various stages of gastrointestinal development. Finally, environmental factors such as stress or nutrient availability also influences gastrointestinal development, thus for the full expression of the genetic endowment an optimal environment is required (Lebenthal, 1989).

Ontogenetic development of intestinal structure and function in mammals has been divided into five phases, three of which occur during gestation (Ménard, 1989 and Colony, 1983). The first phase of gut formation is represented by organogenesis during which the intestine originates from a simple tube derived from endoderm and surrounding mesenchyme (Buddington, 1994). The second phase is marked by the formation of villi and the restriction of cell proliferation to the primordial crypts. The third phase is inscribed by the continuous growth and maturation of the intestine. The fourth phase of gastrointestinal development and maturation relates to the transition from intrauterine life to extrauterine life and is marked by the intake of mammary secretions during suckling. This phase is also marked by differentiation of specific cell types within the intestinal epithelium and the establishment of enzyme patterns

(Buddington, 1994). Finally, during weaning, the intestine matures in preparation for ingestion of an adult-like diet. Each of these successive phases are regulated by dietary inputs which interact with genetic determinants to influence intestinal function either by stimulating the production of a new enterocyte population or by reprogramming existing enterocytes (Buddington, 1994). Additionally, non-nutritive factors (hormones or growth factors) present in amniotic fluid or milk further modulate intestinal growth and maturation during the early developmental period (Buddington, 1994).

1.2 Foetal development of the gastrointestinal tract

1.2.1 Organogenesis and Histogenesis of the gastrointestinal tract

Embryologically, the gastrointestinal tract develops from a simple gut tube formed by the folding of endoderm and an associated outer mesenchymal component, with proliferation occurring throughout the presumptive mucosa (Crelin, 1961). The epithelial lining of the glandular epithelium derives from the endoderm, while the mesoderm gives rise to the muscular layers, blood vessels, connective tissue and the serosa. The gut tube comprises three parts: The foregut which gives rise to the stomach, the descending duodenum and the pancreas; the mid-gut, from which the rest of the small intestine develops; and the hindgut which develops into the large intestine (Crelin, 1961). Extensive proliferation of the epithelium of the intestinal loops formed by the gut tube results in luminal occlusion by epithelial cells followed by recanalization of the intestinal lumen (Crelin, 1961). During the formation of the small intestine, the tube is lined by a single layer of epithelium which later becomes stratified and subsequently assumes the adult simple columnar arrangement (Colony, 1983 and Klein, 1989). In the human, the transition in epithelial cell type occurs early in foetal life, while in rodents this process takes place relatively late in gestation (Colony, 1983, Trier and Moxey,

1979, Mathan et al., 1976 and Kammerad, 1942). For example, cytodifferentiation of paneth cells or endocrine cells has been observed as early as 11 weeks gestation in the human foetus (Moxey and Trier, 1977 and 1978). Regulatory peptides detected in the developing intestine have been implicated to coordinate organogenesis (Aynsley-Green et al., 1990).

1.2.2 Formation of mucosal compartments

Villi formation: In the small intestine, selective proliferative activity appears to be related to developmental processes that accompany the formation of villi and crypts. In the rat, the small intestinal epithelium consists of a single cell layer of undifferentiated cells, and mitotic activity is randomly distributed at the 13th gestational day (Trier and Moxey, 1979). Mitotic activity increases markedly over the next 3-4 days and the epithelium becomes stratified (Trier and Moxey, 1979). At 18 days gestation, pre-villous ridges form in the rat which coincides with a change in the pattern and an overall decrease in mitotic activity, so that at gestational day 20-21, cell proliferation is restricted to the inter-villous space (Trier and Moxey, 1979 and Hermos et al., 1971). Similar developmental processes occur in chicken and humans, although the temporal sequence differs (Grey, 1972, Coulombre and Coulombre, 1958, Burgess, 1975, Arsenault and Ménard, 1987 and Colony, 1983). In the human foetus, the cells lining intestinal villi differentiate into enterocytes with microvilli, brush-border hydrolases and nutrient transporters as early as 22 weeks gestation and the proximo-distal gradient of sugar and amino-acid transporters is already present (Malo, 1991 and Malo and Berteloot, 1987). This developmental pattern also applies to the tissues of the gastric mucosa and the colonic mucosa. As villi maturation and lengthening proceeds in the proximal region of the small intestine, villi start to appear in the more distal regions (Klein and McKenzie, 1983a). Another contributing factor for correct villi formation and differentiation is the endoderm-mesenchyme interaction (Ménard and Calvert, 1991). As shown by Keding et al.

(1986) morphogenesis of both crypt and villi depends on the permissive inductive effect of the mesenchyme.

Crypt formation: Intestinal villus formation precedes crypt formation (Trier and Moxey, 1979). In the human, the first crypts appear to result from simple down-growth into the mesenchyme at approximately 10 to 11 weeks in the proximal small intestine and some time later in the ileum and colon (Colony, 1983). In the foetal rat, a significant number of cells located in the upper third of the villi are capable of tritiated thymidine uptake; however, proliferation becomes completely confined to the incompletely formed crypts just prior to birth (Hermos et al., 1971). Early crypt formation appears to be characteristic of species with long gestation periods (Trahair and Robinson, 1986 and Trahair et al., 1986a and 1986b) and occurs later in species with relatively short gestation periods (Klein, 1989). As the crypts form they become a progenitive zone (Trahair and Robinson, 1986) and dividing cells in the crypt migrate up the villus where they are ultimately lost from the extrusion zone at the tip of the villi (Leblond and Stevens, 1948). Within the crypts, the epithelium comprises of poorly differentiated cells rich in thymidine kinase activity and other enzymes involved in the synthesis of DNA (Herbst et al., 1970). As enterocytes migrate up the villus tip, many proteins characteristic of the process of digestion and absorption are synthesised, including alkaline phosphatase (Moog, 1951 and Lev, 1981), numerous disaccharidases (Auricchio et al., 1965a and 1965b and Auricchio and Sebastio, 1989) and di- and tripeptidases (Auricchio et al., 1981, Lindberg, 1966, Heringova et al., 1966 and Nordström et al., 1968)

1.2.3 Continuous growth and maturation of the foetal intestine

In mammals with long gestation period, such as in pigs, sheep or humans, intestinal organogenesis and the onset of hydrolytic enzyme activity begins early in life. In contrast, in

mice, rats and rabbits, which have a short gestation period, the maturation of the intestine proceeds at slower rates. During foetal development, rapid growth of the gastrointestinal tract is evident. For example, in humans, the intestine elongates approximately 1,000 fold from the 5th to the 40th gestational week (Ménard, 1989). Similarly, a dramatic increase in intestinal diameter is observed (Ménard, 1989). Once villi are formed in the intestine, intestinal function progresses. For example, the human foetus swallows up to 750ml of amniotic fluid a day (Pritchard, 1966). Similarly, foetal lambs also swallow a significant amount of amniotic fluid (Gitlin et al., 1972). Nutrients absorbed by the foetus from amniotic fluid account for approximately 14% of the total nutrient and energy requirement and are important for normal intrauterine intestinal development (Mulvihill et al., 1985). It appears that the development of the upper gastrointestinal tract depends on nutrients swallowed in amniotic fluid (Mulvihill et al., 1985 and Trahair et al., 1986a and 1986b). In addition, amniotic fluid contains many non-nutritive, biologically active substances such as growth factors and hormones which appear to play a pivotal role in gastrointestinal development and function. Epidermal growth factor (EGF), gastric inhibitory peptide and Vitamin D are also present in amniotic fluid and have been shown to stimulate gastrointestinal growth and stimulate the induction of crypts (Calvert et al., 1983, Weaver and Walker, 1988, Aynsley-Green, 1985 and Debiec et al., 1991).

1.3 Development of the gastrointestinal tract during the suckling period

The general pattern of gastrointestinal development is similar in all mammals. As a result of variations in the time of gestation, the period of lactation and the general degree of maturity of the young at birth, however, considerable differences may be observed in the timing of biological events of gastrointestinal development (Henning and Kretchmer, 1973). Nevertheless, gastrointestinal development during intrauterine life in mammals is such that at

the time of birth, the gastrointestinal tract is well equipped to cope with the digestion and absorption of the neonatal diet, namely milk (Henning and Kretchmer, 1973).

1.3.1 Immediate perinatal period

For all mammals, the principle source of nutrition during the immediate perinatal period is milk. There are however marked differences in the milk composition, duration of lactation and the quantity of milk intake between species (Jennes et al., 1964). Generally, the concentration of immunoglobulins, hormones and growth factors in early milk are greater than in later milk (Koldovsky et al., 1992), suggesting that non-nutritive factors derived from milk are important regulators of growth during the perinatal period. Coinciding with the functional demands during the early suckling period is the high rate of lactase and glutamine transport (Buddington, 1994 and Al-Mahroos et al., 1990). Conversely, fructose transport is extremely low during the early suckling period which parallels its concentration in milk (Buddington, 1994).

Immediately after birth, swallowing of mammary secretions increases intestinal motility (Berseht, 1990). Furthermore, the immediate perinatal period is marked by rapid intestinal growth and reorganisation of the mucosa. This can be detected as early as 6 hours after ingestion of colostrum in newborn piglets (Burrin et al., 1992a and 1992b). In addition, during the first 24 hours after birth, a doubling in the weight of the intestinal mucosa occurs in newborn guinea pigs and pigs (Xu et al., 1992, Thompson, 1986 and Widdowson et al., 1976) and in dogs (Heird et al., 1984). In neonatal pigs, the increase in mucosal weight is associated with increases in pinocytosed milk-proteins, an increase in mucosal cellularity and higher concentrations of RNA and greater rates of protein synthesis (Xu, et al., 1992). In newborn humans, the first meal after birth triggers secretion of enteroglucagon, gastrin and other

hormones, however it is not known if the human intestine undergoes similar rapid growth responses. Rapid intestinal growth during the immediate neonatal period is not uniform across species and in newborn kittens, this response is not observed (Buddington, 1994).

1.3.2 Remaining suckling period

The shift from intra-uterine to the extra-uterine environment at the time of birth is marked by the preparation of the intestinal epithelium for absorption and digestion of mammary secretions. In most mammals, this involves an increase in lactase activity that occurs before birth in preparation for the intake of high levels of lactose from milk (Blaxter, 1961 and Kretchmer, 1971). In the rat, lactase activity increases markedly to maximum levels by the first week after birth (Doell and Kretchmer, 1962). In contrast, the levels of α -glucosidases including sucrase-isomaltase, maltase and trehalase are low at birth but increase in concentration just prior to weaning (Rubino et al., 1964 and Henning, 1985). In humans, lactase activity is also higher in newborns compared to adults, reaching maximal levels at the end of gestation and during the immediate perinatal period (Koldovsky, 1969). However, unlike the rat, α -glucosidases levels in humans are well developed during intra-uterine life. They appear by the end of the third month in the human foetus and reach maximal levels during the last trimester of foetal development (Holzel, 1968).

During neonatal development, proliferation of crypt enterocytes, as measured by either the labelling or mitotic index, crypt cell production, cell cycle time or cell migration, remains relatively low until approximately the end of the 2nd postnatal week (Yeh, 1977, Klein, 1989 and Koldovsky et al., 1966). At approximately 16 days *post partum*, a burst of mitotic activity occurs, accompanied by increases in ornithine decarboxylase (an enzyme closely related to cellular proliferation), RNA/DNA ratios and cellular migration, and at the same time, cell cycle

time decreases (Klein, 1977, Yeh, 1977 and Luk et al., 1980). This increased proliferative activity of the small intestine results in a marked increase in villus height, crypt depth, crypt girth and, additionally, the number of crypts per villus (Yeh, 1977 and Clarke, 1972). Thus, the accelerated proliferative activity during the 3rd postnatal week contributes cells for crypt enlargement as well as for cellular replacement. While the number of crypts increase with age, the number of villi remain stable. It has been observed that in chicken and rats, the number of villi at birth are already at the adult level, suggesting that the villus compartment is a stable morphological unit. Nevertheless, there are changes in size of the crypt and villus components. For instance, at 21 days *post partum*, villi and crypt have reached their adult height but they are populated by fewer cells as compared to the adult (Wright and Alison, 1984b).

The genetic determinants responsible for age-related changes in intestinal structure and function can be modulated, but are not exclusively regulated by dietary components. For example, decreasing the litter size in rabbits, enhances body weight gain, intestinal growth and accelerates intestinal maturation (Gall and Chung, 1982). Conversely, restriction of nutrient intake by increasing the litter size, maternal malnutrition by ligation of the mammary gland reduces intestinal protein synthesis, enterocyte proliferation and delays intestinal enzyme maturation (Burrin et al., 1991b, Baker and Camphell, 1989, Hamilton et al., 1983 and Butzner and Gall, 1990).

1.4 Maturation of the gastrointestinal tract during postnatal development

Many functional properties of the intestine in neonatal animals differ from the pattern observed in the adult. In rodents, intestinal maturation occurs at approximately the end of the third week of postnatal life and coincides with weaning (Koldovsky, 1969 and Henning, 1985).

The most striking and significant changes that occur in the intestine during gut development are the closure of the gut to macromolecular uptake and transport, and the distinct patterns of digestive enzyme expression, which involves the shift from β -glucosidases (i.e. lactase) to α -glucosidases (i.e. sucrase). These two developmental processes also occur in other mammals, although there are marked differences between species in the temporal and spatial expression during gastrointestinal development. Macromolecular uptake provides the newborn with passive immunity for the protection from infections in early life. The importance of the shift in digestive enzyme expression, on the other hand, relates to a shift from maternal derived nutrients to a solid adult-like diet.

1.4.1 Macromolecular uptake

In adult mammals, digestion and absorption of proteins involves proteolytic enzymes from the stomach and pancreas and active transport of amino acids in enterocytes of the small intestine, thus in adults, proteins are utilised by extracellular digestion followed by absorption of amino acids and peptides (Henning and Kretchmer, 1973). In the neonate, extracellular protein digestion is minimal and the bulk of proteins and other large macromolecules are absorbed intact (Halliday, 1955). Moreover, most neonatal mammals possess specialised transport pathways in different parts of the gastrointestinal tract for the transfer of macromolecules. Macromolecular uptake occurs over prolonged periods in altricial species (for example rodents) but may be restricted to either the pre-natal period or for a short period after birth, in species that are born comparatively independent and mature, such as ungulates or primates (Weaver and Walker, 1989).

Macromolecular uptake in the newborn rat occurs to some extent antenatally, but the intestinal epithelium in the newborn rat retains the ability to absorb protein macromolecules

from colostrum and milk for 16 to 20 days after birth (Clarke, 1959 and Halliday, 1955). Immunoglobulins, in particular rat immunoglobulin G (rIgG), are preferentially transported in the upper small intestine (Rodenwald, 1973) and involves the binding of the immunoglobulin to Fc receptors present on the microvillous membrane of coated pits at the base of microvilli (Goldstein et al., 1979). These coated pits may be the precursors of the coated vesicles and endosomal tubules that transfer the receptor-bound IgG through the terminal web to the apical enterocyte membrane where IgGs are released (Goldstein et al., 1979 and Weaver and Walker, 1989). In addition to receptors for IgG, Rao et al. (1986) have shown that the microvillous membrane of suckling rats also contain receptors that bind epidermal growth factor (EGF). Thus, the growth factor may be selectively transported across epithelial enterocytes in suckling rats. Transfer of immunoglobulins during the neonatal period relates to the acquisition of passive immunity, which in many other species occurs during intrauterine life. For example, rabbits, guinea pigs and humans acquire passive immunity mainly *in utero*, while cows and sheep and goats acquire it from mammary secretions (Henning and Kretchmer, 1973). Other species (dogs, rats and mice) acquire passive immunity by both routes.

In contrast to the selective uptake in the proximal small intestine in the newborn rat, non-selective uptake of immunoglobulins, proteins, growth factors and/or enzyme occurs in the ileum by non-specific bulk transfer (Rodenwald, 1973, Graney, 1968 and Clarke and Hardy, 1969). In the distal small intestine, epithelial enterocytes in suckling rats have no IgG receptor, and macromolecular uptake occurs mainly by non-selective fluid phase endocytosis (Weaver and Walker, 1989). Enterocytes of the ileum contain an elaborate array of membrane compartments, tubules and a variety of multivesicular bodies and a giant supranuclear vacuole for the transport and degradation of macromolecular proteins (Cornell and Padykula, 1969 and Connella and Neutra, 1984). The capacity for non-selective transfer in the rat remains high

during the first two weeks of postnatal life and then decreases during the third week (Jones, 1972). Concomitantly, the proteolytic activity of the stomach and pancreas increases during the 3rd postnatal week (Jones, 1972). A role for intracellular protein digestion in distal ileum in suckling rats is further supported by studies showing that lysosomal activity is greater in the distal region during the first 3 weeks *post partum* (Patt, 1977 and Henning, 1985).

In humans, meconium corpuscles are analogous to supranuclear vesicles in the distal rat ileum and are present during uterine development until approximately 22 weeks gestation (Trier and Moxey, 1979). Furthermore, specialised apical tubular systems in the human distal small intestine may be responsible for macromolecular absorption from amniotic fluid, similar to macromolecular transport of milk proteins in suckling rats. Thus, as suggested by Trier and Moxey (1979), the structure of the human small intestine at 22 weeks gestation resembles that of the suckling rat just prior to weaning.

The cessation of macromolecular uptake is termed “intestinal closure” and occurs in the rat at the time of weaning. In the rat, intestinal closure appears to be directly related to an increase in cellular proliferation and migration and a repopulation of the immature enterocytes by adult-like cells that are not capable of macromolecular uptake (Rodenwald, 1973 and Simister and Rees, 1983). This process differs in different species and in the rabbit, hamster and guinea pig, enterocyte cell turnover appears partially independent from intestinal closure. Intestinal closure in the rat can be accelerated by administration of corticosteroids, thyroxine (Baintner 1986, Patt 1977), insulin (Harada and Syuto, 1991) and epidermal growth factor (Harada et al., 1990).

1.4.2 Brush border enzymes:

Developmental patterns of brush border enzymes: Ontogenetic development of the small intestine continues in all mammals during adaptation to extrauterine life. Functional adaptation includes the preparation of the intestine for the digestion of dietary carbohydrate. At weaning, changes in digestive enzyme function are especially remarkable in the rat and include a coincidental decrease in lactase-phlorizin hydrolase activity, appearance of sucrase-isomaltase and a rapid increase in maltase, glucoamylase trehalase and alkaline phosphatase activity (Rubino et al., 1964, Henning, 1985 and Ménard and Calvert, 1991). Many brush border peptidase activities such as leucynaphthylamidase, glutamyltranspeptidase and tripeptidase as well as enteropeptidase, the key enzyme in the activation of proteolytic enzymes, are low during the first two weeks of postnatal life in rodents but increase rapidly to adult levels at the time of weaning (reviewed by Ménard and Calvert, 1991). Although the importance of intrinsic timing mechanisms in triggering intestinal maturation at weaning has been well documented, the transition to an adult-like diet is required for normal gastrointestinal development.

1.4.3 Intestinal disaccharidases

The brush border of the small intestine contains 4 glycosidases which split dietary disaccharides to free monosaccharides and oligosaccharides (Auricchio and Sebastio, 1989). Firstly, the lactase-phlorizin hydrolase (LPH) complex is a glycosidase formed by two subunits. LPH is responsible for the hydrolysis of β -glycosidic bonds of lactose, cellobiose and a number of synthetic substances (Auricchio and Sebastio, 1989). LPH contains two active sites which hydrolyse lactose and glycosylceramides present in milk-fat globules, respectively (Schlegel-Haueter et al., 1971 and Auricchio and Sebastio, 1989). Second, the sucrase-isomaltase (SI) forms a complex and in the adult animals represents the major constituent of the brush border

membrane. SI forms two active subunits which hydrolyse maltose/sucrose and maltose/isomaltose and some α -amylases. Furthermore, α -glucosides (up to six glucose residues) are also hydrolysed by SI (Semenza, 1981). Third, the maltase/glucoamylase complex hydrolyses maltase, starch, glycogen and 1-4 and 1-6 linked-oligosaccharides. This complex also hydrolyses isomaltase, although at much lower affinities (Auricchio et al., 1965b). Finally, trehalase is a β -glycosidase responsible for the hydrolyses of α,α' -trehalose, which is a nonreducing disaccharidase occurring mainly in algae and mushrooms (Nakano et al., 1977). In the human intestine, lactase/glycosylceramide and sucrase/isomaltase activities display a distinct gradient along the length of the intestine with highest activities in the jejunum and lower activities in the distal small intestine (Raul et al., 1986). This gradient is reversed for maltase/glucoamylase activity (Raul et al., 1986). Moreover, the intestinal disaccharidases also display a distinct developmental profile in most mammals. Because the SI and LPH complexes have been studied most extensively, some of their characteristics will be reviewed in more detail.

1.4.3.1 Lactase activity in the developing small intestine

LPH, is a type I transmembrane glycoprotein containing both β -galactosidase and β -glucosidase activity within one polypeptide (Auricchio and Sebastio, 1989 and Rings et al., 1994). For many years, LPH has been regarded as a β -galactosidase; however, based on sequence comparison, substrate specificities and mapping of active sites (Wacker et al., 1992), lactase has been shown to be a β -glucosidase (Henrissat, 1991) and as such may have multiple functions beyond the period of lactation. In this thesis, the term lactase will be used for LPH and for general recognition, lactase will be classified as a β -glucosidase.

Lactase is proteolytically processed during the insertion into the apical microvillous membrane, resulting in a membrane bound enzyme and a released enzyme (Yeh et al., 1991d). However, the fate of the released enzyme is still controversial (Rings et al., 1994 and Naim et al., 1991). In most mammalian neonates, the role of lactase is critical, since this enzyme is the only small intestinal brush border hydrolase responsible for the digestion of lactose. Lactase specific activity in the small intestine is high around birth, declining to low levels at the time of weaning (Auricchio and Sebastio, 1989). Lactase activity exhibits a distinct proximo-distal developmental gradient in humans and other mammals (Newcomer and McGill, 1966). In rats, during the first two weeks of postnatal life, lactase (mRNA and protein) is present along the total length of the small intestine and in the proximal colon (Büller et al., 1989). However, on day 21 of postnatal development, lactase mRNA and protein were no longer detectable in the terminal ileum and from day 28 onwards, reduced expression (patchy expression) of lactase has been observed in the duodenum and ileum (Rings et al., 1994).

The developmental decline in lactase activity in the rat appears to be determined by intrinsic genetic determinants (Montgomery et al., 1981). It has been suggested that lactase activity is not regulated by its substrate in experimental animals (Goda et al., 1985 and Leichter, 1973). For instance, in adult rabbits and rats, addition of milk or lactose to the diet did not alter lactase activities (Goda et al., 1985). However, this research group has shown that some regulation occurs in response to high glucose levels. Dietary manipulations in experimental animals have not clearly identified the effects of dietary manipulation on lactase activities, and increases, decreases and no difference in lactase activities have been reported (reviewed by Koldovsky, 1981b). Recent evidence suggests that lactase activity is primarily regulated at the level of gene

transcription so that the total lactase activity correlates well with total lactase mRNA both along the intestine and during development (Büller et al., 1990a and 1990b).

Several studies have suggested that hormones modulate the developmental pattern of lactase at weaning in the rat. For example, in the suckling rat, hypophysectomy and thyroidectomy alter the developmental decline in lactase activity at the time of weaning. This can be restored by thyroxine (T_4) replacement therapy (Yeh and Moog, 1974 and Paul and Flatz, 1983). The involvement of T_4 in the ontogenetic decline of lactase may be related to the fact that this hormone is a potent stimulator of mitotic activity of the intestinal epithelium (Yeh and Moog, 1977). Glucocorticoids appear to influence lactase activity during the early suckling period, and increases in lactase activity have been reported following administration of cortisone, T_4 and cortisone T_4 and in combination (Yeh et al., 1991a, Yeh et al., 1991b and 1991c). For instance, a single dose of cortisone on the 6th postnatal day in suckling rats increased lactase activity and cell turnover rates by 37% and 95%, respectively (Yeh et al., 1991c). Administration of thyroxine alone induced no changes. Administration of cortisone and thyroxine in combination decreased jejunal lactase activity (23%) but increased cell turnover rates by up to 176% (Yeh et al., 1991c). The pattern of lactase expression was similar in the hormone treated and control groups, however, the calculated cell age at half maximum lactase expression was much lower in cortisone and/or cortisone and thyroxine treated rats (Yeh et al., 1991c). Because cell turn-over rates increase markedly between postnatal day 17 and 19 in the rat, the relationship between cell kinetic parameters and lactase expression in suckling rats has been investigated (Yeh et al., 1991c). No direct relationship was however, detected between the lactase activity and enterocyte life span, thus the authors concluded that mechanisms other than enhanced cell turn-over rates are responsible for the decline in lactase expression following hormone treatment. Amongst others, administration of epidermal growth

factor has also been reported to enhance lactase activity in suckling rodents (Malo and Ménard, 1982 and Foltzer-Jourdainne and Raul, 1990 and 1993). In addition, administration of insulin to 2-day old miniature piglets also increased lactase activity, however this was not regulated at the level of its mRNA (Shulman et al., 1992b).

1.4.3.2 Sucrase-isomaltase activity in the developing small intestine

Sucrase-isomaltase is an α -glucosidase located in microvillous membranes of enterocytes, coupled with isomaltase (Koldovsky, 1981a). Cloning of the sucrase-isomaltase from cDNA in rabbits has shown that SI is synthesised as a single precursor. Sucrase isomaltase, thereafter called sucrase, is a glycoprotein responsible for the splitting of sucrose to fructose and glucose and maltose to two glucose molecules (Koldovsky, 1981a). In humans, sucrase is present in the 3 month old foetus with a proximo-distal gradient of activity already established (Koldovsky et al., 1965 and 1981a). In other mammals, including rats, the appearance of sucrase activity varies. For instance, in immature mammals, such as the rat or mouse, sucrase activity is absent in foetal life and during the suckling period but increases at the time of weaning, which is the time of transition to a solid diet containing carbohydrates with α -linkages (Koldovsky, 1981b).

In the foetal small intestine of rodents, the intestine carries a developmental program for sucrase which determines the time of sucrase appearance and also stipulates the proximo-distal developmental gradient (Kendall et al., 1979 and Koldovsky, 1981b). In humans, the foetal development of sucrase and other α -disaccharidases occurs during early foetal development and precedes that of the lactase and cellobiase. Thus, premature infants have been considered lactase-deficient (Koldovsky, 1981b).

The postnatal pattern of sucrase development has been under intense investigation (Herbst and Koldovsky, 1972, Koldovsky, 1981a and 1983, Moog, 1971, Leichter, 1973 and Yeh and Moog, 1975). These studies have shown that in rodents, sucrase activity is practically undetectable at birth and as shown by immunohistochemical staining, appears first during the weaning period in enterocytes near the base of villi (Doell et al., 1965). The developmental pattern of isomaltase (palatinase) parallels that of maltase (Henning, 1985, Rubino et al., 1964 and Moog et al., 1973). Trehalase activity also increases in a similar fashion to that of other α -disaccharidases (Moog et al., 1973).

During the suckling period, sucrase activity can be modulated by nutrients. For instance, feeding a solution containing sucrose or maltose for a 3-4 day period to suckling rat pups increases sucrase activity and at the same time decreases lactase activity (Lenbenthal et al., 1972 and Raul et al., 1986). Similar results are obtained when suckling rats are fed artificially by continuous gastric infusion (Lenbenthal et al., 1972). However, as reported by Henning and Sims (1979), impediment of weaning does not prevent the appearance of sucrase, although a delay in the decline of lactase activity is observed. Sucrose administration by gastrostomy to suckling rats induces precocious appearance of sucrase, but if the suckling rats are previously adrenalectomised, this effect is not observed (Lebenthal et al., 1972), suggesting that the precocious induction of sucrase activity represents a stress-related response. In support of this hypothesis are observations that sucrase activity develops normally in bypassed intestinal segments (Tsuboi et al., 1981). Thus as concluded by Henning (1985), "dietary changes cannot be considered the primary cause of the various enzymic changes that occur in the intestine during the third postnatal week" in rats.

Regulation of intestinal enzymic maturation by glucocorticoids has been studied extensively (Henning, 1978, Yeh and Moog, 1977 and Doell and Kretchmer, 1964). For example, circulating concentrations of corticosterone, which is the principle glucocorticoid in rats, increase at the beginning of the third postnatal week (Redman and Sreebny, 1976) and precedes the increase in sucrase activity by approximately 48 hours. Furthermore, administration of glucocorticoids to suckling rats during the first two postnatal weeks causes precocious induction of sucrase, maltase, trehalase, and amino-peptidase activities (Doell and Kretchmer, 1964, Galand and Forstner, 1974, Henning et al., 1975 and Moog, 1953 and 1971). Conversely, neonatal adrenalectomy or hypophysectomy delays the appearance of several α -glucosidases (Moog, 1953, Koldovsky et al., 1965 and 1981a and 1981b and Yeh and Moog, 1975, 1977 and 1978). On the other hand, administration of cortisone to hypophysectomised suckling rats restores the normal pattern of sucrase activity (Yeh and Moog, 1978). The ability of cortisone to precociously induce sucrase activity appears to be mediated via crypt enterocytes, because it has been observed that administration of hydrocortisone to 9 day old suckling rats induces sucrase activity in enterocytes located near the mouth of the crypt while villus enterocytes appear unaffected (Nordström et al., 1968). As discussed by Henning (1985), developmental changes of enzymes are modulated by, but are not absolutely dependent on, corticosteroids. Thus, glucocorticoids may provide a mechanism for precocious maturation in suckling rats forced to early weaning.

The level of T_4 rises during the second postnatal week (Samel, 1968) and is believed to play a role in intestinal enzyme maturation. For instance, administration of T_4 or triiodothyronine (T_3) not only causes an early decline in lactase activity (Paul and Flatz, 1983) but also precociously increase jejunal sucrase and maltase activity (Koldovsky et al., 1975 and Jumawan and Koldovsky, 1978). Administration of a single dose of T_4 induced precocious

expression of intestinal sucrase-isomaltase protein in both intact and adrenalectomised rats without altering serum cortisone levels, thus T_4 may modulate sucrase expression directly and not by increasing serum cortisone concentrations (Yeh et al., 1989).

1.5 Growth of the mature gastrointestinal tract

Growth in the adult, mature gastrointestinal tract relates to the fact that the tissues of the gastrointestinal tract are able to respond to stimuli by alteration of the proliferative activity of the epithelium. This may occur in response to alteration in the quantity and quality of the diet, following surgical intervention or in disease. This section will review the mechanisms regulating structure and function of the gastrointestinal tract in adult animals. I will mainly review aspects relating to the epithelial lining, because generally speaking, this represents the surface of the mucosa that is covered with the functional cells and also represents the source of new epithelial cells.

1.5.1 Renewal of the intestinal epithelium

In the gastrointestinal tract, the mucosa can be divided into the villi, which form the functional compartment of the intestinal mucosa, and the crypts which represent the proliferative compartment of the mucosa. Cells are produced by stem cells in the proliferative crypt (crypt of Lieberkühn), migrate into the functional compartment and are ultimately lost from the extrusion zones of the intestinal villi (reviewed by Wright and Alison, 1984a and 1984b). Between the proliferative and functional compartment is the transitional zone (maturation compartment) where enterocytes lose their proliferative capacities and acquire their characteristic mature functions. As the cells move from the proliferative compartment to the functional compartment, there is a general increase in cellular organelles, including

mitochondria, microvilli and endoplasmic reticulum (VanDonsen et al., 1976). In the colon, the organisation is similar. However, this region is devoid of villi so that the proliferative and functional compartment are both located in the colonic crypt.

Under normal conditions, the relationship between cell production in the crypt and cell loss from the extrusion zone is maintained in a steady-state (Leblond and Stevens, 1948). This steady-state can be perturbed by a multitude of stimuli including starvation, intestinal resection, coeliac disease or continuous irradiation (Gleeson et al., 1972, Sato et al., 1972, Cairnie, 1967, Al-Dewachi et al., 1977 and Dowling, 1982).

1.5.2 Regulation of intestinal proliferation and mucosal growth

The regulation of mucosal growth in the adult intestine involves numerous nutritional, endocrine, paracrine, neural and vascular factors (Ménard and Calvert, 1991). In addition, second and third messengers that transmit signals to the proliferative crypts are of equal importance in regulating epithelial cell turn over.

1.5.3 Dietary manipulation and endogenous secretions

Like for the suckling animals, nutrients are important stimulators of intestinal growth in the adult animal. For example, the mucosal DNA and total protein content of small intestinal segments reduces by approximately 30% following starvation for a 6 day period (Steiner et al., 1968). Similarly, a 3 day starvation period induces a decrease in duodenal villus height and crypt depth by up to 25% below that observed in control animals. Nevertheless, the proximo-distal gradient in mucosal height is not perturbed by the nutrient deprivation. Concomitant with the decrease in mucosal mass is a reduction in glucose transport and disaccharidase activity (McManus et al., 1970). This suggests that nutritional regulation of mucosal growth

and intestinal absorptive function is region dependent. Further evidence for the stimulation of mucosal growth by lumenally derived nutrients stems from studies in animals maintained on total parenteral nutrition (TPN). In TPN animals, whole body nitrogen balance is maintained but the normal dietary signals and the negative metabolic factors associated with fasting are removed. This leads to a marked intestinal atrophy in TPN fed animals, including rats (Levine, 1991 and Johnson et al., 1975), dogs (Hughes, et al., 1978) and rabbits (Eastwood, 1977). Carbohydrate, amino acid and fat absorption are also perturbed in TPN fed rats (Levine 1986 and Kotler et al., 1980 and 1981), suggesting that in adult animals intestinal proliferation and function are regulated in part by nutrients. An increase in intestinal lactase activity is observed in adult rats following starvation (Nsi-Emvo and Raul, 1984). However, the starvation-induced increase in lactase activity can be prevented by administration of thyroxine (Nsi-Emvo et al., 1986), indicating that factors other than nutrients regulate intestinal function.

In experimental diabetes, Miller et al. (1977) have shown that intestinal crypt cell proliferation and enterocyte migration rates are markedly increased. In pair fed diabetic animals, the increased proliferative activity was persistent which indicates that mechanisms other than increase food intake were responsible. The hyperplasia observed in diabetic animals is usually accompanied by an increase in sugar absorption which has been related to a greater number of sugar binding sites in the intestine of diabetic rats (Schedl and Wilson, 1971). Administration of insulin normalises food intake, intestinal mass and the increased absorptive capacity in diabetic rats (Fedorak et al., 1987), suggesting that insulin may directly regulate intestinal function.

1.5.4 Regulation of intestinal growth and maturation by peptide growth factors

Intestinal growth and enzymic functions in the adult animal are also regulated by numerous peptide growth factors. Because this represents a topic of its own, only a brief discussion will be included.

Endogenous secretion from the stomach, liver, pancreas and small intestine all contribute to mucosal growth. The trophic action of gastrin on pancreatic, gastric and mucosal growth of the small and large intestine is well documented (reviewed by Johnson, 1981). Mucosal hyperplasia has been observed in response to pancreatic secretions (Altmann, 1971). Growth effects in response to cholecystokinin (CCK) have also been demonstrated (Dembinski et al., 1980 and Hughes et al., 1978). For instance, the mucosal hypoplasia that is normally associated with TPN can be prevented by administration of secretin and CCK in rats and dogs (Hughes et al., 1978). Furthermore, in 14-day old rat pups, a marked pancreatic hyperplasia was observed in response to an increased amount of secretin (Harada and Syuto, 1993). In that study, precocious induction of maltase and accelerated cessation of macromolecular transfer have also been observed (Harada and Syuto, 1993). In gut resected rats, administration of secretin and CCK-octapeptide significantly increases mucosal mass which is due to stimulation of pancreatic or biliary secretions (Weser et al., 1977). Similarly, administration of Prostaglandin E₂ (PGE₂) to 16 day old rodents markedly stimulates the maturation pattern of intestinal enzyme expression (Marti and Fernandez-Otera, 1994). For instance, administration of 4mg/kg of PEG₂ increased sucrase, maltase, lactase and aminopeptidase activity. Crypt depth was also increased by up to 35% above control values (Marti and Fernandez-Otera, 1994). Other peptide hormones including bombesin (Karkashan et al., 1992) and insulin (Ménard and Malo, 1979 and Soling and Unger, 1972) have also been shown to stimulate pancreatic and gastrointestinal growth.

Of all peptide hormones, however, EGF effects on the growth and maturation of the intestinal mucosa have been studied most extensively. EGF has been implicated to control intestinal growth and modulate intestinal function. EGF secretions have been shown in salivary, biliary and duodenal epithelium (Cohen, et al., 1982 and Li et al., 1983) and orogastric or intraperitoneal administration of EGF significantly stimulate intestinal H₂O, Na⁺ and glucose transport (Opleta-Madsen et al., 1991). In the hypoproliferative intestine (TPN fed rats), intravenously but not intragastrically administered EGF significantly increase intestinal tissue weight, cellular proliferation and the crypt growth fraction in the gastrointestinal tract (Goodlad et al., 1987a and 1987b and 1992). Gastroprotective effects of EGF in models of mucosal damage have also been described (Tepperman and Soper, 1994).

In summary, it can be concluded that the pattern of gastrointestinal development and maturation is regulated by the interaction of genetic determinants and nutrients, as well as exposure to non-nutritive biologically active factors and endocrine secretions. This process starts in foetal life, proceeds through neonatal life and extends past weaning into adulthood. Although in the adult animal growth and maturation of the gastrointestinal tract have ceased, the tissues of the gut are remarkably adaptive to changes. Furthermore, the intestinal epithelium is continuously replaced. Similar to the immature intestine, mucosal growth and intestinal function in the adult are regulated by nutrients and a multitude of regulatory factors.

Part 2: Insulin-like growth factors, binding proteins and receptors and their role in gastrointestinal growth and function

Insulin-like growth factors (IGFs) are multifunctional anabolic hormones which act throughout life. Their action depends on the responsiveness of the target cell which may be modulated by the presence of circulating or locally produced insulin-like growth factor binding proteins (IGFBPs), specific receptors, their relative expression and ligand affinity. IGFs act in either endocrine, paracrine or autocrine fashion and are capable of stimulating a vast variety of anabolic processes and growth promoting activities (Sara and Hall, 1990).

1.6 Historical aspects

In 1957, Salmon and Daughaday observed that growth hormone (somatotrophin) stimulated $^{35}\text{SO}_4$ incorporation into cartilage *in vivo* but not *in vitro*. The serum from hypophysectomised rats was devoid of the sulfation activity, however treatment of hypophysectomised animals with growth hormone (GH) restored the sulfation activity (Salmon and Daughaday, 1957, Daughaday et al., 1957 and 1972). Based on these observations, Salmon and Daughaday postulated that a “sulfation factor” was necessary in mediating the growth hormone signal to the target tissue. The term somatomedin was designated to substances mediating growth promoting activity of growth hormone. The substance capable of “sulfation activity” in rat cartilage was termed Somatomedin C (Van Wyk et al., 1974).

A second independent line of evidence leading to the discovery of insulin-like growth factors (IGFs) was provided by observations by Frösch et al. (1963). This research group observed that serum elicits insulin-like effects far in excess than would be expected by the immunoreactivity of insulin. Furthermore, only a small fraction of the insulin-like activity on

adipose tissue could be suppressed by anti-insulin antibodies. The non-suppressible part of the insulin-like activity was soluble in acid ethanol and was termed non-suppressible insulin-like activity (NSILA).

A third line of evidence was provided by a series of *in vitro* experiments conducted by Pierson and Temin (1972). These researchers extracted low molecular weight factors from calf serum that showed multiplication stimulating activity (MSA) and also fitted the somatomedin hypothesis. When added to culture medium, these factors stimulated liver cells to replicate. Later, Dulak and Temin (1973) showed that cultured livers cells were also able to secrete MSA into the cultured medium leading to autostimulation of the cultured hepatocytes. It was also recognised that MSA and somatomedin A showed non-suppressible insulin-like activities (Pierson and Temin, 1972); at the same time, NSILA showed growth promoting activities (Morell and Frösch, 1973 and Zingg and Frösch, 1973). When it became apparent that the sulfation activity could not be separated from MSA and NSILA, the individual research groups jointly agreed to introduce the term somatomedins to denote factors that transmit the GH signal in the stimulation of somatic growth and display insulin-like activity.

The term “insulin-like growth factors” was introduced when two biologically active polypeptides, insulin-like growth factor I and II (IGF-I and IGF-II), purified from human plasma and showing structural homology to insulin, were shown to be responsible for the insulin-like effects (Rinderknecht and Humble, 1978a and 1978b). During the last decade, a vast expansion in the field of IGF research has been undertaken. In order to understand the complex interactions of the IGFs as well as to identify the mechanism by which these growth factors promote growth related activities, it is necessary to discuss some aspects of IGF physiology in more detail.

1.7 IGF chemistry and structure

The insulin-like growth factors (IGF-I and IGF-II) belong to a family of polypeptides which show structural homology to insulin and pro-insulin (Rinderknecht and Humbel 1978a and 1978b) and are highly conserved across species. Their overall structure shows similarities to nerve growth factor (NGF), epidermal growth factor (EGF), relaxin and other members of the insulin family (Weinmann and Kiess, 1990). The high sequence homologies between insulin and IGFs indicates that the peptides originated from a common ancestor protoinsulin precursor by means of gene duplication and divergence. The estimated divergence most likely occurred some 600 million years ago, when vertebrates first appeared on earth (Frösch et al., 1985). It is suggested that the divergence between IGF-I and IGF-II occurred some 300 million years later (Frösch et al., 1985).

Using X-ray crystallography, three-dimensional models for IGF-I and IGF-II have been constructed (Blundell et al., 1978). IGF-I is a basic, single chain polypeptide comprising 70 amino acids linked by 3 intra-chain disulfide bridges and a molecular mass of 7646 Da (Rinderknecht and Humbel, 1978b). IGF-II is a slightly acidic single chain molecule made up of 67 amino acids and a molecular mass of 7471 Da. It shows a 65% and 43% sequence homology to IGF-I and insulin, respectively (Rinderknecht and Humbel, 1978b). Like pro-insulin, both IGFs possess an amino-terminal A and B region, separated by a short connecting C region. The C domain of the IGFs is considerably shorter than the C region of pro-insulin and no apparent sequence homology is present within this region (Humbel, 1990). Unlike pro-insulin, the IGFs contain an extension at the carboxyl terminal, which has been labelled the D region (Rinderknecht and Humbel 1978b).

The primary structure of IGF-I is highly conserved as shown from either protein or cDNA studies with bovine, porcine and human IGF-I being identical (Rinderknecht and Humbel, 1978a, Francis et al., 1986, Tavakkol et al., 1988, Shimatsu and Rotwein, 1987). Similarly, rat and mouse IGF-I differ by only one amino acid and from human by 3 and 4 amino acids, respectively (Sara and Hall, 1990). The primary structure of IGF-II is also highly conserved. Differences in the primary structure of IGF-II are observed mainly towards the end of the B domain and within the C region. For instance, bovine IGF-II and human IGF-II differ by only 3 amino acids, while rat and mouse IGF-II differ by 2 amino acids (Sara and Hall, 1990).

1.8 IGF genes, precursors and posttranslational processing

Aspects of the IGF gene structure and the posttranslational processing of IGFs have been recently reviewed by K. Lund (1994). IGF-I genes have been characterised in humans and rats. They consist of more than 80 kilobases of genomic DNA, comprising 6 exons and a number of introns that encode large precursor proteins (Bell et al., 1985, Rotwein et al., 1986, Lund et al., 1989 and Lund, 1994). Exon 3 and 4 comprise the coding sequence of IGF-I and alternate splicing of exon 1, 2, 5 and 6 results in four potentially different IGF-I mRNA species that show different sequences at both the 5' and 3' end (Lund et al., 1989). Alternate splicing thus results in either Class 1 (Ea/Eb) or Class 2 (Ea/Eb) IGF-I mRNAs which show tissue and developmental specific patterns of expression (Lund, 1994). In humans and in the rat, there are at least 4 different precursors which differ in their amino- and carboxyl terminal but contain the same IGF-I sequence (Lund, 1994).

The IGF-II genes have been studied in the mouse, rat and humans. While the mouse and rat gene are similar in organisation, the human IGF-II gene differs from the rodent IGF-II gene in that it comprises 9 exons (30 kilobases of genomic DNA) instead of 6 exons (de Pagter et al., 1988 and Frunzio et al., 1986). In both humans and rodents, exons 4, 5 and 6 are alternately spliced resulting in different IGF-II mRNAs (Nielson et al., 1990 and Soares et al., 1986). As for the IGF-I gene, transcription of the human IGF-II gene can be achieved by different promoters (P1-P4) which are active in a tissue and developmentally specific manner. For example P1 is only active in the adult human liver, while P2, P3 and P4 are active in different foetal tissues at different times in development (Han et al., 1988 and Lund, 1994). Similarly, the three different promoters in the rat are regulated developmentally (reviewed by Lund, 1994).

1.9 Distribution and localisation of IGFs

A wide variety of data implicates the liver as the major source of circulating IGFs. This has been demonstrated in studies of isolated perfused livers (Schwander et al., 1983 and Phillips et al., 1976), foetal and adult liver in organ culture (D'Ercole et al., 1980 and Binoux et al., 1982), rat liver cell lines (Marquardt et al., 1981) and primary hepatocyte cell cultures (Kogawa et al., 1983 and 1992). It has been considered that IGF production by the liver accounts for the major part of the serum IGF content (Baxter, 1986). Studies on the distribution of IGF mRNA in humans and rodents have shown that IGF-I and IGF-II are also synthesised in multiple organs and tissues (D'Ercole et al., 1980 and 1984 and Sara et al., 1983) and are present in several body fluids, including mammary secretions (Baxter et al., 1984a), semen (Baxter et al., 1984b), ovarian follicular fluid (Adashi et al., 1985 and Hammond et al., 1982), amniotic fluid (Merimee et al., 1984), saliva (Costigan et al., 1988), urine (Hizuka et al., 1987), lymph (Cohen et al., 1972) and spinal fluid (Haselbacher and

Humbel, 1982). Although the liver is the major source of IGFs in circulation, the contribution of IGFs by non-hepatic tissues raises the question of the function of circulating IGFs. It has been postulated that the liver and possibly a few other organs act as an endocrine source of IGFs for target tissues at distant sites, while IGFs produced by other non-hepatic tissues act in an autocrine or paracrine fashion (Baxter, 1986). The latter mechanism has been proposed particularly during foetal development, where many of the foetal tissues, including the gastrointestinal tract, produce high levels of IGFs and may act in a paracrine fashion by exerting their biological function on nearby cells (D'Ercole et al., 1980).

1.10 IGFs and their binding proteins

In serum, the IGFs are associated with a heterogenous group of highly specific IGF binding proteins, leaving very little free circulating IGFs (Zapf et al., 1975 and Cohen and Nissley, 1976). During a workshop on the IGFbps in Vancouver, Canada, a proposal was adopted to designate the binding proteins "IGFBP" followed by an arabic numeral and letter prefix to indicate species specificity (Ballard et al., 1989a). This nomenclature has been used throughout this thesis.

The total number of different IGFBPs is not known. So far, six distinct classes of IGFBPs (IGFBP-1 to IGFBP-6) have been identified. The complete primary structures of all six binding proteins have been identified by molecular cloning in the rat and in humans (Rosenfeld et al., 1990, and Shimasaki et al., 1989a and 1989b, 1990, 1991a and 1991b). IGFBPs have been identified in several tissues as well as in serum and other extracellular fluids (Sara et al., 1983, Funk et al., 1992, Lassarre et al., 1991). Considerable sequence homologies exist between the various IGFBPs which suggests that, like the IGFs, the IGFBPs also

also originated from a common ancestral gene. A common feature among their structural properties is the conservation of 18 cysteine residues near the amino and carboxyl termini of the peptides (Sara and Hall, 1990). This implies that a rigid secondary structure is required for interaction of the IGFBPs with either IGFs or with cell surfaces (Clemmons et al., 1991).

Although the IGFBP field is rapidly expanding, our understanding of the function and interactions of IGFBPs is still highly speculative. While most studies have demonstrated that IGFBPs modulate IGF action as inhibitors (Mohan et al., 1989 and Drop et al., 1979), enhancement of biological activities have also been reported (Elgin et al., 1987 and Blum et al., 1989). Some of the already identified characteristics concerning the IGFBPs will be discussed in this review.

1.10.1 IGFBP-3

In postnatal life, IGFBP-3 is the predominant circulating binding protein. The complete primary structure of IGFBP-3 has been identified for humans (Wood et al., 1988), pigs (Shimasaki et al., 1989b) and rats (Shimasaki et al., 1990). In human serum, IGFBP-3 is found in two forms of approximately 40 and 43 KDa, estimated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, which are N-linked glycosylated derivatives of a 29 KDa protein (Wood et al., 1988). In the circulation 90% or more of IGFBP-3 is found in a ternary, high molecular complex of approximately 140 kDa. This large molecular complex comprises a non-IGF-binding, acid labile sub-unit (ALS or α subunit), a glycoprotein of approximately 85 kDa, a 47-53 kDa acid stable β -unit and the 7.5 kDa IGF-I polypeptide (γ -sub-unit) (Baxter and Martin, 1989a). Formation of the 140 kDa complex involves the binding of the β - and γ -sub-unit to form a binary complex before

binding of the α -sub-unit can be accomplished (Baxter and Martin, 1989a and 1989b). In the absence of IGF-I or IGF-II, little interaction occurs between the α - and β -sub-unit (Baxter and Martin, 1989b). Because IGFs are stabilised by binding to IGFBP-3 and the α -sub-unit circulates at a concentration much higher than IGFBP-3, it can be assumed that IGFBP-3 plays a significant role in determining the total concentration of circulating IGFs (Baxter, 1993).

Levels of IGFBP-3 are approximately 20-50 times greater than IGFBP-1 and/or IGFBP-2 and approximately 75% of the IGFs are bound to this carrier protein (Baxter, 1989b, Gargosky et al., 1990). Binding affinities of IGF-I and IGF-II to IGFBP-3 are approximately the same (Martin and Baxter, 1986). The major site of IGFBP-3 synthesis is the liver, however mRNA for IGFBP-3 has also been shown in a variety of human tissues, including the distal small intestine (Albiston et al. 1992). As shown by Naya et al. (1991), IGFBP-3 mRNA expression in adult humans is highest in the liver, ovaries, the spleen and prostate. Several cell lines, including rat hepatocytes, cultured osteoblasts, fibroblasts, chondrocytes and endothelial cells also express the IGFBP-3 gene (Sara and Hall, 1990 and Spencer, 1979).

The three major factors controlling IGFBP-3 concentrations are age, nutrition and growth hormone. In humans, IGFBP-3 levels are developmentally regulated so that plasma IGFBP-3 levels increase from low levels at birth, reaching maximum at puberty, gradually declining to low levels with age (Blum et al., 1990, Baxter and Martin, 1989b). Similarly in the rat, IGFBP-3 levels are low at birth, increasing to adult levels at the time of weaning (Donovan et al., 1989). A developmental pattern of IGFBP-3 expression has also been reported for the pig (Lee et al., 1991). It is generally believed that the 140 kDa is GH dependent, since the 140 kDa complex is enhanced in patients with acromegaly, diminished in patients with hypopituitarism and restored following GH therapy (D'Ercole and Wilkins, 1984,

Baxter and Martin, 1986 and Furlanetto et al., 1977). Further evidence for GH dependence stems from observations that the levels of immunoreactive IGFBP-3 and spontaneous nocturnal secretions of GH show high correlations (Blum and Ranke, 1990). Regulation of IGFBP-3 by the nutritional status has been demonstrated by several investigators. Decreased levels of IGFBP-3 have been reported in humans following food restriction (Blum and Ranke, 1990), in patients in negative nitrogen balance following surgery (Baxter, 1993), in caloric restricted pigs (McCusker et al., 1989) and in rats maintained on a low protein diet (Tomas et al., 1991 and Clemmons et al., 1989). Because age, nutrients and GH are all regulators of IGF-I as well as IGFBP-3, it is still unclear whether GH or IGF-I is the primary stimulus for IGFBP-3 production. Although most clinical studies indicate that GH is the primary stimulus for IGFBP-3, in cultured chondrocytes and fibroblasts the administration of IGF-I increased IGFBP-3 production in the absence of GH (Martin and Baxter, 1990); similarly, IGFBP-3 production increases in hypophysectomised rats following treatment with either IGF-I or GH (Zapf et al., 1989). This suggests that IGF-I may regulate IGFBP-3 directly.

So far, the precise function of IGFBP-3 remains speculative; however, several roles have been implicated. IGFBP-3 prolongs the biological half-life of IGFs from about 10 minutes to approximately 15 hours (Guler et al., 1989b). At the same time, IGF analogues, with greatly reduced affinity to IGFBP-3, have a much shorter serum half-life than IGF-I (Ballard, 1987, Francis et al., 1992, Bastian et al., 1993 and Cascieri et al., 1988). This suggests that IGFBP-3 provides a reservoir for IGFs. At present, it is not clear by which mechanism IGFs in circulation become available to the tissues to elicit their growth related activities. The large IGFBPs complex (ternary complex) does not readily cross the capillary endothelial barrier, however, smaller IGFBPs may do so (Binoux and Hossenlopp, 1988). Thus, for an endocrine role of circulating IGFs, a mechanism to dissociate the ternary complex

or decreasing the binding affinity of the α -sub-unit (involving proteolytic enzymes or proteoglycans) may operate, resulting in the liberation of the binary IGF-IGFBP-3 complex (Clemmons et al., 1983, Baxter 1993 and Blum et al., 1989). Either in complexed form or after liberation of free IGFs, growth related processes may be activated (Baxter, 1993).

1.10.2 IGFBP-1

Whilst the physiological significance of IGFBP-1 is not clear, several potential roles of IGFBP-1 have been identified. IGFBP-1 is abundant in amniotic fluid (Chochinov et al., 1977 and Drop et al., 1979), placental tissues (Koistinen et al., 1986) and conditioned medium from the hepatoma cell line HEP-G2 (Póvoa et al., 1984). IGFBP-1 synthesis has been described in the endometrium (Rutanen et al., 1985), granulosa cells (Jalkanen et al., 1989), foetal liver explants (Lewitt and Baxter, 1989) and HEP-G2 cells (Lee et al., 1988). IGFBP-1 is the most widely studied of the IGFBPs in human serum.

IGFBP-1 is a low molecular BP with a predicted molecular mass of 25 kDa and appears at approximately 28 kDa on non-reduced SDS-PAGE. It has about 20% the affinity for IGFs than IGFBP-3, consists of a non-glycosylated single polypeptide chain and shows approximately equal affinities for both IGFs (Baxter, 1991). IGFBP-1 contains a cysteine rich N-terminus region followed by a region where the amino acids Pro, Ser and Thr are prevalent. This region is normally found in proteins with a short intracellular half life and rapid turnover rates (Holly, 1991). It also contains an Arg-Gly-Asp sequence (RGD region), which is commonly found in matrix proteins involved in cell surface attachment via receptors of the integrin family (Hynes, 1987). As for IGF-I and IGFBP-3, the liver is considered the major

source of IGFBP-1 production. Immunoreactive IGFBP-1 has been shown in foetal as well as adult livers (Baxter, 1993).

In humans, circulating levels of IGFBP-1 are developmentally regulated with high levels at birth falling steadily to low levels at puberty (Baxter, 1993). This pattern is opposite to the pattern of IGF-I or IGFBP-3, but differs during pregnancy where a steady rise in IGFBP-1 and IGF-I occurs (Hall et al., 1986). In humans, a distinct diurnal rhythm of IGFBP-1 has also been described. For example, 24 hour sampling studies in children and adults revealed that IGFBP-1 levels show considerable diurnal variations with a distinct nocturnal peak (Baxter and Cowell, 1987, Busby et al., 1988 and Yeoh and Baxter, 1988). The nocturnal peak levels of IGFBP-1 were dependent on the subject's hormonal and metabolic status, but appeared either independently or inversely related to the subjects GH secretory status (Baxter and Cowell, 1987, Cottrill et al., 1988, Suikkari et al., 1988 and Snyder and Clemmons, 1990). The inverse relationship between GH and IGFBP-1 is demonstrated in patients with acromegaly, where plasma IGFBP-1 levels are usually low (Hall et al., 1988). Conversely, in GH deficient patients, elevated levels of IGFBP-1 have been reported (Busby et al., 1988). Infusion of GH to healthy normal subjects also caused a decline in plasma IGFBP-1 levels and the nocturnal rise in IGFBP-1, which could be blocked by food intake. A rapid decline in IGFBP-1 levels during early morning hours was observed in fasted subjects (Busby et al., 1988).

Regulatory patterns comparable to that of known glucose counter-regulators such as glucagon have been observed for IGFBP-1. For instance, carbohydrate intake suppressed IGFBP-1 levels, while insulin administration (resulting in hypoglycaemia) increased IGFBP-1 levels (Cottrill et al., 1988 and Yeoh and Baxter, 1988). The degree of IGFBP-1 suppression

correlated in clamp studies with the degree of insulin-stimulated glucose transport (Suikkari et al., 1989), further supporting a role of IGFBP-1 in glucose homeostasis. Additionally, IGFBP-1 activity was reduced in patients with insulin-dependent diabetes mellitus which could be restored by insulin replacement therapy (Rieu and Binoux, 1985). Further evidence for a role of IGFBP-1 in glucose homeostasis is derived from in vitro studies. Rat hepatocytes derived from diabetic and insulin treated rats showed increased IGFBP-1 levels (Scott and Baxter, 1986). In human foetal liver explants, the glucose concentration in the medium was inversely related to the IGFBP-1 production, which could be blocked by the addition of insulin to the medium (Lewitt and Baxter, 1989).

From these studies, IGFBP-1 is believed to fulfil several roles. The insulin-induced decrease in IGFBP-1 levels indicate that IGFBP-1 is rapidly turned over. Thus, if insulin levels are low, IGFBP-1 levels may rise in response to the hypoglycaemia to modulate IGF availability and thus further complement a decline in insulin activity (Holly, 1991). Additionally, IGFBP-1 may act as a transport molecule for IGFs from the circulation to the target tissues. The presence of the RGD sequence indicates that IGFBP-1 may attach to the cell surface of certain target cells and consequently modulate IGF activity in a tissue specific manner. This is supported by studies, demonstrating that in perfused rat heart, IGFBP-1 is preferentially located in the cardiac muscle and not in connective tissues (Bar et al., 1990). Finally, since IGFBP-1 shows equal affinity for both IGF ligands, and circulating levels of IGF-II in man are higher than IGF-I levels, IGFBP-1 may play a role in modulating the activity of IGF-II.

1.10.3 IGFBP-2

IGFBP-2 is a single chain non-glycosylated protein and its predicted molecular mass is 31 kDa under non-reducing conditions on SDS-PAGE (Binkert et al., 1989). IGFBP-2 shows approximately equal binding affinities for both IGFs when IGF-I is used as the radioligand; however, a marked preference for IGF-II is exhibited when IGF-II is used as the radioligand (Baxter and Martin, 1989b and Szabo et al., 1988). The complete primary structure was deduced from cDNAs isolated from a rat BRL-3A cell library (Brown et al., 1989), adult rat (Margot et al., 1989) and from human foetal liver libraries (Binkert et al., 1989). IGFBP-2 is developmentally regulated with high levels of IGFBP-2 detectable in foetal rat serum, which decreases rapidly during postnatal life (Orlowski et al., 1990 and Ooi et al., 1990). IGFBP-2 shows little diurnal variation and like IGFBP-1 and -2, also contains a cysteine rich region at the N- and C- termini and a RGD sequence near the carboxyl terminus (Ooi, 1990). The presence of the RGD sequence means that IGFBP-2 may also potentially interact with cell surface integrin receptors (Ooi, 1990).

IGFBP-2 is the major binding protein in serum in the pre- and perinatal period in rats (Glasscock et al., 1991, Babajko et al., 1993 and Chan and Nicoll, 1994). Although plasma levels in adult humans and rats are low, IGFBP-2 is the major binding protein in cerebrospinal fluid and is abundant in the central nervous system (Tseng et al., 1989 and Romanus et al., 1989). It has been determined that IGFBP-3 carries up to 90% of the IGFs; however, IGFBP-2 might also act as a significant IGF carrier protein in the circulation. For instance, IGFBP-2 levels increase under conditions characterised by reduced concentrations of the ternary complex such as during fasting, states of excess IGFs resulting from an IGF-II secreting tumour or following infusion of IGF-I (Zapf et al., 1990, Young et al., 1991 and Thrailkill et al., 1990). Furthermore, in situations of relative IGFBP-3 deprivation, like caloric deficiency

or hypopituitarism in humans (Clemmons et al., 1991) or in rats with hypothyroidism (Näntö-Salonen and Rosenfeld, 1992), IGFBP-2 levels are also elevated. Nutritional regulation of IGFBP-2 has also been documented for rats, where hepatic IGFBP-2 mRNA levels were increased following a protein restricted diet (Strauss and Takemoto, 1990). Regulation of IGFBP-2 by GH has been implicated in studies showing that hepatic IGFBP-2 mRNA was elevated in hypophysectomised rats; however, this could not be restored by GH replacement therapy (Margot et al., 1989).

As suggested by Baxter (1993), IGFBP-2 may provide an intermediate level of IGF regulation under conditions where the larger IGFBP complex is unable to transport all available IGFs. In most conditions listed above, IGFBP-2 would be occupied mainly by IGF-II because of the markedly higher binding affinity of IGF-II to IGFBP-2 and also because in the human circulation, the level of IGF-II exceeds IGF-I concentrations (Baxter, 1993). There may be, however, some species specific exceptions. For example in rodents, this situation is somewhat different because of the extremely low levels of circulating IGF-II during postnatal life.

1.10.4 IGFBP-4, -5, -6

To date, very little is known about the physiology of these smaller molecular weight IGFBPs. Human IGFBP-4 migrates on non-reduced gels with an apparent molecular mass of 24 or 30 kDa, the latter being a N-glycosylated form of the former (Kiefer et al., 1991). IGFBP-4 binds IGF-II with greater affinity than IGF-I (Mohan et al., 1989). In rat serum, IGFBP-4 has been demonstrated on 2 dimensional gel electrophoresis and appears to exist in both glycosylated and non-glycosylated forms, with apparent molecular weights of 28 kDa and

24 kDa respectively (Chan and Nicoll, 1994). IGFBP-4 production has been reported for cultured osteoblasts and other bone-derived cell lines (Tørring et al., 1991), as well as for human fibroblasts (Camacho-Hubner et al., 1992). In the rat, IGFBP-4 mRNA has been demonstrated in several tissues, including adrenals, testis, spleen, heart, lung, kidney, liver stomach and the brain (Shimasaki et al., 1990). Nevertheless, the relative contribution of IGFBP-4 to the circulation is at present not known (reviewed by Baxter, 1993).

At present, little is known about IGFBP-5 in the circulation, but this binding protein appears as a double band on SDS PAGE with an apparent molecular weight of 29 kDa and greater affinity for IGF-II than for IGF-I (Martin and Baxter, 1990). IGFBP-5 has been demonstrated in cerebrospinal fluid and media of various cells in culture (Drop et al., 1992). Likewise, IGFBP-6 has been identified in human cerebrospinal fluid (Roghani et al., 1989) and is also produced by transformed human fibroblasts (Baxter and Saunders, 1992). It appears that IGFBP-6 is regulated by GH because in subjects with acromegaly, a reduction of IGFBP-6 has been observed (Baxter, 1993).

To date, the properties of IGFbps and their interaction with IGFs to regulate cellular events are still somewhat speculative. While most studies suggest that the major role of IGFbps is to prolong the half-life of circulating IGFs, more complex biological activities are being recognised. For example, specialised cell types may be capable of utilising carrier bound IGFs as many cell types and tissues secrete large molecular weight IGFbps endowed with an RGD adhesion sequence near the carboxyl terminus. While some functions of circulating IGFbps have been recognised, the role of the smaller IGFbps in serum are so far presumptuous. In addition, it is not clear why different forms of IGFbps exist and, although there is increasing evidence to suggest distinct roles of IGFbps at different developmental

times or under different physiological conditions, the biological significance still needs to be elucidated.

1.11 IGFs and their receptors

Like other hormones, the action of IGFs on their target cells are mostly initiated by binding to specific cell surface receptors. Examination of receptors by affinity labelling led to the definition of two distinct IGF receptors, the type 1 and type 2 receptor. The type 1 receptor binds IGF-I with high affinity, IGF-II cross-reacts to a lesser degree and insulin binds weakly (Masague and Czech, 1982, and Kasuga et al., 1982). The primary structure of the type 1 receptor has been determined from cDNA and has been identified as a heterotetramer with a molecular mass of 450,000 dalton, comprising two α -subunits (130 kilodaltons) and two β -subunits (95 kilodaltons), joined by interchain disulfide bridges which are dissociable under reducing conditions (Masague and Czech, 1982). The α -subunits contain the extracellular ligand binding site for either IGF-I, IGF-II or insulin, and the β -subunits form the transcellular domain attached to an ATP binding site and an intracellular enzymatic tyrosine kinase domain (Morgan et al., 1986). The binding of the ligand to the extracellular domain results in intracellular signal transmission by autophosphorylation of tyrosine residues within the intracellular β -subunit (Morgan et al., 1986). It is thought that the type 1 receptor mediates the biological effects of both IGF-I and IGF-II (Rechler and Nissley, 1985 and Ullrich et al., 1986). Modifications of the type 1 receptor have been identified on rat and human brain membranes (Gammeltoft et al., 1985 and Roth et al., 1987), representing differences in glycosylation of the α -subunit of the receptor (Heidenreich et al., 1986). It has been proposed that the functional significance of the modified type 1 receptor in the brain relates to the predominance of the truncated IGF-I present in the CNS (Sara and Carlsson-Skwirut, 1988).

A striking feature of the type 1 receptor is its close resemblance in structure to the insulin receptor (Massague and Czech, 1982), even to the extent that some monoclonal human anti-insulin-receptor-antibodies cross-react with the type 1 receptor (Ullrich et al., 1986).

The type 2 receptor (IGF-II receptor) is structurally unrelated to the type 1 receptor and/or the insulin receptor, and binds IGF-II with high affinity and does not recognise insulin (Massague and Czech, 1982 and Nissley et al., 1993). In some tissues, weak affinities for IGF-I have been reported (Rechler and Nissley, 1985). This receptor is a 270 kDa monomer and consists of a large extracellular domain that is approximately 93% of the receptor protein and contains 15 repeat sequences of cysteine residues (Sara and Hall, 1990). The type 2 receptor has a single transmembrane region and a small cytoplasmic domain without tyrosine kinase activity. The sequence of the human IGF-II receptor is identical to the cation independent Mannose-6-phosphate receptor (Tong et al., 1988, Lobel et al., 1987, Morgan and Edelman 1987 and MacDonald et al., 1988). The IGF-II- mannose-6-phosphate (IGF-II/M6P) receptor is located on both the cell surface and golgi apparatus, where it binds and mediates the transport of lysosomal proteins (Von Figura and Hasilik, 1986 and Sahagian, 1984). Although a single receptor mediates the function of two unrelated effects, the receptor contains distinct binding sites for IGF-II and M6P (Kiess et al., 1987a and 1987b, Tong et al., 1980 and 1988 and Braulke et al., 1988). As reported by Kiess et al. (1989a) there are 3 distinct binding sites, two for the mannose 6 phosphate marker and one for IGF-II. Their studies showed that cellular uptake and binding of β -galactosidase by the M6P receptor was inhibited by IGF-II (Kiess et al., 1989b), thus suggesting that IGF-II may regulate the binding and transport of lysosomal enzymes.

In addition, the IGF-II/M6P receptor may also have species specific functions during different times in development. As shown by Kiess et al. (1987a), a circulating IGF-II/M6P receptor, which is 10 kDa smaller than the membrane bound receptor, has been identified in sera of rats ranging from 19 days foetal to 12 month postnatal (Kiess et al., 1987a). The circulating IGF-II/M6P receptor lacks the cytoplasmic domain and appears to be developmentally regulated with sharply declining levels in rats older than 12 months. The developmental pattern of the IGF-II/M6P receptor is similar to that of IGF-II and the mRNA of IGF-II itself (Moses et al., 1980 and Brown et al., 1986). It is believed that the major function of the IGF-II/M6P receptor in foetal life is to target lysosomal enzymes to lysosomes (Kornfeld, 1992) and additionally, to mediate the degradation of IGF-II by receptor-mediated internalisation (Oka et al., 1985 and Kiess et al., 1989b). Finally, specific cellular responses such as Ca⁺⁺ influx (Nishimoto et al., 1987), glycogen synthesis (Hari et al., 1987) or glucose uptake in chondrocytes (Bhaumick and Bala, 1991) have also been attributed to interactions of IGF-II with the IGF-II/M6P receptor, thus suggesting direct signalling functions. As shown by Okamoto, et al., (1990), interaction of IGF-II with the IGF-II/M6P receptor activates GTP-binding proteins via a G-protein, thus stimulating cellular events which may be further enhanced by the interaction of other growth factors such as epidermal growth factor and platelet derived growth factor.

The presence of specific receptors has been shown for a number of foetal and adult tissues, including the heart, lungs, muscle, kidneys, liver, brain and the intestines (Nissley et al., 1993, Cui et al., 1993, Guse, et al., 1992, Adams et al., 1983, Rosenfeld, et al., 1982, Lowe and LeRoith, 1986 and Baskin et al., 1988). Furthermore, subtype receptors, including variant forms of the type 1 receptor, hybrid IGF-I/insulin receptors and anomalous or atypical receptors have also been reported (reviewed by Soos et al., 1991 and Jacobs and Moxham,

1991). The physiological role of the variant receptor forms is at present not clear; however, it cannot be ruled out that these receptors play a significant role in the signal transduction pathway of IGF or insulin.

1.12 Biological activity of IGFs

In vitro action of IGFs: It is well established that IGF-I and IGF-II stimulate DNA synthesis, cellular proliferation and mitotic division in a variety of cell types including fibroblasts, gonadal cells, hepatocytes, epithelial cell, myocytes and hematopoietic cells (reviewed in detail by Van Wyk, 1984). As suggested by Van Wyk (1984), IGFs act as progression factors, in conjunction with competence factors such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF) or epidermal growth factor (EGF). Rapid IGF effects observed on classical insulin target cells are often indistinguishable from insulin effects and include enhanced lipid and glycogen synthesis, glucose uptake and amino acid synthesis (Zapf et al., 1984). Furthermore, IGF inhibits lipolysis and calcium ATPase in the same way as insulin (Frösch et al., 1985). These effects are believed to be mediated through the type 1 receptor or via the insulin receptor (Zapf et al., 1984). The long-term effects, which are mainly mediated through the type 1 receptor, include the stimulation of mitosis, cellular differentiation and associated biosynthetic events such as DNA, RNA and protein synthesis and inhibition of protein breakdown (Sara and Hall, 1990 and Ballard and Gunn, 1985). For example, IGFs have been shown to stimulate erythropoiesis (Claustres et al., 1987), meiotic division in oocytes (El-Eter et al., 1979), differentiation of ovarian function (Adashi et al., 1984) and the differentiation of myoblasts, osteoblasts and oligodendrocytes (Schmid et al., 1983 and 1984, McMorris et al., 1986).

In vivo action of IGFs: *In vivo* studies have confirmed the biological action of IGFs, demonstrating both acute insulin-like action as well as chronic growth promoting effects. In normal and hypophysectomised rats, intravenous bolus injection of IGF-I or IGF-II induced hypoglycaemic effects, enhanced glucose uptake and glycogenesis (Zapf et al., 1986 and Skottner et al., 1987), with IGF-I being more potent than IGF-II. Similar results have been obtained in healthy adult humans (Guler et al., 1987).

Relatively few studies have investigated the *in vivo* growth promoting effects of IGF administration. In hypophysectomised rats, growth promoting effects have been shown following administration of IGF (Guler et al., 1988, Skottner et al., 1987, Zapf et al., 1985 and Schönle, et al., 1982). Similarly, infusion of IGF-I preparations have been shown to promote somatic growth in Snell dwarf mice (Smeets et al., 1983, Van Buul-Offers et al., 1988 and 1986 and 1994), insulin-deficient diabetic rats (Scheiwiller et al., 1986), mutant dwarf rats (Skottner et al., 1989), normal rats (Hizuka et al., 1986) and normal suckling rats (Philipps et al., 1988). Growth promoting action of IGF-I administration on the gastrointestinal tract has been discussed separately.

GH-IGF-I interaction: The relationship between IGF-I and GH is well defined. Circulating levels of IGF-I in humans are dependent upon the GH secretory status. In patients with acromegaly, IGF-I levels are about 4-7-fold higher than in normal subjects (Baxter et al., 1982 and Zapf et al., 1980 and 1981). No elevation is apparent for IGF-II levels (Daughaday, et al., 1981 and Hintz and Liu, 1982). In contrast, in growth hormone retarded or growth hormone deficient subjects (pituitary dwarfs), IGF-I levels are approximately 15-25% those of normal subjects, rising dramatically following GH replacement therapy (Merimee et al., 1982

and Draznin et al., 1980). Similar observations have been made for animals. For instance, IGF-I levels are markedly decreased in hypophysectomised rats (Kaufmann et al., 1978) and dogs (Eigenmann et al., 1985). Conversely, in rats bearing growth hormone-producing tumours high levels of plasma IGF-I are evident, which can be normalised following excision of the tumour (Chochinov et al., 1977). Further evidence for a close relationship between GH and IGF-I stem from the observation that plasma IGF-I levels correlate with birth-weight and gestational age (Gluckmann et al., 1983). Likewise, the GH related growth spurt during puberty coincides with a marked rise in IGF-I levels to values approximately 2-3 times those normally found in adults. During adulthood plasma IGF-I levels decline, which most probably reflect the decline in GH secretion with age (Van Wyk, 1984 and Frösch et al., 1985).

Although these studies have shown that GH and IGFs are interdependent, a distinct pattern of growth promoting activities of GH and IGF-I may exist. For example, in hypophysectomised rats, the kidneys, spleen and thymus are more sensitive to IGF-I administration, whereas skeletal muscles are more sensitive to GH administration (Guler et al., 1988). Similarly, GH shows no anabolic action in insulin-deficient diabetic rats, while IGF-I administration restores their growth (Scheiwiller et al., 1986). In neonatal rats, injection of antiserum raised against either IGF-I, IGF-II or GH has shown that GH but not IGF-I stimulates growth rates (Robinson et al., 1993). Similarly, administration of GH but not IGF-I to neonatal dwarf rats results in increased growth parameters (Ambler et al., 1993).

It appears that at present, distinct GH or IGF-I effects cannot be dissected completely and are obviously further complicated in *in vivo* studies by the interaction of nutritional and other hormonal factors. Some of these questions may be answered by a unique approach of targeted mutagenesis of genes encoding for IGF-I and II and/or their receptor. This approach

has been taken by researchers headed by A. Efstratiadis' research group, investigating the role of IGF-I, IGF-II and the type 1 receptor in embryonic and postnatal growth. These studies indicated that during early embryogenesis, the type 1 receptor serves only *in vivo* signalling of IGF-II, while during later development, both ligand interact. They also showed that until commencement of the involvement of GH in growth, IGF-I functions in growth were independent of GH (Baker et al., 1993).

1.13 IGF-I analogues

To date, several variant forms of IGF-I have been engineered to address aspects of IGF-IGFBP and receptor interactions. Removal of 1 to 5 amino acids from the N-terminus markedly changes the biological potency of IGF-I *in vitro* (Francis et al., 1986). Des(1-3)IGF-I, which represents a truncated form of IGF-I that lacks 3 amino acids (Gly, Pro, Glu) at the N-terminus, shows a greatly increased potency in stimulating DNA and protein synthesis and inhibiting protein degradation in a number of cell lines, including human lung and skin fibroblasts and L6 myocytes (Ballard, et al., 1989b and 1987, Francis et al., 1986 and Szabo et al., 1988). The increased potency of the variant IGF-I molecule was attributed to its difference in binding affinity for IGFBP-2, IGFBP-1 and also to a lesser extent, to IGFBP-3 (Szabo et al., 1988, Ross et al., 1989 and Forbes et al., 1988). A second variant, long R³IGF-I (LR³IGF-I) has been engineered, with Glu³ replacing by Arg³, thus changing the charge at position 3 (Bagley et al., 1989). This variant contains a 13 amino acid residue extension from GH at the N-terminus and also shows greatly enhanced *in vitro* potencies. For example, despite having only about one third of the affinity to the type 1 receptor, LR³IGF-I also shows even lower affinities for IGFBPs (Francis et al., 1992). At the start of this study, the *in vivo* potencies of the two variant forms have been assessed in adult rats following massive small bowel resection

(Lemmey et al., 1991). These studies have shown that the increased potency of the analogues, observed in the *in vitro* studies, were maintained *in vivo*.

1.14 IGFs and the gastrointestinal tract

When this study commenced, very few studies had examined the effect of IGF-I administration on the growth of the gastrointestinal tract. However, a comprehensive review on IGFs and the gut has been recently published by K. Lund (1994). This section summarises major aspects on the distribution, localisation of IGFs, their binding proteins and receptors and their proposed function on gastrointestinal tissues.

1.14.1 IGF immunoreactivity

Studies investigating the immunoreactivity of IGFs in gastrointestinal tissues have supported the hypothesis that IGFs are important regulators of gastrointestinal growth, maintenance and function. In the human foetus, levels of IGF-I immunoreactivity are highest in the stomach compared to other tissue (D'Ercole et al., 1986). As shown by Han et al. (1987), in human foetal stomach and intestine, IGF-immunoreactivity was localised to epithelial cells, with higher concentrations in villus cells than cells from the crypt compartment. Similarly, in foetal rats, immunoreactive IGF has been detected in the gastrointestinal tract, with IGF-II being higher in the intestine than in the liver or other tissues examined (Yang et al., 1985). In neonatal pigs, IGF-I immunoreactivity has been demonstrated throughout the length of the gastrointestinal tract (Schober et al., 1990). Moreover, an increase in IGF-I immunoreactivity in the neonatal pig intestine appeared to coincide with accelerated mucosal growth and maturation of the intestine during the early neonatal period (Schober et al., 1990).

In gut accessory organs, for example in the human foetal pancreas, IGF immunoreactivity to the cells of the duct of islets has also been demonstrated (Hill et al., 1987).

In the adult gastrointestinal tract, IGF immunoreactivity has been demonstrated on the lymphoid M-cells overlying Peyer's patches (Hansson et al., 1988), and also in gastrointestinal exocrine secretions including human saliva (Costigan et al., 1988), gastric and pancreatic secretions and bile (Chaurasia et al., 1994). As suggested by Chaurasia et al. (1994), "no binding proteins were visualised in samples from gastric and pancreatic secretions", suggesting that IGFs contained in the gastrointestinal lumen are either free, or IGFBP concentrations present in these secretions were below the detectable limit of their assay.

1.14.2 mRNA distribution in the gastrointestinal tract

A number of studies have shown mRNA for IGF-I and IGF-II in human and rat gastrointestinal tissues, in particular during the foetal and early neonatal period. In human foetuses, IGF-I mRNA is expressed in the gastrointestinal tract, liver and in the pancreas (Han et al., 1987 and 1988). Likewise, in foetal rat tissues, IGF-I mRNA is also expressed in the gastrointestinal tract, pancreas and liver (Lund et al., 1986 and Hoyt et al., 1988). The expression of mRNA for IGF-II has been documented in human and rat tissues of the gastrointestinal tract, pancreas and the liver, with maximum levels of IGF-II mRNA detectable in foetal tissues (Lund et al., 1986, Han et al., 1988, Adamo et al., 1989, Lowe et al., 1989, Stylianopoulou et al., 1988 and Brown et al., 1986). The high levels of IGF-II mRNA in the gastrointestinal tract decline rapidly during the early postnatal period, paralleled by a decline in liver IGF-II expression (Brown et al., 1986). In contrast, hepatic mRNA levels for IGF-I increase markedly during the neonatal period, resulting in IGF-I mRNA levels of up to 40

times greater in adult rats compared to foetal rats (Adamo et al., 1989 and Lowe et al., 1989). The marked increase in rat hepatic IGF-I mRNA is, however, not paralleled by increases in IGF-I mRNA of gastrointestinal tissues (Adamo et al., 1989 and Lund et al., 1986 and 1994).

In adult rats, IGF-I mRNA expression has been demonstrated in the stomach, small and large intestine as well as in pancreatic tissues. As reported by Lund (1994), in mature rats, the level of IGF-I mRNA in the stomach is several fold greater than in the small or large intestine (Lund, 1994). Expression of IGF-I mRNA in the gastrointestinal tract appears to be nutritionally regulated. For example, fasting of adult rats for 48 hours reduced IGF-I mRNA in the stomach, brain and testes by 30-40% compared to a reduction of 80% in IGF-I mRNA expression in the liver (Lowe et al., 1989).

1.14.3 Gastrointestinal localisation of IGFBPs

In foetal rat stomach, intestine and the pancreas, mRNA transcripts for IGFBP-1 and -2 are expressed at high levels (Wood et al., 1990, Orłowski et al., 1990). IGFBP-1 has been located immunologically, with strong immunostaining of mucosal epithelial cells and cells within the smooth muscle layer (Hill et al., 1989). IGFBP-2 mRNA levels in the foetal rat stomach are expressed at similar levels to IGF-II, with lower levels detected in the intestine compared to the stomach (Orłowski et al., 1990 and Ooi et al., 1990). Like IGF-II, IGFBP-2 mRNA levels also decline with postnatal age (Orłowski et al., 1990). Because IGF-II has a greater affinity for IGFBP-2 and the levels of IGF-II and IGFBP-2 are greater than IGF-I and IGFBP-3 during the foetal period, IGFBP-2 has been considered the foetal binding protein. Other IGFBPs have also been detected. As reported by Lund (1994), recent studies have indicated that IGFBP-4 is expressed at high levels in the myenteric plexus in rat stomach and intestine (Lund, 1994). In the adult rat, Albiston and Herington (1990) have shown that high

levels of IGFBP-3 are expressed in the ileum, which greatly reduced following 70% of jejunoleal resection (Albiston et al., 1992). From the latter studies, it is tempting to speculate that in situations of gut adaptations where gut growth is severely compromised, reduced levels of locally produced IGFBP-3 increases the bioavailability of free IGFs to stimulate the regenerative growth responses observed following gut resection.

1.14.4 Distribution of IGF receptors

Specific receptors (type 1 and type 2) have been identified throughout tissues of the gastrointestinal tract in several species, including humans (Rouyer-Fessard et al., 1990, Korman et al., 1989 and Guo et al., 1992), pigs (Schober et al., 1990), rabbits (Termanini et al., 1990) and rats (Heinz-Erian et al., 1991, Laburthe et al., 1988, Young et al., 1990, MacDonald et al., 1993 and Ryan and Costigan, 1993). As shown by Laburthe et al. (1988), binding of ^{125}I -IGF-I was detectable throughout the entire rat gastrointestinal tract, with the strongest signal being present in the colon. Similarly, Heinz-Erian et al. (1991) have shown that in the rat, IGF-specific receptors were present throughout all tissue layers but were most abundant in the muscularis propria. Within the mucosa, greater receptor densities have been observed in the lamina propria compared to the luminal surface. A proximo-caudal receptor gradient was observed by both research groups (Heinz-Erian et al., 1991 and Laburthe et al., 1988). Along the crypt:villus axis, a greater number of receptors has been observed for the proliferative crypt epithelium than for the villus compartment (Laburthe et al., 1988 and Heinz Erian et al., 1991). In the rabbit, ^{125}I -IGF-I binding is also more pronounced in the muscularis propria compared to the mucosa (Termanini et al., 1990). Receptor concentrations fall progressively from foetal life through adulthood in a similar pattern to that observed for IGF-II production in the gastrointestinal tract, with the type 2 receptor densities declining more rapidly than type 1 receptors (Sklar et al., 1989 and Young et al., 1990). Interpretation of

receptor ligand binding and localisation studies is complicated by the fact that IGF-I and IGF-II interact with receptors as well as their binding proteins so that localisation of the radiolabelled ligand may present a multitude of IGF ligand interactions. However, studies with IGF analogues that show greatly reduced affinities to IGF-BPs and/or receptors provide a unique tool to investigate receptor localisation studies in the near future.

1.14.5 GH-IGF interaction in the gut

As for other organs, a number of studies suggest that pituitary hormones also influence gut growth. In hypophysectomised rats, the mucosa of the small intestine exhibits a marked mucosal hypoplasia with severe stunting of intestinal villi and a decrease in mitotic crypt enterocytes (Bastie et al., 1982, Scow and Hagan, 1965). In addition, the regenerative capacity of the small bowel in these hypophysectomised rats is greatly reduced (Taylor et al., 1979). In hypophysectomised suckling rat pups, the growth of the small intestine is drastically reduced. Growth can be restored by replacement therapy with GH or GH and thyroxine in combination (Yeh and Moog, 1975 and 1978). Trophic effects of GH on the gut have also been reported. For example, GH administration significantly stimulates mucosal growth and lengthening of the small bowel in rats following 80% of jejunoileal resection (Benhamou et al., 1994 and Shulman et al., 1992a). Administration of plerocerooid larvae of the tapeworm *Spirometra*, which produces a GH-like protein, results in stimulation of adaptive mucosal growth in rats following partial small bowel resection (Hart et al., 1987). Enhanced intestinal function following administration of GH has also been reported. In the human small bowel, enhanced uptake of amino acids across the jejunum and ileum following subcutaneous administration of 0.2mg/kg of GH has been shown by Inoue et al. (1994).

Further evidence for GH action on the gut stems from studies in transgenic animals. For example, in growth hormone deficient transgenic mice, the mucosal weight of the duodenum was reduced compared to their litter mates (Behringer et al., 1990). In contrast, transgenic mice, overexpressing bovine GH, showed a massive increase in mucosal mass compared to wild-type litter mates (Ulshen et al., 1993). However, in the first study, circulating IGF-I levels were relatively low while in the latter study, the animals showed elevated levels of circulating IGF-I levels as well as elevation of IGF-I levels in the small intestine (Ulshen et al., 1993). This raises the possibility that the observed mucosal hypo- and hyperplasia in these GH transgenics results from a dual effector mechanism of GH and IGF-I and suggests that the enterotrophic action of GH may be mediated via IGF-I.

1.15 Aim of this thesis

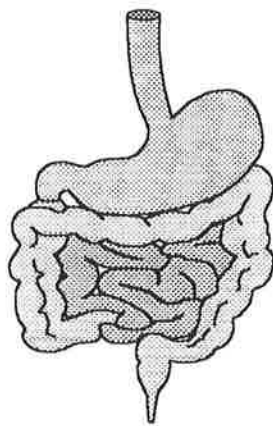
The response of the gastrointestinal tract to IGF-I peptides in situations of compromised gut growth has been addressed by several researchers. For instance, IGF-I peptide effects on the gastrointestinal tract were reported in studies of rats following intestinal resection (Lemmey et al., 1991, Vanderhoof et al., 1992), or in animal models of catabolic weight loss such as dexamethasone-induced catabolism (Read et al., 1992a), after glucocorticoid-treatment or streptozotocin induced diabetes (Read et al., 1991). These studies indicated that IGF-I peptides selectively stimulate gut growth and enhance gastrointestinal function; however, none of these studies conducted a detailed characterisation of the growth response. At the commencement of my PhD studies, very little was known about the effect of IGF-I peptides on the gut. Studies of IGF-I peptide administration in normal rats were sparse, with only a single study reporting gut effects. I therefore decided to characterise in detail the effect of IGF-I peptide administration on the growth and function of the gastrointestinal tract in normal adult rats. Moreover, since differences in responsiveness of the mature gastrointestinal tract and the rapidly growing immature gastrointestinal tract were not elucidated at the time when this study commenced, I decided to define the gut response of IGF-I peptide administration in suckling rats. The role of IGFs on intestinal proliferation and differentiation was not resolved; as such, IGF-I effects on proliferation and cytodifferentiation of the mature and immature intestinal epithelium became the primary aim of this thesis.

The specific aims of this thesis

- 1) *To assess the effects of IGF-I peptide administration on the growth of the gastrointestinal tract in normal adult rats by detailed histological examination of the tissues.*
- 2) *To determine proliferative events following administration of IGF-I peptides and to characterise these events in different intestinal regions.*
- 3) *To determine if the IGF analogues display similar in vivo potency in normal adults as observed in conditions of compromised gut growth.*
- 4) *To assess the responsiveness of suckling rats to IGF-I peptide administration at different time points in development and assess IGF-I specific effects on the tissues of the gastrointestinal tract and other non-gut organs.*
- 5) *To compare the effectiveness of the IGF analogues in suckling rats to those in adult rats.*
- 6) *To assess the effect of IGF-I peptide administration on the disaccharidase enzymes in the immature intestine and determine if IGF-I peptides are able to stimulate the maturation of the digestive enzymes.*

CHAPTER 2:

DEVELOPMENT OF RESEARCH PLANS AND METHODOLOGIES



CHAPTER 2

Development of research plans and methodologies in the study of IGF-I peptide effects on the growth and function of the gastrointestinal tract in adult and suckling rats.

In mammals, vigorous growth and development of organ systems occurs throughout foetal and neonatal life. Furthermore, the tissues of the gastrointestinal tract are unique in that rapid and continuous cell turnover of the mucosal surface is sustained throughout adult life (for reviews see Wright and Alison 1984a and b). Quantitative measurements of gastrointestinal growth and proliferation are therefore complex. Some of the methods used in this thesis, in particular the development of selection criteria for morphometric assessment of histological parameters and the identification of optimal conditions for tissue preparations, required considerable development. The aim of this Chapter is to describe the development of research plans and methodologies to study and assess gastrointestinal growth following administration of IGF-I peptides in adult and suckling rats. Discussion of generally applied methodologies in the assessment of gastrointestinal growth are also briefly outlined in this Chapter. Further details about the application of final protocols are given in the respective Chapters.

2.1 Choice of the animal model

2.1.1 *Species and strain*

The rat is the most commonly used animal model in the study of gastrointestinal growth and intestinal function. In this thesis, the rat was chosen as the appropriate animal model for the following reasons: **1)** they are an inexpensive laboratory animal, **2)** peptide requirements are minimised, **3)** they are easily maintained for breeding, **4)** they give birth to a large number of offspring, thus the large number of suckling animals needed throughout the later parts of the study could be produced by a relatively small breeding stock, **5)** researchers at the Child Health Research Institute had identified the gastrointestinal tract in rats as a

specific target organ for IGF-I peptide action (Read et al., 1991, 1992a and 1992b and Lemmey et al., 1991). Hooded Wistar rats were chosen because these rats are very friendly, making the handling and manipulation of the rat pups an easier task. In addition, female Hooded Wistar rats are highly alert and display excellent nurturing behaviour.

Other animal models were considered. Firstly, larger animals were deemed uneconomical because of the greater costs involved in the purchase and maintenance of the animals. Second, expenses for the recombinant peptides would have significantly increased. Third, in smaller animals such as mice, the surgical manipulations of the suckling animals would have been more difficult and time consuming, which may have jeopardised the well being of these rats. Finally, by using the same species and strain as researchers at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Division of Human Nutrition, Adelaide, archival histological sections of the intestine from adult rats treated with IGF-I peptide were available for examination.

2.1.2 Animal Ethics and Care

The adult female rats used for the studies described in this thesis were purchased from the animal breeding colony at the CSIRO, Glenthorne, South Australia. They were bred in a specific pathogen free environment. In addition, virgin Hooded Wistar rats from the same colony were used for the breeding of the rat pups in the studies described in Chapters 5 and 6. Breeding was carried out at the CSIRO, Glenthorne and the animal holding facilities at the Women's and Children's Hospital.

The experimental protocol for the study described in Chapter 3 was approved by the Animal Care and Ethics Committee of the CSIRO, Division of Human Nutrition and was

conducted in collaboration with Dr. Frank Tomas and associates at the CSIRO. These rats were maintained at the CSIRO animal holding facilities. The animal studies described in Chapters 4, 5 and 6 were approved by the Animal Care and Ethics Committee of the Women's and Children's Hospital. All adult and suckling rats used in these studies were maintained at the animal holding facilities at the Women's and Children's Hospital and all surgical procedures were carried out by myself. Post-operative care was provided mainly by myself with help from Mrs. Kerry Penning and Mrs. Leanne Srpek (animal technicians at the Child Health Research Institute) and the staff from the animal holding facilities at the Women's and Children's Hospital. All experimental protocols were designed with due care to minimise the use of experimental animals and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Recombinant IGF-I peptides and route of IGF-I peptide delivery

2.2.1 IGF-I peptides

The recombinant human IGF-I peptides used in this thesis were provided by Genentech Inc., South San Francisco, CA, USA and by GroPep Ltd., Adelaide, South Australia. The recombinant human analogue LR³IGF-I was supplied by GroPep Pty. Ltd., Adelaide, South Australia. Recombinant human IGF-I peptides (animal/media grade) were synthesised in *Escherichia coli* and exhibit a purity of >95%. The variant LR³IGF-I has Glu³ replaced by Arg³ thus changing the charge at position three (Bagley et al., 1989). Furthermore, LR³IGF-I contains a 13-residue amino acid N-terminal extension from porcine GH, comprising Met-Phe-Pro-Ala-Met-Pro-Leu-Ser-Ser-Leu-Phe-Val-Asn (Francis et al., 1992). The LR³IGF-I variant shows only one third the affinity for the type-1 receptor and shows reduced binding affinity to several of the IGFBPs. However LR³IGF-I has several-fold higher potencies in stimulating protein and DNA synthesis and inhibiting protein breakdown in

rat L6 myoblasts, H35 hepatoma cells and chicken embryo fibroblasts compared to the native IGF-I (Francis et al., 1992). Thus, the increased potency of LR³IGF-I is related to its altered association with the IGF-I receptor and changes in the degree of interaction with IGF-BPs. Both peptides have been used extensively by other researchers in the field, both *in vitro* (Ballard et al., 1987 and 1989b, Francis et al., 1988 and 1992, Bagley et al., 1989, King et al., 1992) and *in vivo* (Lemmey et al., 1991, Read et al., 1992a, Tomas et al., 1992 and 1993 and Bird et al., 1994).

Comparison of the amino acid sequence of IGF-I peptides shows a high degree of similarities between species to the extent that human IGF-I (hIGF-I), bovine IGF-I (bIGF-I) and porcine IGF-I (pIGF-I) have complete identity and hence equal potency (Ballard et al., 1989b, Francis et al., 1986 and Tavakkol et al., 1988). The amino acid sequence between rat IGF-I (rIGF-I) and hIGF-I is also highly conserved (Ballard et al., 1989b) and differs only by one amino-acid. Equipotence in the stimulation of protein synthesis in L6 myoblasts or competition for the binding of ¹²⁵I-labelled IGF-I have been shown for chemically synthesised bIGF-I and IGF-I purified from bovine colostrum (Ballard et al., 1989b). So far, recombinant rIGF-I has not been available in sufficient quantities for large scale infusion studies, but based on the above mentioned information it is expected that infusion of recombinant hIGF-I into rats exhibits similar biological activities, comparable to infusion of rIGF-I.

2.2.2 Continuous subcutaneous infusion of IGF-I peptides

Continuous s.c. delivery was chosen as the means of peptide delivery as this route has been shown to effectively raise plasma IGF-I peptide levels and stimulate tissue growth without significantly influencing blood glucose levels (Scheiwiller et al., 1986 and Schönle et al., 1982 and 1985). In contrast, bolus injections tend to cause acute hypoglycaemia in animals (Zapf et al., 1986, and 1989, and Woodall 1991) and in man (Laron et al., 1989, Guler et al.,

1987, Frösch et al., 1990 and Ranke and Wilton, 1994). Hyperglycaemic effect following s.c. bolus injections of IGF-I have also been reported in sheep (Cottam et al., 1992) and humans (Ranke and Wilton, 1994). Moreover, IGF-I has been shown to be considerably more potent at promoting growth when given by frequent injections rather than once per day (Woodall et al., 1991). Studies from our own laboratory have verified that IGF-I infused continuously into rats is more potent in stimulating gut growth than the same daily dose given by once or twice daily injection (Lemmey, unpublished).

2.2.3 Verification of peptide delivery

The *in vivo* delivery rate of the peptides from mini-osmotic pumps (Alza, Model Alzet 2001) was validated according to the manufacturer's recommendations by Dr. P. C. Owens, CSIRO. For this purpose, 7 adult rats received s.c. implanted mini-osmotic pumps, infusing 7.6 mg IGF-I/ml for a 7 day period. After the peptide treatment period, the pumps were recovered and the residual IGF-I content was measured by RIA as described in Chapter 5, section 5.5.4.5. The average delivery rate determined was 98% of the manufacturer's specification. The recombinant IGF-I peptides used in this thesis retain their full biological activities for at least 18 months when stored at 4°C. At 37°C, the IGF-I peptides remain fully bio-active for up to 3 months (GroPep, Product Specifications, Application Note #101).

2.3 Assessment of gut growth

The most commonly applied measurements to assess gastrointestinal growth are wet weight and length measurements of gastrointestinal tissue components. Although these measurements can be performed quickly and do not require sophisticated equipment, they provide no information on the adaptive changes that occur at the tissue and cellular level during growth. A better understanding of tissue growth can be gained by assessing the

biochemical and morphological status of the gastrointestinal tissues. The usual methods are: **1)** biochemical measurements of gut growth, for example estimation of DNA and protein content of tissue segments; **2)** morphometric measurements in histological sections; and **3)** assessment of the proliferative activity of the tissue.

2.3.1 Biochemical measurements of gut growth

Measurements of DNA and total protein in mucosal scrapings from a defined length of intestine provides an estimate of cell mass (Williamson, 1978). This is a relatively crude measurement in that total measured protein or DNA content cannot distinguish contributions from the epithelium and the lamina propria in the mucosal compartment, or between muscular and connective tissue in the muscularis layer. Considerable amounts of DNA and protein from lymphoid cells are also detected by this method (Wright and Alison, 1984b). Nevertheless, measurements of DNA and protein are easily performed and provide an overall estimation of cellular mass of the mucosal (including submucosal) tissue and muscularis compartments. In this study, the DNA content was measured in tissue homogenates prepared from mucosal scrapings of a 4 cm segment of the jejunum and was determined following the method by Burton (1956). The protein content was measured in the same tissue homogenates by Dulley and Grieve's modification of the Lowry method (Dulley and Grieve, 1975). Both assays were modified to microplates, and optical densities were determined in a Titertek Multiscan MCC microplate reader (Flow Laboratories, North Ryde, Australia). The methodologies for the biochemical assays were already established by others in our laboratories and the application of these methods are described in detail in the respective Chapters.

2.3.2 Morphometric measurements in histological sections

In addition to biochemical measurements of gut growth, changes in the mucosa and the functional cells of the mucosa (epithelium) were assessed. Quantitative evaluation of the

small intestine and in particular the intestinal mucosa cannot be achieved with the measurement of a single parameter because of the complex 3-dimensional configuration of the intestinal mucosa (Wright and Alison, 1984b). For this reason, a number of morphometric measurements were employed to determine histological responses to IGF-peptide administration. These measurements include: **A)** Cross-sectional thickness of mucosal and non-mucosal tissue compartments **B)** measurements of tissue circumference **C)** detailed assessment of the intestinal epithelium.

Cross-sectional thickness measurements: Most researchers measure villus height and crypt depth to detect changes in mucosal mass and neglect changes that may occur in non-mucosal tissue components (ie. muscularis externa). In this study, the cross-sectional thickness of the mucosa and non-mucosal tissue components (submucosa and muscularis externa) have been measured to identify the overall size of these tissue compartments.

Tissue circumference: Measurements of the tissue circumference were taken to detect changes in tissue diameter. In perfectly circular sections, this measurement was used to determine the annular areas of the tissue components. The area was multiplied with the length measurement to estimate tissue volumes.

Detailed assessment of the intestinal epithelium: More detailed measurements on the responses in the epithelial compartment were conducted because of the importance of this compartment in tissue proliferation and absorptive function. Firstly, villus height and crypt depth measurements were taken and the villus height to crypt depth ratio was calculated. Secondly, the number of epithelial cells along the longitudinal crypt axis (crypt column count) and the number of cells around the crypt circumference (crypt row count) were counted in perfectly sectioned tissue material to establish the crypt cell population (calculated as the product of the crypt column count and the crypt row count). An alternative method to estimate the populations of either the crypt or the villi compartment involves microdissection

of crypt/villi and counting the number of epithelial cells following staining of the tissue with the Feulgen method (Wright, 1980, Wright and Alison 1984b). While this method is considered the most accurate and precise, it was not acceptable in the duodenum because of the large ridge-like structures of the villi in this region. However, previous studies by Wright et al. (1989) and Goodlad et al. (1992) have shown that the empirical method employed in this thesis provides an estimation of cell populations comparable to the far more labor intensive microdissection method. The combined information from these measurements provides an estimation of the size as well as the cellularity of the intestinal epithelium.

2.3.3 *Assessment of the proliferative activity*

Changes in mucosal cell population following treatment with IGF-I peptides can be further identified by assessing the proliferative status of the intestinal epithelium. Epithelial proliferation can be studied by a variety of techniques. Counting of mitosis in routine haematoxylin and eosin stained tissues provides the simplest assessment of cell proliferation. This method can be performed in archival paraffin-embedded sections and may be employed in situations where *in vivo* administration of a proliferative marker such as tritiated thymidine is not suitable. However, there are usually only a few mitotic cells per crypt, thus this method provides very limited information on the spatial distribution of proliferative cells within the crypt. The use of flow cytometry is often employed in the study of cell proliferation, but it requires expensive equipment and in order to obtain a complete kinetic profile, it is imperative to ensure that all crypt/villus cells have been completely harvested. More traditionally, cell proliferation has been assessed by measuring the proliferative index, together with the distribution of proliferative cells following administration of an exogenous proliferative marker. For this purpose, the incorporation of an DNA precursor such as tritiated thymidine or bromodeoxyuridine (BrdU) may be employed. Alternatively, cell proliferation can be assessed by immunohistochemical detection of the endogenous proliferative marker, proliferating cell

nuclear antigen (PCNA). PCNA, also known as cyclin, is expressed in proliferating cells and is present in greatest concentration during the S phase of the cell cycle. PCNA is a highly conserved 36-kDa auxiliary protein of DNA polymerase δ (Bravo et al., 1987) and is expressed during G1- and in the S-phase of the cell cycle, declining to low levels during the G2- and M-phase of the cell cycle (Kurki et al., 1988). Immunohistochemical detection of PCNA can be carried out in archival tissue material, thus this technique has an advantage in situations where applications of exogenous proliferation markers is not applicable. On the other hand, tritiated thymidine ($[^3\text{H}]$ thymidine) labelling as a measure of epithelial proliferation has the distinct advantage in that labelling is restricted to S-phase cells and labelled cells are clearly visible if the autoradiographic processing is carefully carried out. Measurement of proliferative activity by this method does, however, require prior administration of $[^3\text{H}]$ thymidine to the animal and cannot be used in studies involving *in vivo* administration of other radiolabelled substances such as labelled amino acids.

In this thesis, the proliferative status of intestinal tissues following administration of IGF-I peptides was monitored using both incorporation of $[^3\text{H}]$ thymidine and immunohistochemical detection of PCNA. $[^3\text{H}]$ thymidine was employed in situations where administration of a proliferative marker did not interfere with the experimental protocol. Alternatively, immunohistochemical detection of PCNA was applied in situations where *in vivo* administration of an exogenous marker was not suitable. Comparisons between $[^3\text{H}]$ thymidine labelling and PCNA labelling in human colorectal tissues have shown that although PCNA is usually over-exposed, the topographic distribution of the label is similar for both markers (Bleiberg et al., 1993). Both methods therefore appear suitable to assess proliferative events following IGF-I peptide administration. The application of methodologies for both proliferative markers are described in the respective Chapters.

2.4 Histological preparations and selection criteria

Before experimental work commenced, a preliminary study was conducted to establish a fixation protocol that would firstly provide good preservation of cytological detail for routine quantitative histological assessment and secondly be suitable for immunohistochemical detection of PCNA. Furthermore, to reduce the variability in histological measurements, strict selection criteria were determined for the measurement of morphometric structures.

2.4.1 Preparation of histological material

Bouin's fluid, which is used routinely at the Child Health Research Institute in the preparation of histological material, was compared to Methacarn fixative to assess its suitability for the detection of PCNA in paraffin embedded tissues. Preservation of PCNA staining in tissue segments fixed for different periods was evaluated. Furthermore, the reproducibility of the immunohistochemical method has been assessed by comparing the PCNA labelling in serially cut sections.

To evaluate the different fixation protocols, multiple duodenal tissue segments were collected from two adult rats and fixed for 2, 4 or 24 hours in either Bouin's fluid or Methacarn fixative (Appendix 2.1). Thereafter, tissue segments were processed for routine paraffin embedding using an automated system (Tissue Tek, Miles Scientific, USA). Five to six transversely orientated tissue segments were embedded in the same mould. Wax blocks were cooled for 2 hours at 4°C prior to sectioning of the tissues at 2µm. From each tissue block, 3 serial-cut sections were prepared. The first of the serially cut section was stained with haematoxylin and eosin (H&E) and mounted with DePex mounting medium (Gurr, BDH Chemicals, Kilsyth, Australia). The remaining two serially cut sections were stored for subsequently detection of PCNA. Tissue sections were examined with a light microscope

(Zeiss, Jenaval, Jena, Germany) and histological measurements were conducted on images captured with a JVC colour video camera. Captured images were digitised by an Image Analysis Software Program (PRISM VIEW, Dapple Systems, Sunyvale, CA), coupled to an Apple Macintosh IICX computer. From each microscopic field, crypt depth and villus height measurements were taken.

2.4.2 Selection criteria

The selection criteria for the measurement of crypt depth included that, a single epithelial cell layer, and the base and neck of the crypt were fully visible in each microscopic field. Selection of villi structures included villi that were cut in a perfect transverse plane with the tip of the villi and the crypt/villus junction clearly visible in each microscopic field. A minimum of 5 perfectly orientated crypt and villus structures were selected and measured in each of the 5-6 tissue segments present on a microscopic slide. For each of the two variables, progressive means and coefficient of variance (CV) were calculated for 30 measurements to determine the minimum number of villi and crypt structures requiring measurement to result in a CV of below 10%.

Good cytological preservation in duodenal tissue segments was achieved with both fixatives. Shrinkage of tissue components was minimal in tissue segments fixed in Bouin's or Methacarn for short periods (2 or 4 hours). Fixation of tissue segments in Methacarn for 24 hours significantly reduced villus height measurements ($P < 0.001$, Student's t-test), as compared to villus height measurements in tissues fixed in Methacarn for 2 or 4 hours (Table 2.1). Conversely, crypt depth in tissue segments fixed for 24 hours in Bouin's fluid was significantly lower compared to the short fixation protocols for both fixatives ($P < 0.0001$, multiple Student's t-test), Table 2.1. Based on these observations, optimal fixation for general

histological morphometry was identified as 4 hours in Bouin's or 2 hours in Methacarn, followed by storage in 70% alcohol for a 24 hour period.

Table 2.1: Measurements of crypt depth and villus height in duodenal sections of normal, female adult rats fixed in either Bouin's fluid or Methacarn fixative.

Fixation Protocol	Villus		Crypt	
	height (μm)	CV	depth(μm)	CV
<i>Bouin's</i> (2hrs)	776 \pm 65(n=13)	8.4%	347 \pm 25(n=15)	7.4%
(4hrs)	765 \pm 64(n=14)	8.4%	341 \pm 24(n=15)	7.1%
(24hrs)	766 \pm 49(n=15)	6.4%	320\pm23(n=10)#	7.1%
<i>Methacarn</i> (2hrs)	770 \pm 49 (n=13)	6.4%	342 \pm 20 (n=15)	5.7%
(4hrs)	684 \pm 58 (n=13)	8.5%	342 \pm 16 (n=13)	4.7%
(24hrs)	621\pm58 (n=17)* f	9.3%	332 \pm 24 (n=14)	7.2%

*All values represent means \pm standard deviations and are derived from up to 30 measurements. The number of observations (n) at which the progressive mean levelled off is shown for each measurement. Villus height in tissue segments fixed for 24 hours in Methacarn was significantly lower than villus height in Methacarn fixed tissue for 2 hours *: $P < 0.05$, or Bouin's fixed tissue for 2, 4 or 24 hours (f: $P < 0.05$). Crypt depth in Bouin's fixed tissue for 24 hours was significantly lower than crypt depth in Methacarn or Bouin's fixed tissue for 2 or 4 hours (#: $P < 0.05$).*

The progressive means and CVs for villus height and crypt depth was assessed and calculated for 30 independent measurements. Results are shown in Table 2.1. For both variables, the progressive mean started to level off after approximately 13-15 measurements. The CVs were approximately 7-8% for crypt depth and villus height for both fixation protocols. In addition to villus height and crypt depth, measurements of the thickness of the muscularis externa and submucosa were evaluated (data not shown). For these variables, 15 measurements in perfectly circular duodenal sections, fixed in Bouin's fluid for 4 hours resulted in a CV of 4.8% (muscularis externa) and 3.8% (submucosa). Measurements of intestinal circumference showed minimal variations between sections and a CV of 6.8% was calculated for six independent measurements (results not shown).

In subsequent studies, tissue segments for standard histological examination were fixed in Bouin's fluid for 4 hours before being transferred to 70% alcohol for a 24 hour period. Morphometric measurements were conducted after carefully scanning the histological preparations to select and measure a minimum of 15 well orientated crypt and villus structures that fulfil the above listed selection criteria. In addition, 15 measurements of the cross-sectional thickness of the muscularis externa and submucosa were taken. Intestinal circumference was traced along the serosa on six transverse embedded, perfectly circular segments at low magnification.

2.5 Detection and evaluation of PCNA

2.5.1 Immunohistochemical detection of PCNA

Two populations of PCNA molecules exist in the nucleus and although both are preserved following formaldehyde fixation (Bravo and MacDonald-Bravo, 1987), only the PCNA molecule that is closely associated with DNA replication sites is preserved in tissues following methanol fixation (Bravo and MacDonald-Bravo, 1987 and Van Dierendonck et al., 1991). In addition, prolonged exposure to fixatives can significantly reduce the intensity of PCNA immunostaining (Hall et al., 1990). I therefore decided to assess the suitability of Methacarn for the detection of PCNA, and to evaluate the reproducibility of PCNA staining intensities in Methacarn fixed tissue. For this purpose, two serially cut sections from each of the above described fixation protocols were assessed. Crypt enterocytes expressing PCNA were detected using a monoclonal anti-PCNA-antibody (murine immunoglobulin M 19A2, Path, Mark Coulter, CA) coupled to a streptavidin-immunoperoxidase reaction, following the methods of Foley et al. 1991 and Garcia et al. 1989. The 19A2 monoclonal PCNA antibody was originally developed by Ogata et al. 1987 and highly significant correlations between the

[³H]thymidine labelling and the PCNA labelling index using this antibody have been demonstrated in archival, paraffin embedded human colorectal tissues (Bleiberg et al., 1993).

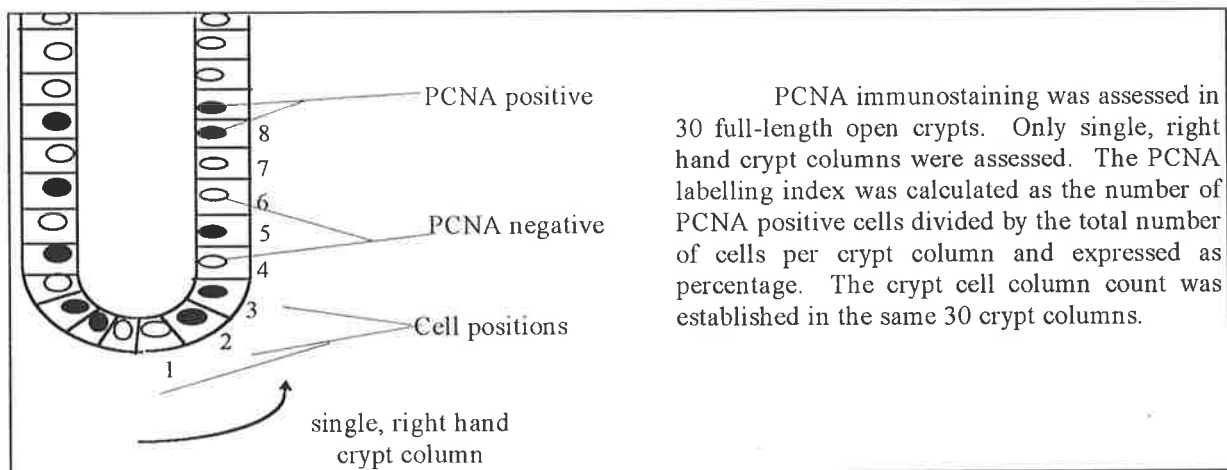
Each of the two serially cut histological sections contained 5-6 duodenal tissue segments and after deparaffinization and rehydration, sections were immersed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxide activity. Sections were then incubated in 10% normal horse serum in phosphate buffered saline (pH 7.4) for 15 min to reduce non-specific staining. This was followed by incubation of the sections with the anti-PCNA antibody (1:50, final dilution) for 90 min and 1.32% normal horse serum. Biotinylated anti-mouse IgM (mu chain specific, Vector Laboratories Inc., Burlingame, CA, U.S.A) was then applied at a dilution of 1:250 in phosphate buffered saline for 30 min, followed by a 30 min incubation with the streptavidin peroxidase conjugate (BioGenex Laboratories, San Ramon, CA, U.S.A.) at a dilution of 1:10. The immunoperoxidase reaction was completed by adding 3,3'-diaminobenzidine (DAB) containing 0.1% hydrogen peroxide (Sigma St. Louis, USA), at a dilution of 1:20 to the sections for 3-6 min. The sections were lightly counter-stained with haematoxylin at a dilution of 1:4 (2 min), dehydrated, cleared and mounted in DePex. Negative control slides from the same tissue were incubated with an irrelevant IgM in diluent in place of the primary antibody. All incubations were carried out at room temperature and reagents were diluted in phosphate buffered saline (pH 7.4). Sections were washed in phosphate buffered saline between incubations.

2.5.2 Assessment of PCNA immunostaining

The quantitative assessment of PCNA labelling was determined in 30 full-length open crypts that fulfilled the selection criteria described above. Crypt epithelial cells were then allocated a position number along the longitudinal axis of the crypt, starting at the base of the crypt (position 0) up to the crypt/villus junction. The presence or absence of PCNA

immunostaining at each position was recorded as illustrated in Figure 2.1. The proportion of crypt epithelial cells expressing PCNA (PCNA labelling index) was determined for the 30 crypts for each of the two serially cut sections and was calculated as the number of PCNA positive cells per crypt column divided by the total number of cells per crypt column. The results are shown in Table 2.2.

Figure 2.1: Schematic representation of an intestinal crypt.



PCNA immunostaining was assessed in 30 full-length open crypts. Only single, right hand crypt columns were assessed. The PCNA labelling index was calculated as the number of PCNA positive cells divided by the total number of cells per crypt column and expressed as percentage. The crypt cell column count was established in the same 30 crypt columns.

PCNA immunostaining in archival tissue material was highly reproducible in both Bouin's and Methacarn fixed tissue segments. However, in Methacarn fixed tissues, PCNA staining intensities reduced from 38.6% after 2 hours of fixation to 31.2% after 24 hours of fixation. It has been previously shown that different fixation protocols result in varying amounts of PCNA labelling intensities in tissue sections (Galand et al., 1989). This may relate to the staining of different populations of PCNA molecules that are preserved differently following formaldehyde or methanol fixation. Despite these differences, the topographic distribution of PCNA positive cells was similar with both fixation protocols and expression of PCNA was confined to the lower two thirds of the proliferative crypt. Compared to tritiated thymidine labelling, PCNA labelling is usually systematically over-expressed, reflecting the fact that a greater proportion of cycling cells and possibly quiescent cells are labelled by the PCNA

antibody. In contrast, administration of a DNA precursor such as tritiated thymidine or BrdU results in a labelling index of approximately 24% and reflects mainly S-phase labelling (Steeb, unpublished data). To reduce any variability in PCNA staining intensities that occur by difference in fixation protocols, tissue segments were fixed for exactly 2 hours in Methacarn fixative before being stored for 24 hours in 70% in all subsequent studies.

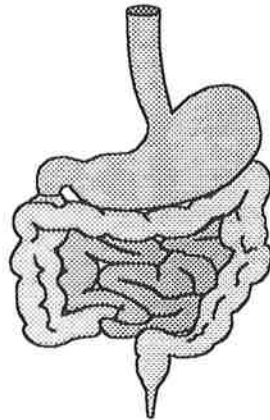
Table 2.2: PCNA labelling indices in duodenal tissue segments fixed in either Bouin's fluid or Methacarn fixative

Fixation Protocol	PCNA labelling(%)	
	<i>Section 1</i>	<i>Section 2</i>
<i>Bouin's (2hrs)</i>	40.4±1.5	42.4±1.5
<i>(4hrs)</i>	38.4±1.3	39.3±1.2
<i>(24hrs)</i>	39.5±0.6	39.9±1.3
<i>Methacarn (2hrs)</i>	38.6±0.9	37.8±0.9
<i>(4hrs)</i>	33.6±1.0**	33.2±1.3
<i>(24hrs)</i>	31.2±1.1**	31.3±1.3

*Values are means±SEM and are derived from 30 measurements for each group. Section 1 and section 2 are serially cut duodenal segments, 2 µm apart. They represent the same intestinal region. For both fixatives, PCNA labelling was similar between section 1 and section 2 at all time points ($P>0.05$, multiple paired t-tests comparison). PCNA labelling in tissue fixed for 4 or 24 hours in Methacarn was significantly lower (**: $P<0.01$, ANOVA) than PCNA labelling in tissues fixed for 2 hours with Methacarn.*

CHAPTER 3:

EFFECTS OF PROLONGED IGF-I PEPTIDE ADMINISTRATION ON THE GROWTH AND FUNCTION OF THE GASTROINTESTINAL TRACT IN ADULT RATS



CHAPTER 3

Effects of prolonged IGF-I peptide administration on the growth and function of the gastrointestinal tract in adult rats.

Contribution to the work

This Chapter describes the effects of prolonged IGF-I peptide administration on the growth and function of the gastrointestinal tract in adult female rats. At the commencement of my PhD candidature, a collaborative study between researchers at the Child Health Research Institute and the CSIRO Division of Human Nutrition was initiated to investigate effects of prolonged IGF-I administration on protein turnover and organ growth in normal adult rats. Dr. Frank Tomas from the CSIRO was the principal investigator of this study, describing the anabolic effects of IGF-I peptide administration in these rats (Tomas et al., 1993). All experimental procedures and surgical manipulation of the animals were carried out by Dr. F. Tomas or by technical staff under his direct supervision. Collection of gastrointestinal tissues was conducted by myself with help from Mrs. Kaylene Pickering, Mrs. Kathryn Davey and Mrs. Kerry Penning. Assessment of gastrointestinal growth responses, histological and immunohistochemical preparations and the collation, assessment and interpretation of the data were carried out by myself.

ABSTRACT

To investigate the effects of prolonged IGF-I peptide infusion on the gastrointestinal tract, adult female rats (115 g, 6 per group) were treated for 14 days with IGF-I or LR³IGF-I at doses of 0, 44, 111, 278 µg/day, delivered by osmotic mini-pumps. Both peptides induced a dose-dependent increase in gastrointestinal tissue weight with LR³IGF-I being several fold more potent than the native IGF-I. Total gut weight, small intestinal weight and small

intestinal length increased by 43, 47 and 13%, respectively, after treatment with 278 $\mu\text{g}/\text{day}$ of LR³IGF-I. Crypt depth and villus height increased after peptide treatment with an associated increase in crypt cell population (+33%), cells per villus column (+34%), and villus cell density (+20%). Proportional increments in proliferating cell nuclear antigen labelling and an unaltered crypt growth fraction indicated a new steady state between cell production and cell loss has been reached. Faecal nitrogen excretion was significantly reduced during the first week of IGF-I peptide treatment, suggesting an increased absorptive capacity of the duodenum. The enhanced potency of LR³IGF-I supports previous findings that the gut is especially responsive to analogues with reduced binding affinity to IGF binding proteins.

3.1 INTRODUCTION

Selective gut growth in response to systemic IGF-I peptide administration has been reported previously. Administration of IGF-I peptides to rats following intestinal resection (Lemmey et al., 1991, Vanderhoof et al., 1992), or in animal models of catabolic weight loss such as dexamethasone-induced catabolism (Read et al., 1992a, Tomas et al., 1993), glucocorticoid treatment or streptozotocin-induced diabetes (Read et al., 1991), stimulates gut growth and enhances gastrointestinal function. In conditions of modified gut growth, the proximal small intestine and the stomach are the most responsive regions and the IGF-I analogues des-(1-3)IGF-I and LR³IGF-I are several-fold more potent in stimulating gastrointestinal growth.

Although these studies have shown that the gastrointestinal tract is a responsive target organ for IGF-I peptides, the mechanism by which IGF-I stimulates gastrointestinal growth in normal animals has not been addressed in great detail. At the commencement of this thesis, only a few studies reported responses to IGF-I administration in normal adult rats and only one included remarks on the gastrointestinal tract. For instance, Hizuka et al. (1986) reported that body weight and longitudinal growth as well as selective organ growth were stimulated by administration of 120µg/day of IGF-I for 7 days to normal adult rats. In suckling rats, Philipps et al. (1988) showed that administration of approximately 30µg/day of IGF-I to neonatal rats selectively increased visceral organ weight. In the only report of gut effects, Young et al. (1990) reported that administration of 1µg/day for a 6 day period to suckling rats stimulated jejunal brush-border enzyme activity, but overall intestinal organ growth was not stimulated at this rather low dose.

The aim of this study was therefore to examine the *in vivo* effects of prolonged s. c. infusion of IGF-I peptides on the growth of the gastrointestinal tissues in normal growing female rats. In view of the selective responsiveness of the proximal small intestine to IGF-I peptide treatment in other animal models (Read et al., 1991 and 1992a and 1992b, Lemmey et al., 1991), a detailed histological and immunohistochemical examination of the duodenal mucosa was conducted.

3.2 MATERIALS AND METHODS

3.2.1 *Recombinant IGF peptides*

Recombinant human IGF-I (rhIGF-I) was provided by Genentech Inc., South San Francisco, CA, U.S.A., and the recombinant human analogue LR³IGF-I was supplied by GroPep Pty. Ltd., Adelaide, S.A., Australia. Specification of the recombinant peptides has been described in Chapter 2.

3.2.2 *Experimental Design*

Choice of animal age group: For this study, 5-6 week old, post-pubertal female rats with a body weight range of 90-110g were used. These rats were still growing, with weight gains of approximately 4g per day. Researchers at the Child Health Research Institute have previously shown that post-pubertal rats are responsive to IGF-I peptide treatment in animal models of compromised gut growth as seen in gut-resected rats (Lemmey et al., 1991) and in dexamethasone treated rats (Read et al., 1992a).

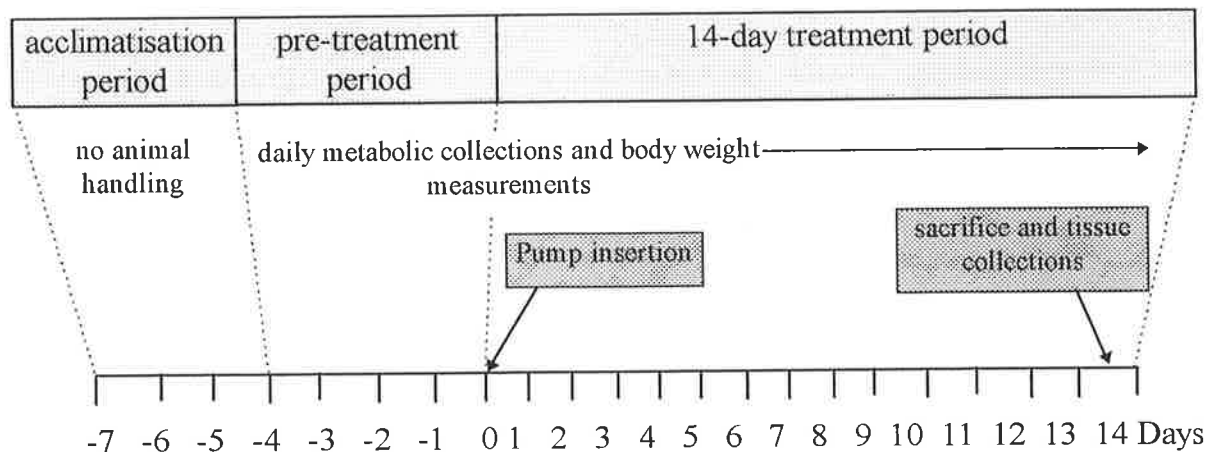
Study design: The rats were allowed to acclimatise to the animal holding facilities (CSIRO) for a 3 day period. To accurately assess the body weight gain, food and faecal output as well as fluid intake and urinary output in a 24 hour period, all animals were housed individually in Techniplast metabolism cages. They were maintained on a 12 h light/dark cycle, at constant room temperature (25°C) and were fed a powdered diet containing 180 g casein plus 2.5 g of methionine/kg body weight as the nitrogen source. Water and food were available *ad libitum*. Daily measurements of body weight, food intake and water intake as well as quantitative collections of faeces and urine were conducted during a 4-day acclimatisation period (**pre-treatment period**) and continued during the 14 day experimental phase

(**treatment period**). The experiment was divided into two animal trials, each trial containing three control rats and three rats in each IGF-I treatment group.

3.2.3 Surgical procedure

At the day of surgery, rats were randomly divided into treatment groups and their body weights were taken. The assignment of rats into treatment groups was carried out by a colleague not involved in any way with the experiment. Associates involved in the study were unaware of the treatment assignment at any stage of the experiment or during data compilation and analyses. On the day of surgery, each animal was anaesthetised with ether and using aseptic techniques, a small incision was made in the pelt within the scapular region of the animal's back. Using a blunt pair of tweezers, the opening was enlarged to enable subcutaneous insertion of a mini-osmotic pump (Model 1007D, Alzet, Alza Palo, CA., USA). Pumps were filled 24 hours before implantation with either 0.1M acetic acid as the vehicle carrier or vehicle containing recombinant human IGF-I or LR³IGF-I, at concentrations that, when delivered at 0.5µl/hour would result in infusion rates of 0 (vehicle), 44, 111 and 278µg/day of IGF-I or LR³IGF-I. The implanted pumps were not primed prior to insertion, so that according to the manufacturer's recommendations, the full pumping rate would have been reached approximately 4 hours after the pump implant. The incision was closed with 2 surgical clips and all animals were returned to their metabolism cages after having gained full consciousness. During the 14-day treatment period, metabolic collections of faeces and urine as well as measurements of body weight and food intake were taken daily at precisely 24 hour intervals. Figure 3.1 shows a generalised protocol of the experimental design.

Figure 3.1: Generalised protocol of the experimental design.



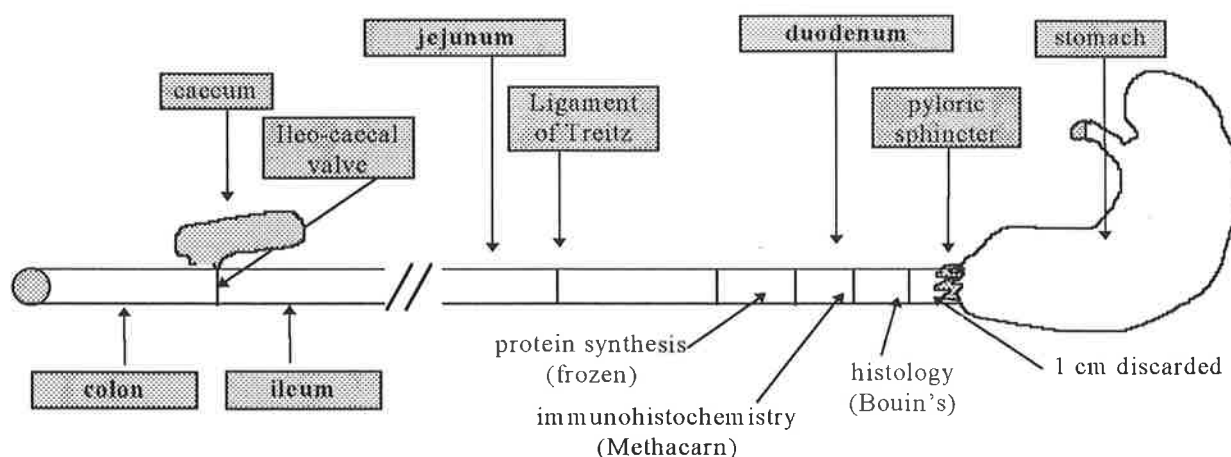
3.2.4 Tissue collections and gut measurements

To measure tissue protein synthesis rates, each animal was injected with 10ml per kg body weight of a solution comprising 0.15 M L-phenylalanine, 0.077 M NaCl and 50mCi L-[2,6-³H] phenylalanine/l into a tail vein at the end of the 14-day treatment period. Exactly 15 minutes later the animal was stunned and decapitated and immediately thereafter a mid-line incision was made and the entire gastrointestinal tract was rapidly excised. The tissue was immediately placed onto an ice-cold glass slab. The stomach, small intestine, colon and caecum were isolated and the duodenum was separated from the remaining small intestine at the Ligament of Treitz. The weight of each region was recorded after removal of gut contents. Length measurements of the small and large intestine were made by placing the tissue horizontally onto a cold glass slab, avoiding stretching of the tissue. Total gut weights and length were calculated as the sum of intestinal components.

Injection of tritiated phenylalanine for assessment of protein synthesis rates of duodenal tissue segments in these animals precluded the injection of tritiated thymidine. Therefore, proliferative activity was assessed by the detection of PCNA as described in Chapter 2 (2.5.1).

In addition to the 1 cm tissue segment collected for routine histological analyses, a second 1-cm segment was collected from the proximal duodenum for immunohistochemical detection of PCNA, starting 1 cm caudal to the pyloric sphincter. These tissue segments were fixed in either Bouin's fluid or Methacarn fixative. Another 2-cm segment of duodenum was rinsed with 0.9% NaCl, blotted dry, weighed, and rapidly frozen in liquid nitrogen for subsequent analyses of protein synthesis rates. A schematic representation of the tissue collection protocol is given in Figure 3.2.

Figure 3.2: Schematic representation of tissue collection protocol



3.2.5 Histological and immunohistochemical analyses

Histological and immunohistochemical analyses were carried out on segments of the duodenum of animals treated with vehicle and the highest dose of IGF-I or LR³IGF-I (278 µg/day). For light microscopy, the tissue segments were fixed in Bouin's fluid for 4 hours, and then processed for routine paraffin embedding using an automated tissue processor (Tissue Tek, Miles Scientific, USA). For each animal, three 2 µm sections cut at 200 µm intervals were stained with haematoxylin and eosin and mounted with DePex mounting medium (Gurr, BDH Chemicals, Kilsyth, Vic., Australia). Sections were examined with a light microscope and quantitative morphometric analysis was conducted on computerised images as described in Chapter 2, section 2.4.1.

Cross-sectional mucosal thickness, submucosal thickness, thickness of the muscularis wall and villus height were measured for each rat on 15 well-orientated transverse duodenal sections at x 125 magnification. Crypt depth was measured (magnification x 250) on 15 well-orientated, full-length open crypts. Circumference of the duodenal sections was traced along the serosa on 6 transverse-embedded segments (magnification x 63). The crypt column count was measured in 30 full-length open crypts and the circumferential cell count was determined in 15 well orientated tissue segments as outlined in Chapter 2, section 2.3.2. The product of the crypt column count and the crypt circumferential cell count was used to estimate the crypt cell population for each animal. Mean cell density of the crypt was calculated by dividing the crypt cell count by the crypt length in 10 sections for each animal. For the villus component, the villus cell count, representing the total number of epithelial cells along the longitudinal axis of the villus, was determined in 15 villi by multiplying the cell density per μm villus epithelium by the total length of the villus. To ensure that the cell densities of all regions along the villus were comparable, a preliminary study was conducted comparing cell densities near the villus base, the mid-villus region and near the tip of the villus. There were no statistically significant differences between the regions sampled (data not shown).

3.2.6 Assessment of intestinal proliferation

In this study, proliferative activity was monitored by the detection of PCNA immunostaining in the crypt epithelium. Following a modification of the method by Foley et al. (1991) and Garcia et al. (1989), a monoclonal anti-PCNA antibody (murine, IgM 19A2, Path, Mark Coulter, CA, U.S.A.), coupled to a streptavidin-immunoperoxidase reaction was employed for the detection of PCNA positive cells. As shown in the preliminary study (Chapter 2, section 2.5), detection of PCNA is maximised in Methacarn fixed tissue. Thus in this study duodenal segments were fixed for exactly 2 hours in Methacarn fixative followed by storage in 70% alcohol for 24 hours. Thereafter, segments were processed for routine paraffin

embedding as described in Chapter 2, section 2.4.1. For each tissue segment a single slide containing 3 tissue sections, 200 μm apart, were prepared at a cutting thickness of 2 μm . Detection of PCNA immunostaining was carried out as described in Chapter 2, section 2.5.

The quantitative assessment of the PCNA sections included the measurement of two parameters. Firstly, the proportion of crypt epithelial cells expressing PCNA was determined for each animal in the same 30 crypts used for measuring the crypt cell count. Secondly, individual PCNA-labelling distribution profiles were established on the same 30 full-length crypts to identify the proliferative profile of the crypt epithelium. For this purpose, epithelial cells were allocated a position number starting at the base of the crypt (position 0) up to the crypt:villus junction and the presence or absence of PCNA immunostaining at each position was recorded as described in Chapter 2, section 2.5. For each animal, the position of half maximum PCNA labelling was calculated from the proliferative distribution curves and divided by the crypt cell column count for that animal to obtain the Crypt Growth Fraction.

3.2.7 *Biochemical analyses*

To estimate the nitrogen content in food or faeces, a weighed sample of approximately 5mg was sealed in a tin container and loaded into the autosampler. The nitrogen content of dried food and faeces was measured by a Carlo Erba NA 1500 Nitrogen Analyser (Milan, Italy) using the Dumas method. Atropine containing 4.84% nitrogen was used as the standard. These methods are well established and have been described previously (Lemmey et al., 1991 and Tomas et al., 1993). Measurements of food and faeces nitrogen content were performed by Mrs. Kerry Penning under the direction of Dr. F. Tomas at the CSIRO.

Methods for the measurements of duodenal protein synthesis rates (calculated from the incorporation of [2,6- ^3H]-phenylalanine) as well as the method for the measurement of the

RNA content have been described elsewhere (Tomas et al., 1991) and were performed by D. F. Tomas, CSIRO Division of Human Nutrition, South Australia.

3.2.8 *Statistical analyses*

All values in Tables and Figures are expressed as means including S.E.M. All groups were compared by a one-way analysis of variance (ANOVA) and where significance was achieved ($P < 0.05$), a post-hoc Fisher's PLSD was applied to identify between group differences. Statistical analyses were performed using the SuperANOVA software package (Abacus Concepts, CA, USA).

3.3 RESULTS

3.3.1 Body weight gain

Body weight at the start of the 14-day treatment period averaged 116 ± 0.2 g ($n=42$) for all animals, with no statistically significant difference between treatment groups. IGF-I and LR³IGF-I administration brought about a dose dependent increase in body weight gain so that the final body weight after 14 days of treatment was significantly greater than the vehicle group for the highest dose of IGF-I and all doses of LR³IGF-I (Table 3.1).

Table 3.1: Final body weight and average food intake of rats treated with vehicle or IGF-I peptides for a 14 day period.

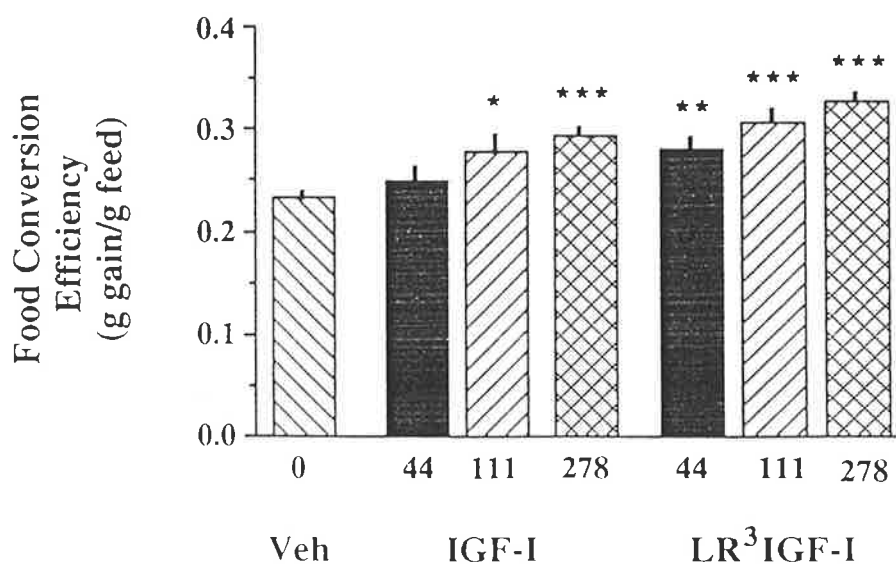
Treatment group	Body weight (g)	Average food intake
	final	(g/day)
Vehicle (0.1M acetic acid)	164±4	14.0±0.5
IGF-I	44µg/day	14.9±0.4
	111µg/day	15.0±0.6
	278µg/day	15.1±0.4
LR ³ IGF-I	44µg/day	15.5±0.2
	111µg/day	15.6±0.3
	278µg/day	15.4±0.3

*Values are expressed as means±SEM with 6 animals/group. Statistical significance from the vehicle-treated control group is indicated by *: P<0.05 and **: P<0.01 by analysis of variance (ANOVA).*

Food intake over the 14-day period was similar for all groups (Table 3.1). The food intake data and protein turnover rates in these rats have been described in detail by Tomas et al. (1993) but are reported here because of the importance of food intake as a determinant of gut mass. These data show that average daily food intake (g/day) was similar across all groups (Fig. 3.1). The average daily food intake expressed as a fraction of the average body

weight over the 14 day period was also similar in all groups except for the highest dose of LR³IGF-I (278µg/day), which showed a significant reduction of the average daily food intake as compared to vehicle treated rats (P<0.01). An increase in body weight gain without a concomitant increase in food intake clearly reflects an improved food conversion efficiency in the rats treated with IGF-I peptides. Indeed, the food conversion efficiency, calculated as the average body weight gain per g food eaten over the 14 day period, was significantly improved in IGF-I and LR³IGF-I treated animals (Fig. 3.3). Administration of 278µg/day of IGF-I increased the food efficiency by 26% above the values obtained for vehicle treated rats (P<0.01). Infusion of 111µg/day and 278µg/day of LR³IGF-I improved food conversion efficiency by 31 and 40%, respectively. Furthermore, all doses of LR³IGF-I were significantly (P<0.01) more potent than the equivalent doses of IGF-I in improving food conversion efficiency (Fig. 3.3).

Figure 3.3: Food conversion efficiency in normal rats treated for 14 days with increasing doses of IGF-I peptides as compared to vehicle treated control rats.



Values represent means±SEM with 6 rat/group. Statistical significance is indicated by *: P<0.05, **: P<0.01 and ***: P<0.001 as compared to vehicle treated group (ANOVA).

3.3.2 *Gastrointestinal growth responses*

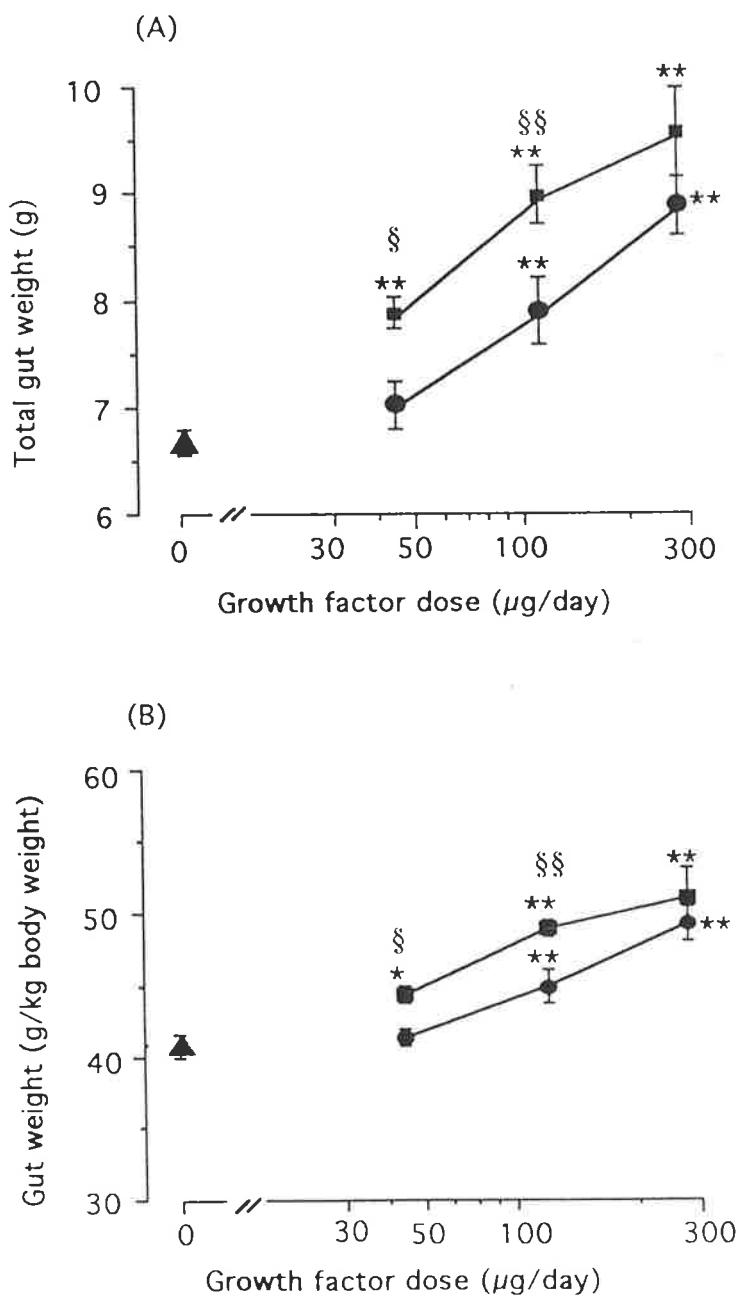
The improved body weight gain with IGF treatment was reflected in all gastrointestinal components, so that gut weight, including the stomach, small and large intestine, was increased in a dose-dependent manner with statistically significant responses apparent at the low dose of LR³IGF-I (44 µg/day) or the medium dose of IGF-I (111 µg/day) (Fig. 3.4 A). The highest peptide doses increased total gut weight by up to 43% above that in vehicle-treated control animals (Fig. 3.4 A). These effects were still apparent when total gut weight was expressed as a fraction of total body weight, indicating a selective action of IGF-I peptides on the gut (Fig. 3.4 B).

Dose responsive increases in tissue wet weight were apparent in individual gastrointestinal segments (Fig. 3.5 A-C), and although the dose-response curves for LR³IGF-I were not parallel to those of IGF-I, LR³IGF-I was more potent in stimulating gut growth than the native peptide. The small intestine was the most responsive region with significant increases in wet tissue weight at all doses of LR³IGF-I and a maximum response of 47% above control values. IGF-I effects were significant at the medium dose, reaching 36% above control at the highest dose.

The increased potency of LR³IGF-I was evident in most regions of the gastrointestinal tract. Total gut weight, fractional gut weight, small intestinal weight and length were all significantly greater in the animals treated with the low and medium dose (44 and 111µg/day) of LR³IGF-I as compared to the animals treated with the equivalent doses of IGF-I (Fig. 3.4 and 3.5). Stomach weight of animals treated with 111µg/day of LR³IGF-I and the large intestinal weight of rats treated with 278µg/day of LR³IGF-I were also significantly greater than the corresponding groups of IGF-I treated animals (Fig. 3.5 A and 3.5 C).

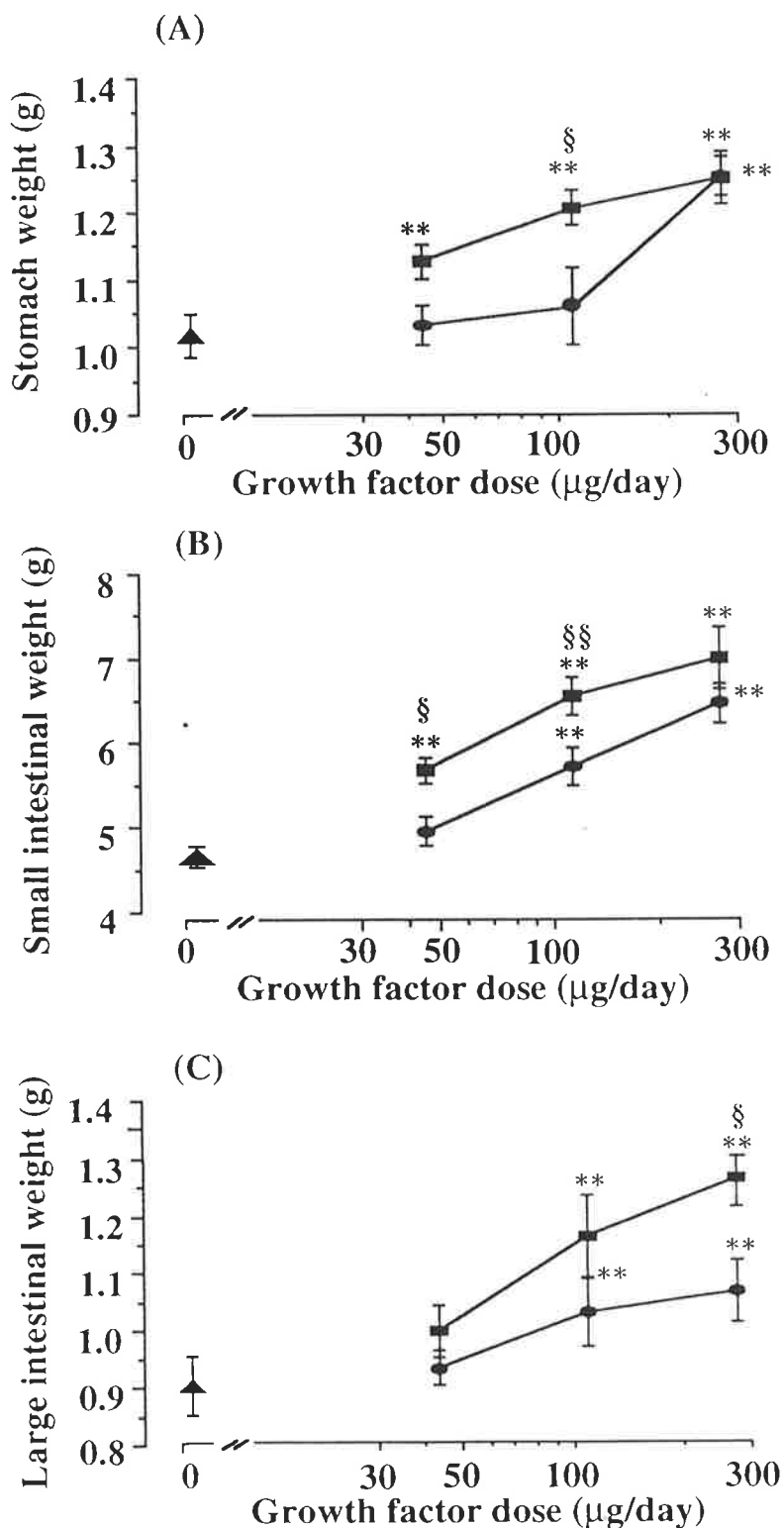
A substantial proportion of the increased total gut weight in IGF-treated rats could be attributed to the combined effect of an increased length together with a greater cross-sectional mass of the small intestine. Thus, LR³IGF-I treatment increased total small intestinal length by approximately 13% even at the low dose, while treatment with IGF-I significantly increased the length of the small intestine of animals in the medium and high treatment groups (Fig. 3.6 A). An increased cross-sectional mass of the small intestine is illustrated by the significantly elevated small intestinal weight to length ratio (Fig. 3.6 B). The large intestinal weight was also significantly increased following administration of IGF-I peptides so that the highest dose of IGF-I or LR³IGF-I induced wet tissue weight increases of 18% and 40%, respectively (Figure 3. 5C). However, in contrast to the marked weight increases, large intestinal length was only affected marginally by the peptide treatment (Fig 3.5 C).

Figure 3.4: Total gut weight (A) and total gut weight as a fraction of body weight (B) in growing female rats after treatment with IGF-I peptides.



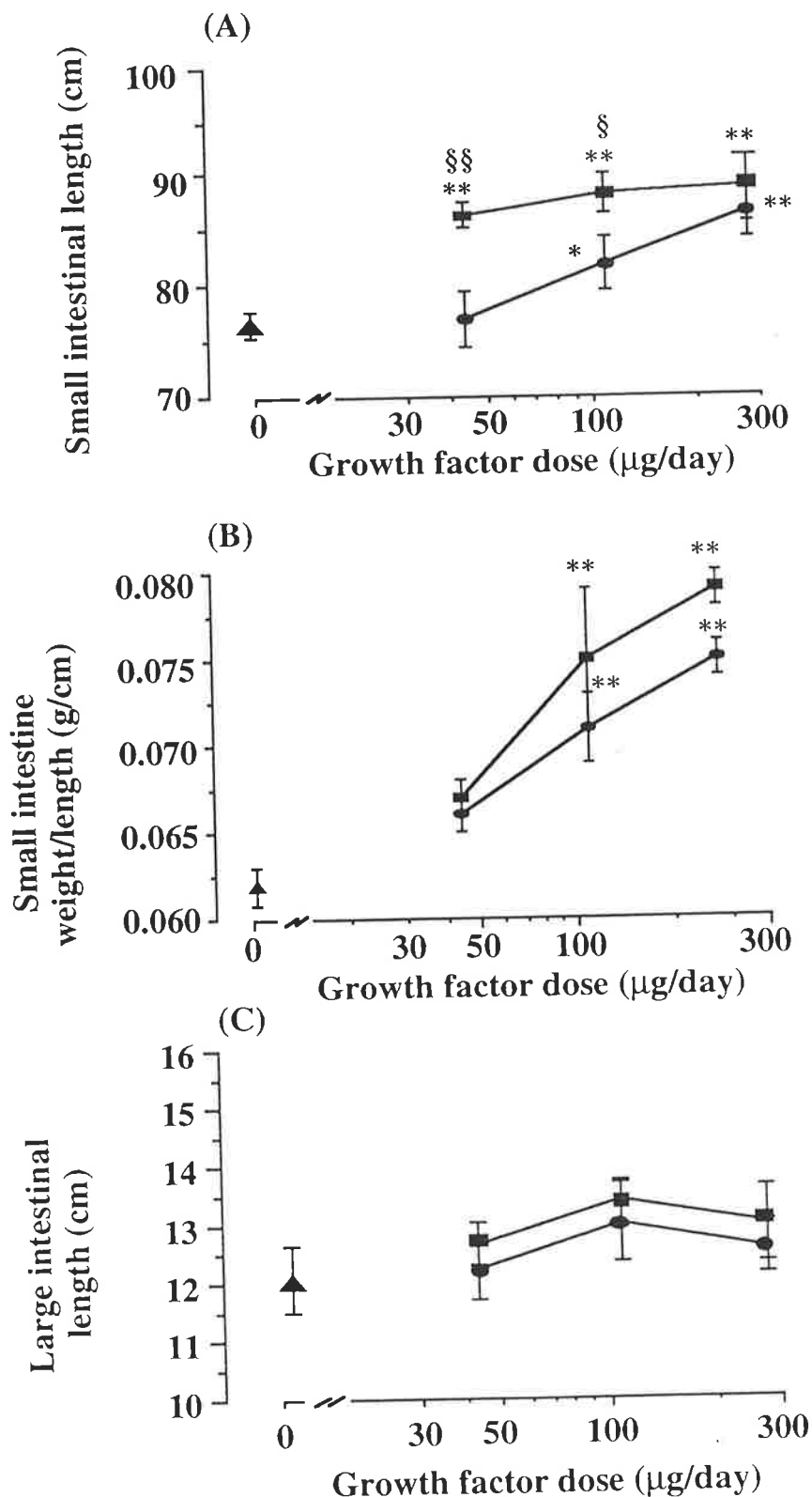
Values are means \pm SEM with 6 rats/group. Significance from the vehicle group \blacktriangle is indicated by *: $P<0.05$ or **: $P<0.01$ for IGF-I treated rats \bullet or LR³IGF-I treated rats \blacksquare . Significant difference of LR³IGF-I treated animals against the same dose of IGF-I treated animals is indicated by \S : $P<0.05$ or $\S\S$: $P<0.01$. Data was analysed by ANOVA.

Figure 3.5: Total weights for stomach (A), small intestine (B) and large intestine (C) of rats treated for 14 days with IGF-I peptides.



Data are expressed as means±SEM for 6 animals per group. Significance from the vehicle treated rats ▲ is indicated by *: $P < 0.05$ or **: $P < 0.01$ (ANOVA) for rats treated with IGF-I ● or rats treated with LR³IGF-I ■. Significant difference of LR³IGF-I treated animals vs. the same dose of IGF-I treated animals is indicated by §: $P < 0.05$ or §§: $P < 0.01$.

Figure 3.6: Total small intestinal length (A), total small intestinal weight per total small intestinal length (B) and large intestinal length (C) of normal female rats treated for 14 days with IGF-I peptides.



Data are expressed as means±SEM for 6 animals per group. IGF-I treated rats ● and LR³IGF-I treated rats ■ were compared to vehicle treated rats ▲. Significance from the vehicle group is indicated by *: P<0.05 or **: P<0.01 as analysed by ANOVA. Significant difference of LR³IGF-I treated animals vs. the same dose of IGF-I treated animals is indicated by §: P<0.05 or §§: P<0.01.

3.3.3 Histological parameters

In this study, the proximal small intestine was identified as one of the most responsive regions of the gut. As shown in Table 3.2, the weight and length of the duodenum were significantly increased after peptide treatment. As for the total small intestine, the weight to length ratio of the duodenum was also significantly elevated in the highest dose of IGF-I and LR³IGF-I treated rats. Accordingly, histological analyses were conducted on the duodenum of the high-dose (278µg/day) IGF-I and LR³IGF-I groups and compared with histological sections from vehicle-treated animals to identify the mechanism by which IGF treatment increased the cross-sectional mass of the proximal small intestine. The circumference as well as the widths of the mucosa, submucosa and the muscularis external layers were measured. Calculations of areas and volumes were conducted for the total wall and mucosa, assuming that the duodenal sections were perfect annular circles (Table 3.3).

Table 3.2: Tissue weight and length measurements of morphological parameters in duodenal sections from rats treated for 14 days with IGF-I or LR³IGF-I (278µg/day).

<i>Duodenum</i>	<i>Treatment group</i>		
	<i>Vehicle</i>	<i>IGF-I</i>	<i>LR³IGF-I</i>
<i>Weight (mg)</i>	636±12	832±23**	857±36**
<i>Length (mm)</i>	81±2	87±4	91±3**
<i>Weight/Length ratio (mg/mm)</i>	7.9±0.2	9.7±0.2**	9.5±0.4**
<i>Circumference</i>	10.1±0.3	10.9±0.2	11.1±0.5
<i>Width (µm) of</i>			
Total wall	1,128±21	1,181±23	1,275±29**
Mucosa	937±21	1,012±24	1,080±32**
Submucosa	62±6	55±4	65±4
Muscularis externa	128±7	114±3	131±6

*Values are expressed as means±SEM, n=6 rats/group. Statistical significance from vehicle-treated control rats is indicated by **: P<0.01, by analysis of variance (ANOVA).*

The circumference of the duodenal sections was not increased by treatment with either IGF-I or LR³IGF-I, although total cross-sectional width of the duodenal wall was significantly higher than in controls for rats treated with LR³IGF-I (Table 3.2). The effect of LR³IGF-I on total wall width could be attributed to responses in the mucosal layer, since the submucosal and muscularis externa layers represented only a small percentage of the total wall width, and appeared unresponsive to IGF treatment. Calculations of the annular area of the duodenal mucosa estimated an overall increase in mucosal area of up to 25% in the LR³IGF-I treated animals (Table 3.3). If duodenal area is multiplied by duodenal length as a measure of volume, it can be shown that treatment with IGF peptides increased the mucosal volume by up to 28% and 41% for the highest dose of IGF-I and LR³IGF-I, respectively. These increases are of similar magnitude than the changes recorded for wet tissue weight gain (Table 3.2).

Table 3.3: Annular tissue areas and tissue volume, calculated from duodenal sections from rats treated with vehicle or 278µg/day of IGF-I or LR³IGF-I.

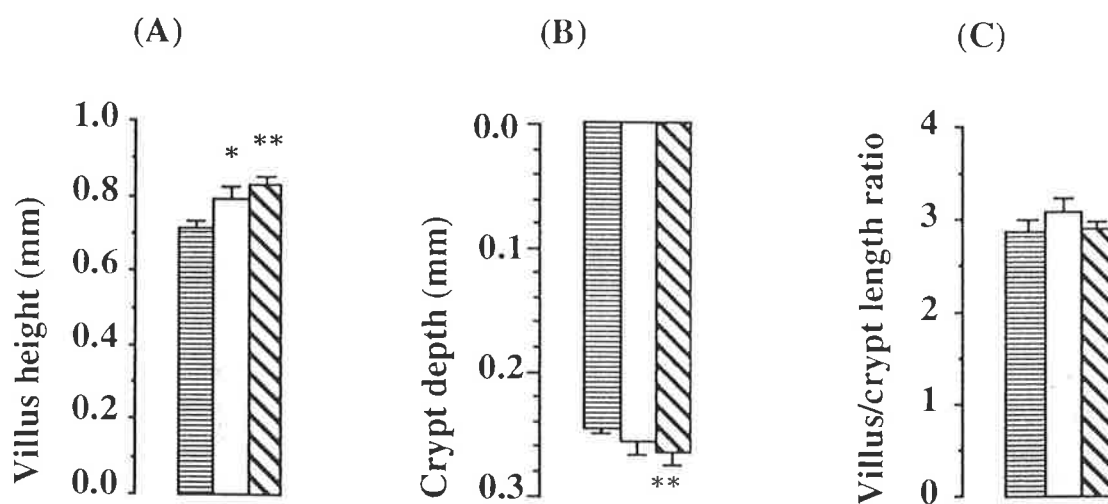
<i>Duodenum</i>		<i>Treatment Group</i>		
		<i>Vehicle</i>	<i>IGF-I</i>	<i>LR³IGF-I</i>
<i>Length (mm)</i>		81±2	87±4	91±3**
<i>Annular area (mm²) of</i>	Total wall	7.41±0.42	8.42±0.33	9.05±0.68
	Mucosa	5.61±0.38	6.67±0.25	7.02±0.65
<i>Volume (mm³) of</i>	Total wall	600.8±39.3	734.0±57.7	827.6±84.6
	Mucosa	453.7±30.5	581.4±44.5	642.9±76.8

*All values are means ±SEM of 6 animals /group. Statistical significant difference from vehicle-treated control animals is indicated by **: P<0.01 as detected by ANOVA.*

To further identify the mechanism of IGF-I action on the mucosal component, a more detailed examination of the functional (villus) and proliferative (crypt) compartment was conducted. The increased cross sectional width of the mucosa was reflected in both the villus

and crypt compartments, so that villus height was significantly increased in rats treated with 278 μ g/day IGF-I or LR³IGF-I, as was crypt depth in the LR³IGF-I treated group (Fig. 3.6A and B). The villus height to crypt depth ratio was not altered after the treatment with IGF-I peptides, demonstrating that the proportionality between the functional and proliferative compartment remained unaltered (Fig. 3.6C). The increased crypt depth in IGF treated rats was accompanied by significant increases in the number of cells per crypt column (crypt cell count) and the number of enterocytes, circumferentially (Table 3.4). As a result, the crypt cell population, as estimated by the product of the crypt cell count and the circumferential count, was increased by 28% and 33% for the high dose IGF-I and LR³IGF-I treated rats, respectively. The density of crypt enterocytes per mm epithelium was not, however, altered by the peptide treatment (Table 3.4). In the villus the total number of cells per column (villus cell count) was also increased with IGF treatment, but in contrast to the crypt, villus cell density was also significantly elevated.

Figure 3.7: Villus height (A), crypt depth (B) and the villus height to crypt depth ratio (C) in the duodenum of female rats treated for 14 days with 278 μ g/day of IGF-I peptides.



The data shows means \pm SEM for 6 rats/group. IGF-I treated animals \square or LR³IGF-I treated animals ▨ were compared to vehicle treated rats ▩ by ANOVA and were considered significant at *: $P < 0.05$ or **: $P < 0.01$.

Table 3.4: Crypt and villus cell counts in duodenal sections of rats treated for 14 days with 278µg/day of IGF-I or LR³IGF-I.

Proliferative parameters	Treatment Group		
	<i>Vehicle</i>	<i>IGF-I</i>	<i>LR³IGF-I</i>
<i>Crypt</i>			
Column count (no. of cells)	33.8±0.7	37.6±0.7*	37.9±0.8**
Circumferential cell count(no. of cells)	21.1±0.4	24.5±0.4**	24.7±0.5**
Cell population (no. of cells)	699±18	899±34**	932±41**
Cell density (cells/mm)	134±2	144±5	132±2
<i>Villus</i>			
Column count (no. of cells)	93±3	125±5*	129±2**
Cell density (cells/mm)	132±3	158±3**	156±4**

*Values are expressed as means±SEM of 6 animals/group. Crypt cell density was calculated by dividing the mean crypt column count by the mean crypt length for each animal. The crypt cell population was calculated as the product of the crypt cell column count and the circumferential cell count. Statistical significant difference from vehicle group is indicated by *: P< 0.05, and **: P<0.01 as detected by ANOVA.*

3.3.4. Proliferative parameters

To characterise the effects of IGF peptides on the proliferative fraction of the duodenal crypt, immunohistochemical techniques were used to detect PCNA. Expression of PCNA declines during the G2 and M phase of the cell cycle and hence PCNA expression can be used as a cell cycle marker, comparable to the use of [³H]thymidine (Bleiberg et al., 1993).

IGF-I and LR³IGF-I increased the number of PCNA positive cells per crypt column (Table 3.5), although statistical significance was not reached (P=0.058, ANOVA). However, because total cell number also increased (Table 3.4), the proportion of PCNA-labelled cells remained at approximately 40% for all groups. (Table 3.5). Maximum PCNA labelling was observed within cell position 5-16 within the crypt and was similar for all groups (Table 3.5). The cell position at which half-maximum PCNA labelling occurred increased proportionally with crypt length in the IGF-I and LR³IGF-I treated animals, so that the crypt growth fraction

also remained similar across all groups (Table 3.5). There was, however, a tendency for the crypt growth fraction to be reduced in the LR³IGF-I treated group (52%) as compared to either the vehicle or the IGF-I treated animals (\approx 56%), which appears to reflect reduced PCNA labelling per crypt column rather than the number of cells per crypt column.

Table 3.5: Proliferative parameters and PCNA labelling in duodenal sections of rats treated for 14 days with 278 μ g/day of IGF-I or LR³IGF-I.

<i>Proliferative parameters</i>	<i>Treatment Group</i>		
	<i>Vehicle</i>	<i>IGF-I</i>	<i>LR³IGF-I</i>
<i>Crypt</i>			
Max. PCNA labelling (%)	78.3 \pm 1.1	81.4 \pm 2.3	78.8 \pm 2.1
Cell position of 1/2 max. labelling	18.8 \pm 0.7	21.4 \pm 1.4	19.7 \pm 0.4
PCNA positive/crypt (no. of cells)	13.3 \pm 0.4	15.7 \pm 0.9	14.3 \pm 0.6
PCNA labelling (%)	38.9 \pm 0.8	40.6 \pm 1.9	38.0 \pm 1.3
Crypt Growth Fraction (%)	55.7 \pm 1.1	56.8 \pm 3.5	51.9 \pm 1.3

All values represent means \pm SEM with 6 rats per group, (n=5 for animals for LR³IGF-I group, PCNA data, only). PCNA labelling index was calculated as the number of PCNA positive cells per crypt column divided by the total number of cells per crypt column. The crypt growth fraction was calculated as the cell position number at which half maximum labelling occurs divided by the total number in the crypt column.

3.3.5 Biochemical parameters

To relate the histological findings with biochemical measures of proliferation, protein and RNA contents were measured in segments of duodenum collected at kill. In addition, the rate of duodenal protein synthesis was measured after injection of the rats with a flooding dose of [2,6-³H]-phenylalanine. Intact segments of duodenum rather than mucosal scrapings were used for these measurements so that tissue samples could be frozen rapidly, thereby reducing variability in the time of incorporation of injected phenylalanine. The increased wet weight per cm of duodenum (Table 3.2) in IGF-treated rats was reflected in proportional increments in protein content, so that the protein content/wet weight were similar in all groups (Table 3.5).

Similarly, the RNA content of the duodenum also changed in proportion to increases in the wet tissue weight of the duodenum (Table 3.6). On the other hand, neither protein synthesis, expressed as either the % of non-collagen protein synthesised per day (estimated from the incorporation of [2,6-³H]phenylalanine), nor the ratio of protein synthesis to RNA content were increased by treatment with IGF-I peptides (Table 3.6). Hence, the increased duodenal tissue mass and protein content in response to IGF infusion could not be attributed to a sustained increase in the rate of protein synthesis.

Table 3.6: Duodenal protein synthesis on day 14 of treatment with vehicle, or 278µg/day of IGF-I or LR³IGF-I.

	<i>Treatment Group</i>		
	<i>Vehicle</i>	<i>IGF-I</i>	<i>LR³IGF-I</i>
Protein (mg/g tissue)	158.4±10.6	158.7±7.2	162.3±12.0
RNA (mg/g tissue)	13.7±1.0	14.1±0.9	13.0±1.0
RNA (mg/g protein)	86.5±2.7	88.3±2.1	79.9±0.6*
Protein synthesis, (%/day)	139.9±7.3	141.0±9.0	132.0±7.0
Synthesis/RNA (g protein/g RNA)	16.2±0.9	15.9±0.8	16.5±0.9

Values are means±SEM for 6 rats/group and were determined from segments of intact duodenum collected 15 minutes after an intravenous injection of a flooding dose of [2,6-³H] phenylalanine at the end of a 14 day peptide treatment period. Protein represents non-collagen protein per gram wet tissue weight. Protein synthesis is expressed as the percent of non-collagen protein synthesised per day. The RNA to protein ratio was significantly reduced in the rats treated with 278µg/day of LR³IGF-I, compared to vehicle treated group (: P<0.05).*

3.3.6 Nitrogen balance measurements

To determine if the increased mucosal mass following IGF treatment was reflected in an improved absorptive function of the small intestine, the nitrogen intake and faecal nitrogen excretion were measured during the two days prior to growth factor infusion and also during the 14 day treatment period. Accurate measurements of nitrogen intake and faecal nitrogen excretion were achieved by keeping the animals in metabolism cages over the entire

experiment. During the 2 days prior to treatment there were no significant differences between groups in nitrogen intake or faecal excretion (Table 3.7). For the 14 day treatment period, average nitrogen intake and excretion were calculated for both the first and second weeks of treatment. During the first week, infusion of IGF-I or LR³IGF-I had no effect on the food intake, so that the average daily nitrogen intake was similar in all groups (Table 3.7). Average faecal nitrogen excretion, however, was significantly reduced by IGF-I or LR³IGF-I treatment, whether expressed as mg/day or as a fraction of nitrogen intake. Similar trends were apparent in the second week, although the effect of IGF peptides on fractional faecal nitrogen excretion failed to reach statistical significance (ANOVA, probability = 0.109).

Table 3.7: Average daily intake and faecal excretion of nitrogen during the pre-treatment period, week 1 and week 2 of rats treated with 278µg/day of IGF-I or LR³IGF-I.

	<i>Treatment Groups</i>		
	<i>Vehicle</i>	<i>IGF-I</i>	<i>LR³IGF-I</i>
<i>Pre-treatment period</i>			
Nitrogen intake (mg/day)	369±12	359±8	364±7
Faecal nitrogen excretion (mg/day)	20.5±2.2	23.2±0.8	23.1±1.0
Fractional faecal nitrogen excretion (%)	5.56±0.61	6.46±0.21	6.36±0.32
<i>Week 1</i>			
Nitrogen intake (mg/day)	400±17	387±9	407±10
Faecal nitrogen excretion (mg/day)	28.1±0.9	23.3±1.1*	22.9±1.5**
Fractional faecal nitrogen excretion (%)	7.06±0.33	6.00±0.18*	5.62±0.35**
<i>Week 2</i>			
Nitrogen intake (mg/day)	413±12	443±13	438±10
Faecal nitrogen excretion (mg/day)	29.9±0.5	30.6±2.1	27.2±1.2
Fractional faecal nitrogen excretion (%)	7.27±0.19	6.88±0.35	6.26±0.38

*Values are means±SEM for 6 rats per group. *: P<0.05 and **: P<0.01 compared with vehicle treated control rats (ANOVA). The fractional faecal nitrogen excretion represents the average nitrogen excretion as a percentage of the average nitrogen intake.*

3.4. DISCUSSION

3.4.1 *IGF-I peptides stimulate growth without concomitant increase in food intake.*

Dietary manipulation (fasting/re-feeding, parenteral nutrition), as well as surgical procedures (resection and intestinal bypass) have provided compelling evidence that nutrient intake regulates gastrointestinal/mucosal growth (for review see Levine, 1991). For example, after intestinal resection, intraluminal nutrients promote adaptive gastrointestinal growth in the dog (Feldman et al., 1976). Mucosal growth may be stimulated either directly or via indirect stimulation of secretory trophic factors (Dworkin et al., 1976 and Spector et al., 1977). In the present study, administration of IGF-I peptides to adult female rats significantly enhanced somatic growth and selectively stimulated gastrointestinal growth without a concomitant increase in food intake. The fact that food conversion efficiency was significantly improved in the absence of an increase in food intake in IGF-I and LR³IGF-I treated rats suggests that IGF-I peptide effects were not only independent of nutrient intake but also improved nutrient utilisation. This findings are in agreement with previous studies, showing that administration of approximately 2mg/kg/day of IGF-I or des(1-3)IGF-I significantly stimulated body weight gain and gastrointestinal growth following intestinal resection without a concomitant increase in food intake (Lemmey et al., 1991 and Vanderhoof et al., 1992). Likewise, nutrient-independent gastrointestinal growth responses following administration of IGF-I peptides have been shown in diabetic (Tomas et al., 1991) and in pair-fed dexamethasone treated rats (Tomas et al., 1992). Further evidence for nutrient independent IGF-I action on the small intestine stems from studies in GH transgenic rodents (Ulshen et al., 1993). These GH transgenics have elevated circulating GH and IGF-I levels as well as elevated expression of IGF-I locally in the small intestine (Lund, 1994). Although GH transgenic mice have a higher food intake than their wild type litter mates, a marked mucosal hyperplasia and hypertrophy of the proximal small intestine was still observed in these animals when pair fed with their wild type litter

mates, demonstrating that the mucosal responses were not due to food intake alone (Ulshen et al., 1993).

3.4.2 IGF-I peptides stimulate gut growth and length

All gastrointestinal regions responded to IGF-I treatment with the upper gastrointestinal tract, with the proximal small intestine and the stomach being the most responsive regions. The marked effects of IGF-I treatment on the gut, particularly the upper gastrointestinal tract, are in accordance with results from other studies. For instance, increases in stomach, duodenum, jejunum and ileum and total gut weight have been described for dexamethasone-treated rats (Read et al., 1992a) and resected rats (Lemmey et al., 1991 and Vanderhoof et al., 1992). IGF-I effects have been shown to increase stomach and total gut weight increased in diabetic rats (Tomas et al., 1991), total gut weight in nephrectomized rats (Martin et al., 1991), duodenal weight in transgenic dwarf mice (Behringer et al., 1990) and stomach weight in lit/lit strain of GH deficient mice (Gillespie et al., 1990). In the present study, large intestinal weights were also significantly affected, in particular following treatment of 278µg/day of LR³IGF-I which induced an increase in total large intestinal weight by 40% above vehicle-treated rats.

In addition to the marked increases in wet tissue weight of the small bowel, IGF-I peptide administration to adult female rats also increased the small intestinal length by up to 16% above control values following administration of 278µg/day of LR³IGF-I. Small intestinal length was increased by the same magnitude when the same dose of LR³IGF-I was infused for 7 days to adult male rats (Read et al., 1992b and Steeb, unpublished). In contrast, lengthening of the small bowel following administration of IGF-I peptides is not observed in

some situations including gut resection (Lemmey et al., 1991) or in diabetic rats (Read et al., 1991). Recently, Bird et al., (1994) observed a shortening of the small intestine by 10% compared to control animals following infusion of 4.5µg/day of LR³IGF-I. However, no adequate explanation was provided for this effect.

Despite the marked increase in large intestinal weight, the length of the large intestine was not affected by the peptide treatment. Similarly, the large intestine in other situations appears to be the least peptide responsive region (Read et al., 1991, 1992a, 1992b and Lemmey et al., 1991). It therefore appears that the mechanism by which IGF-I peptides stimulate intestinal growth or length differs under different physiological conditions and disease and are not uniform throughout the entire length of the gastrointestinal tract and may even differ between species.

3.4.3 IGF-I stimulates growth of mucosal and non-mucosal tissues

In view of the marked growth responses observed in the proximal small intestine, a detailed examination of the duodenum was conducted to determine the growth effects of IGF-I peptides on the mucosal compartment and on non-mucosal tissues. The intestinal mucosa was of major interest because of its role in digestive and absorptive function. In the present study, increased wet tissue weights of the duodenum in IGF-I treated rats were clearly reflected in the histology. Increases in cross-sectional thickness of the mucosa rather than effects on the submucosa and/or muscularis externa were responsible for the observed weight increase. In agreement with the present study, trophic responses of IGF-I on the intestinal mucosa in normal adult rats have been reported by Olanrewaju et al. (1992). Intraileal infusion of a bolus of 10nM of IGF-I or continuous luminal infusion of the same dose at a delivery rate of

1µl/hour significantly increased the mucosal wet tissue weight, DNA, RNA and protein content of ileal mucosa homogenates. Besides, the activity of ornithine decarboxylase, an enzyme associated with cell proliferation, was significantly stimulated. In contrast, Young et al. (1990) did not observe increases in mucosal wet weights following s.c. administration of 1µg/day of IGF-I to 6 day old suckling rats, although some jejunal brush border enzyme activities were increased at this low dose. In a study by Bird et al. (1994), administration of 4.5µg/day of LR³IGF-I to adult rats failed to stimulate gastrointestinal growth. In view of the low dose of IGF-I peptides used by Young et al. (1990) and Bird et al. (1994), which are approximately 40 and 10-fold lower than the lowest dose of IGF-I peptides used in the present study, as well as the route of peptide delivery, the lack of gut growth responses in their studies does not seem surprising.

Mucosal growth has also been analysed in transgenic mice over-expressing IGF-I (Mathews et al., 1988). In these transgenic mice, circulating levels of IGF-I are elevated while circulating GH levels are almost undetectable, thus these animals represent a useful model to address IGF-I action on mucosal growth independent of GH. The intestinal mucosa in these IGF-I transgenics does not exhibit the mucosal hypertrophy and hyperplasia of the small intestine as observed in GH transgenic mice (Ulshen et al., 1993). Nevertheless, proliferative indices were enhanced and ornithine decarboxylase activity was elevated. Lund, (1994) speculated that the mitogenic response of the mucosa in IGF-I transgenic mice may be attributed to an increased cell turnover stimulated by IGF-I and the lack of mucosal hyperplasia may be a result of the GH deficiency. Thus, enhanced mucosal growth may be the result of the interaction between GH and IGF-I.

3.4.4 Proliferative effects

Morphometric analyses and immunohistochemical data were used to identify the mechanism by which mucosal mass increased following IGF-I administration. Morphometric assessment of the mucosa confirmed that IGF-I peptide administration resulted in a hyperplasia of the crypt and villus epithelium. Within the crypt compartment, both the length and the width of the crypts were increased, resulting in an overall increase in crypt cell population of up to 33%. Calculations on the size of the proliferative compartment of the crypt, using PCNA as the proliferative marker, indicated that the number of cells labelled with PCNA increased proportionally with crypt size so that the labelling index of IGF-I peptide treated rats was similar to vehicle treated rats. At the same time, the crypt growth fraction, which defines the proportion of proliferative cells in the crypt column, remained similar across all groups. The small intestinal villi and its component crypts are a stable structural unit in a steady state, which is maintained by a balance between the production of new enterocytes in the crypt and cell loss from the villus tip (Wright and Alison, 1984b). It was of interest to note that in the present study, despite the marked increase in the absolute size of the crypt and villus compartments, the relative proportions between the villus compartment and the crypt compartment were preserved after 14 days of peptide administration. Moreover, the proportionality between the non-proliferative segment of the crypt (maturation zone) and the proliferative zone of the crypt was also maintained. This suggests that in the small intestinal mucosa of rats treated for 14 days of IGF-I peptides, a new steady state between cell production and cell loss has been established.

Other growth factors have been considered potential growth regulators of the intestinal epithelium, including EGF (Goodlad et al., 1992 and Ulshen et al., 1986), enteroglucagon (Jacobs et al., 1976, Bloom et al., 1987 and Gornacz et al., 1984), TGF α (Beauchamp et al.,



and Sarraf 1994 and Hoosein et al., 1989). EGF, which has been studied most extensively, appears to have direct *in vivo* effects on epithelial cell renewal (Goodlad et al., 1992). Orogastrically administered EGF exhibits trophic effects on the gastrointestinal tract in newborn rabbits and suckling rats (Koldovsky and Thornburg, 1987 and Koldovsky, 1989) and in adult rats, intraluminal administered EGF stimulates ornithine decarboxylase activity and DNA synthesis in the small intestine (Ulshen, et al., 1986). In addition, EGF is also a potent stimulator for epithelial cell production in the intestine of adult rats (Goodlad and Wright, 1990 and Goodlad et al., 1992), and of humans (Walker-Smith et al., 1985 and Sullivan et al., 1991). The mechanism by which EGF stimulates cell proliferation of the intestinal epithelium appears to be similar to IGF-I induced proliferation. Goodlad et al. (1992) examined the proliferative response of intravenously administered EGF on the small and large intestinal epithelium in rats in which cell proliferation was reduced to a steady state basal level by maintaining the rats on intravenous derived nutrients. Their study showed that administration of 60µg/day of EGF for a 8-day period to adult TPN fed Hooded Wistar rats significantly increased crypt column and crypt row counts compared to control rats. Furthermore, mitotic activity as assessed by the incorporation of [³H]thymidine in EGF treated rats increased proportionally to crypt length to values comparable to the orally-fed control rats. [³H]thymidine labelling was, however, significantly increased in the colon of the TPN plus EGF treated animals compared to oral fed control animals (Goodlad et al., 1992). The crypt growth fraction in the small intestine was not altered by EGF, but in the large intestine a significant reduction in the crypt growth fraction was observed (Goodlad et al., 1992).

3.4.5 Proposed mechanisms of IGF-I induced proliferation

Hyperproliferative growth of the crypt epithelium reflects an increased crypt cell production rate, which in turn could be influenced by changes in one or more of several

3.4.5 Proposed mechanisms of IGF-I induced proliferation

Hyperproliferative growth of the crypt epithelium reflects an increased crypt cell production rate, which in turn could be influenced by changes in one or more of several parameters, including the crypt growth fraction, the stem cell population, the crypt cell cycle time, or any combination of the above (Wright and Alison, 1984b). In the present study, changes in the crypt growth fraction are unlikely to contribute to the mechanism of IGF-induced crypt proliferation in view of the similar percentage and distribution of PCNA-labelled cells in sections from control and IGF-treated rats. To determine if cell cycle time, usually measured by fraction labelled mitosis, contributed to the altered proliferative status observed in this study requires further investigation. Minor alterations in the cell cycle time of epithelial cells located at the crypt base (stem cells) could lead to a marked increase in the crypt cell production rate and thus alter the proliferative status. For example, changes in the cell cycle time in the human small intestinal mucosa in coeliac disease (Wright et al., 1973), or in rats during recovery from irradiation (Leshner and Bauman 1969), the crypt cell production rate can double even in the absence of any other changes. Changes to the stem cell population are somewhat less defined because discrete histological identification of stem cells has so far been unsuccessful and little is known about their growth properties (Potten and Hendry, 1985).

In addition to the changes in the proliferative compartment, morphometric measurements of the functional (villus) compartment indicated that villus height increased in proportion to the crypt compartment. Interestingly, IGF-I peptide administration also increased villus cell density, indicating that changes in enterocyte life-span and/or alterations in enterocyte extrusion rates may have occurred. Measurements of enterocyte transit times can be achieved by continuous labelling, while the rate of exfoliation of intestinal enterocytes can be estimated by measuring the amount DNA lost into the bowel lumen by perfusion of a segment (Goldsmith, 1973). The latter method has its drawback in that some of the exfoliated

intestine the number of crypts contributing to one villus can be as high as 35. In addition, while the number of villi seems to be highly conserved (Clarke, 1967), new crypts are continuously formed by longitudinal division of existing crypts (crypt fission) occurring at a rate of approximately 0.0013-0.006 per day (Löffler and Grossmann, 1991 and Totafurno et al., 1987). In the present investigation, an attempt was made to define morphologically the ratio of crypt number to villus number by measuring the area occupied by crypts in a constant area of mucosa at the crypt/villus junction. This method was, however, unsuccessful because newly formed crypts can only be identified clearly in serially sectioned material. Thus to determine if the villus/crypt ratio has been altered by administration of IGF-I peptides, one must either employ autolysed preparations that remove the epithelial layer followed by scanning electron micrography (Loehry and Creamer, 1969) or carefully scan serially sectioned material (Smith and Jarvis, 1979).

3.4.6 LR³IGF-I is more potent than IGF-I

The responsiveness of the intestinal regions to IGF-I peptide administration was dose dependent and the IGF-I analogue was several-fold more potent than the native IGF-I. The increased potency of the IGF-I analogue may be best explained by the reduced binding affinity of LR³IGF-I to several of the IGF binding proteins found in rat plasma (Francis et al., 1992). The reduced binding affinity of LR³IGF-I to IGF binding proteins is likely to increase the overall “free” IGF pool in rat plasma and thereby enhance the delivery of IGFs from the circulation to the target tissues. Although the binding affinity of LR³IGF-I to the receptor is three- to fourfold lower than IGF-I (Francis et al., 1992), the greater potency observed with LR³IGF-I may be brought about by a larger proportion of free LR³IGF-I accumulating in gastrointestinal tissues. In support of this, Ballard et al. (1991) have shown that radiolabelled des(1-3)IGF-I, a truncated IGF-I analogue that also shows reduced binding affinity to several

of the IGF binding proteins, was cleared more rapidly from the circulation than the radiolabelled native IGF-I, but accumulated to a greater extent in gastrointestinal tissues including the stomach and small and large intestine. Improved potency and enhanced growth of the gastrointestinal tract have also been observed when the IGF-I variant, des(1-3)IGF-I is administered to gut resected rats (Lemmey et al., 1991 and Vanderhoof et al., 1992). Bovine des(1-3)IGF-I has the last 3 N-terminal residues (Gly-Pro-Glu) missing and is 4-50 times more effective in stimulating DNA and protein synthesis and inhibiting protein degradation in cell culture systems, compared to bIGF-I or hIGF-I (Ballard et al., 1988, Francis et al., 1986 and Szabo et al., 1988). As for LR³IGF-I, the increased potency of des(1-3)IGF-I can be attributed to the altered affinities of the truncated IGF to the type 1 receptor, which in human lung and skin fibroblasts and L6 myoblasts is several-fold greater (Ballard et al., 1988). Moreover, the truncated IGF-I shows a reduced affinity for IGFBP-2 (Szabo et al., 1988), IGFBP-1 (Ross et al., 1989) and to a lesser extent to IGFBP-3 (Forbes et al., 1988).

3.4.7 Direct or indirect action of IGF-I peptides?

The present study was not designed to distinguish direct or indirect effects of IGF-I peptides on the gastrointestinal tract, so that indirect action of IGFs on the gastro-intestinal tissue can not be dismissed. In addition, it is possible that IGF-I peptides also synergistically interact with other intestinal growth modulators such as EGF. For example, IGF-I mRNA was 2-3 fold lower in the jejunum and ileum of EGF infused rats compared to saline infused control rats, suggesting that EGF down-regulates IGF-I mRNA expression (Selub et al., 1991). Synergistic action of IGF-I and EGF to stimulate proliferation of cultured intestinal epithelial cells has also been shown by Duncan et al., (1994). This group suggested that EGF may act as a competence factor by increasing the transition of proliferating cells from G0 to G1, thus allowing IGF-I to act as a progression factor on the cycling cell population. Additionally, as discussed in Chapter 1, an intact GH/IGF-I axis may be necessary to optimise

gastrointestinal growth responses. Conversely, the pattern of type 1 IGF-I receptors in the intestinal epithelium of a number of species including rat (Heinz-Erian et al., 1991, Laburthe et al., 1988, Young et al., 1990, MacDonald et al., 1993 and Costigan, 1993), pig (Schober et al., 1990), rabbit (Termanini et al., 1990) and human (Rouyer-Fessard et al., 1990, Korman et al., 1989 and Guo et al., 1992) as well as on IEC-6 cells, a rat jejunal intestinal crypt cell line (Park et al., 1990), strongly supports a direct action of IGFs on the gut. A study by Laburthe et al. (1988) has shown that binding of ^{125}I -IGF-I was detected throughout the entire gastrointestinal tract in rats and was highest in the colon. Heinz-Erian et al. (1991) have shown that IGF-I specific receptors are found throughout all tissue layers but are most abundant in the muscularis propria. Within the mucosa, greater receptor densities were observed in the lamina propria than on the luminal surface. In agreement with Laburthe's study, Heinz-Erian et al. (1991) also showed a proximo-caudal distribution gradient of receptors with greater receptor densities in the ileum and colon compared to the proximal intestine (Heinz-Erian et al., 1991). Both studies showed that a distinct receptor gradient also exists along the vertical axis of the gut with a greater number of receptors in the proliferative crypt than along the villus axis. In the rabbit gastrointestinal tract ^{125}I -IGF-I binding is also more pronounced in the muscularis propria than in the mucosa (Termanini et al., 1990), which suggests that intestinal proliferation may be stimulated via endocrine or paracrine pathways rather than by agents present in the gut lumen. Further evidence for direct IGF-I action stems from *in vitro* studies. Cell lines established from gastro-intestinal carcinomas or from epithelial cells clearly proliferate in response to IGF-I administration (Park et al., 1990 and Thompson et al., 1990). In view of the localisation of IGF-I specific receptors along the longitudinal axis of the gut (small to large intestine), it can be speculated that systemically derived IGF-I peptides elicit their mitogenic response by binding of the IGF-I ligand to specific receptors located in the proliferative crypt. While it is not clear at present to what extent systemically IGF binds to IGF-I specific

receptors located on enterocytes along the functional villus, locally produced IGFs may play a role in modulating intestinal function via autocrine or paracrine stimulation.

3.4.8 *Absorptive function*

Although the villus surface area was not directly measured in the present study, it is anticipated that the increased cellularity of the small intestinal mucosa greatly amplified the surface area, which, combined with a lengthening of the small intestine, would be expected to greatly improve absorptive function. This was supported by the finding that fractional faecal nitrogen excretion was reduced by up to 20% during the first week of treatment with IGF peptides. Although we cannot exclude a contribution from non-dietary sources, such as secreted protein or sloughed intestinal cells, to faecal nitrogen excretion, net intestinal loss of nitrogen in faeces was clearly improved. Similarly, administration of IGF-I peptides significantly increased the nitrogen balance in rats that underwent a 80% jeunoileal resection (Lemmey et al., 1991). In the present investigation, the rats absorbed approximately 95% of their net nitrogen intake. It might therefore be expected that even greater effects on absorptive function will be observed in a modified design such as high dietary fat or high nitrogen.

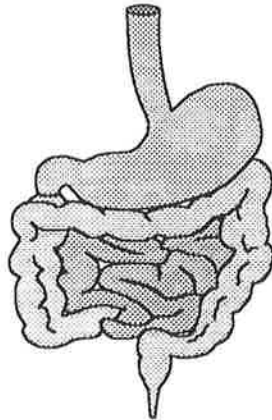
3.4.9 *Where to go from here?*

In conclusion, the present study has shown that IGF-I, and in particular LR³IGF-I, stimulates the growth of the gastrointestinal tissues in normal adult rats. The mechanism by which IGF peptides induce gastrointestinal tissue growth appears to involve changes in both the proliferative compartment and the functional compartment of the mucosa. Possible mechanisms by which a proliferative response of the intestinal epithelium is evoked include alteration of crypt cell population and possibly changes in cell cycle time and/or modulations of villus enterocyte life span. Despite the marked mucosal hyperplasia, which involved a massive

increase in cellularity in both the crypt and villus compartment, the proportionality between these two compartments was maintained. At the start of the present study, a steady state presumably existed within the proliferative crypt and between the crypt and the villus compartment, because the villus and crypt compartment are a stable unit and cell production and cell loss are in a steady state. Yet treatment with IGF-I peptides appears to have stimulated mucosal growth in such a way that after the 14 day treatment a new equilibrium was reached despite a massive cellular increase. This implies that IGF-I peptides have initiated proliferative events which are responsible for the transition from one steady state to the other. If this is the case, it should be possible to demonstrate, at an earlier time-point, an increase in the proliferative activity of the intestinal crypt epithelium without a concomitant changes in crypt cellularity. It is therefore hypothesised that IGF-I peptide administration for a 3 day period initiates a rapid increase in cellular proliferation. At present it is not clear if LR³IGF-I and IGF-I differ in their potency to evoke proliferative responses of the intestinal epithelium in a short-term infusion protocol and if so, whether proliferative effects are similar in different intestinal regions. These questions will be addressed in the next Chapter.

CHAPTER 4:

**ADMINISTRATION OF IGF-I PEPTIDES FOR A 3 DAY PERIOD
STIMULATES PROLIFERATION OF THE INTESTINAL
EPITHELIUM IN NORMAL ADULT RATS.**



CHAPTER 4

Administration of IGF-I peptides for a 3 day period stimulates proliferation of the intestinal epithelium in normal adult rats.

Contribution to the work

This Chapter describes the growth and mitogenic responses of the intestinal mucosa in normal adult rats following administration of IGF-I peptides for a 3-day period. The planning and execution of the study, as well as all data collation and analyses were conducted by myself. Mr. Callum Gillespie and Mrs. Kathryn Davey provided assistance during the collection of gastrointestinal tissues.

ABSTRACT

The previous Chapter described the long-term effects of IGF-I peptide administration on body weight gain and gastrointestinal growth. In this study, the short-term effects of IGF-I administration on intestinal proliferation have been investigated. Female rats (110g, 5-6/group) were infused for 3 days with 278 μ g/day of either IGF-I or LR³IGF-I and compared to either vehicle-treated or control rats. LR³IGF-I but not IGF-I increased body weight and wet tissue weight of the small and large intestine (+20%), compared to controls. Tissue weight responses were independent of food intake and were reflected in the histology of the tissue. Duodenal and ileal crypts length in LR³IGF-I treated animals were increased by 13% and 22%, respectively, and were associated with an increase in crypt cell number. No such histological changes were observed in IGF-I treated rats. On the other hand, tritiated thymidine labelling indices were significantly elevated (up to 14%) following administration of either IGF-I or LR³IGF-I (up to 14%) in both the duodenum and ileum. Thus, IGF-I administration for 3 days stimulated intestinal proliferation without increasing the crypt size.

In contrast, LR³IGF-I induced proportional increments in thymidine labelling and crypt size, suggesting that LR³IGF-I is not only more potent in stimulating intestinal proliferation in a long-term infusion protocol but also induces proliferative effects more rapidly than the native IGF-I. In the colon, wet tissue weights were increased similarly in magnitude to the small intestine, yet thymidine labelling in IGF-I peptide treated rats was not increased in this region. It is possible that proliferative effects by IGF-I peptides were disguised by the low inherent mitotic activity of the colon. These results suggest that although both IGF-I peptides stimulate intestinal proliferation, intestinal mitogenesis is more advanced in rats treated with the IGF-I analogue, indicating that LR³IGF-I induces proliferative responses more rapidly.

4.1. INTRODUCTION

The study in Chapter 3 has indicated that IGF-I peptides play an important role in gastrointestinal growth and function. IGF-I peptide administration induced a proportional increase in potentially proliferative enterocytes as demonstrated by immunohistochemical detection of cells positive for proliferative cell nuclear antigen (PCNA). The proportionality between the proliferative and maturation compartment of the crypt as well as between the crypts and the villi was maintained. These observations have led to the conclusion that administration of either IGF-I or LR³IGF-I peptide for a prolonged period stimulate mucosal growth in such a way that after 14 days of administration of the peptide, a new steady state between cell production and cell loss has already been reached.

The current study was designed to identify the early proliferative responses of the intestinal epithelium that led to the new steady state following IGF-I or LR³IGF-I administration. IGF-I and LR³IGF-I were compared to determine if the two peptides differed in their potency to evoke proliferative responses of the intestinal epithelium during this early period. Furthermore, I compared the early proliferative responses of the two peptides in the small intestine to that in the colon. To examine the early proliferative responses, rats were infused for only 3 days commencing at the same body weight as the rats described in Chapter 3 and under an identical peptide dose and infusion protocol so that direct comparisons could be made between the two studies.

4.2. MATERIALS AND METHODS

4.2.1 *Recombinant IGF-I peptides*

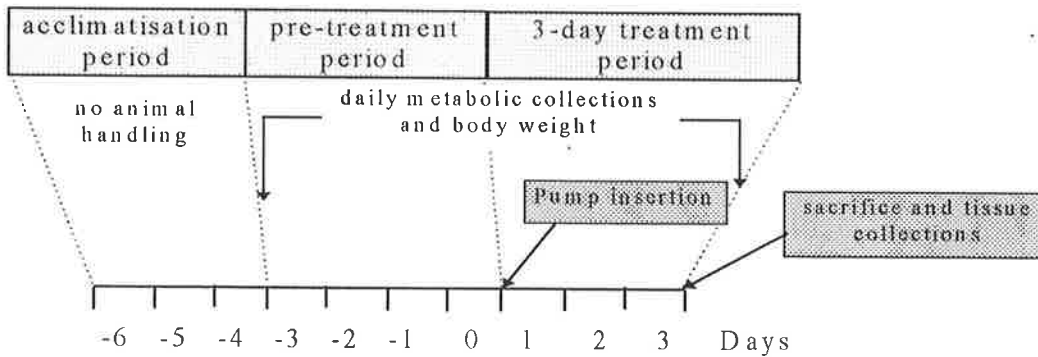
Recombinant human IGF-I and the recombinant analogue LR³IGF-I were provided by GroPep Ltd., Adelaide, South Australia. Specification of the peptides has been provided in Chapter 3, section 3.2.1.

4.2.2 *Experimental design*

Animals: For direct comparisons between this study and the study described in Chapter 3, female Hooded Wistar rats of approximately 100g were obtained from the same breeding colony (CSIRO). All animals were housed individually in Techniplast metabolism cages and the maintenance and care of the animals closely followed the protocol as outlined in Chapter 3, section 3.2.2.

As in the previous study, all animals were allowed to acclimatise for a 3-day period, before being placed into the metabolism cages for a 4-day pre-treatment period. This was followed by a 3-day peptide treatment period. Daily measurements of body weight, food and water intake as well as quantitative collections of faeces and urine were taken at precisely 24 hour intervals and conducted throughout the pre-treatment and treatment period of the experiment. Figure 4.1 illustrates a general outline of the experimental design.

Figure 4.1: Generalised protocol of the experimental design.



4.2.3 Surgical procedures

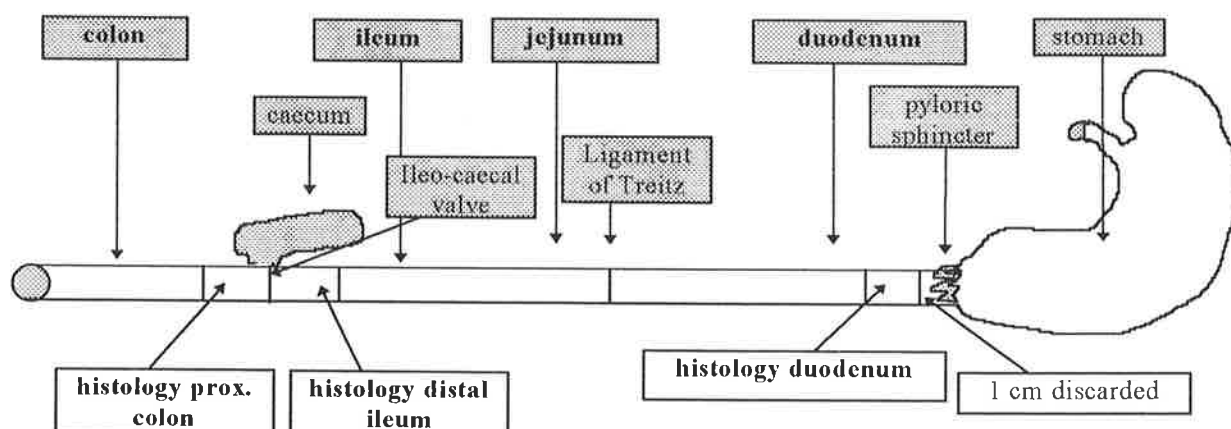
IGF-I peptides were infused subcutaneously for a 3-day period. Between 900 and 1200 hours on the morning following the pre-treatment period, the rats were anaesthetised with 0.04ml/kg Brietal for osmotic mini pump (Alzet, Model 1003D, Alza, Palo, CA., USA) implantation within the subcutaneous scapular region. The pumps were filled with either IGF-I or LR³IGF-I or contained the vehicle alone (0.1M acetic acid). At a mean pumping rate of 0.99 μ l/h, the peptides were delivered at a rate of 278 μ g/day for the 3-day period. There were 6 rats in each of the vehicle IGF-I and LR³IGF-I treated groups and a control group (N=5), receiving no treatment or pump, was also included. The study was divided into 2 animal trials. The first trial contained 3 rats from each of the two peptide treatment groups, 3 vehicle treated rats and 3 untreated control animals. The second trial contained 3 rats from each of the peptide treated and vehicle treated groups and 2 untreated control animals. The trials were staggered by 1 day. The pumps were not primed prior to implantation; the full pumping rate was reached approximately 4 hours after insertion of the pumps. No special post-operative care of the animals was required. During the 3 days of peptide infusion, daily measurements of body weights and metabolic collections were continued. At the end of the 3-day treatment period between 1300 and 1600 hours, each animal was injected with a single intra-peritoneal injection of 0.5 μ Ci/g body weight of tritiated thymidine (Amersham International,

Buckinghamshire, England, specific activity 25Ci/mol) in the same order of pump implantation. Thus all rats received IGF-I peptides for the same length of time.

4.2.4 Tissue collections and measurements

Exactly one hour after the injection of the isotope, the animal was stunned and decapitated. The abdomen was opened by a mid-line incision and the entire gastrointestinal tract was rapidly excised and placed onto a ice-cold glass slab. Removal of gastrointestinal tissue components followed closely the protocol described in Chapter 3, section 3.2.4. Weight and length measurements were taken for the stomach, small and large intestine and the caecum. For histological analyses, multiple tissue sections were collected from the proximal duodenum (stating 1 cm caudal to the pyloric sphincter) and the distal ileum, while large intestinal samples were collected from the proximal colon. The segments were rinsed in cold 0.9% w/v NaCl and immediately fixed in Bouin's fluid. Total gut weights and length were calculated as the sum of all intestinal components. Figure 4.2 shows a representation of the tissue collection protocol.

Figure 4.2: Schematic representation of tissue collection protocol.



4.2.5 Histology and autoradiography

For quantitative histological morphometry, tissue segments of the duodenum, ileum and colon were fixed in Bouin's fluid for 4 hours and stored in 70% ethanol prior to processing for

routine paraffin embedding. From each of the intestinal regions sampled, 4-6 tissue segments were embedded in transverse orientation in the same mould and 6 serially cut sections of 2 μ m thickness were prepared for each animal. The first of the serially cut sections from each animal was de-waxed, re-hydrated, stained with haematoxylin and eosin and mounted with DePex (Gurr, BDH Chemicals, Kilsyth, Australia) for histological analyses. The remaining 5 serially cut sections were used for autoradiography, following the general description by Rogers (1979). For this purpose, they were de-waxed, re-hydrated and briefly dipped in 10% lithium carbonate to reduce chemography during autoradiographic processing. The sections were then incubated in the darkroom at room temperature for 30 seconds in autoradiographic emulsion (LM-1, Amersham, Australia) at a dilution of 1:1 with distilled water. After incubation, the slides were chilled on a pre-cooled tray for 10 minutes and air-dried for 2-3 hours. All slides were stored at 4°C in light-tight photographic slide containers and kept for 2-20 days. After exposure for 2, 4, 6, 10 or 20 days, the slides were developed in Ilford Phenisol Developer (Amersham, Australia) at room temperature (dilution 1:4) for 6 minutes and rinsed in sodium thiosulphate (BDH Chemicals, Australia) for 4 minutes. The developed slides were rinsed for 15 minutes in de-ionised water, counterstained with haematoxylin and mounted with DePex. Negative control slides were included from animals not injected with the isotope.

In addition to the transversely embedded tissue material, a single, 2 cm long tissue segment from each region was embedded facing serosal side down, so that tissue sections orientated longitudinal to the bowel lumen could be obtained. From serially cut sections, tissue segments representing the crypt/villus junction were identified and utilised to count the number of enterocytes located around the circumference of the crypt (crypt row count).

4.2.6 Histological assessment and evaluation of autoradiographs

Histological sections were examined with an Olympus BH-2 light microscope. Quantitative morphometric analysis was conducted using a drawing tube attached to the microscope and measurements were taken using a digitising tablet (Summa Sketch II, Summa graphics), coupled to an Apple Macintosh II CI computer. In the duodenum and ileum, crypt depth was measured in 15 well-oriented crypts as described in Chapter 3, section 3.2.5. The depth of the colonic crypts was measured in 15 crypts randomly selected, with care taken to avoid sections containing Peyer's patches.

The dose of tritiated thymidine used in this study produced clear labelling of S-phase nuclei, showing numerous black grains deposited over the nuclei and a negligible background after 10-20 days of exposure. Before analytical evaluation began, the optimal time of exposure of the autoradiographic sections was evaluated. Sections exposed for 2 days showed weak staining and 69% of the positive cells had less than 4 silver grains deposited over their nuclei. After 4 days of exposure, the mean grain density was 9.3 grains (range 5-13 grains), increasing to 21.8 grains (range 11-35) after 10 days of exposure; at the same time, background staining was still negligible after 10 days of exposure. After 20 days of exposure, the grain density increased to form a conglomerate in approximately 21% of the positive cells, so that differentiation between individual nuclei became difficult.

On this basis, I decided to analyse all proliferative parameters in tissue sections exposed for 10 days. A minimum of 6 grains per nuclei was used to define positively labelled cells. Proliferative parameters were assessed in 30 crypts from each animal in the duodenum, ileum and colon. Analyses were confined to crypts where the entire length could be completely visualised and which contained a single layer of epithelial cells only. In each crypt, a single column (right hand column) along the longitudinal axis of the crypt was assessed and the total

number of cells and the number and position of tritiated thymidine labelled cells was recorded. For each of the intestinal regions, the labelling index was calculated as the ratio of labelled cells to total cell number for each crypt column, in the same manner described for PCNA labelling. In addition, the circumferential cell count (crypt row count), measured as the number of epithelial cells around the circumference at the crypt:villus junction was measured in the duodenum and ileum in serial cut sections from tangentially embedded material. The product of the crypt column count and the crypt row count was used to estimate the total crypt cell population in all small intestinal regions.

For each animal, thymidine labelling index distribution profiles were established for the duodenum and the ileum. From these curves, the cell position within the crypt at which maximal thymidine labelling occurs and the cell position of half maximum thymidine labelling was identified. The maturation compartment of the crypt in which epithelial cells have lost their proliferative capabilities and acquire their mature, functional properties was identified from these curves as the region above the last labelled cells within the crypt. To determine if IGF-I peptides increase thymidine incorporation into enterocytes in the lower and mid-crypt region, the number of cells labelled with tritiated thymidine up to cell position 19 was calculated. The crypt growth fraction (CGF), which identifies the proportion of proliferating cells within the crypt, was calculated for each animal from the thymidine labelling distribution profiles of 30 perfectly orientated crypts by dividing the cell position at which half maximum labelling occurred by the total number of cells per cell column.

In the colon, the number of cells per colonic crypt were counted in sections from the proximal colon. Crypts at both the base and at the apex of mucosal folds were included in the analysis. The number of cells labelled with tritiated thymidine was recorded; however, labelling distribution profiles were not established.

4.2.7 *Statistical analyses*

All values in Tables and Figures are expressed as means including standard error of the mean (SEM). All groups were compared by a one-way analysis of variance (ANOVA) and where significance was achieved ($P < 0.05$) a post-hoc Dunnett's test (Super ANOVA, Abacus Concepts Inc., Berkeley CA) was applied to identify variations between treatment groups and either vehicle treated or control animals. To examine the degree to which body weight gain and fluid balance vary following IGF peptide treatment, data was analysed by product-moment correlations and significance was tested with a *t*-test with $n-2$ degrees of freedom (Pearson, Sokal and Rohlf, 1987).

4.3 RESULTS

4.3.1 *Body weight responses*

The body weight at the start of the study averaged 97.9 ± 1.0 g (N=23) for all rats. After 4 days of acclimatisation to the metabolism cages, the average body weight increased to 109.9 ± 1.0 g. Rats were then randomised into treatment groups such that no statistically significant differences in body weight were apparent between treatment groups (Table 4.1).

Infusion of LR³IGF-I for a 3-day period resulted in a significantly higher body weight (134.9 ± 1.1 g) as compared to animals treated with vehicle (127.0 ± 2.8 g) or normal rats (control group, 122.3 ± 2.0 g) without an implanted mini pump (Table 4.1). The body weight gain in LR³IGF-I treated animals could not be attributed to an increase in food intake, as food consumption was approximately 13g/day for all groups (Table 4.1). Accordingly, food conversion efficiency, calculated as the ratio of average daily body weight gain to food consumption for the 3 day treatment period was highly significant in the LR³IGF-I treated group ($P < 0.0001$) when compared to either vehicle, control or IGF-I treated animals (Fig. 4.3). To determine if the accelerated weight gain in the LR³IGF-I treated animals could be attributed to fluid retention, the fluid balance (fluid intake/24 hours minus fluid output for the same 24 hour period) was estimated for all animals. Although fluid intake was highly variable throughout the 3-day treatment period in all groups, no statistically significant difference was detected in either fluid intake or urinary output over the experimental period, arguing against fluid retention as a possible mechanism for the extra weight gain observed in LR³IGF-I treated rats ($P = 0.74$, ANOVA). To examine the degree to which body weight gain and the fluid balance correlate following IGF-I peptide treatment, product-moment correlation coefficients were calculated for the combined data (Fig. 4.4 A) and for each treatment group individually (Fig. 4.4 B). No statistical significant correlation was found between body weight gain and the

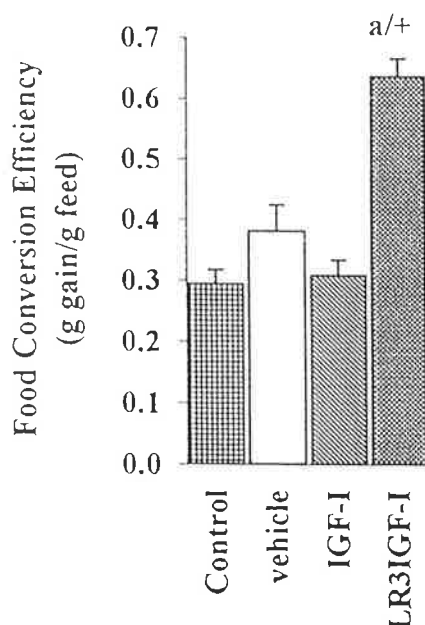
fluid balance during the 3-day treatment period in either treated or untreated rats ($r_{\text{obtained}} = 0.26$, $r_{\text{critical}} = 0.41$ at $P < 0.05$, Fig 4.4 A). Furthermore, no significant correlation between these two variables was found when the data was analysed for each treatment group individually (r values for each group are shown in Fig. 4.4 B).

Table 4.1: Body weight (g), and body weight gain (g/3 days) and average food consumption (g/24 hours) in rats treated for 3 days with $278\mu\text{g/day}$ of either IGF-I or LR³IGF-I as compared to vehicle treated or untreated control rats.

Treatment Group	Body weight			Average food
	starting (g)	final (g)	gain (g/3 days)	intake (g/24 hours)
Control (N=5)	110.5±2.1	122.3±2.0	11.8±1.4	13.7±0.5
Vehicle (N=6)	111.2±2.6	127.0±2.8	15.4±1.9	13.6±0.3
IGF-I (N=6)	108.6±2.6	122.1±2.7	13.5±2.1	13.4±0.4
LR ³ IGF-I (N=6)	109.7±0.6	134.9±1.3*/a	25.2±1.4*/a	12.9±0.1

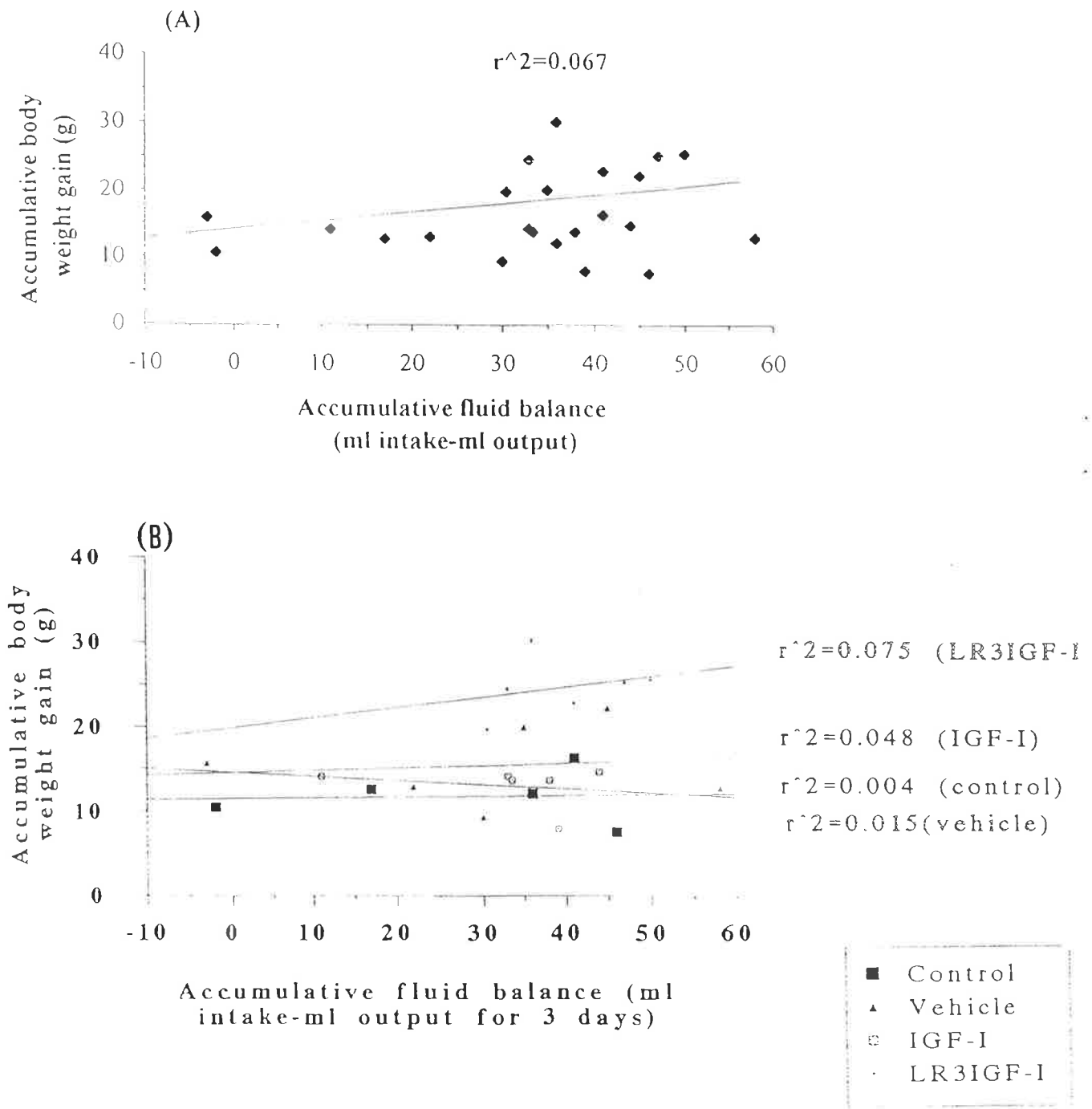
All values are expressed as means ±SEM. Statistical significance from the vehicle treated group is indicated by *: $P < 0.01$ and from the untreated control group by a: $P < 0.01$, ANOVA, Dunnett's post hoc test (two-tailed).

Figure 4.3: Food conversion efficiency in normal adult rats treated for 3 days with $278\mu\text{g/day}$ of IGF-I or LR³IGF-I compared to vehicle or untreated control rats.



Values are means±SEM for 6 rats/group (N=5, control group). Statistical significance from vehicle treated rats is indicated by *: $P < 0.0001$ and from the untreated control group +: $P < 0.0001$ as detected by ANOVA.

Figure 4.4: Correlation between total accumulated body weight gain (g) and the accumulated fluid balance (fluid intake-urinary output (ml)) in rats treated for 3 days with IGF-I peptides.



Graph A displays the correlation analysis of the combined data set ($N=23$), r obtained=0.26, r critical=0.41, $df=21$. Graph B shows the correlation analysis with calculations of r^2 for each group. No statistically significant correlations were obtained between accumulated body weight and accumulated fluid balance for control ($r=0.06$), vehicle ($r=0.12$), IGF-I ($r=0.22$) and LR³IGF-I ($r=0.28$) treated rats. Critical r values for vehicle, IGF-I and LR³IGF-I group is 0.81, $df=4$ and for the control group r critical is 0.75, $df=3$. In both graphs, each data point represents a single animal.

4.3.2 Gastrointestinal responses

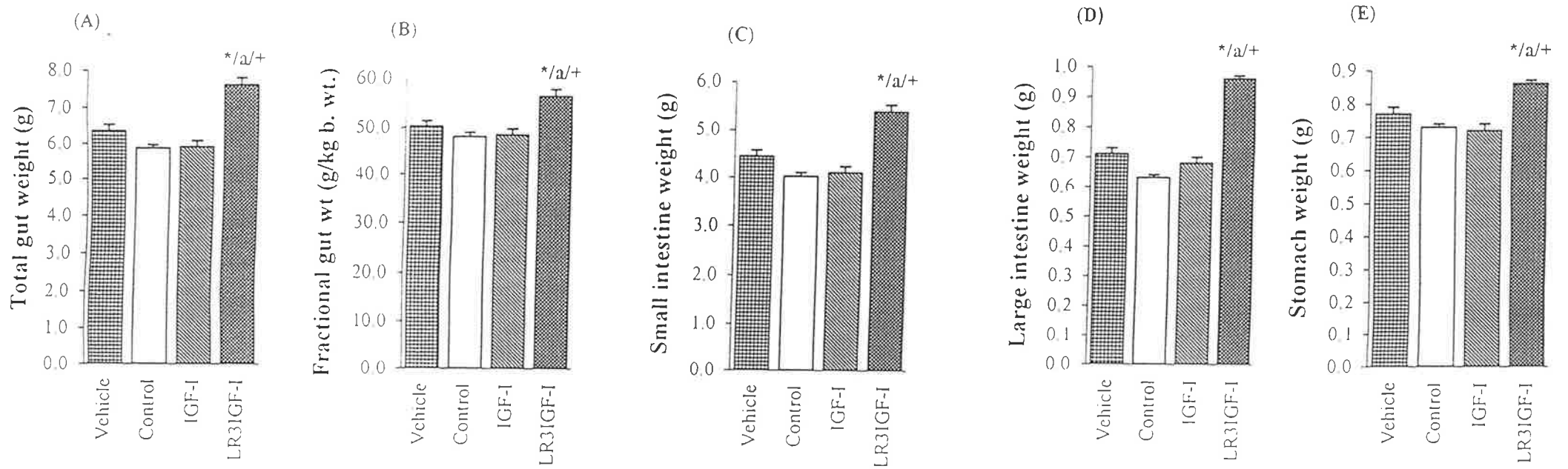
Gastrointestinal weight in the animals fitted with the vehicle pump was similar to the gut weight of untreated control rats, indicating that implantation of the pumps had no effect on gastrointestinal tissue growth. However comparison between the vehicle group and the animals treated with 278 μ g/day of LR³IGF-I showed that the increased body weight gain in the LR³IGF-I group was reflected in the wet tissue weights of their gastrointestinal tissues. Total gut weight, small and large intestinal weight, were increased by 19%, 22% and 21%, respectively in LR³IGF-I treated rats compared to vehicle treated animals (Figure 4.5 A, C and D). Stomach weight increased by 12% following treatment with LR³IGF-I (Figure 4.5 E). Selective action of LR³IGF-I on the gut was evident when corrections for body weight gain were made, so that fractional gut weight (total gut weight/kg body weight) was significantly elevated in LR³IGF-I treated animals (56.6 ± 1.4 , $P < 0.01$) compared to either IGF-I (48.5 ± 1.3) or vehicle treated control animals (50.3 ± 1.1), Fig. 4.5 B. The increase in gastrointestinal tissue weight in LR³IGF-I treated animals contrasts markedly with the results obtained for IGF-I treated animals, so that infusion of 278 μ g/day of recombinant IGF-I did not affect body weight gain or gastrointestinal tissue weights (Table 4.1 and Figure 4.5 A-E). Increases in small and large intestinal length were not observed in any of the animals treated for 3 days with the IGF-I peptides (Figure 4.6).

4.3.3 Mitogenic response of the intestinal mucosa

The study in Chapter 3 has shown that administration of 278 μ g/day of IGF-I peptides to normal female rats, for 14 days, selectively stimulated the growth of intestinal mucosa, so the main focus here was to assess the mitogenicity of IGF peptides during the initial period of peptide administration. In this study, administration of LR³IGF-I for only 3 days resulted in a statistically significant increase ($P < 0.01$) in crypt depth, both in the duodenum (+8%) and in the ileum (+13%), compared to vehicle treated animals (Table 4.2). The increased crypt depth

in this group was associated with a proportional increase in the number of cells per crypt column (Table 4.2). Furthermore, the circumferential cell count (crypt row count) was also significantly increased in both small intestinal segments, resulting in an overall increase in crypt cell population by approximately 30% (Table 4.2). Administration of LR³IGF-I also stimulated proliferation of the colonic mucosa within the 3-day infusion period. Colonic crypt depth in LR³IGF-I treated animals was marginally increased in comparison to vehicle treated animals but not control treated animals; as in the small intestine, colonic crypt hyperplasia was accompanied by a statistically significant increase in the crypt cell column count (Table 4.2).

Figure 4.5: Total gut weight (A), fractional gut weight (B), small intestinal weight (C), large intestinal weight (D) and stomach weight (E) in normal female rats treated for 3 days with either 278 μ g/day of IGF-I or LR³IGF-I as compared to vehicle treated or untreated control rats.







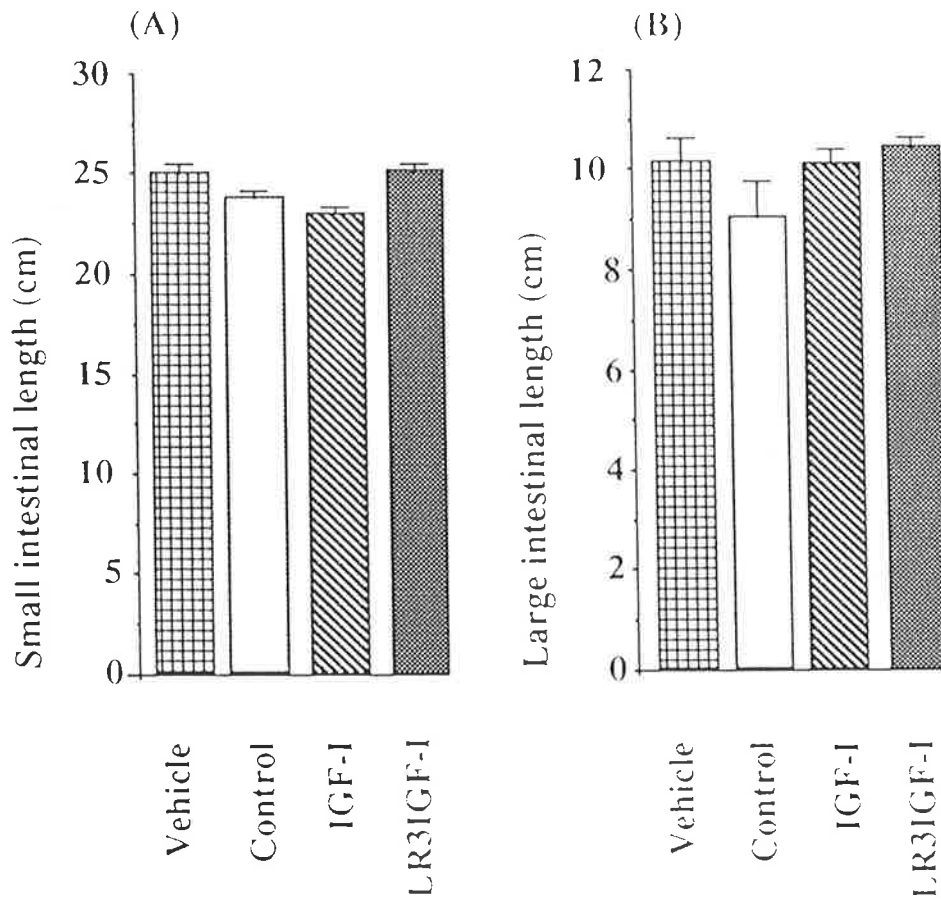
Values are means \pm SEM with 6 rats/group (N=5, for control group). IGF-I  LR³IGF-I  treated rats are compared to either vehicle treated rats  or untreated control rats . Significance from the vehicle group is indicated by a: P<0.01. Significance from untreated control group is indicated by +: P<0.01, as determined by ANOVA. Significant difference in wet tissue weights between IGF-I and LR³IGF-I treated rats is shown by *: P<0.01.

Figure 4.6: Total small intestinal length (A) and total large intestinal length (B) in normal female rats treated for 3 days with vehicle or 278 μ g/day of IGF-I or LR³IGF-I.



Values are means \pm SEM with 6 rats per group. The control group show data of 5 animals. No statistical significant difference was detected between the peptide treated rats and either untreated controls or vehicle treated rats (ANOVA).

Table 4.2: Histological parameters in the duodenum, ileum and colon of female rats treated for 3 days with or without 278µg/day of IGF-I or LR³IGF-I.

Histological Parameter	Treatment Group			
	Control	Vehicle	IGF-I	LR ³ IGF-I
Duodenum				
Crypt depth (µm)	260±7	255±5	257±5	293±14 (a/*)
Crypt column count (no. cells)	34.1±1.1	35.1±0.2	33.7±0.5	41.7±0.9 (b/**)
Crypt row count (no. cells)	19.3±0.3	20.0±0.3	19.8±0.3	21.8±0.5 (b/**)
Crypt population (no. cells)	656±14	702±13	668±9	910±22 (b/**)
Ileum				
Crypt depth (µm)	190±8	203±7	192±5	231±4 (b/**)
Crypt column count (no. cells)	29.4±0.4	29.2±0.6	29.3±0.6	33.5±0.8 (b/**)
Crypt row count (no. cells)	20.3±0.4	20.0±0.1	20.1±0.3	21.6±0.4 (b/**)
Crypt population (no. cells)	596±16	582±11	588±16	723±26 (b/**)
Proximal colon				
Crypt depth (µm)	191±1.0(c)	171±5	173±6	198±9 (a)
Crypt column count (no. cells)	25.6±1.9	22.4±0.7	25.1±0.7	28.5±0.6 (b)

*Values are expressed as means±SEM. Statistically significant difference from the vehicle group is indicated by a: P<0.05 and b :P<0.01. Difference from the control group is indicated by *: P<0.05 and **: P<0.01. N=6 animals per group, except for the control group where N=5. Crypt depth measurements in untreated control rats were significantly greater than in vehicle and IGF-I treated rats (c: P<0.05).*

To further assess the mitogenic properties of the IGF-I peptides on the intestinal epithelium, I constructed thymidine labelling distribution profiles of the duodenum and ileum for each animal. In the duodenum of control or vehicle treated animals, a total of 29-30% of the crypt cells were labelled with the isotope (crypt labelling index), Table 4.3. In both groups, the thymidine labelling indices were low in the basal cell positions (1-4). However, from cell position 5-16, labelling indices increased, reaching a maximal labelling of 58% and 61% for control and vehicle treated animals, respectively (Table 4.3 and Fig. 4.7 A and B). After cell position 16, proliferative indices declined to reach half maximum labelling at cell position 20 and from cell position 29-35 in the crypt column, no labelled cells were apparent (Fig. 4.7 A and B). Thus, the maturation compartment of the crypt was identified from cell position 29 upwards. The crypt growth fraction for the vehicle and untreated control rats was calculated at approximately 60%.

In the duodenum of the LR³IGF-I treated animals, the crypt cell labelling index was significantly increased (32.7%) as compared to the control groups (29-30%), Table 4.3 and Plate 4.1C. As for the control and vehicle groups, low proliferative indices were evident in the first few cell positions, however increased crypt cell labelling was reflected in an increased maximum labelling, calculated at 66%, at cell positions 5-16 (Table 4.3 and Fig. 4.7 D). Furthermore, the number of thymidine labelled cells up to cell position 19 was also significantly greater in LR³IGF-I treated rats as compared to either vehicle or untreated control rats (Plate 4.1C). After cell position 16, the percent thymidine labelling in the LR³IGF-I treated rats declined more slowly than in the vehicle or control group, so that half maximum labelling was not reached until cell position 24. Thymidine labelling was detectable up to cell position 36 in this group, indicating that the maturation compartment (cell position 36-42) was shifted upwards, in proportion to with the lengthening of the crypt. This indicates that LR³IGF-I administration for 3 days led to a significant increase in the number of crypt cells, associated with an increase in the proportion of cells labelled, in particular in the lower and mid-crypt

region (up to cell position 19) and an increase in maximal labelling. Most importantly, however, LR³IGF-I also increased the cell position of half maximum labelling in proportion to the total number of cells per crypt column and hence the crypt growth fraction remained at approximately 60%, Table 4.3 and Fig. 4.7 D.

The labelling distribution profiles in IGF-I treated rats also showed an increase in the overall crypt cell labelling index which was virtually identical to that in LR³IGF-I treated rats (Table 4.3). In this group, maximal labelling was increased to 67% as compared to approximately 60% in the control groups (Fig. 4.3 and 4.7 C), and furthermore, the proportion of cells accumulating the nuclear label up to cell position 19 was increased from 7.32 ± 0.09 cells (vehicle group) to 8.45 ± 0.07 in LR³IGF-I treated rats. Unlike in the LR³IGF-I group, thymidine labelling declined more rapidly (similar to the vehicle and control group) so that half maximum labelling was reached at cell position 20 in IGF-I treated rats, which was similar to control values (Table 4.3 and Figure 4.7 C). Since the total number of cells per crypt column was not altered in this group, the overall crypt growth fraction was maintained at 58% and comparable to the crypt growth fraction of the control groups (Table 4.3). A microphotograph of the thymidine incorporation in IGF-I treated rats is shown in Plate 4.1B.

Thus, the mechanism by which IGF-I induced epithelial proliferation in this short-term administration protocol differed from that of the LR³IGF-I group. Despite the marked increase in the proliferative pool, short term IGF-I administration did not increase the cellularity of the crypt. Increased crypt labelling was achieved by increasing the proportion of proliferative cells in lower and mid-crypt cell positions leading to a higher maximal labelling, rather than a recruitment of proliferative cells in higher cell positions, as seen in the LR³IGF-I group.

In the ileum, the overall crypt labelling index was lower than in the duodenum (Table 3). This was reflected by reduced nuclear labelling in lower cell positions (Fig. 4.8 A-D) as well as a lower maximal labelling (Table 4.3). On the other hand, the position of half

maximum labelling in the two intestinal regions was similar, occurring at approximately cell position 20 for the vehicle, control and IGF-I treated animals and at cell position 24 for animals treated with LR³IGF-I (Table 3). As for the duodenum, in LR³IGF-I treated animals cells in higher position retained their proliferative activities longer (as indicated by the upward-shift in the 1/2 maximum labelling). On the other hand, in IGF-I treated rats, the proportion of proliferative enterocytes in mid-crypt cell positions remained high (Table 4.3 and Figure 4.8), reaching a maximum thymidine labelling index of 60% as compared to 54-57% in the control groups (Table 4.3). This led to a non-significant increase in the crypt growth fraction in this group (P=0.08 as detected by ANOVA, Table 4.3).

Finally, the mitogenic response of the colonic mucosa to IGF-I peptides was assessed in tissue segments from the proximal colon. Although similar changes in wet tissue weight in LR³IGF-I treated rats were observed for the small and large intestine, thymidine labelling indices were not increased by IGF-I peptides (Table 4.4). Nevertheless, in LR³IGF-I treated rats, an average of 3.7 cells per crypt were labelled as compared to 2.9 cells/crypt in vehicle or untreated control rats (Table 4.4). Although statistical significance was not reached, it is possible that IGF-I peptide effects in the colon have been disguised by the overall low inherent proliferative activity of the colon. In the present study, thymidine labelling indices were 11-13% for all groups with no statistical difference between treatment groups. The histological samples for the colon were taken from the proximal large intestine and a great deal of heterogeneity in crypt morphology was observed. For example, the proximal colon contains mucosal rugae and the crypts at the apex of mucosal folds are longer than the crypts at the base of the crypts. Consequently, the colonic crypt cell population could not be established because serial cut sections of longitudinal embedded material did not provide a uniform display of crypts suitable for circumferential crypt row counts in this region. Because of the low proliferative activity in the colon, labelling distribution curves were not established for the colon.

Table 4.3: Proliferative parameters in the duodenum and ileum of rats treated for 3 days with 278µg/day of IGF or LR³IGF-I as compared to vehicle and untreated control rats.

Proliferative Parameter	Treatment Group			
	Control	Vehicle	IGF-I	LR ³ IGF-I
Duodenum				
Crypt labelling index (%)	29.9±1.4	29.1±0.6	33.3±0.7 (b/**)	32.7±0.7 (a/*)
Maximal labelling (%)	58.3±2.9	61.1±2.2	66.7±1.9	65.6±2.0
Crypt labelling up to cell position 19 (no. cells)	7.12±0.38	7.32±0.09	8.45±0.07 (a/**)	8.34±0.21 (a/**)
Cell position at 1/2 max. labelling	19.6±0.6	20.1±0.5	20.0±0.9	24.3±0.8 (a/**)
Crypt Growth Fraction (%)	59.0±0.7	57.4.0±1.3	58.5±2.1	58.4±1.9
Ileum				
Crypt labelling index (%)	26.2±0.7	26.0±0.6	29.2±0.6 (b/**)	29.4±0.6 (b/**)
Maximal labelling (%)	56.7±3.5	54.6±1.5	60.0±2.0	55.2±2.1
Crypt labelling up to cell position 19 (no. cells)	6.88±0.08	6.92±0.02	7.33±0.06 (b/*)	7.58±0.13 (b/*)
Cell position at 1/2 max. labelling	19.3±0.6	18.7±0.9	20.0±0.3	23.3±0.7 (b/**)
Crypt Growth Fraction (%)	66.3±2.4	64.2±1.6	69.5±0.5	69.5±1.6

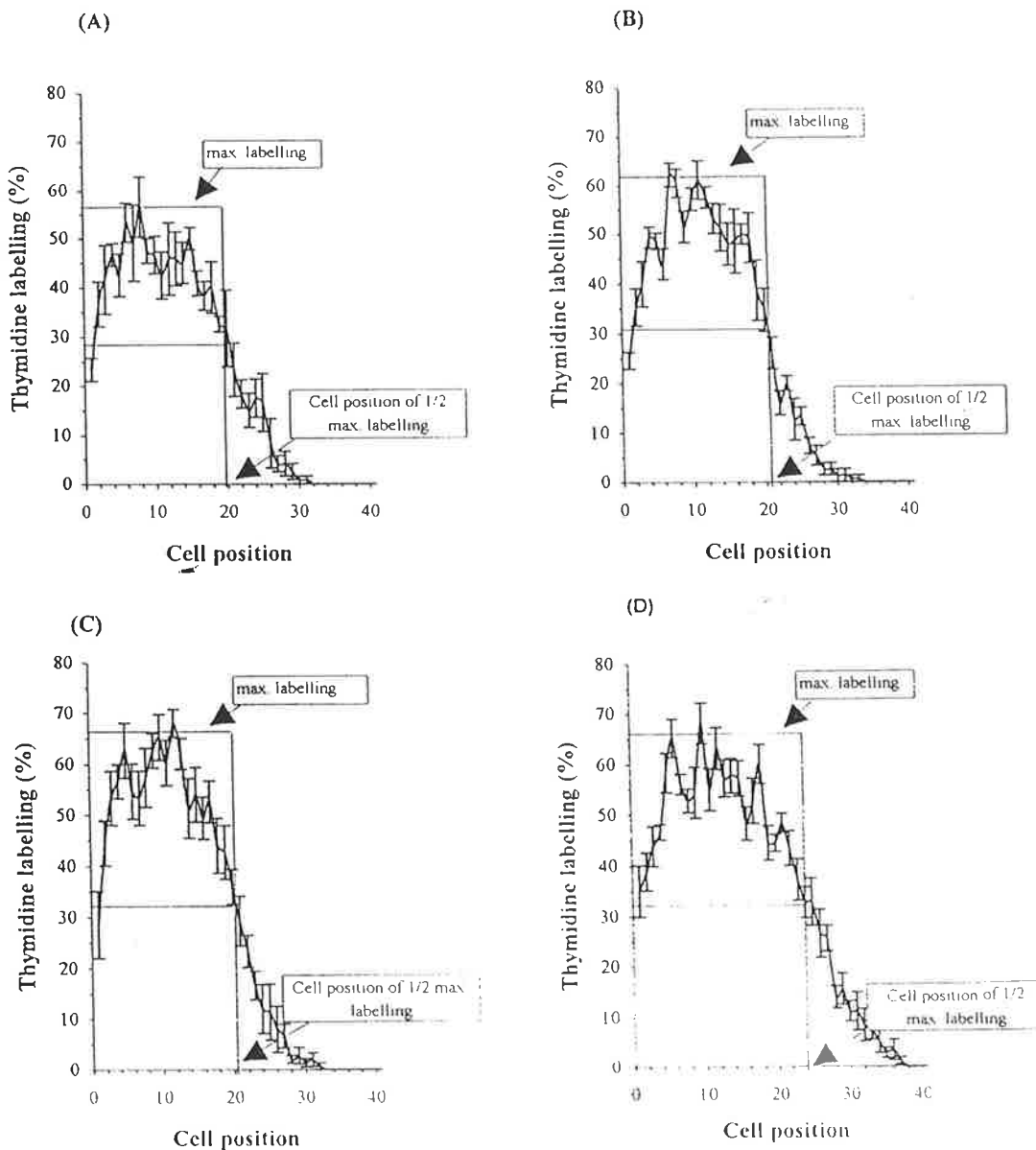
*Values are expressed as means±SEM. Statistically significant difference from the vehicle group is indicated by a: P<0.05 and b: P<0.01. Differences from the untreated control rats is indicated by *: P<0.05 and **: P<0.01. N=6 animals per group except for control group (N=5). The crypt labelling indices represent the proportion of tritiated thymidine labelled cells per crypt column and was calculated from 30 full-length open crypt columns for each animal. Maximal labelling occurred in enterocytes in mid-crypt cell positions and was calculated from the top 3 thymidine labelled enterocytes within cell positions 5-16.*

Table 4.4: Proliferative parameters in the colon of rats treated for 3 days with 278µg/day of LR³IGF-I and IGF-I as compared to vehicle treated and untreated control rats.

Proliferative Parameter	Treatment Group			
	Control	Vehicle	IGF-I	LR ³ IGF-I
Colon				
Crypt column count (no. cells)	25.6±1.8	22.4±0.7	25.1±0.7	28.5±0.6a/*
No. cells labelled/crypt	2.9±0.3	2.9±0.2	2.9±0.2	3.7±0.2
Crypt labelling index (%)	11.6±1.6	13.1±0.6	11.8±0.8	12.8±0.9

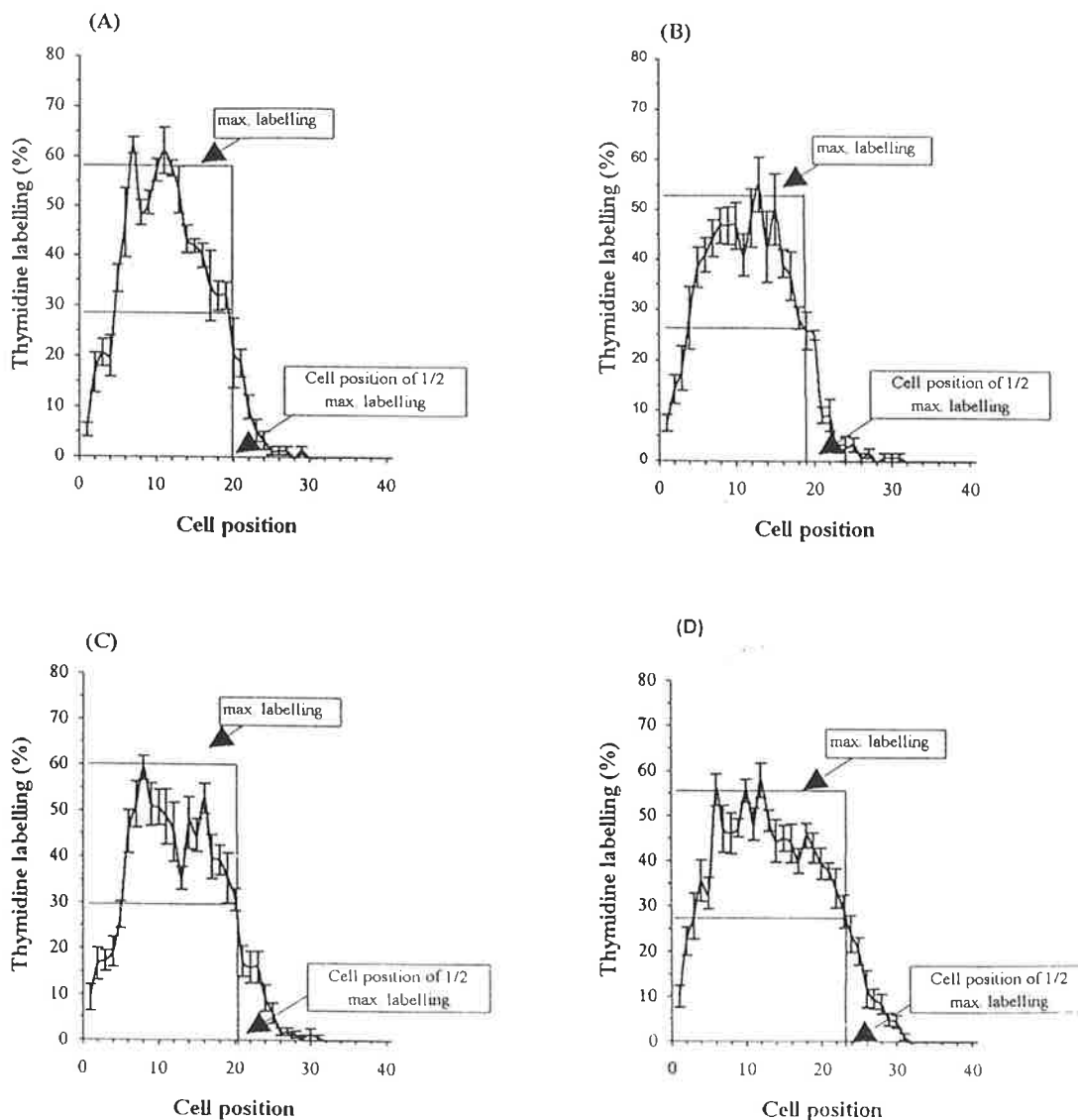
*All values are means±SEM. Statistical significance from the vehicle group is indicated by a: P<0.05. Difference from the untreated control group is indicated by *: P<0.05. Each group comprised 6 animals, except the IGF-I and control group (N=5). The crypt labelling index represents the proportion of labelled cells per crypt column as calculated from 30 full-length open colonic crypts in tissue sections from the proximal colon.*

Figure 4.7: Tritiated thymidine labelling distribution profiles in tissue sections from the duodenum in untreated control rats (A), rats treated with vehicle (B) or rats treated with 278 μ g/day of either IGF-I (C) or LR³IGF-I (D).



All values are represented as means \pm SEM of 6 rats per group ($N=5$, control and IGF-I). Sections from one animal in the IGF-I group were excluded because of very weak incorporation of the isotope. For all animals the mean tritiated labelling index (%) was calculated from 30 full-length open crypts. Maximal labelling (%) represents the average percentage of labelling in the top three labelled enterocytes within cell position 5-16. The crypt growth fraction was calculated for each animal individually as the cell position of half maximum labelling divided by the total number of cells per crypt column.

Figure 4.8: Tritiated thymidine labelling distribution profiles in tissue sections from the ileum in untreated control rats (A), rats treated with vehicle (B) or rats treated with 278 μ g/day of either IGF-I (C) or LR³IGF-I (D).



All values are represented as means \pm SEM of 6 rats per group (N=5, control). For each animal the mean tritiated labelling index (%) was calculated from 30 full-length open crypts. Maximal labelling (%) represents the average percentage of labelling in the top three labelled enterocytes within cell position 5-16. The crypt growth fraction was calculated for each animal individually as the cell position of half maximum labelling divided by the total number of cells per crypt column.

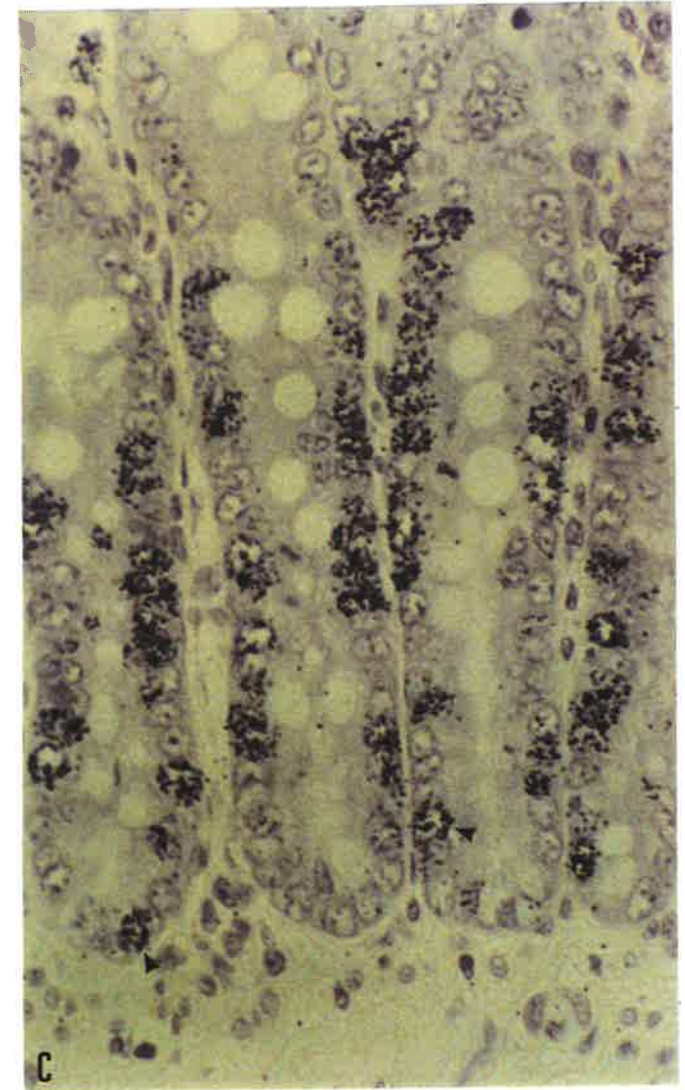
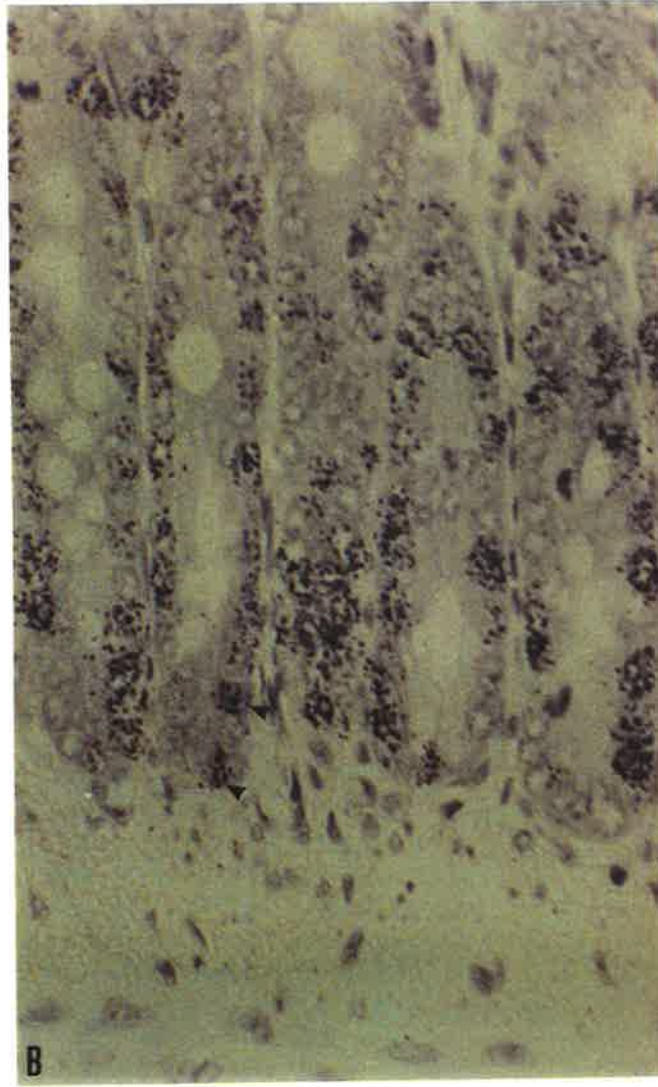
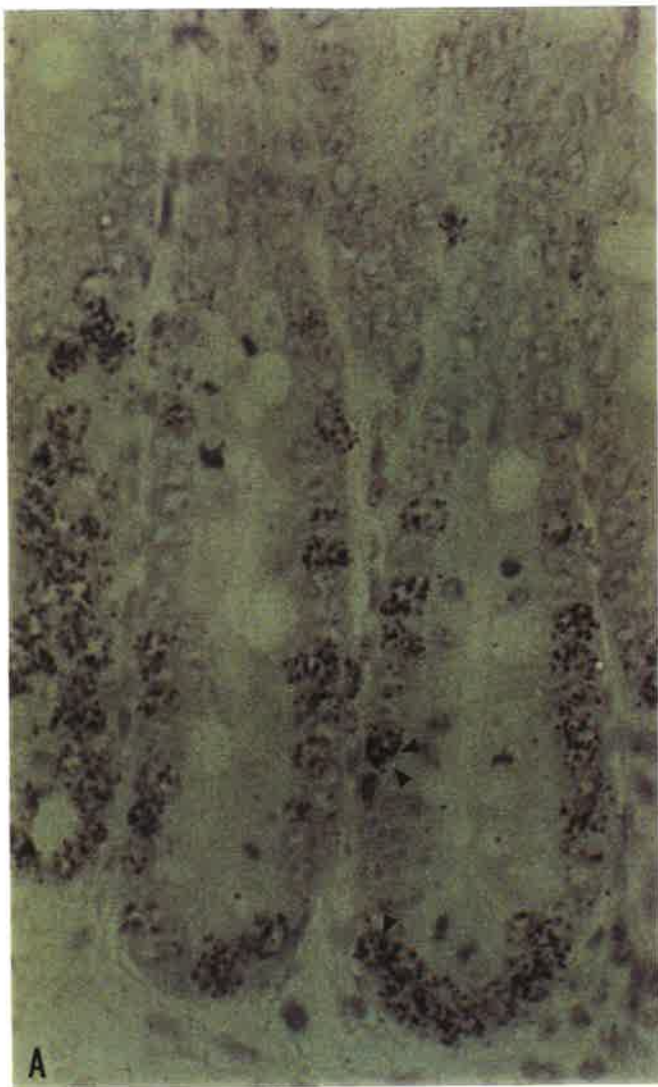


Plate 4.1: Tritiated thymidine labelled duodenal crypt epithelium in normal adult rats treated for 3 days with either vehicle (A), 278µg/day of IGF-I (B) or 278µg/day LR³IGF-I (C). Examples of thymidine incorporation into crypt enterocytes is indicated by arrows. Sections were exposed for 20 days and were photographed at a magnification of x 400 before picture enlargement. In rats treated with either 278µg/day of IGF-I (B) or the same dose of LR³IGF-I (C), thymidine incorporation was significantly increased.

4.4 DISCUSSION

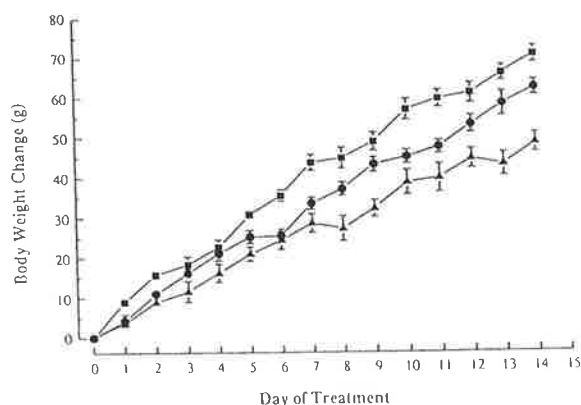
In the study described in Chapter 3, infusion of IGF-I and in particular LR³IGF-I significantly enhanced body weight gain and mucosal growth in normal female rats. Treatment with either peptide resulted in a crypt hyperplasia with proportional increments in the percentage of cells labelled with PCNA, indicating that after 14 days of treatment with IGF-I peptides a new balance between crypt cell production and cell loss had been established. The present study clearly demonstrated that administration of IGF-I or LR³IGF-I for 3 days to adult female rats elicits early proliferative events that lead to the massive increase in mucosal mass observed in the long-term infusion study. Moreover, this study has also shown that, although both peptides stimulate intestinal proliferation, proliferative events in LR³IGF-I treated rats are more advanced as compared to the IGF-I treated rats.

4.4.1 *Short- and long term effects of IGF-I peptides.*

Administration of LR³IGF-I for only 3 days significantly increased body weight in normal female rats, whereas the same dose of IGF-I had no apparent effect on body weight gain. These results are in accordance with the early body weight response observed in the 14 day treatment study. As shown in Figure 4.9 (adopted from Tomas et al., 1993), body weight gain during the first 3 days of IGF treated rats in the 14 day study paralleled the body weight gain of vehicle infused rats (Fig. 4.9). Divergence in the growth curve occurred after approximately 8 days of IGF-I treatment (Fig. 4.9). In contrast, following treatment of 278µg/day of LR³IGF-I for only 1 day, rats had gained an average of 8.6±1.2g as compared to 4.6±0.9g in the vehicle treated rats. These values are very similar to those observed in the 3 day study, where LR³IGF-I treated rats gained 8.2±1.8g on the first day following implantation of the peptide pump. The vehicle treated rats in the 3 day study gained on average 5.1±1.3g during the same 24 hour period.

In the rats treated for only 3 days with LR³IGF-I, the body weight gain was not accompanied by an increase in the food intake which averaged approximately 13g per day for all groups. Likewise, in the rats treated for 14 days with IGF-I and LR³IGF-I, body weight gain was independent of nutrient intake. Furthermore, the rats treated with 278µg/day of LR³IGF-I for 14 days also showed a markedly improved food conversion efficiency over the 14 day period (Chapter 3, Figure 3.3). Whilst average food intake and initial body weight gain were virtually identical for the rats in either study, the rats treated for 3 days with LR³IGF-I showed markedly improved food conversion efficiency, even to a greater extent than the average food conversion efficiency of the rats treated for 14 days. Thus, in order to compare the food conversion efficiencies of the rats from the 14 day study with that of the 3 day study, food conversion efficiencies were calculated for the first three days for both studies. As shown in Table 4.5, the average food conversion efficiencies in rats treated for either 14 or 3 days were similar during the first 3 days of treatment. This demonstrates that the overall lower food conversion efficiencies obtained for the rats treated for 14 days reflect their reduced growth rate during the second week of peptide treatment.

Figure 4.9: Body weight gain in normal female rats treated for 14 days with either vehicle or 278µg/day of IGF-I or LR³IGF-I.



All values are means±SEM for Vehicle treated animals ▲, 278µg/day of IGF-I ● and 278µg/day of LR³IGF-I ■ with 6 animals on each day. The graph has been adopted from Tomas et al., 1993.

Table 4.5: Food conversion efficiencies during the first 3 days of treatment with 278µg/day of LR³IGF-I for either 3 or 14 days in normal adult rats.

Treatment group	Food conversion efficiency	
	3 day study	14 day study
Vehicle	0.38±0.04	0.33±0.02
LR ³ IGF-I	0.64±0.03**	0.59±0.02a

Values represent means±SEM, with 6 rats per group. Food conversion efficiency has been calculated as the ratio of average daily body weight gain to food consumption for the same period for the first 3 days of treatment with 278µg/day of LR³IGF-I for either 3 or 14 days. Statistical significance from respective vehicle groups are indicated by *: P<0.01 (3 day study) and a: P<0.01 (14 day study).

The possibility that the marked body weight gain in rats treated with LR³IGF-I may be attributed to additional factors such as fluid retention were explored because recent research suggests that IGF-I mediates fluid retention, in particular in human subjects with growth hormone deficiency undergoing GH (rhGH) replacement therapy. These patients may experience transient fluid retention (Ranke and Wilton, 1994 and Rosen et al., 1994), which may be mediated by the antinatriuretic effects of GH, whereby GH is acting on the sodium pump (Herlitz et al., 1992), or alternatively, effects may be mediated indirectly via IGF-I.

Although the fluid balance was crudely assessed in the present study, the marked increase in body weight gain in the rats treated with 278µg/day of LR³ IGF-I for 3 days could not be explained by an increase in fluid retention. Furthermore, there was no correlation between the accumulative body weight gain for the 3 days and the fluid balance for the same period. Similarly, the analyses on the carcass content of the rats treated for 14 days with 278µg/day of LR³IGF-I showed no change in the water content as compared to vehicle treated rats (Tomas et al., 1993).

In the animals treated with LR³IGF-I, a marked increase in body weight was reflected in a substantial increase in the total gut weight. Moreover, comparing the total gut weight (wet tissue weight) of the rats treated for 3 days with 278µg/day of LR³IGF-I with the total gut weight of rats treated for 14 days with the same peptide dose indicates that 44% of the total weight gain occurred during the 3 day treatment period. This affirms that selective growth of the gut was very rapidly induced by administration of LR³IGF-I.

4.4.2 Differences in proliferative effects between IGF-I and LR³IGF-I

Although the wet tissue weight of gastrointestinal components was increased only in the LR³IGF-I treated rats, activation of proliferative responses was also observed in IGF-I treated animals at the end of the 3 day treatment period. Rapid stimulation of intestinal epithelial cell proliferation following administration of IGF-I has been demonstrated in a number of *in vitro* studies. For example, IGF-I is a potent mitogen for human foetal small intestinal cells (Duncan et al., 1990). Administration of IGF-I to IEC-6 cells, a cell line derived from rat jejunal crypts, stimulates DNA and protein synthesis and in RIE-1 cells, an epithelial cell line derived from rat small intestine, IGF-I, also stimulates cellular proliferation (Corps et al., 1987 and Park et al., 1989). The tritiated thymidine uptake by canine fundic epithelial cells is also stimulated by IGF-I administration and although EGF and insulin also elicit a mitogenic

response in these cells, much higher concentration of these factors are however needed to achieve an equivalent effect (Chen et al., 1989). In the present study, administration of 278µg/day of IGF-I or LR³IGF-I for a 3 day period significantly stimulated the incorporation of tritiated thymidine into crypt enterocytes *in vivo*, indicating that IGF-I peptides are important mitogens for the intestinal epithelium in adult rats.

Although both peptides increased the incorporation of tritiated thymidine in intestinal enterocytes in the duodenum and in the ileum, a concomitant increase in the size of the proliferative compartment was only observed in the rats treated with the analogue. This aspect was initially confusing, because in the 14 day study, administration of either peptide resulted in an increase of the crypt compartment accompanied by proportional increments of potentially proliferative cells. The proliferative labelling profiles were therefore examined in detail to determine the difference in proliferative response of the two peptides.

From the thymidine labelling distribution profiles in the control groups, it was evident that maximal thymidine incorporation was greatest in the mid-crypt region (cell position 5-16), which represents the zone of greatest cell production. As shown by Wright (1980), this is the "proliferative compartment proper" and the labelling index obtained in this region in the present study compares well to the theoretical labelling index of 60%. Following treatment with IGF-I, an increase in the number of proliferative cells in lower and mid crypt cell positions indicated that more cells in this region of the crypt had entered the cell cycle, leading to the observed increase in the thymidine labelling index in this group. In addition, the maximum labelling also increased, which demonstrates that more cells in the proliferative compartment proper were cycling. The most likely mechanism by which such a rapid increase in proliferative activity may have been achieved is a reduction in the cell cycle in enterocytes in basal cell positions. Cell cycle times in basal cell positions, as measured by FLC (fraction

labelled mitosis), stathmokinetic or continuous labelling methods, are prolonged compared to cell cycle times of enterocytes in the upper-crypt region (Al-Dewachi et al., 1974) so that a fractional decrease in the cell cycle time in basal positions would greatly increase the proliferative pool. At present it is not clear whether controlling and stimulating agents act directly upon stem cells or on dividing progeny, however observation from gut resection studies and from hydroxyurea induced crypt cell death (Al-Dewachi et al., 1977) suggest that stem cells are an important target to increase the crypt cell production rate. Alternatively, stimulation of transient (mature) cells in the gut can not be excluded.

The most interesting finding of this study was the fact that although both IGF peptides significantly increased the thymidine incorporation into the crypt enterocytes, increased proliferative activity associated with an increase in the size of the crypt compartment was only observed in the LR³IGF-I treated animals. Although I can not exclude the possibility that the mechanism by which the two peptides induce proliferative events differs, it is highly likely that the proliferative responses observed in animals treated with LR³IGF-I represent a more “advanced” stage of intestinal proliferation as compared to the IGF-I treated rats. This is supported by the fact that in rats treated for 14 days with either IGF-I or LR³IGF-I a marked increase in size of the crypt compartment was observed, in both groups. This suggests that although both peptides initiate increased thymidine incorporation after 3 days, in LR³IGF-I treated animals this has already been translated into an increase in crypt size, which in IGF-I treated animals occurs some time later. Thus, the proliferative effect observed in the rats infused with IGF-I should be detectable after administration of LR³IGF-I for only 1 or 2 days.

Another point of interest was the finding that administration of LR³IGF-I resulted in a greater proportion of cycling cells in upper-crypt cell positions, indicating that migrating enterocytes had retained their proliferative capacities had not entered the maturation

compartment of the crypt. This may have been achieved by alteration in enterocyte transit time.

4.4.3 Mechanisms of IGF-I peptide induced proliferation

The accelerated proliferative effects of LR³IGF-I are most likely the result of several interacting factors. LR³IGF-I has approximately 1000 fold lower affinity towards IGFBP-3, IGFBP-4, total rat plasma and L6 myoblast binding proteins (Ballard et al., 1993). However, despite the reduced affinity to binding proteins, LR³IGF-I has shown a substantially greater bioactivity than IGF-I in several functional assays associated with growth in L6 myoblasts and H35 hepatoma cells (Francis et al., 1992). This increased potency is observed despite the fact that the analogue binds with approximately 4-fold lower affinity to the type 1 receptor (Francis et al., 1992). It is possible that infusion of 278µg/day of LR³IGF-I may have increased the free IGF pool in plasma to a greater extent than infusion of the IGF-I. This would lead to a greater level of IGF molecules free to interact with their respective receptors stimulating intestinal mitogenesis through signal transduction pathways, thus in LR³IGF-I treated animals proliferative events may have induced more rapidly than in IGF-I treated rats.

Another contributing factor is the fact that LR³IGF-I is also cleared more rapidly from the circulation (Bastian et al., 1993). For example, the metabolic clearance rate for LR³IGF-I is approximately 11 fold higher than for IGF-I in adult female Sprague Dawley rats (Bastian et al., 1993). Furthermore, location of radiolabelled IGF-I or LR³IGF-I in visceral organs and tissues differs between the two peptides (Bastian et al., 1993). In addition to differences in clearance and tissue distribution between the two peptides, the increased potency observed with LR³IGF-I may also result from reduced interaction of the analogue with locally produced IGFBPs. Most likely, a combination of complex interactions between the IGF-I ligand,

endogenous IGFBPs present in the serum and tissues and the availability to the IGF receptor determine the mitogenic response.

4.4.4 Regional effects

The estimated crypt cell population in the ileum was lower compared to the duodenum. This was mainly a reflection of a decrease in the number of cells per crypt column rather than a reduction in the number of cells around the crypt circumference. The progressive decrease in crypt cell population with distance down the intestine (proximo-caudal gradient) is consistent with observations by other researchers. For example, in the mouse, crypt cell populations in the duodenum have been estimated at approximately 500 cells/crypt, falling to approximately 360 cells/crypt in the ileum (Wright and Alston, 1984b). The proximo-caudal gradient was not altered by the peptide treatment so that crypt cell populations were still greater in the duodenum than in the ileum. However, following treatment with 278 μ g/day of LR³IGF-I, the proximo-caudal gradient, which in vehicle treated animals accounts to approximately 13%, increased to 26%, thus indicating the relatively greater responsiveness of the proximal small intestine to the IGF-I analogue.

The number of thymidine labelled cells was also lower in the ileum compared to the duodenum, but in view of the proximo-caudal fall in the crypt cell population, the labelling index in the two intestinal regions remained equitably constant. The overall tritiated thymidine labelling distribution profiles in the ileum were similar to the profiles obtained for the duodenum. Yet in the ileum, lower thymidine labelling indices were observed in the lower crypt cell positions compared to the duodenum. To my knowledge, no systematic study of variations in proliferative indices with cell position in different anatomical sites have been reported but Cheng and Leblond (1974) proposed that "no great deal of variation from the pattern observed in the proximal small intestine should be expected". Thus to eliminate the

fact that the variations observed in the present study are a reflection of the rather small sample size (30 crypt measurements) or the fact that the labelling distribution profiles were obtained from measurements in sectioned material rather than from microdissected crypts, a more detailed systematic study needs to be conducted.

In the present study, the proximal large intestine appeared also responsive to IGF-I peptide treatment. The crypt depth in the colon was increased by approximately 15% and 4% in LR³IGF-I treated rats when compared to the vehicle and control treated animals respectively. Although administration of LR³IGF-I increased the wet tissue weight of the colon to a similar extent to that of the small intestine, an increase in thymidine labelling was not observed. The proliferative effect of IGF-I peptides may have been somewhat disguised by the inherent low proliferative activity of the colon. In the colon variation in crypt morphology, in particular in the proximal colon, lead to a great deal of heterogeneity in morphometric and cell kinetic parameters. For example, in the rat, colonic crypts become longer and more slender with distance away from the ileo-caecal junction. Furthermore, mucosal rugae are prominent in the proximal colon but not in the distal colon and crypts at the apex of the mucosal folds are longer than the crypts at the base of the folds (reviewed by Wright and Alison, 1984a and 1984b). In the present study, the thymidine labelling index in the proximal colon was calculated at 11-13%, which compares well with the thymidine labelling index of 11% in normal adult rats, obtained by Sunter et al. 1979a and 1979b. To obtain more conclusive data on the proliferative response of IGF-I peptides on the colonic mucosa, direct measurements of cell cycle time and/or the crypt cell production rates need to be taken from a well defined region.

4.4.5 *Other comparative studies*

In agreement with the findings of the present study, Olanrewaju et al. (1992) have shown that administration of IGF-I to IEC-6 cells effectively induced the growth related enzyme ornithine decarboxylase (ODC). In addition, this group also reports that intraileal administration of 200µl of 10nM of IGF-I for a 3-day period into adult male Sprague-Dawley rats significantly increased ODC activities, mucosal mass and mucosal cellularity (as measured by DNA, RNA and protein content of tissue homogenates). In contrast, in the present study mucosal mass was increased in LR³IGF-I treated animals but not in rats treated with the native IGF-I. Several possibilities may account for the difference in results. Firstly, it is feasible that the increased mucosal mass observed in Olanrewaju's study relates to the fact that their rats were fasted for 66 hours following surgical implantation of the intraileal catheter (for delivery of IGF-I). It is well established that in the rat, a progressive mucosal atrophy occurs after a period of fasting (Altmann, 1972 and Goodlad and Wright, 1984). Most prominently, the villus population is reduced by up to 20-30% and a massive reduction in the crypt cell population also contributes significantly to the hypoproliferative status of the mucosal epithelium following starvation of up to 72 hours (Clarke, 1972, Al-Dewachi et al., 1975 and Hopper et al., 1968). The hypoproliferative status is thus marked by a reduction in the DNA and protein content which relates to the diminished crypt and villus population. Within the crypt, the rate at which new crypt cells are produced is therefore greatly diminished and results from a marked decline in cell cycle time and crypt growth fraction (Wright and Alison, 1984b) As a consequence, the intestinal mucosa is more sensitive to stimulation by a mitogen, such as IGF-I, because of the low basal proliferative activity of the intestinal mucosa in this hypoproliferative status.

Second, it is likely that gut effects in response to intraluminal delivery of IGF-I effects differ from those seen after systemic administration of IGF-I. For IGF-I to exert trophic action

on the intestinal mucosa via paracrine or autocrine pathways requires the survival of IGF-I in the gut lumen. Although Olanrewaju et al. (1992) claim that “the gut lumen is free of IGF binding proteins” and thus “IGF-I found in the gut lumen may be biologically active”, recent research from our own laboratory suggests that in adult rats IGF-I is rapidly degraded in the upper gastrointestinal tract (Xian et al., 1994). For example, the half life of IGF-I in the upper gastrointestinal tract (duodenum and ileum) as assessed by trichloroacetic acid, immuno- and receptor precipitation studies was calculated at approximately 2 min. It therefore seems unlikely that intraluminally infused IGF-I remains biologically active in the upper gastrointestinal tract. Finally, the marked gut responses observed by Olanrewaju et al. (1992) are even more surprising given that the dose of IGF-I used in their study was approximately 720pg/day of IGF-I, which represents approximately 1/150,000th of the IGF-I dose used in the present study.

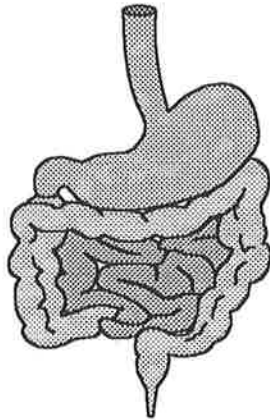
4.4.6 Where to go from here?

In summary, the present study has shown that administration of 278µg/day of IGF-I or LR³IGF-I to normal adult rats for a 3 day period initiate proliferation events that leads to the massive increase in mucosal mass observed in rats treated with the same dose of IGF-I peptides for 14 days. The initial proliferative responses of the epithelium in the duodenum and ileum include an increase in mitotic activity, which in IGF-I treated rats, was achieved by increasing the proliferative pool of enterocytes in the lower portion of the crypt. In LR³IGF-I treated rats, crypt cells in the upper portion of the proliferative crypt were recruited. It appeared that proliferative events in LR³IGF-I treated rats were more advanced because the increased proliferative activity was associated with a crypt hyperplasia, comparable to responses in rats treated for 14 days with IGF-I peptides. This indicates that IGF-I peptides are important mitogens that rapidly stimulate proliferation of the intestinal epithelium in normal growing rats. To investigate if IGF-I peptides also play a significant role in mediating cell

proliferation and more importantly cell differentiation in the immature gut will be addressed in the next two Chapters. Firstly, the effects of IGF-I peptides on the growth of the gastrointestinal tract will be studied in 6 day old neonatal rats to investigate if IGF-I peptides stimulate gut growth at a time at which organ growth occurs at a maximum rate. Second, it is not clear if IGF-I peptides influence the maturation of intestinal function, which is essential for the successful transition from milk to a solid diet at the time of weaning. Thus, the effects of IGF-I peptide administration on the reciprocal ontogeny of the disaccharidase system from Lactase-Phlorizin Hydrolase (LPH) to the alpha - glucosidase (sucrase - isomaltase, maltase and trehalase), that occurs at the time of weaning (day 19-21 *post partum*) will be addressed in 12 day old suckling rats. These studies are described in Chapters 5 and 6.

CHAPTER 5:

IGF-I PEPTIDES STIMULATE GASTROINTESTINAL GROWTH AND INTESTINAL PROLIFERATION IN SUCKLING RATS DURING THE EARLY POSTNATAL PERIOD.



CHAPTER 5

IGF-I peptides stimulate gastrointestinal growth and intestinal proliferation in suckling rats during the early postnatal period.

Contribution to the work

This Chapter describes a series of experiments that were designed to characterise the effects of IGF-I peptide administration to 6-day old neonatal rats for a 6.5 day period on the growth and function of the gastrointestinal tract. This Chapter also includes the results from preliminary studies which were conducted to establish the feasibility of implantation of mini-osmotic pumps for subcutaneous delivery of IGF-I peptides in 6 day-old neonatal rats, as well as preliminary studies to identify the optimal dose range of IGF-I peptide in neonatal rats. The planning and execution of all experiments described in the present Chapter, including surgical manipulation and handling of animals, collection and preparation of tissues for histological, biochemical and histochemical analyses, were carried out by myself. All analytical work including computer assisted image analysis, collation and interpretation of data was also conducted by myself. Assistance was provided by Mrs. Kerry Penning and Mrs. Leanne Srpek during animals handling and tissue collection. Mrs. Anna-Maria Mercorella assisted in the cutting of histological material, and Mrs. Carolyn Mardell with the histochemical work. Mrs Cheryl Shoubridge provided valuable assistance with the biochemical assays and plasma IGF-I radioimmunoassays. Mr. Michael Conlon helped in the collection and weighing of non-gut organs. Plasma insulin measurements were conducted by Dr. John Oliver, Flinders Medical Center, South Australia. Iodination of IGF-I was carried out by Mr. Spencer Knowles.

ABSTRACT

To investigate the effects of IGF-I peptide administration on the gastrointestinal growth and function in neonatal rats, 6-day old neonatal rats (6-8 rat pups /group) were treated for 6.5 days with IGF-I or LR³IGF-I delivered via s.c. implanted mini-osmotic pumps. Before commencement of the study, optimal peptide doses were established. Based on these results, IGF-I was infused at doses of 2, 5 and 12.5µg/g body weight per day. LR³IGF-I was administered at 2 or 5µg/g/day. IGF-I plasma concentrations were significantly increased following infusion of 5 and 12.5µg/g/day of IGF-I. No apparent change in the IGFBP profile was observed in rats receiving recombinant IGF-I peptides. Body weight gain was significantly increased in rat pups treated with 5µg/g/day of LR³IGF-I. While body weight gain was not altered in IGF-I treated rats, the weight of several non-gut organs, most noticeably the kidneys and spleen, were increased following administration of either IGF-I and LR³IGF-I. These effects were still apparent after correction for body weight gains. Selective action of IGF-I peptides on gastrointestinal tissues was evident with increases in total gut weight of up to 60% above control values in the highest dose of LR³IGF-I treated rat pups. Small intestinal but not large intestinal length was also increased by IGF-I peptides. Histologically, villus height, crypt depth and the thickness of the muscularis externa were increased following IGF-I peptide infusion. Responses were more pronounced in the duodenum compared to the ileum. Within the crypt epithelium, proliferative activity was increased in proportion to crypt size in IGF-I and LR³IGF-I treated rat pups. Duodenal lactase activity was also enhanced in proportion to duodenal mass, as indicated by biochemical and histochemical analyses. Conversely, sucrase activity was not precociously induced by IGF-peptides. These studies suggest that IGF-I peptides influence gastrointestinal growth and maturation of intestinal epithelium during early postnatal development, without affecting the normal developmental pattern of disaccharidase activities.

5.1 INTRODUCTION

The studies described in Chapters 3 and 4 have clearly shown that continuous subcutaneous infusion of IGF-I and in particular LR³IGF-I influences gastrointestinal growth and function in normal adult rats. The present Chapter describes a series of *in vivo* experiments that were carried out firstly, to investigate if growth responses to IGF-I administration in the immature gut are comparable to those observed for the adult gastrointestinal tract. Second, these experiments also investigated the role of IGF-I on the maturation of intestinal disaccharidases in suckling rats. Finally, they determined if the IGF-I analog (LR³IGF-I) was also more potent than the native IGF-I in stimulating growth and maturation of the immature intestine given that the majority of the IGF-BPs in neonatal serum are low molecular weight binding proteins, mainly IGF-BP-2 and -1 (Donovan et al., 1989).

At the commencement of my PhD studies, information on the effects of IGF-I administration on somatic growth during the postnatal developmental period were sparse. Of the few papers published prior to 1991, only 1 paper reported specific gut responses. While most researchers adopted a protocol whereby IGF-I peptides were administered by daily intraperitoneal injection, continuous infusion of IGF-I by s.c. implanted mini-osmotic pumps was the choice of peptide delivery in a series of studies in hypophysectomised rats. These studies are briefly summarised.

Philipps et al. (1988) demonstrated that twice daily injection of 0.4µg/g body weight of IGF-I to 3 day old rat pups for a period of 12 days significantly stimulated body weight gain, tail length and selective organ growth. In addition, significant increases in bone marrow erythropoietic precursor cells were apparent. In the same study, IGF-I stimulation of erythropoietic precursor cells was maintained, although the somatic growth effect of IGF-I was diminished in rat pups exposed to postnatal nutrient deprivation (Philipps et al., 1988). The

only study reporting gut effects in normal suckling rats following administration of IGF-I was conducted by Young et al. (1990). In their study, once daily injection or oral instillation of 1µg/day of IGF-I to 10 day old rat pups for a period of 6 days resulted in the stimulation of jejunal brush border enzymes. However, no increase in body weight gain was reported.

Infusion of IGF-I and IGF-II by mini-osmotic pumps has been reported in hypophysectomised rats (Glasscock et al., 1992). Continuous infusion of 1.9µg/g/day to 10 day old hypophysectomised rat pups for a period of 8 days restored serum IGF-I levels but failed to increase body weight gain. However, similar to the study by Philipps, et al. (1988), selective organ growth was stimulated by the infusion of IGF-I (Glasscock et al., 1992). In neonatal growth hormone deficient rats, twice daily injection of 3.3µg/g body weight for a period of 10 days failed to increase body weight gain, body composition and nose-tail length (Ambler et al., 1993). Furthermore, no weight changes were recorded for organs, including the heart and spleen (Ambler et al., 1993). Unlike the study by Glasscock et al. (1992), serum IGF-I levels in the neonatal growth hormone deficient rats were not elevated following administration of IGF-I, indicating that the route of peptide administration is important.

In a later study, Robinson et al.(1993), showed that immunoneutralisation of IGF-I or IGF-II had no effect on body weight gain in neonatal suckling rats. Their study showed that injection of antiserum raised against IGF-I or IGF-II to 2 day old suckling rats for 3 days did not alter the body weight growth curves. However, specific depletion of GH by passive immunoneutralisation significantly lowered plasma IGF-I levels and impeded the body weight gain in the neonatal rat pups, suggesting that a functional GH/IGF-I axis is important during postnatal growth in the rat. Their study failed, however, to examine the specific organ growth.

None of the above-mentioned studies investigated in detail the effects of IGF-I administration on gastrointestinal growth and maturation of the intestinal epithelium, or assessed the development of disaccharidase activities. Moreover, gut effects in response to administration of the far more potent IGF-I analog have not been studied. Accordingly, the present study investigated the effects of IGF-I peptide administration on the immature rat intestine. Two age groups were chosen. Firstly, the effects of IGF-I peptide administration on gut development have been studied during the early lactation period, starting IGF-I treatment at 6 days *post partum*. This time point was chosen because growth of gastrointestinal tissues occurs at an accelerating rate, hence the effects of IGF-I peptide administration on gut growth and proliferation were addressed. As shown by Woodall et al. (1991) and by researchers from our own laboratory (Lemmey et al., unpublished) optimum growth responses with IGF-I are achieved by either frequent subcutaneous injections, sufficient to maintain plasma IGF-I levels, or by continuous infusion of the peptides. For constancy with the adult rat studies described in Chapters 3 and 4, I chose continuous infusion as the method of IGF delivery. This precluded the use of animals younger than 6 days of age, in which surgical implantation of osmotic pumps would have been too stressful.

A second time point was chosen during the later lactation period. In the latter study, IGF-I infusion commenced at 12 days *post partum*, so that cessation of peptide treatment at 18.5 days coincided with the time of weaning. In the rat, a reciprocal shift in digestive enzyme expression from β -glucosidases (lactase-phlorizin hydrolase) to the α -glucosidases (a mixture of sucrase-isomaltase, maltase II and III and trehalase disaccharidases) occurs at the time of weaning and therefore the effects of IGF-I peptide infusion on the regulation of the developmental expression of disaccharidase activities were addressed. This study is described in Chapter 6.

5.2 PRELIMINARY STUDY TO ESTABLISH PROTOCOLS, ANIMAL HANDLING AND PEPTIDE DOSE RESPONSE

5.2.1 *Animal handling*

The single most important factor to obtain litters with a minimal spread in birth-weights was the care and maintenance of the pregnant dam. For this reason, I have provided a detailed account of the protocols required to obtain reproducible experimental data. The time after conception, when the pregnant rats were delivered from the CSIRO breeding colony to the animal holding facilities at the Women's and Children's Hospital, was crucial. Rats delivered from the colony less than 7 days after conception often resorbed their young. For instance on two occasions, only 1 out of the 4 pregnant rats delivered actually gave birth. On the other hand, delivery of pregnant rats at an advanced stage of pregnancy disturbed the pregnant dams so that their subsequent nurturing behaviour was influenced. In these rats, still birth and aggressive behaviour towards their young was commonly observed, leading to unacceptably large spreads in birth weight and body weight gain during the first two weeks *post partum*. I therefore decided to have the pregnant rats delivered 10 days after confirmed conception thereby allowing the rats to settle in their new environment. The pregnant rats were not handled during the first 3 days after delivery.

To further reduce any stress that could interfere with the experimental design, a protocol was devised for the pre- and post-natal care of the dam and the pups. This included delivery of no more than 4 pregnant rats at any one time to the animal holding facilities at the Women's and Children's Hospital and housing of rats with minimal disturbances by other users of the holding facilities. The pregnant rats were handled with utmost care and all handling was restricted to one operator. A daily routine was strictly maintained in which pregnant dams were handled for 10 minutes prior to weighing of the animal at the same time each morning. The pregnant rats were encouraged to explore and smell the hands of the operator. This

seemed to reduce nervous behaviour of the dams and made subsequent handling of their young easier. Approximately 2-4 days before the expected date of birth, the handling routine was shortened to 2 minutes. All rats were monitored closely so that the time of birth was accurate to within 1-2 hours, except for two rats which gave birth during the early morning hours. Birth-dates for these rats are accurate to within 12 hours. The day of birth was designated "day 0".

5.2.2 *Postnatal care of the dam and rat-pups*

To assure adequate feeding of the pups, all litters were reduced to 9 pups/dam on the second postnatal day. Litters of less than 9 pups were supplemented with a cross-fostered pup from another litter. Successful cross-fostering was achieved by rubbing the fostered pup against the new litter mates and rolling the young in the nesting material of the new cage, thus masking the smell of their natural mother. All cross-fostered pups were successfully adopted by their "new mothers". Starting on day 4 *post partum*, all pups were marked for identification. Body weight gain data was collected daily at exactly 24 hour intervals for both the rat pups and the dams for the period before pump implantation (**pre-treatment period**) and during the peptide infusion period (**treatment period**).

5.3 PRELIMINARY DOSE RESPONSE OF IGF PEPTIDES

Before commencing the systemic infusion studies in the neonatal rats, preliminary studies were conducted to establish firstly, if implantation of mini-osmotic pumps was feasible in 6 day-old neonatal rats and if so, whether implantation of the pumps affected the behaviour of either the rat pups and/or the dams. Secondly, based on the adult infusion studies, I tested a range of peptide doses to determine IGF-I peptide doses that would result in gastrointestinal stimulation.

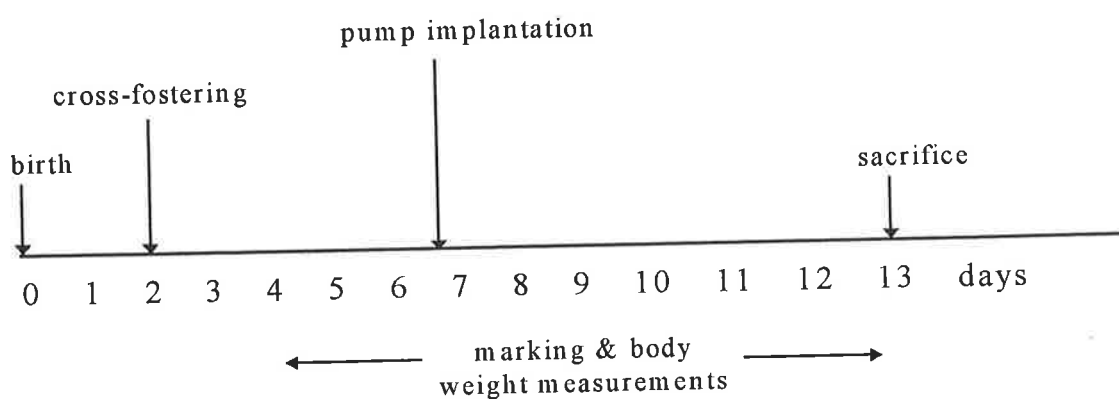
5.3.1 *Anaesthesia and pump implantation in 6 day old rats*

Two Hooded Wistar virgin rats were mated with a male rat and pregnancy was confirmed by vaginal smear. Pregnant rats were handled as described above. These rats gave birth on the same day approximately 2 hours apart to 8 and 10 pups, respectively. On day 2 *post partum*, one rat pup was cross-fostered (outlined in section 5.2.2), resulting in 9 pups per dam. On day 5 *post partum*, both litters were removed from their mothers and placed on a heated blanket. Optimal concentration and flow rate of the Fluothane inhalation anaesthetic (Halothane Ph. Eur., ICI Pharmaceutical, Macclesfield, Cheshire, England) for implantation of the mini-osmotic pumps was determined in 10 rat pups (5 from each litter). Exposure of the suckling rat pups to increasing doses of Fluothane (1.5% to 5%) identified that inhalation of 3-3.5% Fluothane at a flow rate of 1.25 l/min resulted in very few deaths under anaesthesia (~2.5%). At the same time, all anaesthetised rat pups exhibited deep muscular relaxation and quick recovery from anaesthesia after approximately 2 minutes. All rat pups were returned to their mothers after having gained full consciousness. The dams showed a great interest in the pups, sniffing and licking their pups extensively. I therefore decided to mask the "surgery smell" by rolling the pups in nesting material prior to the return to their mothers, which greatly reduced the mother's interest in the pups. For all subsequent pump implantation, Fluothane was administered at 3%, increasing to 3.5% if required, at a flow rate of 1.25l/min.

On day 6 *post partum*, seven rats, 4 from litter 1 and 3 from litter 2, were selected for implantation of a mini-osmotic pump. Using aseptic techniques, a 1cm incision was made with iris scissors within the scapular region of the pups receiving a pump. A mini-osmotic Alzet pump (Model 1007D, (0.5 μ l/hour), Alza, Palo, CA), filled with 0.1M acetic acid, was then inserted s.c. into each rat pup in a manner similar to that described for the adult rats (Chapter 3, section 3.2.3). To reduce the risk of infections, the site of incision was washed with 0.2ml of a solution containing 0.32mg trimethoprim with 1.6mg sulphamethaxazole in saline (David

Bull Laboratories, Melbourne, Australia), before being closed using a monofilamentous suture (Dermalon, CE-2, size 6.0, Needle size 12 mm, Davis and Geck, Cyanamid, Montreal, Canada). The surgical procedure was completed in less than 5 minutes for each pup. Six rats (three from each litter) were anaesthetised for the same period and the remaining 3 untreated rats that received neither anaesthesia nor a pump, were also included as untreated controls. To reduce disturbance of the mother and to standardise the period over which suckling was interrupted, all rat pups were initially returned to their litter mates. After regaining full consciousness, the entire litter was returned to the respective mothers. All rats were monitored closely at 30 minute intervals for the remainder of the day after pump implantation. Thereafter, rats were monitored in the morning and evening for the remaining treatment period. Starting on the first post-operative day, the rat pups and the dams were weighed at exactly 24 hour intervals as described for the adult rats. After a 7 day treatment period, rats were sacrificed to establish the tissue collection protocol for the peptide infusion study. The experimental protocol for the preliminary study is illustrated in Figure 5.1.

Figure 5.1: Experimental protocol for the establishment of anaesthesia and pump implantation in 6 day old neonatal rats



5.3.2 Body weight response

The body weights of rat pups with and without surgically implanted pumps were compared at birth; the day of pump implantation and at sacrifice and are shown in Table 5.2. The average body weight of the rat pups on the second postnatal day from the two litters were 7.2 ± 0.1 g and $7.1 \text{g} \pm 0.2$ g, (mean \pm SEM) respectively, with no statistical difference between the two litters ($P > 0.05$, Student's t-test). All pups appeared well throughout the treatment period and gained weight at a rate of approximately 1.5-3g per day. On day 6 and day 13 *post partum*, the rat pups weighed approximately 15 and 30g, respectively, with no statistically significant difference between the control animals and the rats with or without a surgically implanted mini-osmotic pump (Table 5.2). I concluded that subcutaneous implantation of mini-osmotic pumps to 6 day old rat pups did not adversely affect the well being of the rat pups. Moreover, dams and pups did not appear stressed by the implantation of pumps.

Table 5.1: Comparison of body weights of rats with and without a surgically implanted mini-osmotic pump.

<i>Rat pups</i>	Body weight		
	Initial weight (day 2 <i>post partum</i>)	Surgery/anaesthesia (6 days <i>post partum</i>)	Final (13 days <i>post partum</i>)
pumps (N=7)	7.36 ± 0.11	15.61 ± 0.48	29.43 ± 1.07
no pumps (N=6)	6.96 ± 0.27	14.96 ± 0.74	29.75 ± 1.35
untreated controls (N=3)	7.28 ± 0.91	15.80 ± 1.20	30.10 ± 1.70

Values are means \pm SEM. No statistical difference in body weight at any time point was detected between rats with and without surgically implanted pumps (ANOVA, $P > 0.05$). The weight of the pumps has been subtracted from the body weight.

5.3.3 Peptide dosage in suckling rat studies

Satisfied that the pump implant did not adversely affect the well being of the pups as well as the behaviour of the dams, a preliminary experiment was conducted to determine the optimal peptide dose for suckling rats. Based on the studies described earlier (section 5.1),

and on concurrent studies in adult rats by collaborators within the Cooperative Research Center, growth responses of the gastrointestinal tract have been shown for doses ranging from 0.04 $\mu\text{g/g/day}$ of IGF-I (Young et al., 1990) up to 2.0 $\mu\text{g/g/day}$ (Lemmey et al., 1991). In the studies described in Chapters 3 and 4, the adult rats were infused with IGF-I peptides at doses ranging from 44 $\mu\text{g/day}$ to 278 $\mu\text{g/day}$. On a per g body weight basis, these rats received on average 0.4, 1.0 and 2.5 $\mu\text{g/g/day}$ of IGF-I or LR³IGF-I. Accordingly, 2 $\mu\text{g/g/day}$ was chosen as the lowest dose for this study. In the remaining two experimental Chapters, peptide doses are reported as $\mu\text{g/g/day}$ so that comparisons are possible.

To determine gut responsive IGF-I doses suitable for continuous infusion, 2 female Hooded Wistar rats were mated to obtain a total of 18 rat pups. Between 1600 and 1700 hours on day 6 *post partum*, the rats were fitted with a mini-osmotic pump (Alza 1007D, 0.47 $\mu\text{l/hour}$, Alza, Palo, CA) containing either vehicle (0.1M acetic acid), IGF-I or LR³IGF-I at doses of 2, 5 or 12.5 $\mu\text{g/g}$ body weight per day. One rat from each litter was assigned to either treatment with vehicle or IGF-I peptides and in addition, 2 rats from each litter were included as untreated controls. Administration of anaesthesia, surgical implantation of the mini-osmotic pump, pre- and postnatal care of the rat pups and dams followed the protocol described in section 5.3.1. The un-primed pumps were implanted so that, according to the manufacturer's instruction, the full pumping rate was reached 4 hours after pump implantation. Peptides were infused for a 6.5 day period. In the early morning hours on the 13th postnatal day, all rats were removed from their mothers and sacrificed so that growth responses of the gastrointestinal tissues and non-gut organs could be assessed.

5.3.4 IGF-I peptide effects on body weight

Throughout the 6.5 day treatment period, all animals treated with 2, 5 and 12.5µg/g body weight per day of IGF-I and 2 and 5µg/g body weight per day of LR³IGF-I were healthy and well. However, the two rats treated with 12.5µg/g/day of LR³IGF-I died on the first and second day following pump implantation showing severe symptoms of hypoglycaemia that could be reversed by a bolus injection of a solution containing 5% glucose.

As shown in Table 5.3, infusion of 5µg/g/day of the IGF-I analog but not IGF-I appeared to stimulate body weight gain. As observed in the preliminary pump implantation study, body weights of untreated control animals and vehicle treated rats were remarkably similar. It seemed therefore redundant to include untreated control animals in future growth studies.

Table 5.2: Body weights in 6 day-old suckling rats, treated for 6.5 days with vehicle or IGF-I peptides.

Treatment groups	Body weights		
	Birth (g)	Surgery (g)	Final (g)
Vehicle (N=2)	6.9-7.1	14.4-14.6	23.7-24.1
Control (N=4)	6.8-7.2	13.9-16.0	22.5-24.7
IGF-I 2µg/g/day	5.7-5.9	14.8-15.2	24.1-24.7
5µg/g/day	6.3-7.0	14.1-14.6	25.1-26.3
12.5µg/g/day	6.6-7.2	14.5-15.2	23.7-24.0
LR ³ IGF-I 2µg/g/day	7.9-7.0	14.0-14.7	28.1-28.4
5µg/g/day	6.3-7.0	14.0-14.3	28.9-29.1
12.5µg/g/day	6.5-6.9	13.9-14.2	N/A

All values are expressed as the range of body weights from 2 rats per group, except for control (N=4). N/A= not available, the two rat pups treated with 12.5µg/g/day of LR³-IGF-I died on the first and second day following pump implantation.

5.3.5 IGF-I peptide effects on gastrointestinal tissue weight.

To determine the effect of IGF peptides on the growth of the gastrointestinal tract, the gut was removed and weight and length measurements of each tissue component were recorded following the protocol described for the adult rats (Chapter 4, section 4.3.). Additionally, non gut organs including the liver, lungs, heart, brain, kidneys, thymus and the spleen were rapidly excised, rinsed in physiological saline, dried and weighed.

Statistical comparison between the treatment groups was not conducted because of the small sample size. Nevertheless, treatment with 5µg/g/day of LR³IGF-I appeared to increase gastrointestinal wet tissue weights. For example, total gut weight and small intestinal weight were increased by 51% and 53% above values obtained for vehicle treated animals (Table 5.4). Similarly, small intestinal length in this group increased by 18% compared to length measurements of vehicle treated controls. Although the wet tissue weight increases were less striking in the IGF-I treated rats, marginal increases in gastrointestinal tissue components were also observed in IGF-I treated rat pups (Table 5.4). However, unlike the rat pups treated with LR³IGF-I, the dose response to IGF-I was very flat with similar increases in wet tissue weight obtained for all 3 doses.

5.3.6 IGF-I peptide effects on organ weights

Non-gut organ weights were recorded as either absolute organ weight (g) or as relative organ weight (organ weight (g) per kg body weight) and are shown in Table 5.5. Infusion of IGF-I and in particular LR³IGF-I to suckling rats selectively increased the wet tissue weights of the kidneys, thymus and spleen. Liver weights decreased following administration of 5µg/g/day of LR³IGF-I. In agreement with these results, increased wet tissue weight for the kidneys, spleen, thymus, liver, brain, adrenals and the heart following administration of IGF-I peptides have been previously reported (Philipps et al., 1988, Hizuka et al., 1986, Guler et al., 1988, Glasscock et al., 1992 and Asakawa et al., 1992).

Table 5.3: Gastrointestinal weight and length measurements in suckling rats following 6.5 days of IGF-I peptide treatment.

Treatment groups	Gut weights				Gut length	
	Total (mg)	Small intestine(mg)	Large intestine(mg)	Stomach (mg)	Small intestine (mm)	Large intestine(mm)
Vehicle	1008-1195	761-911	146-120	128-137	320-352	51-53
IGF-I 2µg/g/day	1160-1203	873-910	146-153	140-141	323-336	49-51
5µg/g/day	1105-1261	941-816	145-150	144-175	324-331	55-56
12.5µg/g/day	1169-1222	865-899	149-160	155-163	340	51-52
LR ³ IGF-I 2µg/g/day	1098-1163	827-870	143-145	126-150	339-340	46-52
5µg/g/day	1594-1734	1220-1334	199-204	179-196	390-404	52-61

The table shows the range of gastrointestinal tissue weight (mg) and length (mm) for 2 rat pups per treatment group.

Table 5.4: Organ weights in 6 day old suckling rats following treatment with either vehicle or IGF-I peptides for 6.5 days.

A) Absolute organ weights (mg)

Treatment groups	<i>Liver</i>	<i>Lungs</i>	<i>Heart</i>	<i>Brain</i>	<i>Kidneys</i>	<i>Thymus</i>	<i>Spleen</i>
Vehicle	815-892	390-494	143-173	1049-1079	300-391	67-69	138-182
IGF-I 2µg/g/day	863-921	388-416	175-188	1069-1076	398-422	62-68	164-172
5µg/g/day	915-962	449-450	170-178	1100-1143	340-459	92-110	154-161
12.5µg/g/day	895-912	455-496	171-177	1138-1196	456-474	89-97	173-183
LR ³ IGF-I 2µg/g/day	931-976	448	126-169	0993-1131	451-467	68-84	148-169
5µg/g/day	815-819	494-512	176-177	1031-1139	591-644	92-121	212-219

B) Relative organ weights (g/kg body weight)

Treatment groups	<i>Liver</i>	<i>Lungs</i>	<i>Heart</i>	<i>Brain</i>	<i>Kidneys</i>	<i>Thymus</i>	<i>Spleen</i>
Vehicle	33.9-37.7	16.2-20.9	5.9-7.3	43.6-45.6	12.5-16.5	2.8-2.9	5.7-7.7
IGF-I 2µg/g/day	34.9-38.3	15.7-17.3	7.2-7.6	43.2-44.7	16.1-17.5	2.5-2.8	6.8-6.9
5µg/g/day	34.8-38.4	17.1-17.9	6.8-6.9	41.8-45.6	13.5-17.5	3.7-4.2	5.8-6.4
12.5µg/g/day	37.3-38.4	18.9-20.9	7.1-4-7.4	47.4-50.4	18.9-19.9	3.7-4.1	7.2-7.9
LR ³ IGF-I 2µg/g/day	32.8-34.7	15.7-15.9	4.5-5.9	35.3-39.8	16.0-16.4	2.4-2.9	5.2-5.9
5µg/g/day	28.1-28.2	17.0-17.7	6.0-6.1	35.4-39.3	20.4-22.1	3.2-4.2	7.3-7.5

Values represent the range of organ weights for 2 animals per treatment group. Statistical analysis was not conducted due to the small sample size.

5.3.7 Concluding remarks on preliminary study

Doses of LR³IGF-I were clearly defined. Administration of 2 and 5µg/g/day of LR³IGF-I resulted in dose dependent increases in the weight of the gastrointestinal tract, non-gut organs and body weight. The maximum tolerated dose of the potent analogue was identified as 5µg/g/day. Administration of 12.5µg/g/day of LR³IGF-I induced a excessive state of hypoglycaemia in the two rats pups treated with this dose within 24 hours after the start of infusion, indicating that the dose was toxic to the suckling rat pups. In contrast, no toxic effects were observed with IGF-I doses up to 12.5µg/g/day. Although body weight did not respond, some enhancement of gut weights and non-gut organ weights was apparent, even at 2µg/g/day. Dose responses appeared very flat (for example in the stomach), so it seemed unlikely that marked growth responses would be apparent with higher doses. Hence, doses of 2 to 12.5µg/g/day of IGF-I were used in the future studies.

5.4 SYSTEMIC INFUSION OF IGF-I PEPTIDES TO 6 DAY OLD SUCKLING RATS.

5.4.1 *Recombinant IGF peptides*

Recombinant hIGF-I and recombinant hLR³IGF-I were purchased from GroPep Pty. Ltd., Adelaide, South Australia. Specifications of the recombinant peptides have been described in Chapter 2. To minimise variations that may occur as a result of differences in peptide batch, the total quantity of lyophilised IGF-I and LR³IGF-I needed for completion of the present study was purchased and stored at -70°C for a maximum of 3 months at the final concentrations used in this study. Based on the results from the preliminary studies, the average body weight of 6 day old neonatal Hooded Wistar rat pup is approximately 15g. Thus, the 6 day old rat pups receiving IGF-I peptides at 2, 5 or 12.5µg/g body weight per day received on average 30, 75 and 188µg/day of peptide, respectively.

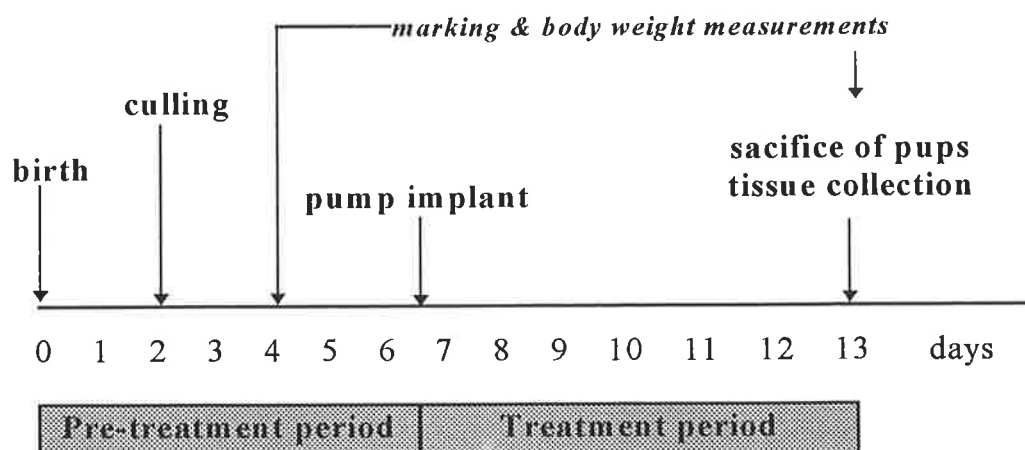
5.4.2 *Experimental design*

To obtain sufficient rat pups with a minimal spread in birth weights, 3 batches with 3 pregnant rats each were used in the present study. The first two batches were treated simultaneously while the third batch was used to make up numbers where losses occurred. Pregnant rats were delivered to the animal holding facilities at the Women's and Children's Hospital from the CSIRO Division of Human Nutrition breeding colony. Mating of each batch was staggered by 3 weeks, so that at any one time no more than 27 rat pups were handled. Care of the pregnant rats and rat pups has been described above. Both female and male rat pups were included in this study.

On day 6 *post partum*, 1 rat pup from each of the 3 litters was randomly assigned to each of the 6 treatment groups, leaving 3 spare animals per litter. Typical distribution of body

weights across 3 litters of rat pups and assignment to treatment groups are shown in Appendix 5.1. For surgical insertion of the pump, the entire litter (including the spare animals) was separated from their mothers and placed on a heating blanket. Rats receiving a mini-osmotic pump were anaesthetised with 3-3.5% Fluothane inhalation anaesthetic at a flow rate of 1.25l/min (Halothane Ph. Eur., ICI Pharmaceutical, Macclesfield, Cheshire, England). The insertion of the mini-osmotic Alzet pumps (Model 1007D, (0.47 μ l/hour), Alza, Palo, CA) and the post-operative care of the rat pups and the dam were conducted in a manner similar to that described above (5.3.3). On the first post-operative day, the daily routine of animal handling and weighing continued throughout the peptide treatment period. The implanted pumps were not primed prior to insertion, thus, peptide infusion commenced approximately 4 hours after the pump implant (at approximately 2000-2100 hours on the day of surgery). To ensure that peptide infusion occurred for the same length of time for all animals, rat pups were sacrificed in the order of pump implantation in the early morning hours (600-800 hours) on the 13th postnatal day. Therefore, the peptides were infused for 6 days and 9-10 hours (called 6.5 days thereafter). Figure 5.2 illustrates schematically the experimental design. Assignment of the rat pups to the treatment groups was carried out by an associate not involved in any way with the present study. All analysis was carried out using coded samples so that at any one stage of the experiment treatment groups were not disclosed to the operator.

Figure 5.2: Experimental design of the IGF-I infusion study in 6 day old rats.



A total of 3 rat pups were excluded from in the study, one because of infection at the site of pump implantation and the other two because the sutures had completely opened on the day following pump implant. For completion of the present study, a total of 45 rats received a subcutaneously implanted pump with 8 rats receiving acetic acid, 7 rats receiving LR³IGF-I at a dose of 2µg/g/day. All other treatment groups comprised 6 rat pups.

5.4.3 Tissue collections and blood sampling

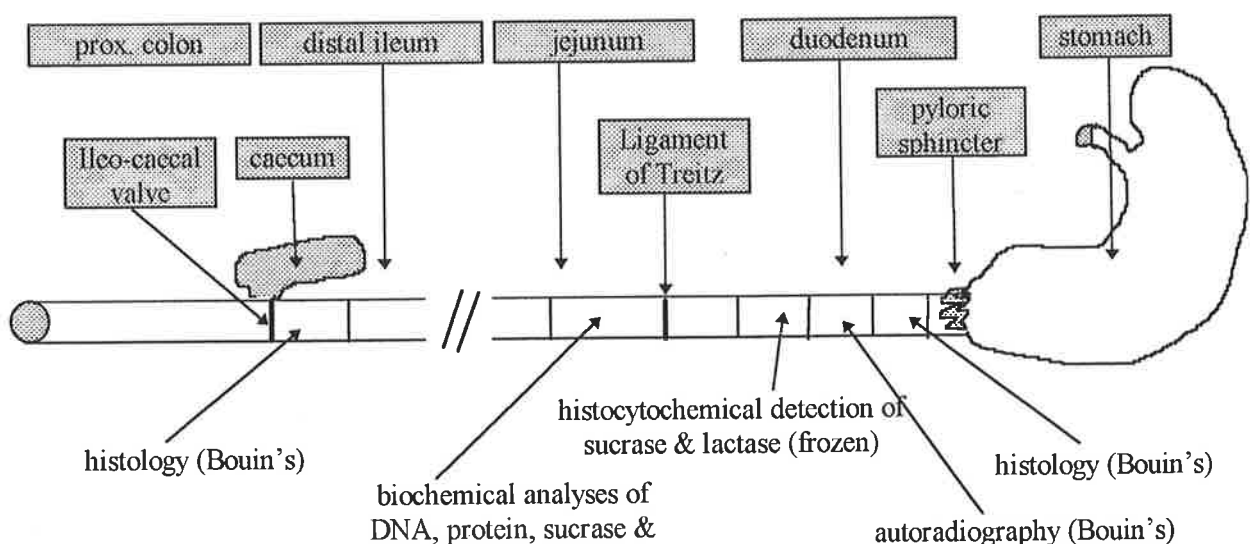
After the peptide infusion period, all rat pups were separated from their mothers and kept warm on a heated blanket. Exactly 1 hour prior to the sacrifice of the animals, each rat was injected into the intraperitoneal cavity with 0.5µCi/g body weight of tritiated thymidine (Amersham, International, Buckinghamshire, England, specific activity 25Ci/mol). The tracer was injected into 2 sites, left and right from the mid-line, with care taken to avoid injecting into visceral organs. Exactly one hour after injection of the isotope, the animals were stunned and killed by decapitation. For subsequent biochemical analysis (IGF and IGFBP profiles and insulin concentrations in plasma), trunk blood was collected into heparinised Eppendorf tubes, immediately centrifuged at 400g for 1 min using a benchtop centrifuge, snap frozen in liquid nitrogen and stored at -70°C. The entire gastrointestinal tract was then rapidly excised. Wet tissue weights and length measurements of intestinal components were taken as described for the adult rats (Chapter 3, section 3.4.2). For histological and autoradiographic evaluation of the growth response, multiple transverse tissue segments from the duodenum and ileum were collected and fixed in Bouin's fixative for 4 hours as described in Chapter 4, section 4.2.5.

In addition, a 4 cm jejunal segment, collected adjacent to the Ligament of Treitz, was collected for measurements of disaccharidase activities, as well as the mucosal DNA and protein content. For this purpose, the jejunal tissue segment was opened longitudinally by cutting along the mesenteric border, rinsed in saline, blotted dry and weighed. Placing the

serosal side onto an ice-cold glass slab, the mucosa and submucosa were scraped from the underlying muscularis externa using a glass slide. The mucosal scrapings (plus submucosa) were then weighed, immediately frozen in liquid nitrogen and kept until required for biochemical analyses.

To determine if IGF-I peptide administration influences the spatial distribution pattern of intestinal disaccharidases, a 1cm duodenal tissue segment was collected for subsequent histocytochemical detection and localisation of sucrase and lactase along the length of duodenal villi. The 1 cm duodenal tissue segment was placed in transverse orientation onto a small piece of balsa wood, for embedding in O.C.T. (embedding compound, Miles Inc. Diagnostic Division, Australia). Thereafter, the tissue sections were immediately frozen in liquid nitrogen and stored at -70°C until required for histocytochemical analysis. Finally, to evaluate the growth response of IGF-I peptide infusion on the growth of non- gut organs, the thymus, spleen, kidneys, liver, adrenals, lungs and the heart were removed, rinsed in physiological saline, blotted dry and weighed. Figure 5.3 illustrates the gastrointestinal tissue collection and fixation protocol.

Figure 5.3: Schematic illustration of gastrointestinal tissue collection protocol



5.4.4 Analytical measurements

5.4.4.1 Histology

Quantitative morphometric analysis was conducted in paraffin embedded tissues of the duodenum and ileum. Measurements included villus height, crypt depth and the thickness of the muscularis externa. The methodologies for these measurements have been described in detail in Chapter 4, section 4.2.5. All morphometric measurements were conducted on video captured images. For all histological parameters, 15 measurements were taken in selected, well oriented duodenal or ileal sections (described in Chapter 4, section 4.2.5).

5.4.4.2 Autoradiography

The proliferative activity of the intestinal epithelium following administration of IGF-I peptides was determined by tritiated thymidine incorporation in multiple duodenal and ileal tissue segments fixed for 4 hours in Bouin's. The methodology for the autoradiographic detection and quantisation was carried out as described for the adult animals (Chapter 4, section 4.2.5). The proliferative activity was assessed in 30 full-length open crypts of duodenal sections and follows closely the methodology described in Chapter 4, section 4.2.6.

5.4.4.3 Measurements of DNA, protein and disaccharidases in jejunal tissue homogenates

The jejunal tissue samples were thawed and diluted 1:80 with 50mM phosphate buffer. The diluent sample was then homogenised at 20°C for 30 seconds using an ultraturex tissue homogeniser at 15000g. 50µl of the diluted sample was then incubated with an equivalent volume of 0.2M sucrose (lactose) for 30 minutes at 37°C, before adding a Tris-buffered glucose oxidase reagent (Sigma Chemicals, St. Louis USA) to achieve a final concentration of 0.17M. The sample was then incubated for a further 30 min at 37°C. All methods for sucrase and lactase measurements have been adapted to microplates. Optical density readings were determined in a Titertek Multiscan MCC microplate reader (Flow

Laboratories, North Ryde, Australia) at a wavelength of 492nm with a background filter of 690nm. Sucrase and lactase activities were calculated as the amount of glucose (nmol of glucose) liberated per cm tissue per minute. Assay protocols are described in detail in Appendix 5.3 .

To avoid thawing tissue samples more than once, all biochemical assays were carried out on the same day. To measure the jejunal DNA and protein content, 1.5ml of 1M NaOH was added to the thawed mucosal samples. The mixture was then homogenised at 20°C for 30 seconds at 15000g. The mucosal DNA content in jejunal tissue segments was measured by the method of Burton (1956) in homogenate samples extracted with 0.3M perchloric acid and finally dissolved in 0.5M perchloric acid. The protein content was measured by Dulley and Grieve's modification of the Lowry method (Dulley and Grieve, 1975). Both methods have been adapted to microplates. Optical density readings were determined in a Titertek Multiscan MCC microplate reader (Flow Laboratories, North Ryde, Australia). For the DNA and protein content, readings were taken at a wavelength of 620 nm and 690 nm, respectively. The assay protocols are outlined in Appendix 5.2 and 5.3.

5.4.4.4 Histocytochemical detection of sucrase and lactase

Tissue preparation: Frozen tissue sections collected from the proximal duodenum were cut with a cold microtome into 8µm thick sections. The sections were transferred onto coverslips rather than glass slides, so that all incubations could be carried out in 5ml Colombia Jars. All sections were kept at -18°C until processing. To prevent sections floating off during incubations, all coverslips were previously coated in gelatin (Appendix 2.1). From each section of duodenum, two coverslips with 4 sections each were prepared so that lactase and sucrase activities could be measured from virtually identical sites (less than 10µm apart). Tissue sections were then thawed for 10 minutes at room temperature. Histocytochemical detection of lactase activity was carried out following the general method described by Lodja

and Kraml (1971) and Lojda et al., (1973). Sucrase activity was measured as α -glucosidase activity, which represents a combination of sucrase, isomaltase, maltase II and maltase III and trehalase, and was detected as described by Gutschmidt et al., (1979). In a preliminary series of experiments, I determined the optimal sample treatment and incubation times for the neonatal rat tissue samples. These experiments have shown that the enzyme reaction took place in the presence of saturating conditions of the artificial substrate under initial rate conditions (Figure 5.4).

Localisation of β -glucosidases (lactase): The thawed sections were incubated for 15 min. at 37°C in a medium comprising 1.1mM 5-bromo-4chloro-3-indolyl- α -frucopyranoside (Sigma Chemicals, St. Louis USA), 3mM ferricyanide, 3mM ferrocyanide (Sigma Chemicals, St. Louis, USA) in 0.1M citric acid phosphate buffer (pH 6.0). Gutschmidt and Emde (1981) have determined that these conditions ensure maximal rates of hydrolysis. Following incubation for exactly 15 min, the enzyme reaction was stopped by rinsing the sections in 4% formal calcium (Appendix 2.1). While wet, the coverslips were mounted onto glass-slides using Glycergel mounting medium (Dako, Dako Corporation, Carpenteria, CA USA). No counterstain was applied. The enzyme reaction gave a blue colour reaction product.

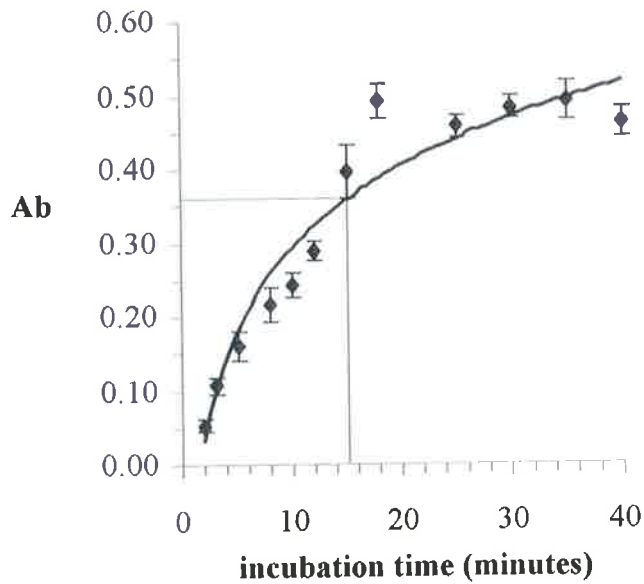
Localisation of α -glucosidases (sucrase): α -Glucosidases activity was detected in 8 μ m cryostat sections from the duodenum using saturating substrate concentrations as described by Gutschmidt et al., (1979) under initial rate conditions. To prevent the diffusion of naphthol substrate enzyme reaction product from its site, the thawed tissues were fixed in 4% formal calcium for 5 minutes at 4°C prior to incubation with the artificial substrate. The sections were then incubated for exactly 18 min at 37°C in 12mM 2-naphthyl- α -D-glucopyranoside (Sigma Chemicals, St. Louis USA) and 0.6% hexazonium-p-rosaniline in 0.1M citric acid phosphate buffer (pH 6.0). The hydrolysis was stopped exactly 18 min. later by rinsing the sections for 5 minutes in isotonic saline followed by post-fixation with 4% formal calcium. The coupling

agent hexazonium-p-rosaniline produces a orange colour reaction of the enzyme product. No counterstain was applied.

Measurement of enzyme products: All sections were examined with an Olympus BH-2 light microscope (magnification $\times 250$), mounted with a monochrome, high resolution CCD camera (TM-7 camera, Pulnix Industrial Products Division, Clayton, Vic. Australia). Maximum absorption for the enzyme reaction colour product of 2-naphthyl-D-glucopyranoside occurs over a wavelength range of 480nm and 530nm (Gutschmidt et al., 1979). Thus, for densitometric estimation of α -glucosidases (sucrase) activity, the microscope was equipped with a neutral density filter (1.0) and a FS 500nm circular microscopic field filter. Maximum absorption for the indolyl substrate product has been determined by spectral analyses and identified maximum absorption of the reaction product at 590nm-660nm (personal communication, Dr. D. Tivey, Waite Institute, The University of Adelaide). For the densitometric scanning of β -glucosidases (lactase), the microscope was equipped with a neutral density filter (1.0) and a FS 600nm circular field filter.

Analysis of enzyme profile: For each animal, the Absorbency (Ab) of the colour enzyme product for both enzymes was measured along the length of 6 perfectly orientated villi using an image analysis software program (Video Pro, Leading Edge, Adelaide, Australia). For each villi, Ab readings were computed on the right side of the villus structure, by tracing a 1 pixel thick line ($1.67\mu\text{m}$) along the right hand side of each villus. Starting at the base of the villi (crypt:villus junction), Ab readings were recorded at 4 pixel intervals ($6.7\mu\text{m}$) and the corresponding distance along the villus structure was computed. Thus, the data have been expressed as Ab as a function of distance along the villus axis, where the base of the villi is the starting point and the tip of the villi is the end point. Figure 5.5. schematically depicts the methodology for Ab measurements of sucrase and lactase along duodenal villi.

Figure 5.4: Incubation of rat duodenal tissue sections with 5-bromo-4chloro-3-indolyl- α -frucopyranoside for establishment of incubation time.



To establish the optimal incubation time, duodenal sections were incubated for up to 40 minutes. From 6 perfectly orientated sections, absorbency readings were taken from the mid-villus region (region of most intense enzyme colour reaction). Figure 5.4 shows that absorbency readings were taken under initial rate conditions. For all future studies, tissue sections were incubated for 15 minutes. A similar curve was obtained for tissue sections incubated with 2-naphthyl- α -D-glucopyranoside for detection of sucrase (data not shown).

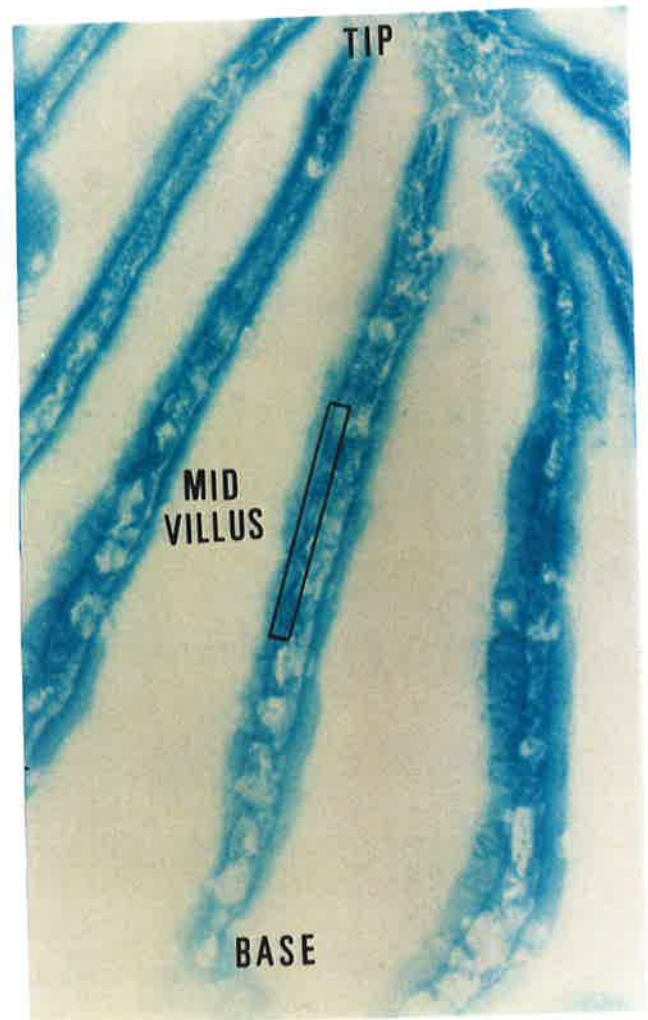
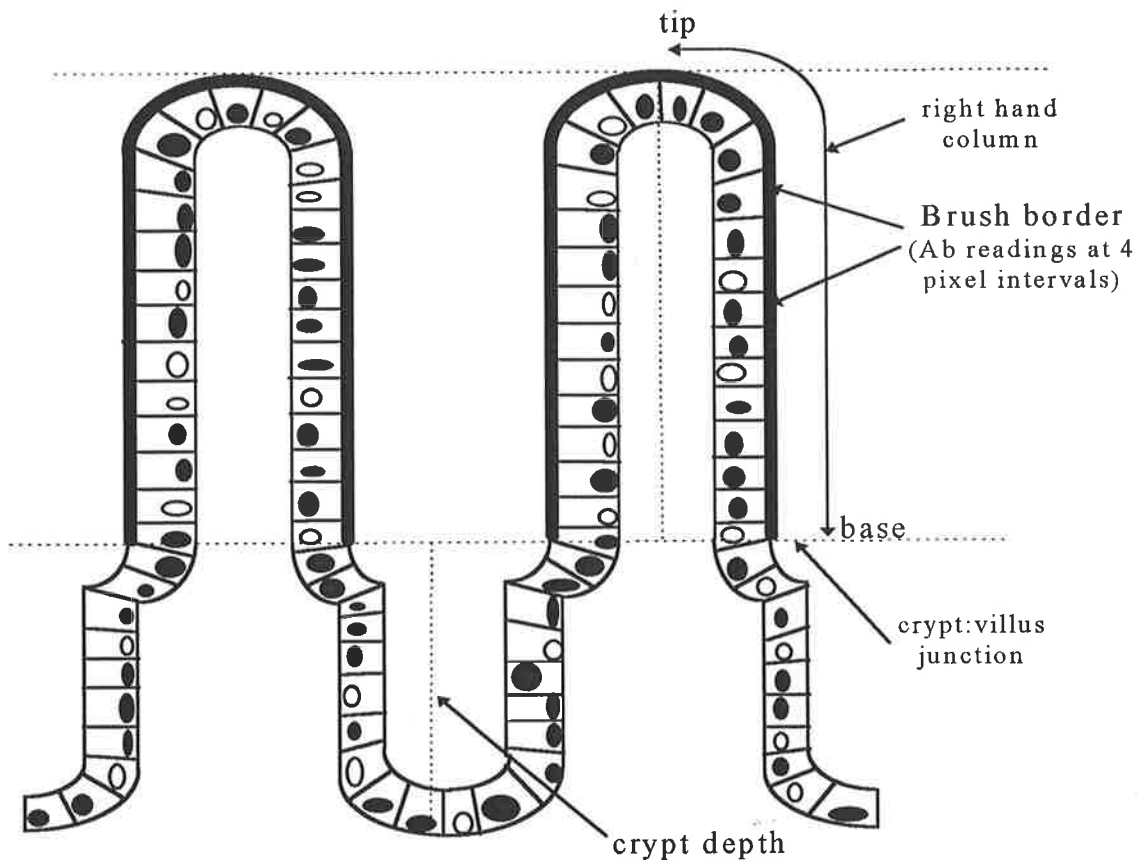


Figure 5.5: Measurements of the enzyme colour products for lactase and α -glucosidases along the length of duodenal villi by computer assisted image analysis.



The Ab reading program was set up to record arbitrary Ab units between 0 and 1, where a value close to 0 represented the background reading of the microscopic slide and values close to 1 represented maximal (black) colour development. For each animal, differences in villus length for the 6 villi measured were accounted for by standardising each villi to the average villus length (Appendix 5.5), keeping the base of the villi as the reference starting point. Each distance point recorded was multiplied by a correction factor, calculated as the average villus height divided by the absolute villus height. Ab was then expressed along the corrected villus height so that an Ab reading at 50% along the length of the villi represented the mid-villus region (half way) in all rat pups. To establish enzyme distribution profiles along the length of duodenal villi for each group, the mean of the standardised villus length was plotted against the corresponding average Ab measurement of each animal for all

groups. Because of the time consuming nature of the analysis, enzyme profiles were established only for the vehicle treated pups, and the pups treated with highest dose of IGF-I (12.5µg/kg/day) and the pups treated with 5µg/g/day of LR³IGF-I. Enzyme activity (Ab) along the length of duodenal villi was statistically compared at pre-determined distances along villi (50, 100, 250, 400 and 500µm).

5.4.4.5 Plasma IGF-I radioimmunoassays

Chromatography: Plasma from individual rats was chromatographed under acid conditions to separate IGFBPs from the IGF ligands. From each sample, 40µl of plasma was diluted with running buffer to obtain 400µl of a solution (pH 2.5) containing acetic acid (200mM), trimethylamine (50mM) and Tween (0.005%). An equal amount of freon (1,1,2-trichloro-1,2,2-trifluoroethane), AR grade (Mallinckrodt, Paris, KY., USA), was then added to each sample to extract lipids from the diluted plasma samples. Thereafter, all samples were thoroughly mixed and after centrifugation at 10,000g for 10 minutes, the aqueous layer was collected from each sample. A 200µl aliquot of the defatted sample was then applied to a Protein-Pak 125 molecular sieve chromatography column (Waters-Millipore, Lane Cove, NSW, Australia) using a calibrated auto-injector. The column was previously equilibrated with the running buffer described above. Using an automated fraction collector, 0.5ml fractions were collected and protein elution was measured at 280nm. Initially, all fractions were analysed separately to determine elution volumes of IGFs and IGFBPs (Appendix 5.6). On this basis, pools eluting between approximately 6.25 -8.25ml (pool 1) contained IGFBPs, while pools eluting between 8.25-8.75 ml (pool 2) represented the intermediate region with very little RIA activity. Pool 3 eluting between 8.75-10.25ml contained IGF-I, and pool 4 eluting between 10.25-10.75 contained insignificant IGF activity.

Radioimmunoassay for IGF-I. For the radioimmunoassay, hIGF-I was used as standard and radioligand. The IGF-I ligand was iodinated with Na ¹²⁵I (Amersham Australia, Pty. Ltd. North Ryde, NSW, Australia) to specific activities between 63-84Ci/g by chloramine T and purified from fraction components by chromatography through Sephadex G-50 (Pharmacia, North Ryde, NSW, Australia) in 50mM sodium phosphate (pH 6.5), containing 1mg/ml bovine serum albumin (described previously by Francis et al., 1990). 200µl of running buffer and 60µl of Tris base (0.4M) was added to each tube of the pooled sample, the standard, the non-specific binding tubes (no primary antibody) and the quality control tubes. To each tube containing the sample, standard or the quality control sample, 50µl of the primary antibody (rabbit anti bovine IGF-I) was added at a dilution of 1:80,000. The antiserum was prepared by immunising rabbits with a conjugate of bovine IGF-I and albumin as described by Dawe et al. (1988). After adding the primary antibody, 50µl of radiolabelled IGF-I (18-25,000cpm) was added, the mixture was then vortexed and tubes were incubated for 16 hours at 4°C. The following was then added to the mixture: 50µl of the secondary antibody (sheep anti rabbit IgG, Silenus, Hawthorn, Australia) at a 1:20 dilution, 10µl of a carrier protein (rabbit IgG, Silenus, Hawthorn, Australia) at a dilution of 1:200 and 1ml of chilled 5.5% polyethylene glycol (PEG 6000, Fluka Chemica AG, Buchs, Switzerland) in 0.9% NaCl. Tubes were vortexed for 10 seconds and centrifuged at 2500g for 20 minutes at 4°C, prior to aspiration of the supernatant. The radioactivity in the pellet was measured for 3 minutes. Radioactivity in the tubes containing no IGF-I antibody was subtracted from each value. Minimal detectable levels were approximately 21ng/ml of the samples assayed and 50% inhibition of radiolabelled IGF-I binding was achieved by approximately 180pg. The rabbit anti-bovine antibody used in this assay cross-reacts equally well with bovine and human IGF-I since the two peptides are structurally the same, but cross-react only 25% as well with rat IGF-I (P. C. Owens, personal communication).

5.4.4.6 Western ligand blot analysis

The IGFBP profile in the plasma was determined by Western ligand blots. Because only a small quantity of blood was extracted from the 6 day old rat pups at the time of sacrifice, the rat plasma was pooled for each of the treatment groups and the vehicle treated rats. Adult rat plasma was included in the analyses. From each animal, 10 μ l of plasma was collected and pooled. A total of 20 μ l of the pooled sample was then diluted in concentrated sodium dodecylsulphate (SDS)-loading buffer to a total volume of 0.2ml and incubated at 65°C for 15 minutes. Thereafter, 20 μ l of this mixture was loaded onto a 1.5mm thick SDS-polyacrylamide gel for electrophoresis (SDS-PAGE). The SDS-PAGE consisted of a 10% separating gel atop of a 4% stacking gel as described by Laemmli (1970). Samples were running in lanes 2-8. In Lane 9, ¹⁴C-labelled Rainbow markers (Amersham International, Amersham, Bucks UK), which were previously incubated with 50 μ l of concentrated SDS loading buffers, were run. The electrophoresis was carried out at 10mA for 16 hours. Proteins were transferred onto nitrocellulose sheets (0.45 μ m, Schleicher and Schuell, Dassel, Germany) at 300mA for a 2 hour period (Hoefer Transphor TE 42 apparatus, Hoefer Scientific, San Fransisco, CA, USA). The nitrocellulose sheets were dried overnight and the dried nitrocellulose sheets were then washed in 500ml of Tris buffer (35ml 2M NaCl, 10ml of 0.5M Tris, 1% Triton X-100, at pH 7.4) for 30 minutes. This was followed by a wash in buffer with 1% bovine serum albumin (w/v) for 90 minutes followed by a wash in buffer containing 0.1% Tween 20 (v/v) for 10 minutes. The washed nitrocellulose was then incubated for 3 hours with 1% serum albumin buffer containing 0.1% Tween 20 and ¹²⁵I-IGF-I (1x10⁷ cpm). Thereafter, the nitrocellulose sheets were washed every 20 minutes with fresh 0.1% Tween 20 buffer for a period of 3 hours. The washing steps and incubation with the radiolabelled ligand were conducted in a shaking water bath at 30°C. The nitrocellulose was dried and exposed to X-ray film (A8323, Konica, Tokyo, Japan) at -70°C for a minimum of 15 days, before being developed.

5.4.4.7 Plasma insulin measurements

The plasma insulin concentration was measured for each animal in trunk blood samples collected at the end of the 6.5 day peptide treatment period using a Pharmacia Phadeseph radioimmunoassay kit. These measurements were performed by Dr. J. Oliver, Flinders Medical Center, South Australia.

5.4.5 Statistical analysis

All values in Figures and Tables are reported as either the range of the data or means \pm SEM. Treatment groups were compared by one-way analysis of variance (ANOVA) and when significance was attained at $P < 0.05$, a Fisher's Protected Least Significant Difference *post hoc* was applied to the data to identify the between group difference. All statistical analysis was performed using the Super ANOVA software package (Abacus Conceptus, CA USA).

5.5 RESULTS

5.5.1 Plasma IGF-I concentrations

IGF-I concentrations were measured for each animal in plasma samples collected at the time of sacrifice. Pre-treatment IGF-I concentrations were not measured because tail vein collections were not possible in these young animals. However, plasma samples from 3 untreated control rats sacrificed on the 13th postnatal day were included in the analyses.

Table 5.5: IGF-I concentrations in plasma samples from rats collected at the end of a 6.5 day peptide treatment period compared to vehicle treated and untreated control rats. IGF-I concentrations were measured in chromatographed plasma samples by radioimmunoassay with a polyclonal anti-bovine IGF-I antibody.

Treatment group	IGF-I concentrations (ng/ml)
Control	58.7±9.3
Vehicle	39.0±4.3
IGF-I 2µg/g/day	79.0±9.3
5µg/g/day	153.4±18.3**
12.5µg/g/day	236.5±26.1**
LR ³ IGF-I 2µg/g/day	45.8±13.9
5µg/g/day	45.1±6.9

*Values are means±SEM with 6-8 rats per group. One plasma sample was excluded from analysis from the 12.5µg/g/day of IGF-I group. Control values represent a pooled plasma sample from 3 untreated control rats. Statistical significance from the vehicle treated rats is indicated by **: P<0.0001 as detected by ANOVA.*

IGF-I concentrations in plasma samples collected from the untreated control rats and vehicle treated rats were 58.7±9.3ng/ml and 39±4.3ng/ml of plasma sample, respectively, with no statistically significant difference between the two groups. The IGF-I concentration in plasma from rats treated with recombinant hIGF-I increased in a dose dependent manner, so that infusion of the highest dose (12.5µg/g/day) of IGF-I significantly raised plasma IGF-I

concentrations ($P < 0.01$) to 6 times that of vehicle treated rats (Table 5.6). In contrast, administration of either dose of LR³IGF-I failed to increase IGF-I concentrations in plasma above the level detected in vehicle treated or untreated control rats (Table 5.6). Specific antibodies for the detection of rhLR³IGF-I were not available at the time when these assays were conducted, thus accurate measurement of the analogue in rats plasma could not be made. Furthermore, the cross-reactivity of the polyclonal anti-bovine IGF-I antibody to LR³IGF-I is approximately 10%. Estimation of the relative amounts of IGF-I in the circulation of rat pups infused with LR³IGF-I are therefore unknown.

5.5.2 *IGFBP profiles*

Unfortunately, the Western Ligand blot is very faint and could not be repeated because of the small quantity of plasma available (Plate 5.1). However, an attempt was made to interpret the blot. In the adult rat plasma, which was running in Lane 8, the most intense band was the doublet migrating at 40-46 kDa. This band has been previously identified as rIGFBP-3 (Zapf et al., 1988, Donovan et al., 1991). The faint doublet, identified at approximately 30 kDa most likely represents proteolytic fragments of rIGFBP-3 and/or rIGFBP-2/IGFBP-1 (Donovan et al., 1989 and 1991 and Yang et al., 1990). In addition, a band migrating at approximately 24 kDa was observed, and most likely represents the smaller molecular weight binding proteins, for example, rIGFBP-4 (Donovan et al., 1991).

By contrast, the 40-46 kDa band in neonatal rat plasma (Lane 3-7) was virtually absent, which is in agreement with previous reports (Donovan, et al., 1989 and Glasscock et al., 1990). In the present study, the major neonatal BP was found in the 30 kDa region and, as previously shown, this most probably represents the non-glycosylated rIGFBP-2 (Glasscock et al., 1990). A faint band migrating at approximately 24 kDa was also observed and most likely represents rIGFBP-4. Because of the poor quality of the ligand plot, it is not possible to

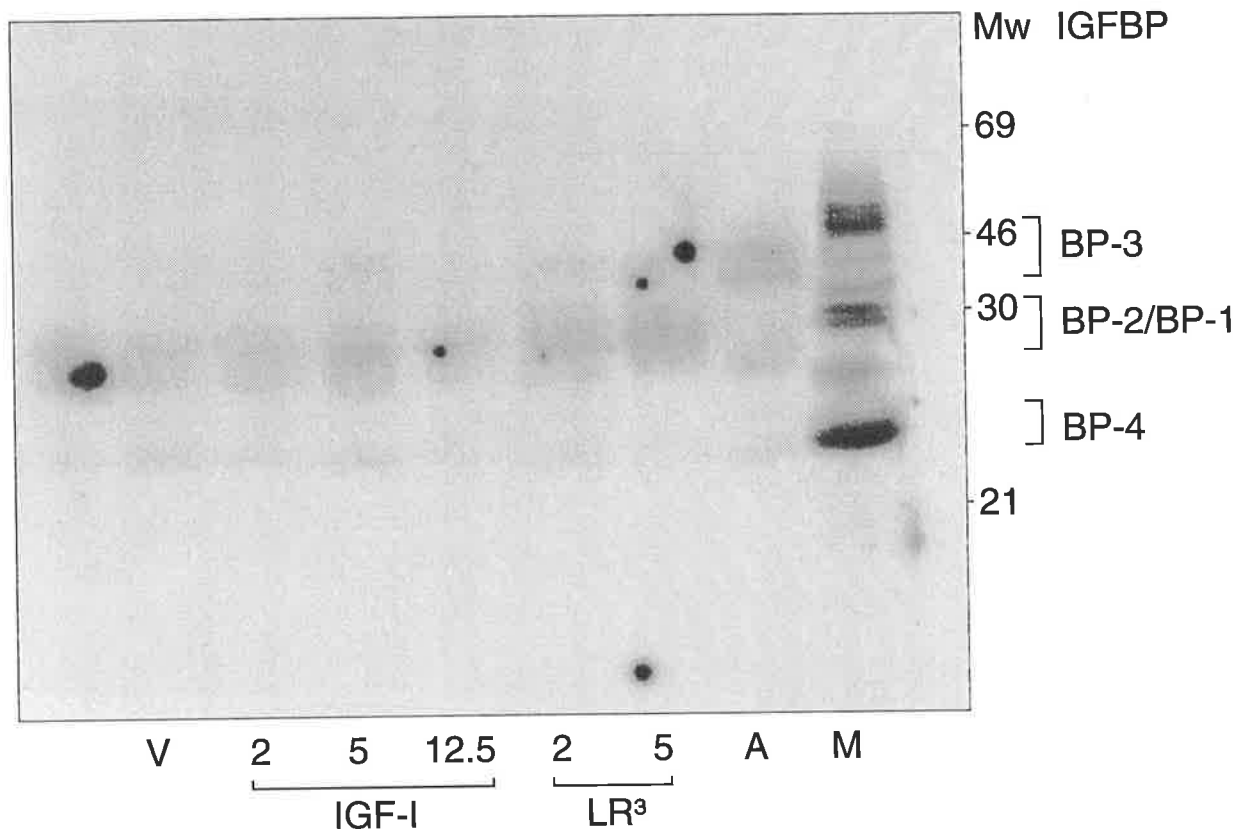


Plate 5.1: A representative ligand blot showing IGFBP profiles in 13 day old rat pups treated with or without IGF-I peptides. 20 μ l of pooled plasma sample from 6-8 animals per group were diluted with Laemmli sample buffers (Laemmli, 1970) to a total volume of 0.2ml and heated at 65°C for 15 minutes. 20 μ l of the sample were then separated on 10% SDS-polyacrylamide gel by electrophoresis and then electroblotted onto nitrocellulose as described in Material and Methods. Membranes were incubated with about 1×10^7 cpm of 125 I-IGF-I for 3 hours in a shaking water bath. 125 I-IGF-I bands were visualised by autoradiography. Migration position of molecular weight markers (M) are shown on the right hand side (Lane 9). Proposed IGFFBPs are also indicated. Pooled plasma from vehicle treated rats is shown in Lane 2. Plasma pools from rats treated with 2, 5 or 12.5 μ g/g/day of IGF-I are shown in Lane 3, 4 and 5, respectively. Plasma pools from rats treated with 2 or 5 μ g/g/day of LR³IGF-I are shown in Lane 6 and 7, while Lane 8 depicts pooled adult rat plasma.

determine if administration of IGF-I peptides altered the binding protein profile. However, it appears that the apparent binding protein profile was similar for all pooled neonatal plasma samples. The quality of the blot does, however, not permit further qualification of this statement. A representation of the Western Ligand blot is shown in Plate 5.1.

5.5.3 Somatic growth response

Body weight changes

On the day of pump implantation (6th postnatal day), the body weights ranged from 13.6g to 15.9g, with no statistically significant difference between treatment groups (Table 5.7). After the 6.5 day treatment period with IGF-I peptides or vehicle (13th postnatal day), only rats treated with the highest dose of LR³IGF-I (5µg/g/day) showed a significantly greater body weight gain compared to vehicle treated rats (Table 5.7).

Table 5.6: Body weights in suckling rats treated with and without IGF-I peptides.

Treatment group	Initial body weight (g) (day 6 <i>post partum</i>)	Final body weight (g) (day 13 <i>post partum</i>)	Body weight gain (g)
Vehicle	13.6±0.4	25.0±0.9	11.4±0.5
IGF-I 2µg/g/day	15.9±0.3	27.3±1.0	11.4±0.9
5µg/g/day	14.3±0.5	27.2±1.0	12.9±0.8
12.5µg/g/day	14.5±0.3	26.6±0.7	12.2±0.7
LR ³ IGF-I 2µg/g/day	14.1±0.5	25.0±1.3	11.0±0.8
5µg/g/day	14.9±0.4	29.6±0.7*	14.7±0.5*

*All values represent means±SEM with 6-8 animals per group. Statistical significance from the vehicle group is indicated by *: P<0.05, ANOVA.*

IGF-I peptide effects on non-gut organs

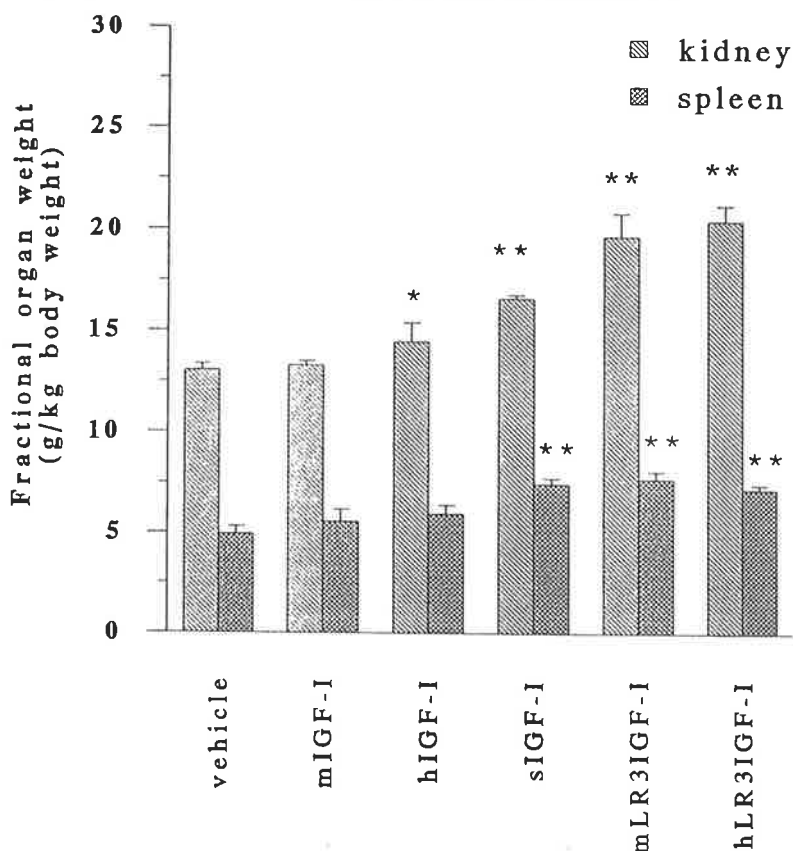
To determine the effect of IGF-I peptide administration on the growth response of non-gut organs, the wet tissue weights of the brain, heart, kidneys, liver, lungs spleen and the thymus were taken. Most significantly, the wet tissue weights of the kidney and spleen were increased in rat pups treated with 5 and 12.5µg/g/day of IGF-I and with 2 and 5µg/g/day of LR³IGF-I, so that treatment with the highest dose LR³IGF-I resulted in an increase in kidney weight of 85% above vehicle treated rats (Table 5.8). Spleen weights in the same group increased by 76% (Table 5.8). Tissue weights were still significantly elevated when corrections for body weight gain was made (Figure 5.6).

Table 5.7: Absolute organ weights on day 13 post partum in suckling rat pups treated for 6.5 days with IGF-I peptides.

Treatment group	<i>Heart</i> (mg)	<i>Liver</i> (mg)	<i>Lungs</i> (mg)	<i>Thymus</i> (mg)	<i>Kidneys</i> (mg)	<i>Spleen</i> (mg)	<i>Brain</i> (g)
Vehicle (0.1M acetic acid)	160±6	804±38	433±13	72±3	328±18	124±10	1.01±0.06
IGF-I 2µg/g/day	170±5	991±42*	474±21	73±6	326±13	152±18	1.05±0.02
5µg/g/day	163±5	855±42	451±13	89±6	389±19*	160±6*	1.05±0.03
12.5µg/g/day	169±5	874±31	472±20	84±6	443±13**	198±9**	1.01±0.01
LR³IGF-I 2µg/g/day	163±9	767±43	467±42	75±7	484±28**	188±9**	0.98±0.02
5µg/g/day	187±7	844±29	528±37*	106±13*	607±32**	218±7**	1.04±0.02

Values are means±SEM for 6-8 rat per group. Statistically significant difference from the vehicle group is indicated by *: P<0.05 and **: P<0.001 as detected by ANOVA.

Figure 5.6: Fractional organ weights of kidneys and spleen on day 13 post partum in suckling rats treated for 6.5 days with IGF-I peptides.



Values are means \pm SEM with 6-8 rats per group. Significant difference from the vehicle treated rat pups is indicated by *: $P < 0.05$ or **: $P < 0.01$, ANOVA. Vehicle treated rats received 0.1M acetic acid, mIGF-I = 2 μ g/g/day, hIGF-I = 5 μ g/g/day and sIGF-I = 12.5 μ g/g/day. LR³IGF-I was infused at 2 μ g/g/day (mLR³IGF-I) or 5 μ g/g/day (hLR³IGF-I).

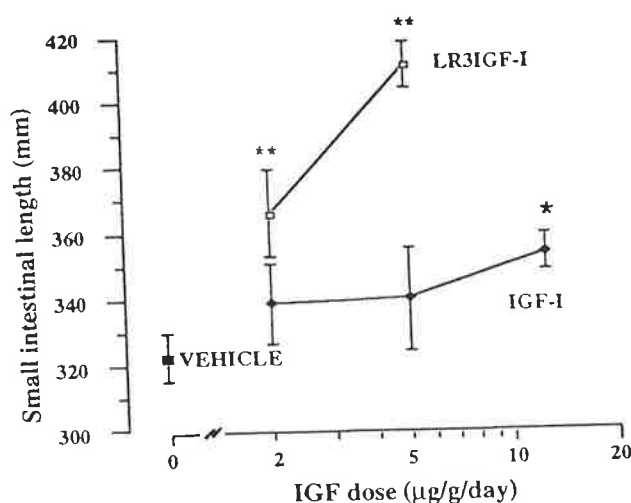
5.5.4 IGF-I effects on gastrointestinal wet tissue weight and length

Although body weight gain in rat pups treated with IGF-I was similar to the body weight gain in vehicle treated rats, the wet tissue weights of gastrointestinal components were selectively increased in rat pups treated with either IGF-I or the IGF-I analog. For example, total gut weight, small intestinal weight, large intestinal weight and stomach weight were significantly increased by 17%, 18%, 15% and 10% respectively in rat pups treated with 12.5 μ g/g/day of IGF-I (Figure 5.7 A-D). The IGF-I analog was markedly more potent than the native IGF-I, so that the wet tissue weight of the total gut, small and large intestine increased by approximately 56-60% following infusion of 5 μ g/g/day of LR³IGF-I (Figure

5.7A-D). It was interesting to note that in addition to the IGF-I effects on the small and large intestine, the caecum was also responsive to the IGF-I treatment; administration of 5µg/g/day of LR³IGF-I and 12.5µg/g/day of IGF-I increased the weight of the caecum from 37mg in the vehicle treated rats to 54mg in the rats treated with 5µg/g/day of LR³IGF-I (data not shown). Selective action on gastrointestinal tissue by IGF-I peptides was demonstrated when tissue weight was expressed as fraction of body weight (Table 5.9). In contrast to the adult gut, however, the small intestine and the large intestine were equally responsive in suckling rats. As in the adult rat studies, the duodenum was the most peptide responsive region with increases in duodenal wet tissue weight of up to 60% following administration of 5µg/g/day of LR³IGF-I (data not shown).

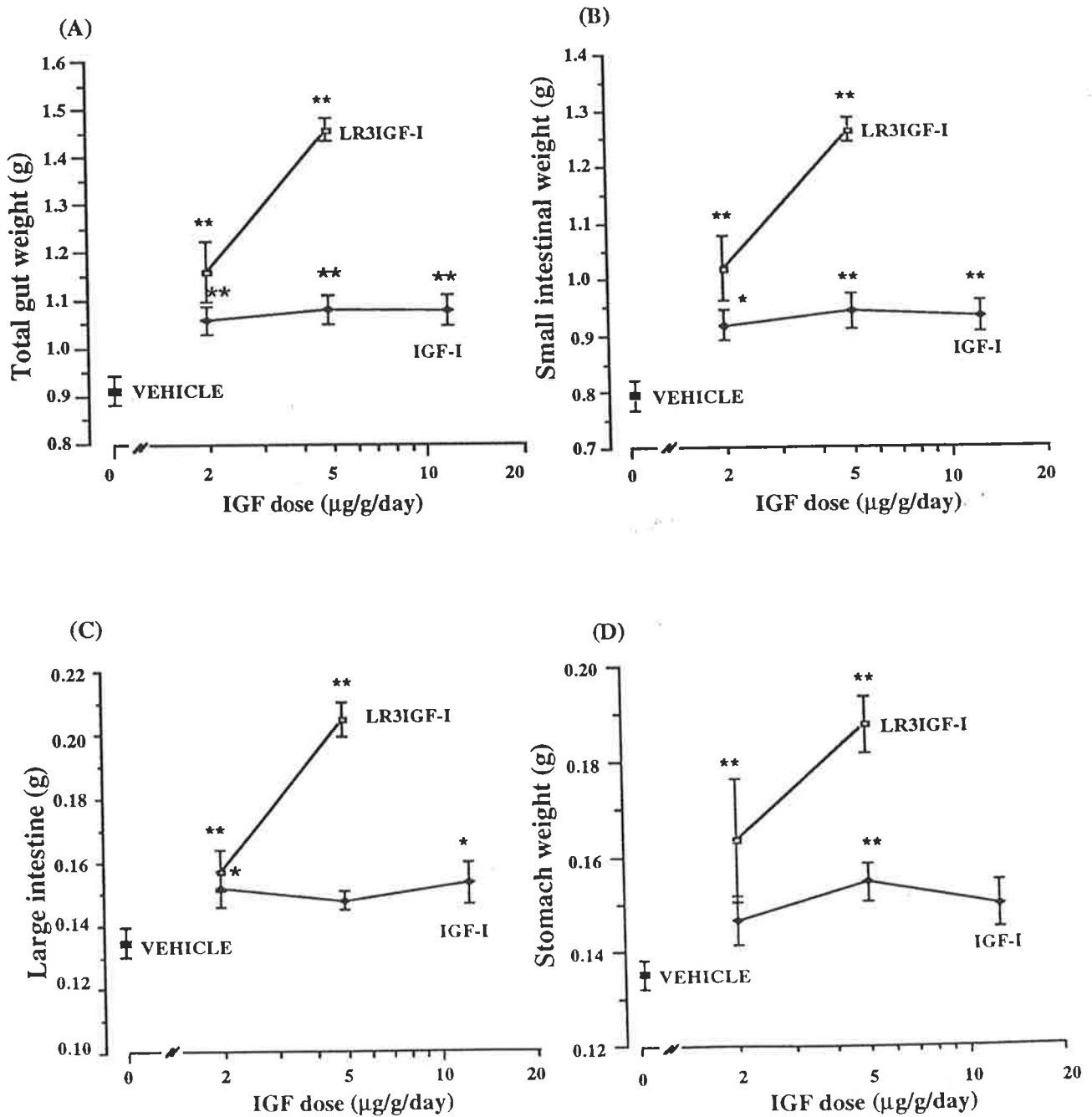
Infusion of IGF-I peptides also significantly increased intestinal length by up to 25% in the rat pups treated with the highest dose of LR³IGF-I (Figure 5.8 A). The large intestinal length was marginally increased in rat pups treated with the highest dose of LR³IGF-I, however statistically significant difference was not detected between treatment groups (ANOVA, P<0.28).

Figure 5.8: Small intestinal length in suckling rats treated for 6.5 days with IGF-I peptides.



Each data point represents the mean of 6-8 rat pups in each group. Error bar indicates the SEM. Statistically significant difference from the vehicle group was determined by ANOVA and is indicated by *: P<0.05 and **: P<0.01.

Figure 5.7: Total gut weight (A), small intestinal weight (B), large intestinal weight (C) and stomach weight (D) in suckling rat pups at 13 days post partum following treatment with IGF-I peptides for 6.5 days.



All values are means ± SEM for 6-8 rat pups per treatment group. Statistical significance from the vehicle treated rat pups is indicated by *: P < 0.05 and **: P < 0.01 as detected by ANOVA.

Table 5.8: Fractional weight (g per kg body weight) of gastrointestinal components in suckling rat pups following continuous infusion of IGF-I peptides for a 6.5 day period.

Treatment group	<i>Fractional gut weights (g/kg body weight)</i>			
	Stomach	S. intestine	L. intestine	Total gut wt.
Vehicle	5.45±0.18	31.95±0.89	5.28±0.08	37.23±0.9
IGF-I 2µg/g/day	5.39±0.17	34.02±1.28	5.51±0.09	39.52±1.29
5µg/g/day	5.74±0.27	35.28±2.17	5.42±0.32	40.70±2.5
12.5µg/g/day	5.64±0.24	35.34±1.00	5.72±0.23	41.05±1.12
LR ³ IGF-I 2µg/g/day	6.55±0.33**	40.97±1.33**	6.18±0.32**	47.15±1.59**
5µg/g/day	6.36±0.15**	42.92±0.89**	7.06±0.32**	49.99±1.19**

*Values are means±SEM for 6-8 animals per group. Statistical significance from the vehicle treated rat pups is indicated by *: P<0.05 and **: P<0.01 as determined by ANOVA.*

5.5.5 IGF-I effects on histological parameters

To determine if the increase in wet tissue weight was reflected in the histology of the intestinal tissue, morphometric analysis of the mucosa and non-mucosal tissue layers was carried out on tissue segments from the duodenum and ileum. As in the adult rat studies, administration of IGF-I peptides significantly increased the mucosal thickness exemplified by a significant increase in both villus height and crypt depth. For example, administration of 2, 5 and 12.5µg/g/day of IGF-I increased villus height in the duodenum between 9 and 18% above control values (Table 5.10). At the same time, administration of 2 and 5µg/g/day of LR³IGF-I increased villus height by 8% and 24%, respectively, above values obtained for vehicle treated rat pups. Similarly, crypt depth was significantly increased in the rat pups treated with the highest dose of IGF-I and both doses of LR³IGF-I (Table 5.10). Administration of IGF-I peptides also stimulated growth of the muscularis externa so that treatment with the highest dose of LR³IGF-I induced a 31% increase in the thickness of the muscularis externa layer (Table 5.10).

Table 5.9: Histological measurements in the duodenum and ileum of suckling rat pups treated for 6.5 days with IGF-I peptides.

Treatment group	<i>Duodenum</i>			<i>Ileum</i>		
	Villus (μm)	Crypt (μm)	muscularis externa (μm)	Villus (μm)	Crypt (μm)	muscularis externa (μm)
Vehicle)	432 \pm 12	72 \pm 3	62 \pm 2	412 \pm 22	63 \pm 3	50 \pm 3
IGF-I 2 $\mu\text{g/g/day}$	488 \pm 16*	79 \pm 2	63 \pm 3	421 \pm 27	65 \pm 2	52 \pm 2
5 $\mu\text{g/g/day}$	471 \pm 12*	77 \pm 2	64 \pm 5	453 \pm 20	66 \pm 4	49 \pm 4
12.5 $\mu\text{g/g/day}$	512 \pm 7**	84 \pm 1*	68 \pm 3	456 \pm 17	66 \pm 2	55 \pm 3
LR ³ IGF-I 2 $\mu\text{g/g/day}$	468 \pm 10**	91 \pm 3**	72 \pm 4*	449 \pm 9	70 \pm 3	57 \pm 2**
5 $\mu\text{g/g/day}$	535 \pm 10**	92 \pm 3**	81 \pm 3**	505 \pm 11*	77 \pm 3*	68 \pm 2**

*Values are means \pm SEM for 6-8 rat pups per treatment group. Statistical significance from the vehicle groups has been determined by ANOVA. Significance is indicated by *: $P < 0.05$ and **: $P < 0.01$. For each animal, measurements were taken for villus height, crypt depth and thickness of the muscularis externa from 5-6 tissue segments of the proximal duodenum and distal ileum.*

IGF-I effects were less pronounced in the ileum as increases in mucosal and non-mucosal tissue thickness were only observed in the rat pups treated with LR³IGF-I (Table 5.10). As described by Altmann and Enesco (1967), the small intestine exhibits a proximo-distal gradient with villus size and crypt depth decreasing in caudal direction. Similarly, in the present study villus height, crypt depth and the thickness of the muscularis externa in the distal ileum were lower compared to the duodenum. This indicates that although mucosal growth was stimulated by administration of IGF-I, the negative proximo-distal gradient was not perturbed (Table 5.10).

5.5.6 IGF-I effects on proliferative parameters

The tritiated thymidine labelling index and the crypt cell population were calculated for the duodenum and the ileum of all animals. This was achieved by counting the number of cells along the longitudinal crypt axis (crypt column count) and around the circumference in serial sectioned material as outlined in Chapter 4, section 4.2.6. The thymidine labelling index was

calculated for 30 full-length open crypts as described in Chapter 4, section 4.2.6. Following administration of IGF-I peptides, the number of cells per crypt column in the duodenum increased from 11.8 ± 0.3 in the vehicle treated rat pups to 13.0 ± 0.3 in the IGF-I treated rats ($12 \mu\text{g}/\text{g}/\text{day}$). In the highest dose of LR³IGF-I treated rats, the crypt column count increased to 14.7 ± 0.6 cells (Table 5.11). Similarly, in the ileum, the number of cells per crypt column increased from 11.3 ± 0.6 cells in the vehicle treated group to 12.6 ± 0.2 cells in the rat pups treated with $5 \mu\text{g}/\text{g}/\text{day}$ of LR³IGF-I (Table 5.11). The circumferential cell count was not altered in either the duodenum or in the ileum. This contrasts to the findings in the adult rat studies, where the circumferential cell count was significantly increased following IGF-I peptide treatment. The thymidine labelling index in the duodenum was 26% for the vehicle treated and up to 30% for the rats treated with LR³IGF-I (Table 5.11). Although thymidine labelling was increased, statistical significance was not achieved ($P=0.4$, ANOVA). Similarly in the ileum, thymidine labelling increased from 28% (vehicle) to 30% in rats treated with the highest dose of LR³IGF-I (Table 5.11) with no statistically significant difference between treatment groups. It appears that although the crypt cell population in the duodenum and ileum significantly increased in the rat pups treated with IGF-I peptide, the incorporation of tritiated thymidine increased in proportion to crypt size (Table 5.11), so that the proliferative response of the intestinal mucosa in suckling rats is similar to that observed for the adult rats.

Table 5.10: Proliferative parameters in the duodenum and ileum of suckling rats treated for 6.5 days with IGF-I peptides.

Treatment group	Vehicle 0.1M acetic acid	IGF-I 2µg/g/day	IGF-I 5µg/g/day	IGF-I 12.5µg/g/day	LR ³ IGF-I 2µg/g/day	LR ³ IGF-I 5µg/g/day
<i>Duodenum</i>						
crypt column count (no. cells)	11.8±0.3	12.4±0.4	13.1±0.2**	13.0±0.3*	13.1±0.3**	14.7±0.6**
crypt row count (no. cells)	11.2±0.2	11.1±0.1	11.6±0.3	11.3±0.2	12.0±0.4	11.6±0.4
crypt cell population (no. cells)	131.5±3.6	137.2±4.3	152.5±4.4*	147.6±3.3*	153.4±6.0**	171.3±8.0**
thymidine labelling index (%)	26.2±0.6	28.0±1.1	27.8±1.6	27.5±1.4	28.2±1.2	30.2±2.0
<i>Ileum</i>						
crypt column count (no. cells)	11.3±0.3	12.0±0.4	11.6±0.1	10.9±0.1	11.7±0.2	12.6±0.2**
crypt row count (no. cells)	10.6±0.1	10.3±0.1	10.3±0.2	10.3±0.1	10.6±0.2	10.5±0.2
crypt cell population (no. cells)	119.5±2.6	123.7±5.3	119.1±2.2	111.9±1.6	124.6±2.5	131.8±1.9**
thymidine labelling index (%)	28.2±1.2	30.9±1.2	27.4±1.1	27.7±1.6	27.7±1.1	30.0±2.3

Values are means±SEM with 6-8 rat pups per treatment group. Statistical significance from the vehicle treated rats is indicated by *: $P<0.05$ and **: $P<0.01$ as determined by ANOVA.

5.5.7 Biochemical estimation of disaccharidase activity

To determine if administration of IGF-I peptides to suckling rat pups alters the digestive capacity of the intestine, the activities of the disaccharidases lactase and sucrase were measured in mucosal scrapings from a 4 cm jejunal tissue segment. Lactase activity, which is high at birth and during nursing when milk is the sole nutrient, declines to low levels at the time of weaning (approximately day 20 *post partum*), Doell and Kretchmer, (1962). In contrast, at birth, the activities of sucrase activity as well as other α -disaccharidases is minimal and then increases to reflect functional maturation of the intestine to handle carbohydrates in the adult diet (Henning et al., 1975, Rubino et al., 1964).

In the present study, a 60% increase in lactase activity, measured as nmol of glucose produced per minute per cm of jejunal mucosa, was recorded following administration of 5 μ g/g/day of LR³IGF-I (Table 5.12). At the same time, sucrase activity remained low in all groups, indicating that IGF-I peptides did not induce enterocytes to express their mature digestive enzyme pattern at this stage (Table 5.12). An attempt was made to measure the DNA and protein content in the mucosal scrapings so that sucrase and lactase activities could be expressed as specific activities (per DNA or protein content). However, the amount of mucosal tissue obtained from the 4 cm scrapings (60-70mg) precluded accurate measurements of DNA and protein even when samples were minimally diluted during the assay procedures. Because the amount of DNA and proteins measured in were near minimal detectable levels, I considered the data un-reliable. In addition, expression of sucrase and lactase activities per mg of mucosal protein or DNA may be somewhat deceiving because the two disaccharidases are expressed exclusively in the brush border and may be better compared to the protein content of the same specific cellular compartment (for examples brush border vesicle preparations) rather than total mucosa plus submucosa of which it represents only a small fraction.

Table 5.11: Lactase and sucrase activity in jejunal tissue homogenates from suckling rats treated for 6.5 days with IGF-I peptides.

Treatment group	Lactase (nmol Gluc./min/cm)	Sucrase (nmol Gluc./min/cm)
Vehicle (<i>0.1M acetic acid</i>)	290±32	10.7±0.9
IGF-I 2µg/g/day	309±62	7.53±2.0
5µg/g/day	288±28	8.4±1.2
12.5µg/g/day	315±20	6.5±1.4
LR³IGF-I 2µg/g/day	347±21	19.3±12.3
5µg/g/day	469±61*	9.0±1.8

Values are means±SEM for 6-8 rat pups per treatment group. Statistical significant difference from the vehicle group is indicated by *: $P < 0.05$ as detected by ANOVA.

5.5.8 *Histochemical detection of sucrase and lactase activities along the villus axis*

To determine if administration of IGF-I peptides altered the pattern of lactase and sucrase activities along the villus axis, a histochemical detection assay was employed. In vehicle treated rats, lactase activity was low in enterocytes located near the crypt:villus junction. Lactase activity then increased rapidly to reach maximum expression in the mid-villus region, in enterocytes between 100µm and 400µm. Expression of enzyme activity declined towards the tip of the villi (Figure 5.9 A). Similarly, in rat pups treated with IGF-I, lactase activity was also low in enterocytes positioned at the base of the villi. The lactase activity profile in this group differed slightly from the profile obtained in the vehicle treated rats in that enzyme activity, as measured by the Ab of the enzyme colour product, was marginally lower in enterocytes located up to 200µm, however no statistically significant difference was detectable by ANOVA (Figure 5.9 B). Enterocytes located at approximately 300µm showed maximal enzyme activity which also reached marginally higher values than those observed for the vehicle group (Figure 5.9 B).

The enzyme distribution profile for the rat pups treated with 5 μ g/g/day of LR³IGF-I was similar to the profiles obtained for the rats treated with 12.5 μ g/g/day of IGF-I (Figure 5.9 C). However, because the villi were significantly longer in this group (629.5 \pm 13.3) when compared to either the vehicle treated rat pups (495.8 \pm 15.0) or the IGF-I group (582.3 \pm 7.7), lactase activity was still detectable in enterocytes located above 650 μ m. This is clearly demonstrated in Figure 5.9 C. Therefore, when comparing the biochemical lactase data with the histochemical lactase profiles, the increase in lactase activity in the LR³IGF-I rats, detected by biochemical analysis, reflects the increase in mucosal mass and is most likely related to a greater pool of enterocytes expressing lactase activity. Although the villus cell population was not determined, it can be assumed that the cellularity of the villus compartment greatly increased in LR³IGF-I treated rats. No statistically significant difference in lactase activities (Ab) at the pre-determined distances (50, 100, 250, 400, or 500 μ m) was detected between the three groups by ANOVA (Figure 5.9 A-D). A representative photomicrograph of histochemical detection of lactase activity along duodenal villi is shown in Plate 5.2. Sucrase activities were not detectable in the frozen section of rat pups treated with either vehicle or IGF-I peptides, indicating that sucrase activity was not precociously induced at this stage of gut maturation by administration of IGF-I peptides. These results are in agreement with the biochemical data.

In the paraffin embedded tissue material villus height measurements were approximately 15% lower compared to villus height measurements in frozen sections. The magnitude of the difference in measurements was, however, similar for all 3 groups (15%, 14% and 17% for the vehicle, IGF-I and LR³IGF-I group, respectively). The discrepancies in measurements are the results of a number of factors, including shrinkage of the tissue during processing for paraffin embedding, and the fact that in the frozen sections villus height was measured along the periphery rather than through the villus core.

5.5.9 Plasma insulin levels

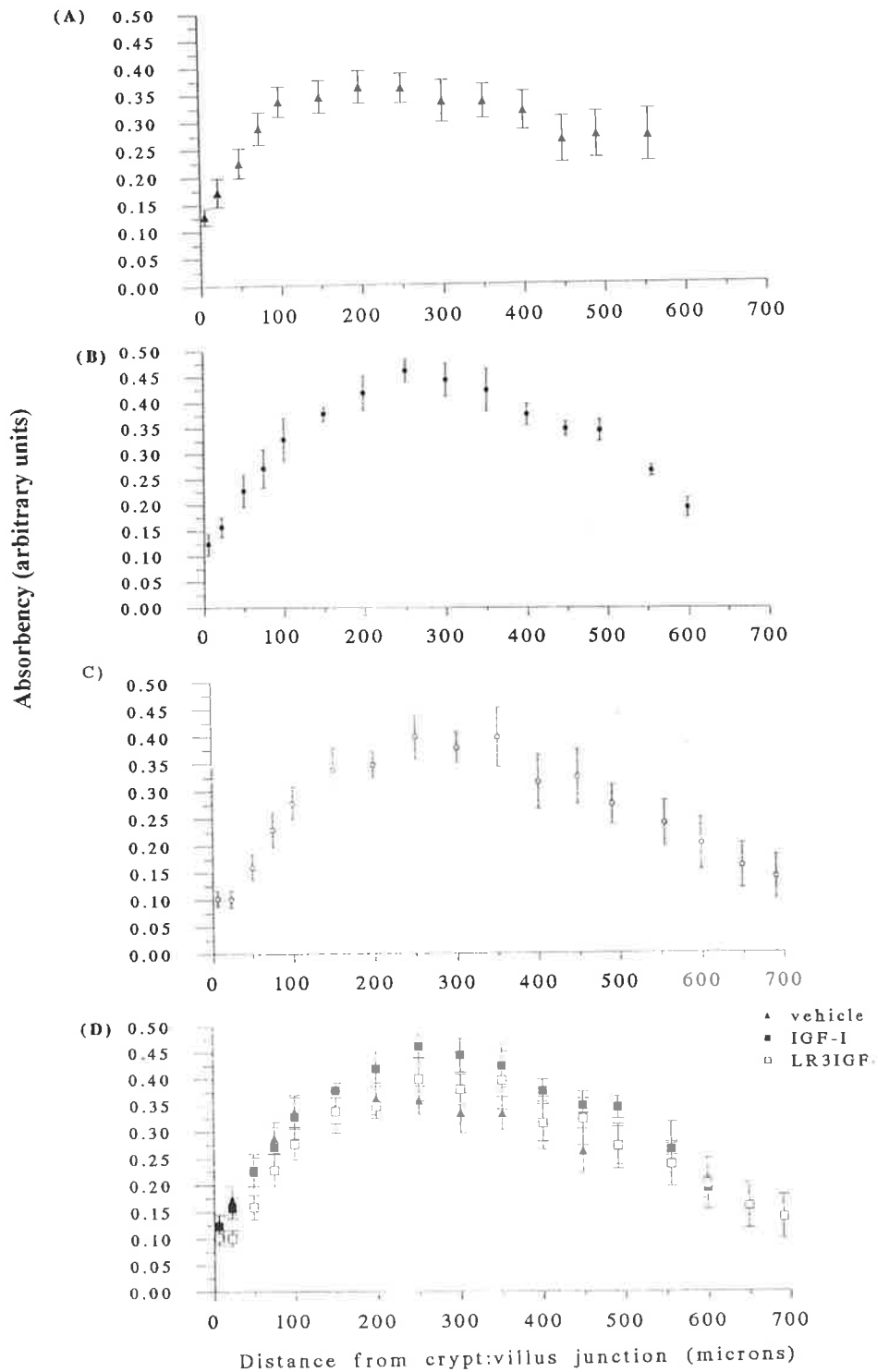
To determine if administration of IGF-I or LR³IGF-I influences changes in insulin secretion, plasma insulin levels were measured for each animal at the end of the 6.5 day peptide treatment period. Serial sampling of blood by tail vein bleeding was not carried out in these young rats as this invasive technique may have greatly influenced the outcome of the proliferative data and enzyme activity data due to stress-related stimulation. Although in all treatment groups plasma insulin levels were much lower compared to adult levels (which range between 10-13 μ U/ml), administration of 5 μ g/g/day of LR³IGF-I significantly reduced plasma insulin levels compared to vehicle treated rats (Table 5.12). In contrast, administration of the same dose of IGF-I significantly elevated plasma insulin levels well above values measured for the vehicle treated rats as well as all other peptide treated groups (Table 5.12).

Table 5.12: Insulin levels measured in plasma from suckling rats treated with vehicle or IGF-I peptides.

	Vehicle	IGF-I 2 μ g/g/day	IGF-I 5 μ g/g/day	IGF-I 12.5 μ g/g/day	LR ³ IGF-I 2 μ g/g/day	LR ³ IGF-I 5 μ g/g/day
Plasma insulin (μ U/ml)	5.95 \pm 0.85	7.87 \pm 0.50 ^a	4.97 \pm 0.4	5.37 \pm 0.73	4.30 \pm 0.57	3.87 \pm 0.38 ^b

Values are means \pm SEM for 6-8 rats per group. A significantly greater plasma insulin level compared to either vehicle treated rats or all other peptide treated groups is indicated by a: $P < 0.01$. "b" indicates a significantly lower plasma insulin level compared to the vehicle rats receiving 0.1M acetic acid.

Figure 5.9: Distribution profile of lactase activity along duodenal villi in suckling rat pups treated with vehicle (A), 12.5 μ g/g/day of IGF-I (B) or 5 μ g/g/day of LR³IGF-I (C).



Values are means \pm SEM for 6-8 rats per group. The Ab of the enzyme reaction product was measured under initial rate conditions from the base of crypt:villus junction to the villus tip in 6 right hand villi structures as described in the method section. Figure 5.8D shows the profiles for vehicle (\blacktriangle) IGF-I (\blacksquare) and LR³IGF-I (\square) superimposed for comparison.

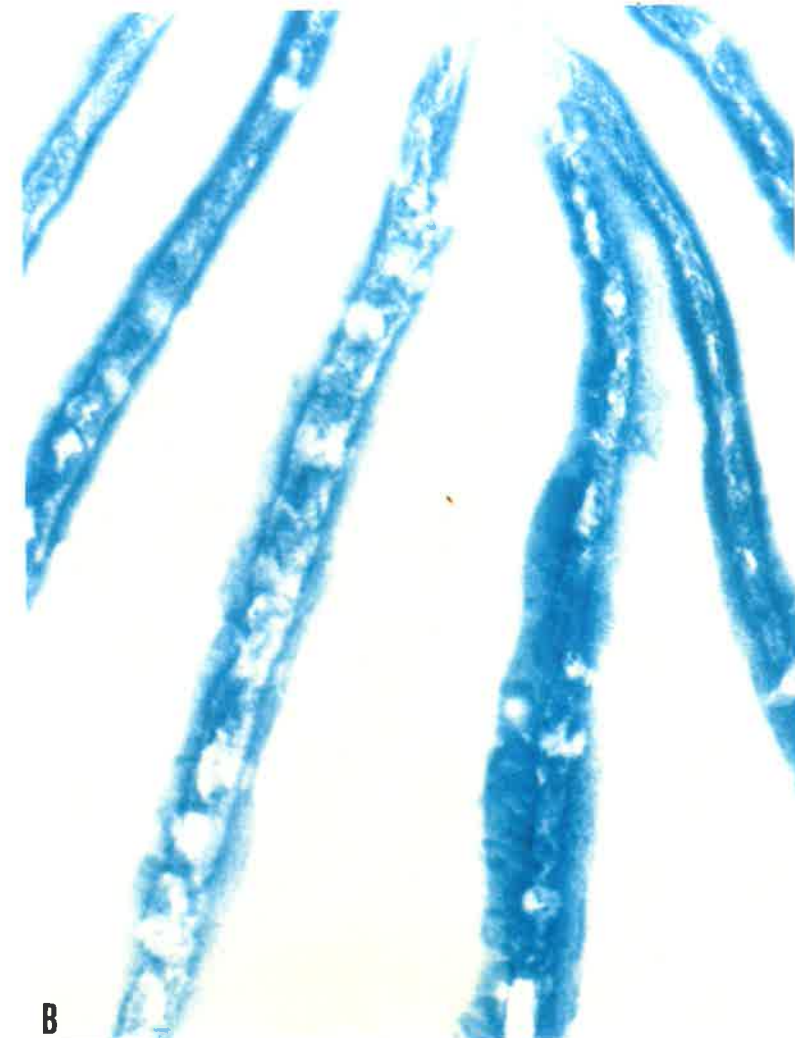
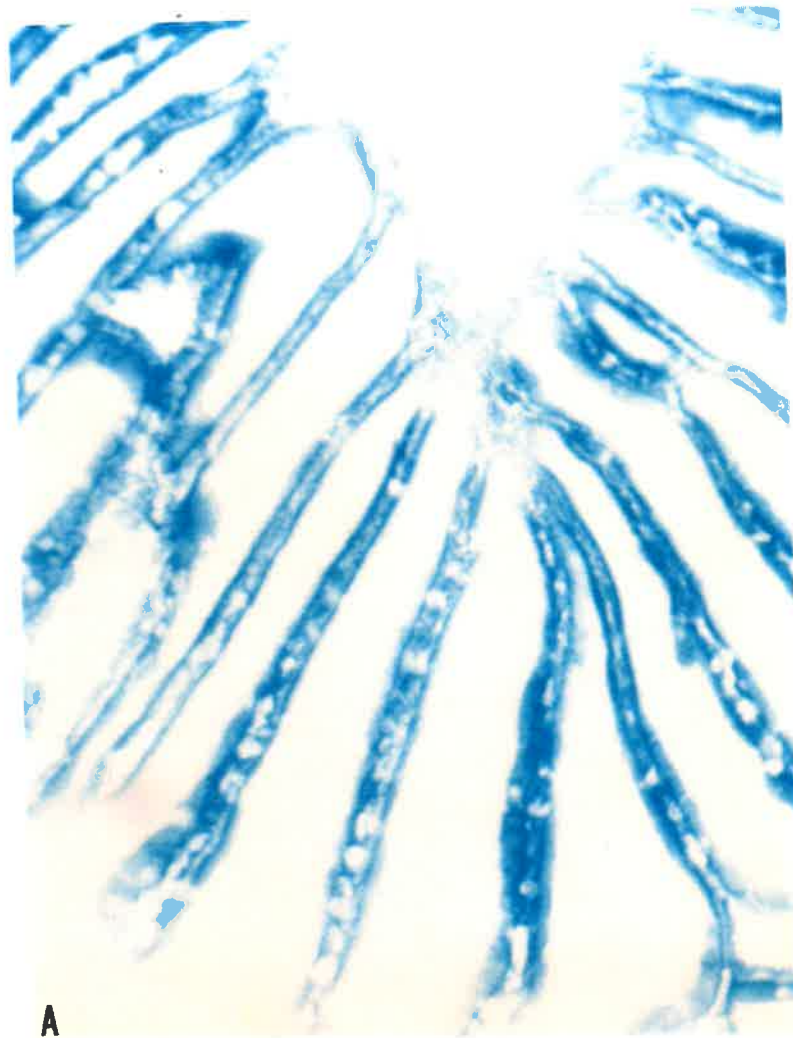


Plate 5.2: Representative micrographs of histochemical detection of lactase along duodenal villi in 13 day old suckling rats treated for 6.5 days with 12.5 μ g/g/day of IGF-I (**A and B**). Cryostat sectioned duodenum was incubated in saturating concentrations of 5-Br-4-Cl-3-indolyl- α -frucopyranoside for 15 minutes. Lactase activity, was detected in the brush border of villus enterocytes and appeared blue. Enzyme activity was measured by measuring the intensity of the enzyme reaction colour product along duodenal villi from the base to the tip of duodenal villi. Photographs were taken at a magnification of x 125 for (**A**) and x 400 (**B**).

5.6.1 IGF-I peptides responses in suckling rats compared to adult rats

The present study has demonstrated that administration of IGF-I peptides, in particular the LR³IGF-I analog, selectively stimulates gastrointestinal growth and maturation of intestinal function during the early suckling period in rats. Some of the IGF-I effects were similar between the adult and suckling rats; however, others differed considerably.

5.6.1.1 Plasma IGF-I levels in suckling and adult rats

Plasma IGF-I levels measured in rat plasma of untreated or vehicle infused 13 day old suckling rats were markedly lower than plasma IGF-I levels measured in adult rats. This is consistent with reports showing that IGF-I levels in neonatal rat plasma vary considerably with developmental age. For example, IGF-I levels rise from approximately 80ng/ml in late foetal rat serum (19 days gestation) to approximately 500ng/ml by postnatal day 25 (Glasscock et al., 1991). In the present investigation, plasma IGF-I levels in untreated and vehicle infused rat pups on the 13th postnatal day were determined at 40-80ng/ml. Similar IGF-I levels were measured by Robinson et al. (1993) in normal neonatal rat pups of the same strain. In their study, IGF-I concentration was also below 50ng/ml of plasma for 7 day old rat pups, increasing to approximately 75ng/ml by day 15.

Plasma IGF-I levels from the IGF-I treated rats described in Chapter 3 have been reported by Tomas et al. (1993). In their control or vehicle treated rats, plasma IGF-I concentrations, as measured by radioimmunoassays in acid-gel filtered, chromatographed plasma samples, were calculated between 400 to 440ng/ml (Tomas et al., 1993). Similar levels (350-800ng/ml) of plasma IGF-I in adult rats have been reported by others (Donovan et al., 1990, Benedict et al., 1994, Lemmey et al., 1991 and Vanderhoof et al., 1992).

IGF-I levels increased in a dose-dependent manner up to 6 fold following administration of the highest dose of IGF-I (12.5µg/g/day). In congruence, in adult rats, IGF-I levels in plasma were also increased in proportion with the infused IGF-I doses (Appendix 5.6). Most interestingly, however, infusion of comparable doses of IGF-I (2.8µg/g/day in the adult rats and 2 µg/g/day in the suckling rats) resulted in a similar increase in plasma IGF-I levels for both groups. This point is of interest, because plasma binding profiles in suckling rats differ markedly from the plasma binding profile in adult rat. This will be discussed later.

Infusion of LR³IGF-I did not produce any significant changes in the measured IGF-I levels in both the suckling rat and the adult rat studies. Interpretation of IGF-I levels as determined by radioimmunoassays employed in this thesis is difficult because of the difference in cross-reactivities of the different types of IGF-I. In the IGF-I infused rats, endogenous rat IGF-I and rhIGF-I were present in combination that may have varied with the different dose rates due to suppression of endogenous IGF-I by the infused peptide. Similarly, plasma of LR³IGF-I infused rats would contain a mixture of endogenous rat IGF-I and infused human recombinant LR³IGF-I in proportions that could not be estimated accurately by RIA. Thus, it is not possible to determine the relative amount of IGF-I present in the circulation following infusion of IGF-I peptides. Even so, it is clear that infusion of rhIGF-I significantly elevated plasma IGF-I levels in suckling rats in a dose dependent manner, while it is highly unlikely total IGF-I was increased by the rapidly cleared analogue LR³IGF-I (Bastian et al., 1993). Another contributing factor is the greatly reduced affinity of LR³IGF-I to IGF-BPs, so that despite a three-fold lower affinity to the type 1 receptor, rapid transfer of IGF-I from the circulation to the tissues would greatly enhance stimulation of cellular events.

5.6.1.2 *Body weight and organ weights:*

Despite the marked elevation in plasma IGF-I in the IGF-I treated rat pups, administration of the rhIGF-I preparation for 6.5 days did not stimulate body weight gain. In contrast, administration of the far more potent IGF-I analog, LR³IGF-I, stimulated body gain when administered at 5µg/g/day. Body weight gain in response to IGF-I administration have been previously reported. An increase in body weight gain following twice daily s.c. injection of 1.8µg/g body of IGF-I for a 12 day period has been demonstrated by Philipps et al. (1988). However, upon closer examination of their data, almost identical increments in body weight gain were recorded for the peptide and vehicle treated animals during the first 9 days of treatment; statistically significant divergence in the growth curve only became evident from the 10th treatment day onwards. At the same time, a second, less potent IGF-I preparation, produced in yeast rather than *Escherichia coli*, failed to improve body weight gain in the same study (Philipps et al., 1988). In congruence with the results from the present study, in hypophysectomised neonatal rats, continuous infusion of approximately 1.9µg/g/day for a 8 day period also failed to improve body weight gain above that observed for their sham hypophysectomised litter mates (Glasscock et al., 1992).

The lack of body weight gain in the suckling rat pups contrasts the marked stimulation of body weight gain in adult rats described in Chapter 3, where administration of IGF-I peptides, at doses ranging from 1-3µg/g/day, significantly improved body weight gain. Likewise, normal adult rats injected for 7 days with approximately 1.2µg/g/day of IGF-I also showed markedly improved body weight gain compared to saline treated controls (Hizuka et al., 1986). Data from our own laboratory has shown that administration of up to 3µg/g/day of IGF-I or LR³IGF-I for 7 days to adult male rats also significantly stimulates body weight gain (Steeb, unpublished). It therefore appears that in suckling rats stimulation of somatic growth depends on the route of peptide administration as well as the potency of the preparation and

the developmental stage at which IGF-I peptide administration is started. The latter may be related to the increasing GH dependency with increasing developmental age in suckling rats.

5.6.1.3 Selective action on non-gut organs

Although body weight gain was not observed in IGF-I treated rat pups, selective effect on organ growth by IGF-I peptides was demonstrated in the present study. In agreement, the weight of the liver, brain, heart and testes increased in response to IGF-I administration in normal neonatal rats (Philipps et al., 1988). Increased weights of the spleen, kidney and lungs have been reported in hypophysectomised neonatal rats infused with IGF-I (Glasscock et al., 1992). These effects are, however, often diminished when fractional organ weights are calculated (organ weight expressed per kg of body weight). Under various experimental conditions in adult rats, *in vivo* administration of IGF-I peptides also selectively increases the weight of several organs (Asakawa et al., 1992, Van Buul-Offers et al., 1988, Skottner et al., 1989, Tomas et al., 1991 and 1993, Martin, et al., 1991 and Lemmey et al., 1991, Guler et al., 1988 and Binz et al., 1990). Moreover, in normal adult rats, selective increase in organ weights has been reported (Hizuka et al., 1986). The organs most regularly reported to respond are the kidney, spleen, thymus, adrenals, testes and the heart.

In the present investigation, the two organs that most noticeably increased following IGF-I peptide administration were the spleen and the kidney. Evidence exists to show that splenic growth is mediated in part by IGF-I. For example, in hypophysectomised rats, splenic growth was greater following infusion of hrIGF-I, compared to GH replacement (Guler et al., 1988). Thus, in a pituitary intact animals, GH effects on splenic growth seem to be mediated by IGF-I. A massive increase in the fractional weight of the kidneys following administration of LR³IGF-I was also observed in the present study. Since clearance studies have indicated that the distribution of des(1-3)IGF-I, which also shows a greatly reduced binding to several of

the IGF-BPs, was greatest in the kidneys compared to other organs, it is tempting to speculate that in the LR³IGF-I infused rats more free IGF-I (LR³IGF-I and/or displaced endogenous IGF-I) may have preferentially stimulated growth of the renal tissues.

5.6.1.4 Gastrointestinal response to IGF-I peptides in the neonatal period

The present study has shown that although body weight gain was altered only in the rat pups treated with the highest dose of the IGF-I analogue, stimulation of gastrointestinal growth was also seen in the rats treated with the native IGF-I. While a dose dependent increase in wet tissue weight of most gastrointestinal components was observed for the more potent IGF-I analogue, a rather flat dose response was observed with IGF-I. Furthermore, in the rats treated with IGF-I, the weight of gastrointestinal components increased in proportion with body weight gain so that the fractional gut weight was not different from the fractional gut weight in vehicle treated rats. As for the adult rats, the proximal small intestine, in particular the duodenum, was the most peptide responsive region.

A marked increase in large intestinal weight was observed in LR³IGF-I treated rats. This reflects an increase in cross-sectional mass as large intestinal length of suckling rat pups was only marginally affected by peptide treatment. Similarly, in normal adult rats, treated for either 3 or 14 days with IGF-I peptides, large intestinal length was also unaltered after administration of IGF-I peptides. This is somewhat surprising because in both, suckling and adult rats, IGF-I receptors density is greatest in the colon and terminal ileum compared to the proximal small intestine (Heinz-Erian et al., 1991, Laburthe, et al., 1988 and Young et al., 1990). Moreover, both receptors (type 1 and type 2) are present in suckling and adult rats and IGF-I binding to the immature intestine appears to be 2-2.5 times greater than to the adult intestinal tissue (Young et al., 1990).

Consistent with the data from the adult rat studies, increases in tissue mass were reflected in the histology of the tissues. The intestinal epithelium of the suckling rats showed a high level of structural development, characterised by villi lined with a single epithelial layer and a well defined brush border, but villi and crypts were much shorter in the suckling rats as in the adult rat intestine. A relative increase in the cross-sectional thickness of the mucosa was, however, evident even at the lower doses of IGF-I peptide. In addition, the cross-sectional thickness of the muscularis externa was also significantly increased in the suckling rats treated with IGF-I peptides. Conversely, in the adult rats, the muscularis externa appears unresponsive to the peptide treatment. In the immature intestine, IGF-I may therefore act as a more general growth promoter on a variety of intestinal tissues, while in the mature intestine, IGF-I action may be restricted more specifically to the maintenance of intestinal proliferation.

5.6.1.5 Histological changes in the rat intestine following administration of IGF-I peptides

During postnatal development of the rat intestine, distinct morphological changes of the intestinal epithelium occur. These result in an increase in the number of cells per crypt (crypt depth), cells per villus (villus height), the crypt girth (crypt circumference) and the number of crypts per villi (crypt villus ratio) (Herbst and Sunshine, 1969). For example, there is a 23% increase in duodenal villus length between postnatal day 6 and 22 (Yeh, 1977) and a 50% increase in villus number between the 10th and 30th postnatal day (Klein and McKenzie, 1983a). The underlying connective tissues and the muscularis externa follow a similar pattern. For example, intraepithelial lymphocytes increase several hundred fold between birth and 3 weeks *post partum* (Orlic and Lev, 1977).

In agreement with previous studies, duodenal villi and crypts in the suckling rats in the present study were approximately 40% and 72% shorter than those obtained in the adult rats. Although IGF-I peptide administration significantly increased these parameters, both

compartments were still significantly smaller than those in the adults. In addition, in the suckling rats, duodenal villi were long finger-like structures with similar width at the villus base and tip. In comparison, the adult rat duodenum exhibited typical leaf-like villus structures. Following treatment with IGF-I peptides, the number of cells per crypt column were markedly increased in suckling rat pups. On the other hand, the circumferential cell count, which was significantly greater in the adult IGF-I peptide treated rats, appeared unaltered in the suckling rats. This is not surprising, because previous research has demonstrated that in the duodenum of suckling rats, a 120% increase in crypt depth occurred between postnatal day 6 and 22, while crypt girth increased simultaneously by only 33% (Yeh, 1977a). It therefore appears that crypt elongation precedes expansion of the crypt and this contingency was not perturbed by the peptide treatment.

5.6.1.6 Proliferative response of the immature small intestinal epithelium

Kinetic differences of the suckling rat intestine compared to the adult include lower crypt cell birth rates and mitotic indices and markedly slower cell migration rates; furthermore, cell cycle times during the early neonatal period are also significantly longer (Klein, 1977, and Altmann and Enesco, 1967). For instance, cellular migration is 48 hours in adult rats and mice, while in suckling rats migration is characteristically much slower and transit time along the length of the villus has been approximated at 96 hours (Koldovsky et al., 1966 and Herbst and Sunshine, 1969). Therefore, the mechanism of growth in the neonatal intestine differs immensely from that in adult rats. In the suckling rat, the intestinal tissues need to continuously grow and at the same the dynamic equilibrium between crypt cell production and cell loss needs to be maintained.

As demonstrated in the adult rat studies, administration of IGF-I peptides resulted in an increase in proliferative activity in the duodenum and ileum which was proportional to

increases in crypt size. Nevertheless, in suckling rats the intestinal thymidine labelling indices were consistently lower compared to these in the adult rats. For instance, in the duodenum, thymidine incorporation was approximately 26% in the suckling rats; conversely, in the adult rats approximately 30% of duodenal enterocytes incorporated the thymidine label. In agreement with these findings, Klein, (1977) has shown that following a pulse of tritiated thymidine administration, the labelling index in the ileum of 8 day old suckling rats was approximately 27%. Similarly, Yeh, (1977a) has shown that in 16 day old rat pups 27% of duodenal enterocytes were labelled with tritiated thymidine. In the adult rats, the data is less uniform and thymidine labelling indices between 30 to 40% have been reported (Messier and Leblond, 1960 and Klein and Torres, 1978).

Increased proliferative activity in proportion to increasing crypt size following administration of IGF-I peptides indicates that the normal equilibrium between cell loss and cell production has been maintained in these otherwise rapidly growing animals. Furthermore, it also indicates that similar to the observation in the adult rats, a new steady state between cell production and cell loss was reached after only 6.5 days of administration of IGF-I peptide (discussed in detail in Chapters 3 and 4). The growth pattern during the postnatal period differs from the adult rats in that organ growth in the suckling rats is still incomplete and takes place at an accelerating rate. Nevertheless, the present results indicated that during the early suckling period, IGF-I activity was directed not only to maintained the homeostasis of the continuously renewing intestinal epithelial, but also to promote normal intestinal growth and functional re-differentiation.

5.6.2 Plasma binding protein profiles

In the present investigation, an attempt was made to determine the IGF-BPs in plasma samples from 13 day old suckling rats, treated for 6.5 days with IGF-I peptides. The total

amount of plasma that could be collected was very small so that only pooled samples from each treatment group were available for Western ligand blot analysis. Unfortunately, the quality of the blot is not the best, thus interpretation of the ^{125}I -IGF-I bands was difficult.

Overall, it appeared that the IGFBP pattern of bands seen on the Western ligand blot was typical for those previously reported for rodent serum (Clemmons et al., 1989, Zapf et al., 1989, Breese et al., 1991, Benedict et al., 1994, Donovan et al., 1989 and Glasscock et al., 1990 and 1991). The apparent intensities of the IGFBP bands were difficult to assess, because of their low intensities which could not be further enhanced by prolonged exposure. The two major bands in the pooled plasma samples from the suckling rats migrated at approximately 28-30 kDa and 24 kDa. The 28,000-30,000 Mw protein most likely represents IGFBP-2. In agreement, Margot et al. (1989) have shown that the predominant IGFBP in neonatal rat plasma is a non-glycosylated protein with an apparent molecular weight of 29,500, identified as IGFBP-2 by immunoblot (Donovan et al., 1989). In the rat at the time of weaning, IGFBP-2 disappears and simultaneously the levels of the major adult binding protein, IGFBP-3, increase (Donovan et al., 1991 and Shimasaki et al., 1989a). IGFBP-1 in rat plasma is difficult to identify by Western Ligand blot since its approximate molecular weight (28,000) is similar to rIGFBP-2 (Yang et al., 1990). Due to its apparent molecular weight, the 24,000 kDa band visible on the Western ligand blots in the present study most likely represented rIGFBP-4 (Shimasaki et al., 1990).

The bands representing glycosylated forms of rIGFBP-3 were virtually not detectable; however, in rat serum from older suckling rats, juvenile and adult rats, rIGFBP-3 appears as a doublet migrating at approximately 40,000-46,000 Mw (discussed in Chapter 6). It has been previously reported that in normal suckling rat pups, these bands become apparent at approximately postnatal day 19 (Donovan et al., 1991), but this may be delayed following

nutrient restriction. For example, in GH intact, nutrient restricted rat pups, the expression of rIGFBP-3 as well as serum IGF-I and IGF-II levels were reduced whereas IGFBP-1 and IGFBP-2 levels were enhanced (Donovan et al., 1991). This indicated that the nutritional status is important in the regulation of IGFBPs. Thus, if the action of IGFBPs is to inhibit the bio-availability of IGFs to the tissues, then a reduction in serum IGF-I/II levels combined with an increase in IGFBPs as observed in Donovan's study may limit somatic growth so that nutrients are available for metabolism. Provided that in the present study, nutrient availability was similar for all rat pups, it may be hypothesised that treatment of rat pups with IGF-I at a later stage in lactation may precociously induce the expression of IGFBP-3 compared to vehicle treated rats of the same age. This hypothesis has been tested in the next Chapter.

5.6.3 Growth effect or metabolic effects?

The design of the study did not permit the quantification of the amount of milk suckled by the pups, but all rat pups appeared very well nourished throughout the experiment. In addition, the treatment groups were randomised across the litter. Since all litters comprised 9 rat pups per dam, litter specific artefacts can be discounted. However, the possibility that milk intake in the suckling rats may have varied between treatment groups cannot be excluded. Nevertheless, IGF-I treatment of adult rats does not increase food intake as shown in Chapters 3 and 4 and also reported by others (Martin et al., 1991, Lemmey et al., 1991 and Tomas et al., 1993).

In the present study glucose end-point measurements were not taken because of the extremely small amount of blood sample available. Although in the preliminary study infusion of 12.5 µg/g/day of LR³IGF-I induced a severe state of hypoglycaemia that was toxic to the rat pups, none of the rats treated with up to 12.5 µg/g/day of IGF-I or with doses up to 5 µg/g/day of LR³IGF-I showed visible signs of hypoglycaemia. In the LR³IGF-I treated rat pups, a dose-

dependent reduction in plasma insulin was evident at the end of the treatment period, suggesting that insulin-like metabolic effects may have been activated in these rats. For example, lowering of blood glucose levels and free fatty acids (Guler et al., 1987), and a decrease in insulin secretion (Guler et al., 1989a and Jacob et al., 1989) have been reported following administration of IGF-I. While it is believed that these effects are mediated mainly by the interaction of IGF-I with the type 1 receptor, recent work in cultured hepatocytes from adult rats has shown that short-term and long-term metabolic effects of IGF-I are also mediated through the insulin receptor (Hartmann et al., 1990).

In the rat pups treated with 2 μ g/g/day of IGF-I a significant increase in plasma insulin was recorded. This effect was not dose dependent so that rat pups treated with either 5 or 12.5 μ g/g/day of IGF-I showed plasma insulin levels comparable to those of vehicle treated rats. At present it is not clear if the increase in plasma insulin levels in these rats represents a real observation. Because the amount of milk intake by the rat pups could not be controlled in this study. Furthermore, because the suckling pups would not have suckled continuously, it is possible that, by chance, some rat pups in the IGF-I treatment group had been feeding for longer periods just prior to sacrifice. To establish if insulin levels in rat pups treated with 2 μ g/g/day of IGF-I were indeed increased by the infusion of the peptide or whether this increase is related to the feeding regime needs further investigation.

5.6.4 *Maturation of digestive enzymes*

The most striking changes that occur in the intestine during development are the decline of lactase activity and the increase in sucrase and maltase activities for the digestion of dietary carbohydrates (Henning and Kretchmer, 1973). The major carbohydrate in the diet of suckling rats is lactose (Jenness et al., 1964) and activities of lactase are detectable on the eighteenth day of gestation (Henning and Kretchmer, 1973). Lactase activity in suckling rats

reaches maximum during the early perinatal period and starts to decline to adult-like low levels at the end of the 3rd postnatal week (Doell and Kretchmer, 1962). In many species, including humans, lactase activities are lower in the adult compared to the newborn (Koldovsky, 1969).

In the present study, total lactase activity (measured biochemically in mucosal scraping from the jejunum) was significantly increased in the rat pups treated with the highest dose of LR³IGF-I. At the same time, mucosal mass was also increased in this group, so that the increase in lactase activity reflects the increase in mucosal mass. In congruence with the biochemical findings, histochemical distribution profiles showed that maximum lactase activity was similar in IGF-I treated and vehicle treated rats. Because the villus structures were significantly longer in LR³IGF-I treated rats, lactase activity was of course detectable, covering a greater distance along the longitudinal gut axis (crypt-villus axis), thus also reflecting an increase in total lactase activity. The low lactase activities at the base and towards the tip of the villi in all groups presumably reflect differences in lactase synthesis and degradation in intestinal enterocytes located at these positions. For example, Alpers et al. (1972) have shown that larger molecular weight proteins, including disaccharidases, have the fastest turnover rates. This implies that variations in localisation where synthesis begins and differences in the rate of lactase turnover may lead to distinctions in enzyme distribution along the villus-crypt axis. In agreement with the present study, Boyle et al. (1980) have shown that lactase activity is expressed maximally in the mid-villus region in rat jejunum.

IGF-I peptide treatment ceased on the 13th postnatal day, and in all groups lactase activities in the duodenum remained high. Because the developmental decline in lactase activity occurs at approximately the end of the 3rd postnatal week in rats, I did not expect to observe the developmental decline in lactase activity associated with weaning. Similarly, sucrase activity, was not detected histochemically in the duodenal section of rats treated

with either peptides or vehicle. In agreement with these findings, Lund et al. (1987) demonstrated that in 16-day-old suckling rat pups only trace amounts of α -glucosidase activity were detected histochemically at the base of villi. I therefore concluded that treatment of 6 day old suckling rat pups for 6.5 days with IGF-I peptides did not alter the normal developmental pattern of lactase and did not precociously induce sucrase activity. Although minimal sucrase activities were detected biochemically, this may be a reflection of hydrolysis of sucrose by disaccharidases other than sucrase and may also relate to the lower sensitivity of the assay procedure compared to the localisation studies. Regulatory factors that modulate intestinal lactase and sucrase activity will be discussed in detail in Chapter 6.

5.6.5 *Where to go from here?*

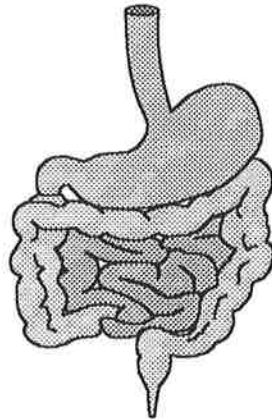
In summary, the present study has shown that administration of IGF-I peptides, and in particular LR³IGF-I, to normal, 6 day old suckling rats for a 6.5 day period selectively stimulated gastrointestinal growth. Mucosal and non-mucosal responses were evident. Within the mucosa, marked increases in the cellularity of the crypt and villus compartment were observed which were related to an increase in proliferative activity. The thymidine labelling indices of the duodenum and ileum were increased in proportion with crypt size. At the same time, lactase activity increased proportionally to villus length and sucrase activity was not precociously induced. Despite elevation in IGF-I plasma levels following administration of IGF-I, the IGFBP profile appeared unaltered by the peptide treatment. Furthermore, the normal balance between cell production and cell loss was not perturbed by the peptide treatment, indicating that IGF-I maintains intestinal proliferation in a manner similar to that observed for the adult rats. In addition, administration of IGF-I peptides did not alter the developmental profile of disaccharidase activity, suggesting that IGF-I does not influence the intrinsic maturation pattern of disaccharidase activity expression in the early neonatal period.

Thus, IGF-I peptides promote gut growth and epithelial proliferation at a time when overall organ growth occurs at its maximum.

Several questions remained un-answered. Firstly, does IGF-I affect gut growth in suckling rats that become increasingly GH and exhibit a more adult-like pattern of gastrointestinal growth and if so, are IGF-I responses more or less pronounced during this period compared to the early suckling period? Second, although the pattern of disaccharidase activity was not altered in rat pups treated during the earlier period, it is not clear if administration of IGF-I peptides influences the developmental decline in lactase activity that occurs at the end of the 3rd postnatal week. Finally, the effect of IGF-I peptide administration on the postnatal increase in intestinal sucrase activity will be investigated. The effect of IGF-I peptide administration on gut growth and maturation just prior to weaning will be addressed in a series of experiments described in Chapter 6.

CHAPTER 6:

ENHANCED GASTROINTESTINAL GROWTH AND INTESTINAL DISACCHARIDASE ACTIVITIES IN 12 DAY OLD SUCKLING RATS TREATED WITH IGF-I PEPTIDES



CHAPTER 6

Enhanced gastrointestinal growth and intestinal disaccharidase activities in 12 day old suckling rats treated for 6.5 days with IGF-I peptides

Contribution to the work

This Chapter describes a series of experiments that were conducted to elucidate the effect of IGF-I peptide administration on the gastrointestinal growth and maturation of intestinal disaccharidase activities in older suckling rats. The planning and execution all experiments including animal handling, surgical manipulation, gut tissue collection and preparation of histological tissue material was conducted by myself. Furthermore, the histocytochemical detection and Western Ligand blot analyses were also carried out by myself. In addition, I performed all analytical work, computer assisted morphometry, data comparison, analyses and interpretation. Assistance was provided by Mrs. Kerry Penning and Mrs. Leanne Srpek during animals handling and tissue collection. Mrs. Anna-Maria Mercorella assisted in the cutting of histological material and Mrs. Carolyn Mardell with the histocytochemical work. Mrs Cheryl Shoubridge performed the biochemical assays and the RIAs for plasma IGF-I. Mr. Michael Conlon helped in the collection and weighing of non-gut organs. Plasma insulin measurements were conducted by Dr. John Oliver, Flinders Medical Center, South Australia. Iodination of IGF-I was carried out by Mr. Spencer Knowles.

ABSTRACT

To determine the effects of IGF-I peptide administration on the growth of the gastrointestinal tract and on the maturation of intestinal function, 12-day old suckling rats (6-9 rat pups/group) were infused with IGF-I or LR³IGF-I at doses of 0, 2, 5 or 12.5 µg/g/day via s.c. implanted mini-osmotic pumps. Infusion of 5 and 12.5 µg/g/day of IGF-I significantly

increased plasma IGF-I concentration. Western ligand blot analysis showed enhanced expression of IGFBP-2/-1 and IGFBP-3 in rats infused with the highest dose of IGF-I and both doses of LR³IGF-I. Body weight gain increased in a dose-dependent manner by up to 41% (5µg/g/day of LR³IGF-I) above control values. Highly significant increases in wet tissue weights of visceral organs, most noticeably the kidneys and spleen, were recorded for all peptide treated rats. Similarly, gastrointestinal tissues selectively increased in response to IGF-I peptide infusion. Total gut weight, small and large intestinal weight as well as stomach weights increased by 57%, 58%, 46% and 61%, respectively, following treatment with 5µg/g/day of LR³IGF-I. Consistent with results from previous Chapters, the duodenum was the most peptide sensitive gut region and increased by up to 86% above control values. Small and large intestinal length contributed significantly to the massive increase in intestinal weight. Evidently, histological parameters reflected this increase and cross-sectional thickness was increased in mucosal and non-mucosal tissue compartments. As for the younger rats, the proximo-distal gradient was maintained so that increases in morphological parameters were less pronounced in the ileum. IGF-I peptides also influenced intestinal maturation. Lactase activity was markedly reduced in jejunal tissue homogenates of rats treated with 5µg/g/day of LR³IGF-I. Correspondingly, sucrase activity was precociously increased in the same treatment group. This findings were confirmed histochemically. Lactase activity was virtually undetectable in cryostat sectioned duodenum of LR³IGF-I treated rats. In analogous tissue sections, sucrase activity was enhanced and expressed in enterocytes located in higher positions along the villus axis. These results indicate that infusion of IGF-I peptides selectively increases somatic and visceral organ growth. This study also shows that rats are more sensitive to IGF-I infusion immediately prior to weaning, than during the early suckling period. This study has confirmed that IGF-I peptides selectively stimulate gastrointestinal growth and intestinal proliferation. Most importantly, however, IGF-I peptides also accelerate the reciprocal shift in disaccharidase activities of lactase and sucrase immediately prior to weaning.

6.1 INTRODUCTION

The previous Chapter has shown that continuous s.c. infusion of IGF-I peptides selectively stimulate gastrointestinal growth in neonatal rats. Moreover, several non-gut organs, including the kidney and spleen were amongst the most peptide sensitive tissues. The present Chapter describes a series of experiments, investigating the effect of IGF-I peptide administration on the growth of the gastrointestinal tract in older suckling rats. Firstly, the major focus of this study was to investigate the role of IGF-I peptide administration on the reciprocal shift in disaccharidase activities that occurs at the time of weaning in rats. Second, this study addressed, in general, whether the gastrointestinal tract and other non-gut organs are similarly responsive to treatment with IGF-I peptides in older suckling rats compared to the responses observed in rats during the early suckling period.

One of the major difference between younger rat pups and older suckling rat pups is their attenuating dependency on maternally derived nutrients with increasing age. Thus, in preparation for digestion of an adult-like diet at the time of weaning, functional adaptation of the immature intestine takes place involving the re-organisation of the intestinal epithelium. The shift from the infantile to the mature state of the intestinal epithelium is not gradual but rather occurs over a relatively short critical period. In the rat this process takes place between 19-24 days *post partum* (Yeh and Moog, 1975). The changes associated with functional maturation include a coincidental decrease in lactase activity, appearance of sucrase and a rapid increase in maltase, glucoamylase, trehalase and alkaline phosphatase (Henning, 1985 and Ménard and Calvert, 1991). Hence, the distribution and activity of enzymes associated with the microvillous membrane reflect functional demands throughout development.

There is ample evidence to show that intrinsic timing mechanisms as well as nutritive and hormonal factors regulated the changes associated with weaning (Doell and Kretchmer,

1964, Herbst and Koldovsky, 1972, Galand and Forstner, 1974 and Yeh and Moog, 1975). Most noticeably, EGF has been implicated to play an important role in the regulation of intestinal disaccharidases activity and expression (Malo and Ménard, 1982 and Foltzer-Jourdainne and Raul, 1990). In addition, a study conducted by Young et al. (1990) reported changes in disaccharidase activities following *in vivo* administration of IGF-I or IGF-II in rats. This group showed that administration of 1µg/day of IGF-I or IGF-II by either oral instillation or by once daily intraperitoneal (i.p.) injection significantly increased intestinal enzymes. Specific enzyme activity of maltase, lactase, alkaline phosphatase and aminopeptidase in the jejunum but not the duodenum or ileum were increased following administration of IGF-I or IGF-II by either route. On the other hand, orally but not i.p. administered IGF-II increased duodenal maltase, lactase and aminopeptidase activity as well as jejunal lactase activity. Surprisingly, administration of IGF-II by either route decreased aminopeptidase activity in the ileum, only. Although these findings were somewhat inconsistent, they suggested that IGF peptides may influence functional maturation and activities of disaccharidases and peptidases in the rat intestine. However, because peptide treatment was terminated well before weaning, the regulation of the developmental decline in lactase activity and the appearance of sucrase activity by IGF-I peptides were not addressed. Accordingly, the major aim of the study described in the present Chapter was to evaluate the effects of IGF-I peptide administration on gastrointestinal growth and in particular assess the pattern of intestinal lactase and sucrase activities in response to peptide administration just prior to weaning.

6.2 MATERIALS AND METHODS

6.2.1 *Recombinant IGF peptides*

Recombinant hIGF-I and hLR³IGF-I were purchased from GroPep Pty. Ltd., Adelaide, South Australia. Specifications of the recombinant peptides have been summarised in Chapter 2. Peptides were infused at 2, 5 or 12.5µg/g/day in a manner similar as described for the 6 day old suckling rat pups (Chapter 5, section 5.4.1). The average body weight in the 12 day old rat pups was approximately 22g, so that rat pups were infused at doses of 45, 114 or 285µg/day (IGF-I) and 45 or 114µg/day for LR³IGF-I.

6.2.2 *Experimental design*

All animal handling and experimental protocols employed in this Chapter followed closely the procedures described for the younger rat pups except that IGF-I peptide infusion started at day 12 *post partum* rather than on day 6. (Chapter 5, section 5.4.2). Accordingly, all rat pups in this study were treated for 6.5 days and the tissue and blood collection protocols were maintained as described in the Materials and Methods section in Chapter 5, sections 5.4.2-5.4.3. As summarised previously, non-gut organ weights were recorded for all animals at the time of death.

6.2.3 *Analytical measurements for the assessment of gut growth*

6.2.3.1 *Histology*

Quantitative morphometric analyses of villus height, crypt depth and the thickness of the muscularis externa was conducted in paraffin embedded tissues from the duodenum and ileum in vehicle and IGF-I and LR³IGF-I treated rats.. The methodologies and selection criteria for these measurements have been outlined in detail in Chapter 2, section 2.3.2. All

morphometric measurements were conducted on video captured images. For all histological parameters, 15 measurements were selected from well oriented duodenal sections as described in Chapter 4, section 4.2.5.

6.2.3.3 Measurements of mucosal DNA, protein and disaccharidase activities in jejunal tissue homogenates.

Biochemical analyses of the mucosal DNA and protein content as well as measurements of lactase and sucrase activities were carried out in mucosal (plus submucosal) scrapings from a 4cm jejunal tissue segment. Methodologies followed the protocols described in Chapter 5, section 5.4.4.3.

6.2.3.4 Histochemical detection of lactase and sucrase activities

Lactase and sucrase activity has been localised by histochemical detection of the disaccharidases in cryostat sectioned duodenum cut at 8 micron thickness. The preparation of the tissue segments, and the techniques for the localisation of lactase and sucrase were carried out as outlined for the younger rat pups in Chapter 5, section 5.4.4.4.

6.2.4 Measurements of plasma IGF-I levels and insulin

Similar to the study described in Chapter 5, plasma IGF-I concentrations were measured in individual plasma samples collected at the end of the peptide treatment period, on day 19 of postnatal life. Plasma IGF-I concentrations were measured by radioimmunoassay using a rabbit anti bovine IGF-I antibody after extraction of plasma IGFBPs by acid gel permeation chromatography. These procedures have been summarised in Chapter 5, section 5.4.4.5. The plasma insulin concentrations were measured for each animal in trunk blood at

the end of the peptide treatment period using a Pharmacia Phadeseeph radioimmunoassay kit. These measurements were performed by Dr. J. Oliver.

6.2.5 Western ligand blot analysis

The binding protein profiles in rat pups treated for 6.5 days with IGF-I peptides or vehicle were determined in pooled plasma samples from each of the treatment groups collected at the end of the treatment period on day 19. IGFBPs were determined by Western ligand blot analysis after electrophoresis of the pooled samples on SDS-PAGE following the general procedure adopted in Chapter 5, section 5.4.4.6.

6.2.6 Statistical analysis

Means \pm SEM have been reported in all Tables and Figures. Treatment groups were compared by one-way analysis of variance (ANOVA) and where significance was attained at $P < 0.05$, a Fisher's Protected Least Significant Difference *post hoc* was applied to the data to identify the between group difference. All statistical analysis was performed using the Super ANOVA software package (Abacus Conceptus, CA USA).

6.3 RESULTS

6.3.1 Plasma IGF-I levels

At the start of the peptide treatment period, the IGF-I plasma level measured in pooled sample from 3 untreated control rats on day 19 was $49.8 \pm 7 \text{ ng/ml}$. Infusion of IGF-I peptides for 6.5 days, significantly elevated plasma IGF-I levels ($P < 0.0005$) in the rat pups treated with 5 or $12.5 \mu\text{g/g/day}$ of IGF-I (Table 6.1). IGF-I plasma levels in rat pups treated with either 2 or $5 \mu\text{g/g/day}$ of LR³IGF-I were marginally higher compared to the IGF-I levels in vehicle treated rats but no statistically significant difference was obtained (Table 6.1).

Table 6.1: Plasma IGF-I concentrations in rat plasma samples collected at the end of a 6.5 day peptide treatment period compared to vehicle treated and pre-treatment levels. IGF-I concentrations were measured in chromatographed plasma samples by radioimmunoassay using a polyclonal anti-bovine IGF-I antibody.

Treatment group	IGF-I concentrations(ng/ml)
Control (N=3)	49.8 ± 7.0
Vehicle (N=7)	52.0 ± 5.1
IGF-I $2 \mu\text{g/g/day}$ (N=5)	143.8 ± 9.6
$5 \mu\text{g/g/day}$ (N=6)	$219.8 \pm 21.0^{**}$
$12.5 \mu\text{g/g/day}$ (N=6)	$229.8 \pm 61.4^{**}$
LR ³ IGF-I $2 \mu\text{g/g/day}$ (N=6)	78.9 ± 15.9
$5 \mu\text{g/g/day}$ (N=6)	75.7 ± 10.9

*Values are means \pm SEM. Pre-treatment sample represents pooled plasma from 3 rat pups sacrificed at the beginning of the treatment period (day 12). Statistical significance from the vehicle treated rats is indicated by **: $P < 0.0001$, as detected by ANOVA.*

Plasma IGF-I concentrations ranged from as low as 59.8ng/ml up to 396ng/ml in the group infused with 12.5µg/g/day of IGF-I. To assure that the results were representative, all assays were repeated. Intra-assay coefficient of variation was estimated at 4.2% for the first assay run and 4.5% for the second run. Inter-assay coefficients were 10.2% and 12.3% for the first and second set of assays, respectively. IGF-I levels of the re-assayed samples were of similar magnitude to those obtained in the first assay. I therefore concluded that IGF-I infusion in older suckling rats increases plasma IGF-I levels, but this may vary greatly amongst individual samples.

6.3.2 Plasma IGFBP profile in older suckling rats treated with and without IGF-I peptides

A representative Western ligand blot from rat plasma of 19 day old rat pups treated with or without IGF-I peptides is shown in Plate 6.1. As for the younger rat pups, the major IGFBP in plasma was a 30 kDa protein, presumably IGFBP-2/1. The relative intensities of this band seemed to vary between vehicle and IGF-I peptide treated rat pups, appearing more intense in rat plasma from rats treated with 5 and 12.5µg/g/day of IGF-I and 2 or 5µg/g/day of LR³IGF-I compared to the corresponding band in vehicle treated rat plasma (Plate 6.1). On the other hand, the intensity of a protein band migrating at approximately 24 kDa seemed similar for all groups, including the adult plasma sample running in Lane 8. This band most likely represents rat IGFBP-4. The most striking difference between this blot and the Western ligand blot for the 13 day old rat pups was the appearance of the larger molecular weight IGFBP migrating at 40 to 46 kDa (IGFBP-3) in the pooled samples from IGF-I and LR³IGF-I treated rat pups. This was most noticeable in the plasma samples from rat pups treated with LR³IGF-I. As expected, this band represents the major adult IGFBP and appeared as a distinct doublet in the pooled plasma samples from adult rats (Lane 8). Therefore, infusion of IGF-I and in particular LR³IGF-I to 12 day old suckling rats for 6.5 days may enhance the expression

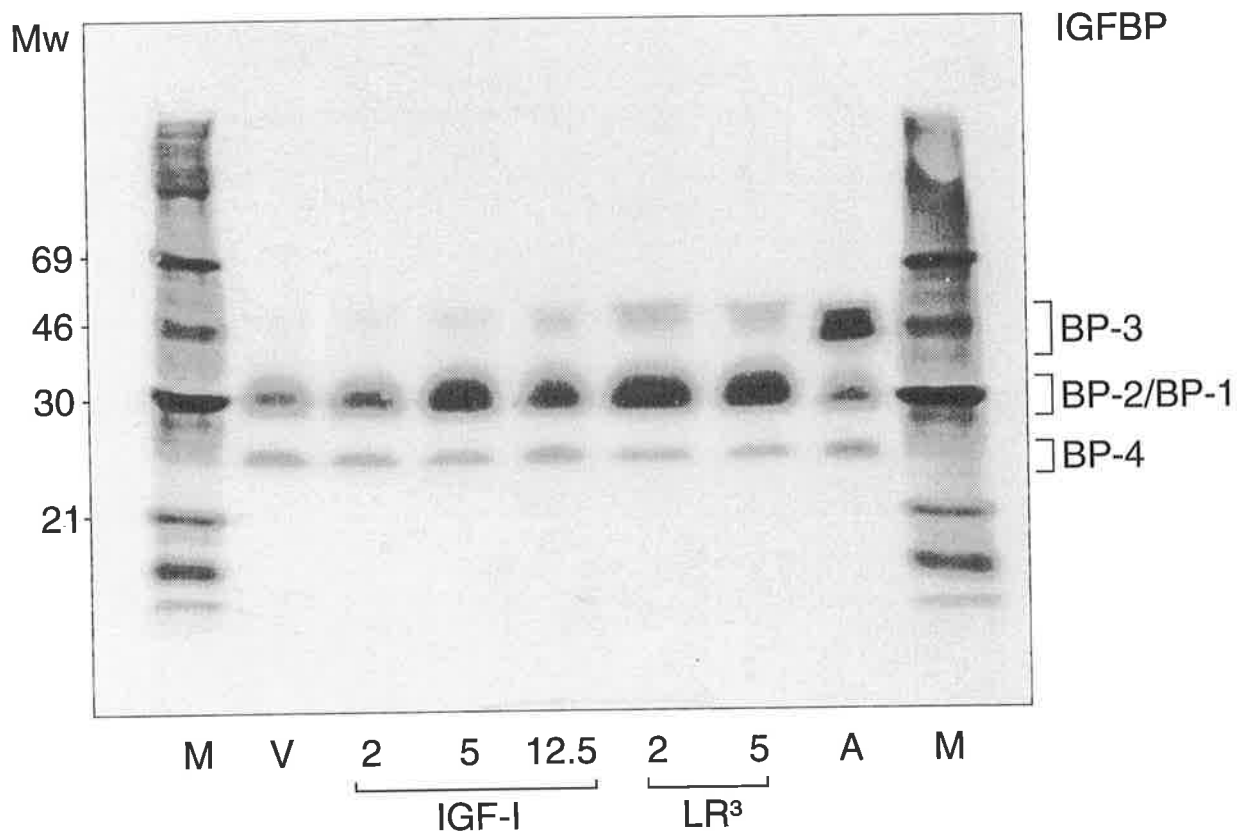


Plate 6.1: A representative ligand blot showing IGFBP profiles in 19 day old rat pups treated with or without IGF-I peptides. 20 μ l of pooled plasma sample from 6-8 animals per group were diluted with Laemmli sample buffers (Laemmli, 1970) to a total volume of 0.2ml and heated at 65°C for 15 minutes. 20 μ l of the sample were then separated on 10% SDS-polyacrylamide gel by electrophoresis and then electroblotted onto nitrocellulose as described in Material and Methods. Membranes were incubated with about 1×10^7 cpm of 125 I-IGF-I for 3 hours in a shaking water bath. 125 I-IGF-I bands were visualised by autoradiography. Migration position of molecular weight markers (M) are shown on the right and left hand side (Lane 1 and 9). Proposed IGFBPs are also indicated. Pooled plasma from vehicle treated rats is shown in Lane 2. Plasma pools from rats treated with 2, 5 or 12.5 μ g/g/day of IGF-I are shown in Lane 3, 4 and 5, respectively. Plasma pools from rats treated with 2 or 5 μ g/g/day of LR³IGF-I are shown in Lane 6 and 7, while Lane 8 depicts pooled adult rat plasma.

of IGFBP2/-1 and may accelerate the developmentally regulated appearance of IGFBP-3 in rat plasma.

6.3.3 Plasma insulin levels

Endpoint measurements of plasma insulin concentrations were taken from rats treated with vehicle or with IGF-I peptides. At the end of the 6.5 day peptide infusion period, no statistically difference was detected in plasma insulin levels between treatment groups ($P=0.62$, ANOVA), indicating that at the end of the peptide treatment period, plasma insulin levels were not altered in rat pups infused with IGF-I peptide (Table 6.2). However, because blood was not sampled throughout the peptide treatment period, these measurements do not reflect the dynamics of insulin secretion in response to continuous infusion of IGF-I peptides.

Table 6.2: Plasma insulin levels measured in 19 day old suckling rats treated with vehicle or IGF-I peptides .

	Vehicle	IGF-I <i>2µg/g/day</i>	IGF-I <i>5µg/g/day</i>	IGF-I <i>12.5µg/g/day</i>	LR ³ IGF-I <i>2µg/g/day</i>	LR ³ IGF-I <i>5µg/g/day</i>
Plasma insulin (µU/ml)	5.71±0.18	5.23±0.55	5.03±0.52	5.47±0.33	5.4±1.1	4.37±0.3

Values are means±SEM for 6-9 rats per group. No statistically significant difference was observed between treatment groups.

6.3.4 Growth parameters

Body weight gain

As shown in Table 6.3, the body weights were similar for all treatment groups at the start of treatment period on day 12. Following infusion of the growth factors for 6.5 days, rat pups treated with 5 or 12.5µg/g/day of IGF-I and 2 or 5µg/g/day of LR³IGF-I gained significantly more weight than their vehicle treated litter mates (Table 6.3). Rat pups treated

with vehicle gained $9.2\pm 0.5\text{g}$ during the experimental period, while the rats treated with the highest dose of LR³IGF-I gained $13.0\pm 0.4\text{g}$ during the same duration (Table 6.3).

Table 6.3: Body weights in suckling rats treated for 6.5 days with and without IGF-I peptides.

Treatment group	Initial body weight (g) (day 12 <i>post partum</i>)	Final body weight (g) (day 19 <i>post partum</i>)	Body weight gain (g)
Vehicle	21.5±0.3	30.7±0.5	9.2±0.5
IGF-I 2µg/g/day	21.4±0.6	31.2±0.6	9.7±0.4
5µg/g/day	22.3±0.3	33.05±0.*7	10.7±0.6
12.5µg/g/day	22.4±0.4	36.6±1.1**	14.2±1.1**
LR ³ IGF-I 2µg/g/day	22.1±0.8	34.8±1.4*	12.7±0.9*
5µg/g/day	21.8±0.2	34.8±0.5**	13.0±0.4**

*All values represent means±SEM with 6-9 animals per group. Statistical significance from the vehicle group is indicated by *: P<0.05 and **: P<0.01 as detected by ANOVA.*

Organ growth

Similar to the results obtained in the younger rat pups, administration of IGF-I peptides selectively stimulated growth of several non-gut organs. The wet tissue weights of the heart, spleen and kidneys increased in a dose dependent manner (Table 6.4). Most strikingly, the weight of the kidneys increased by 28% even at the low dose of IGF-I, while a 92% increase in kidney wet tissue weight was observed following treatment with 5µg/g/day of LR³IGF-I (Table 6.4). Near parallel responses were observed for the spleen (Table 6.4). The weights of the liver were increased in rat pups treated with the highest dose of IGF-I but not with LR³IGF-I (Table 6.4). On the other hand, thymus weight increased in rats treated with 2µg/g/day of LR³IGF-I but surprisingly, a significant reduction (-21%) in thymus weight was observed in rats treated with 5µg/g/day of LR³IGF-I (Table 6.4). Careful examination of individual data from animals in this group did not provide a reasonable explanation. The brain and lungs appeared not affected by the growth factor treatment. Organ weights were still significantly

elevated after corrections for body weight gains, so that the fractional organ weight were greater in IGF-I peptide treated rat pups compared to vehicle treated litter mates. For instance, fractional kidney weight in rat pups treated with the highest dose of IGF-I and the highest dose of LR³IGF-I were 49% and 69% greater compared than fractional kidney weights in vehicle treated rats (Figure 6.1). Similarly, fractional spleen weights increased by up to 81% above control values following treatment with IGF-I peptides (Figure 6.1).

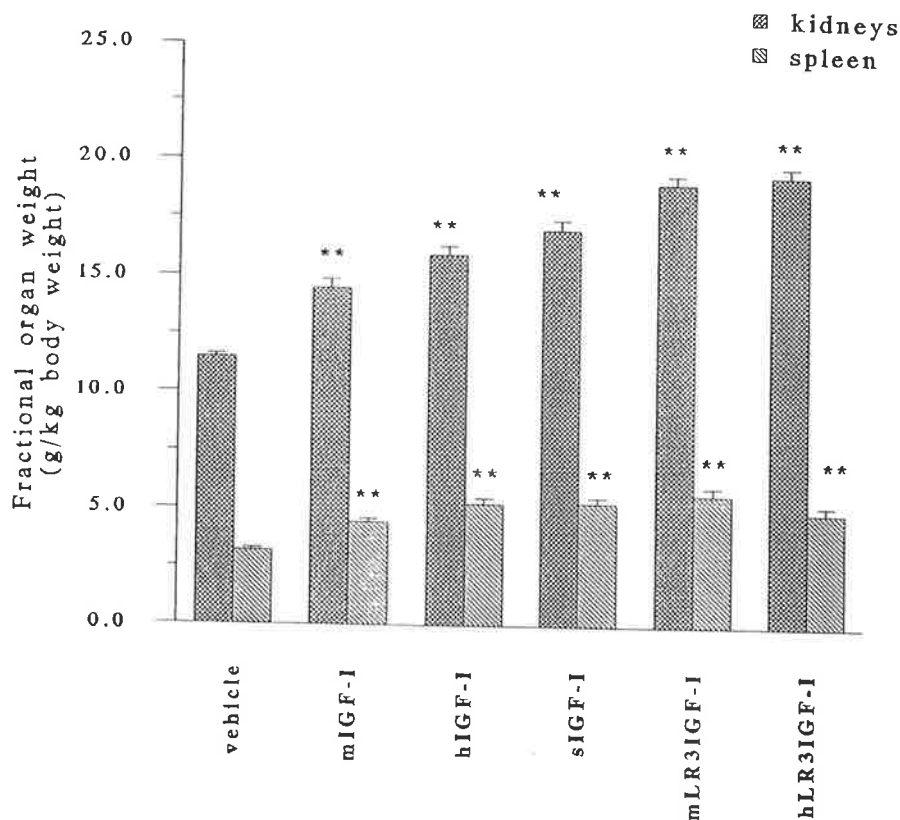
Table 6.4: Absolute organ weights on day 19 post partum in suckling rat pups treated for 6.5 days with or without IGF-I peptides.

Treatment group	<i>Heart</i> (mg)	<i>Liver</i> (g)	<i>Lungs</i> (mg)	<i>Thymus</i> (mg)	<i>Kidneys</i> (mg)	<i>Spleen</i> (mg)	<i>Brain</i> (g)
Vehicle	181±3	1.11±0.03	469±8	105±7	353±6	97±5	1.17±0.14
IGF-I 2µg/g/day	188±5	1.11±0.03	485±18	106±4	451±8**	138±6**	1.21±0.13
5µg/g/day	213±6**	1.13±0.05	500±17	113±6	528±20**	174±9**	1.17±0.21
12.5µg/g/day	218±10**	1.35±0.09*	538±42	126±5	628±33**	196±14**	1.15±0.18
LR ³ IGF-I 2µg/g/day	213±9**	1.09±0.09	526±9	149±17**	663±26**	199±17**	1.20±0.28
5µg/g/day	202±4*	1.09±0.03	510±12	83±10^a	676±10**	172±10**	1.20±0.91

Values are means±SEM for 6-9 rat per group. Statistically significant difference from the vehicle group is indicated by *: P<0.05 and **: P<0.001 as detected by ANOVA.

"a" indicates a statistically significant lower thymus weight in LR³IGF-I treated rats compared to vehicle treated rat pups.

Figure 6.1: Fractional organ weights of kidneys and spleen on day 19 post partum in suckling rats treated for 6.5 days with or without IGF-I peptides.



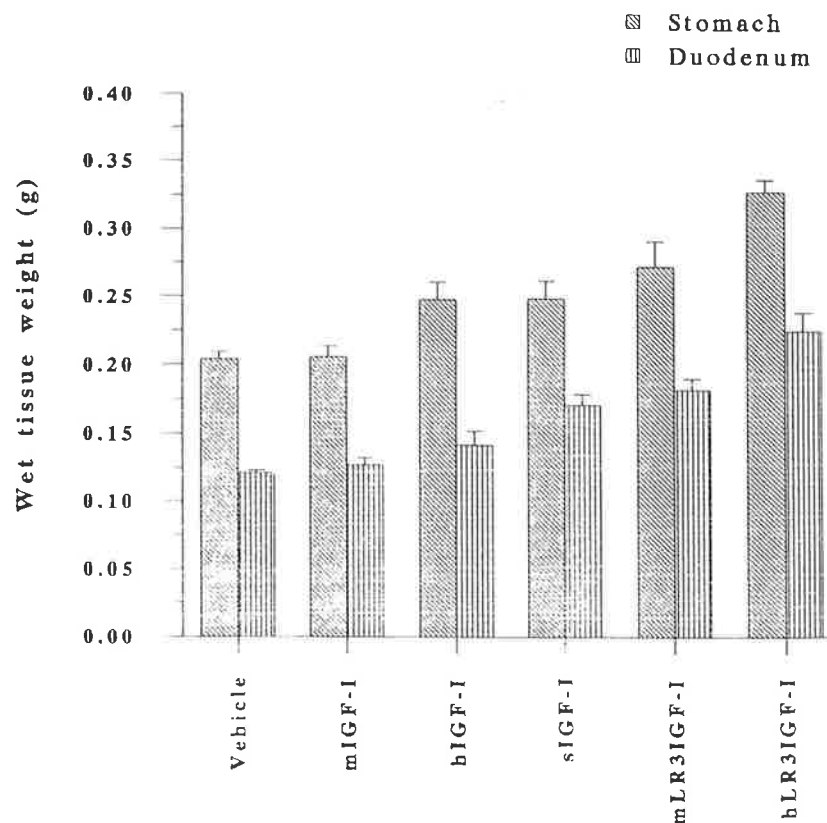
Values are means \pm SEM with 6-9 rats per group. Significant difference from the vehicle treated rat pups is indicated by **: $P < 0.001$, ANOVA. Vehicle treated rats received 0.1M acetic acid, mIGF-I = 2 μ g/g/day, hIGF-I = 5 μ g/g/day and sIGF-I = 12.5 μ g/g/day. LR³IGF-I was infused at 2 μ g/g/day (mLR³IGF-I) or 5 μ g/g/day (hLR³IGF-I).

6.3.5 Gastrointestinal response to IGF-I peptides

In agreement with the results from the previous studies, administration of IGF-I peptides to 12 day old suckling rats for a period of 6.5 days significantly increased the weight of the gastrointestinal tract. Growth responses were dose-dependent and were apparent in all regions of the gut. For instance, in the rat pups treated with 5 μ g/g/day of LR³IGF-I, total gut weight, small intestinal weight and large intestinal weight increased by 57%, 58% and 46%,

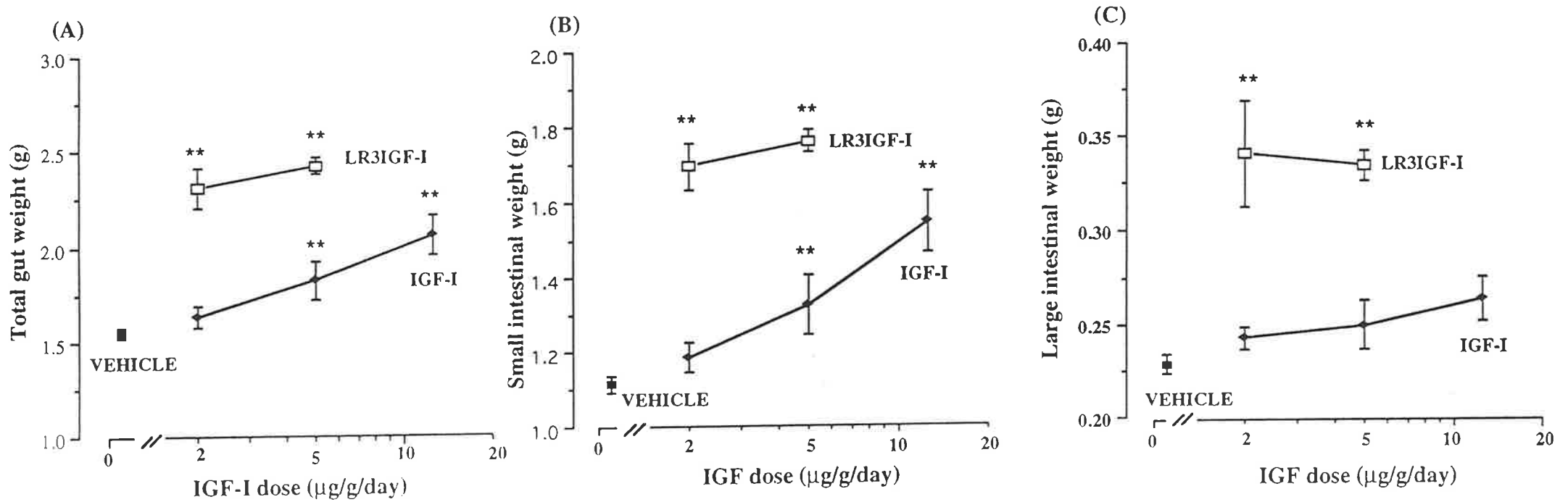
respectively (Figure 6.2 A-C). Consistent with the findings from the studies described in the previous Chapters, the proximal regions of the gut, in particular the stomach and duodenum, were the most peptide responsive regions in the present study. Wet tissue weights of the duodenum increased by 86% and 41% following administration of the highest dose of LR³IGF-I and IGF-I, respectively (Figure 6.3). Similarly, the wet tissue weight of the stomach increased by 61% and 22% following infusion of 5µg/g/day of LR³IGF-I and 12.5µg/g/day of IGF-I, respectively (Figure 6.3). As for the younger rat pups, the fractional weights of the intestinal components were still significantly elevated when corrections for body weight gain were made (data not shown).

Figure 6.3: Wet tissue weights of the stomach and duodenum in 12 day old suckling rat pups treated with or without increasing doses of IGF-I peptides.



All values are Means±SEM for 6-9 rat pups per group. Statistically significant difference from the vehicle group are shown by **: $P < 0.01$ as indicated by ANOVA. mIGF-I=2µg/g/day, hIGF-I=5µg/g/day and sIGF-I=12.5µg/g/day. LR³IGF-I treated rats have been infused with 2µg/g/day (mLR³IGF-I) or 5µg/g/day (hLR³IGF-I).

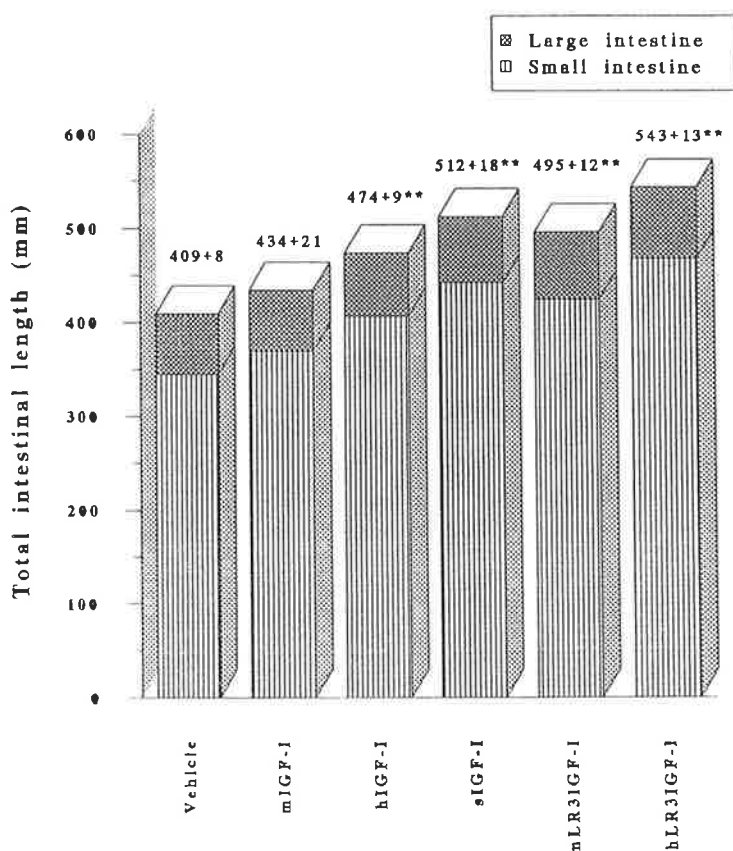
Figure 6.2: Total gut weight (A), small intestinal weight (B) and large intestinal weight (C) in 19 day old rat suckling pups treated with vehicle or IGF-I peptides.



All values represent means \pm SEM for 6-9 rats per group. Statistically significant difference from the vehicle treated rat pups is indicated by *P: <0.05 or **P: <0.01 as detected by ANOVA.

Increases in small and large intestinal length contributed significantly to the massive increase in intestinal mass. Total intestinal length was increased by up to 38% in rat pups treated with the highest dose of LR³IGF-I (Figure 6.4). Most interestingly, lengthening of the large intestine was also observed, so that treatment with 5µg/g/day of LR³IGF-I or 12.5µg/g/day of IGF-I significantly increased the length of the large intestine by 17% and 10%, respectively.

Figure 6.4: Total intestinal length in 19 day old suckling rat pups treated for 6.5 days with or without IGF-I peptides.



*Values represent means ± SEM for 6-8 rat pups per group. Total small intestinal length has been calculated as the sum of the small intestinal length plus the large intestinal length. Statistical significance from the vehicle treated group is indicated by **: P < 0.01 (ANOVA). IGF-I was infused at 2µg/g/day (mIGF-I), 5µg/g/day (hIGF-I) or 12.5µg/g/day (sIGF-I). The analogue was infused at 2µg/g/day (mLR³IGF-I) or 5µg/g/day (hLR³IGF-I).*

6.3.6 Histological parameters

Quantitative histological morphometry was carried out in tissue segments from the duodenum and ileum of vehicle and IGF-I peptide treated rat pups. As observed in the adult and the younger suckling rats, the increase in wet tissue weights was reflected in the histology of both mucosal and non-mucosal tissue compartments. In the duodenum, villus height was increased even in the rat pups treated with the lowest dose of IGF-I (Table 6.5). As for the younger rats, the proximo-distal gradient was maintained so that increases in villus height in the ileum were less pronounced compared to the duodenum. Nevertheless, in rat pups treated with the highest doses of IGF-I and LR³IGF-I ileal villus height was still increased by 9% and 12%, respectively, above control values (Table 6.5). Crypt depth in the duodenum increased by up to 57% in rats treated with 5µg/g/day of LR³IGF-I; correspondingly, the same peptide dose induced a 28% increase in crypt depth in the ileal segment (Table 6.5). As has been noticed for the suckling rats described in Chapter 5, IGF-I administration also stimulated non-mucosal tissue components. For instance in the present study, the thickness of the muscularis externa in the duodenum increased from 86±4µm in the vehicle treated rats to 117±7µm and 118±5µm in the rat pups treated with the highest dose of IGF-I and LR³IGF-I, respectively (Table 6.5).

Table 6.5: Histological measurements in the duodenum and ileum of 19 day old rat pups following treatment with IGF-I peptides for 6.5 days.

Treatment group	<i>Duodenum</i>			<i>Ileum</i>		
	Villus (µm)	Crypt (µm)	muscularis externa (µm)	Villus (µm)	Crypt (µm)	muscularis externa (µm)
Vehicle)	409±7	107±4	85±4	397±10	102±2	71±4
IGF-I 2µg/g/day	450±7**	115±6	87±3	399±7	104±2	75±2
5µg/g/day	449±10**	125±6**	92.8±5	388±8	108±2	77±1
12.5µg/g/day	453±8**	129±3**	99±3	431±15*	116±5**	84±3*
LR ³ IGF-I 2µg/g/day	439±6*	127±4**	117±7**	431±7*	114±4*	89±4**
5µg/g/day	468±9**	168±6**	118±5**	443±10**	132±5**	96±6**

*Values are means±SEM for 6-9 rat pups per treatment group. Statistical significance from the vehicle groups has been determined by ANOVA. Significance is indicated by *: P<0.05 and **: P<0.01*

6.3.7 Biochemical estimation of gut growth

Because of the time consuming nature of autoradiographic localisation studies, proliferative activity was not directly assessed in the present study. An overall estimation of cellular mass of the mucosal component was, however, obtained from biochemical estimations of the DNA and total protein content in mucosal scrapings from a defined length of jejunum. To assure accurate measurements of mucosal protein and DNA content, a greater tissue mass was scraped than in the study described in Chapter 5.

In the present study, the protein content in mucosal scrapings from a 4 cm jejunal segment increased in proportion with mucosal mass so that protein (mg) per g of jejunal mucosa was similar for all groups (Table 6.6). Similarly, the DNA content also increased in proportion to mucosal mass, resulting in similar amounts of DNA per g mucosa for rat pups treated with either vehicle or IGF-I peptides (Table 6.6).

Table 6.6: Jejunal protein and DNA content in mucosa collected from rat pups treated with vehicle or IGF-I peptides on day 19.

Treatment group	Wet tissue weight (mg)	Protein (mg/g tissue)	DNA ($\mu\text{g/g}$ tissue)
Vehicle)	65 \pm 2	104.6 \pm 3.4	5.7 \pm 0.2
IGF-I 2 $\mu\text{g/g/day}$	58 \pm 4	105.7 \pm 7.8	5.4 \pm 0.1
5 $\mu\text{g/g/day}$	70 \pm 4	113.6 \pm 7.2	6.0 \pm 0.2
12.5 $\mu\text{g/g/day}$	82 \pm 7*	94.2 \pm 7.2	5.1 \pm 0.3
LR ³ IGF-I 2 $\mu\text{g/g/day}$	74 \pm 4	103.3 \pm 7.0	5.7 \pm 0.3
5 $\mu\text{g/g/day}$	81 \pm 3*	117.7 \pm 5.8	5.6 \pm 0.3

Values are expressed as means \pm SEM. Each group comprises 6-9 rat pups. Statistically significant difference is indicated by *: $P < 0.05$ as determined by ANOVA.

6.3.8 Biochemical estimation of disaccharidase activity

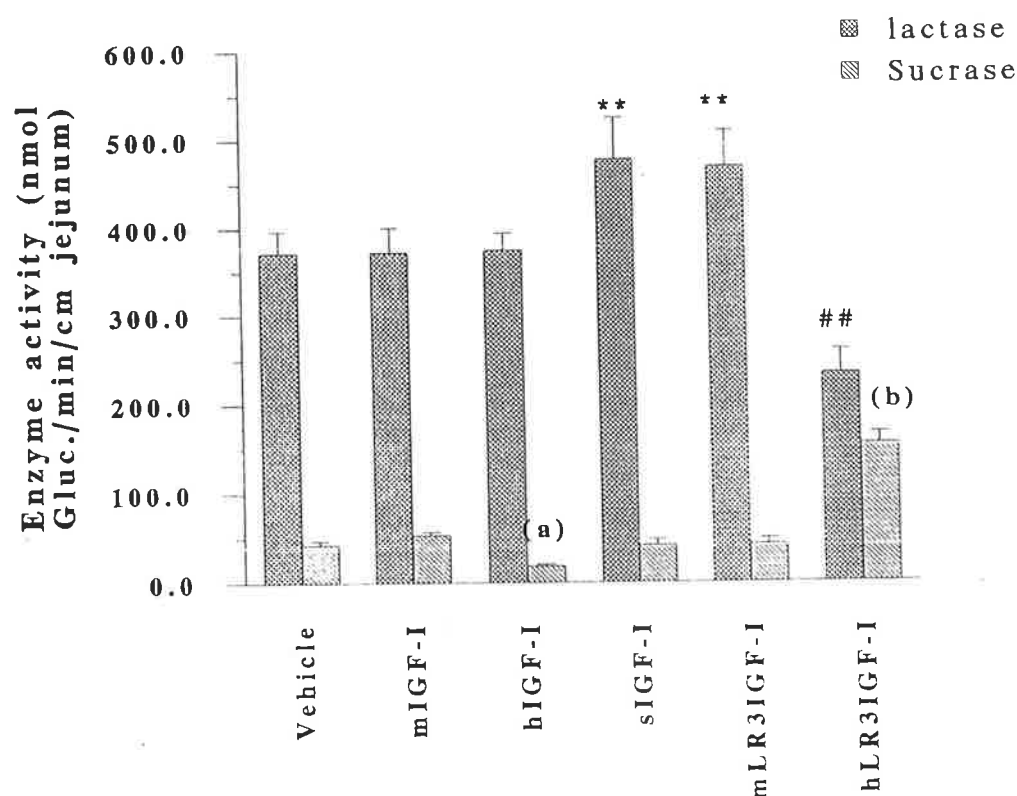
Lactase activity in the rat intestine declines to low levels while sucrase and other α -glucosidases increase at the end of the 3rd postnatal week (Henning, 1985). Because peptide administration ceased on the 19th postnatal day in the present study, the effect of IGF-I peptide administration of the normal developmental pattern of disaccharidase activity in the rat intestine were investigated. In the previous Chapter, administration of LR³IGF-I to suckling rats during the early postnatal period (day 6 to day 13) increased lactase activity proportional to increases in mucosal weight, indicating that peptide treatment did not accelerate gut maturation at this early time. In contrast, a developmentally regulated decline in lactase activity and precocious appearance of sucrase was observed in the present study.

Total lactase activity in tissue homogenates prepared from a 4cm jejunal tissue segments was significantly increased in rat pups treated with 12.5 μ g/g/day of IGF-I and in the rats treated with 2 μ g/g/day of LR³IGF-I (Figure 6.5), reflecting the increase in the mucosal and in particular the epithelial tissue mass in these groups. In contrast, despite the marked increase in mucosal mass in rats infused with 5 μ g/g/day LR³IGF-I, a significant reduction in total lactase activity was recorded (Figure 6.5). Moreover, lactase activity was still significantly lower when the data was expressed as specific lactase activity (per μ g of DNA). For example, in the pups treated with 5 μ g/g/day of LR³IGF-I specific lactase activity was 2.5 ± 0.3 nmol Glu./min/cm/ μ g DNA compared to 3.9 ± 0.2 nmol Glu./min/cm/ μ g DNA in vehicle treated rat pups (Table 6.7). This suggests that administration of 5 μ g/g/day of LR³IGF-I stimulates the premature decline in lactase activity during the later suckling period in rat pups.

Sucrase activity expressed as either total or specific sucrase activity remained low in all groups except for the rat pups treated with 5 μ g/g/day of LR³IGF-I (Figure 6.5 and Table 6.7).

In this group, total sucrase activity increased by 62% above the values obtained for vehicle treated rats. When the data was expressed per μg mucosal DNA, sucrase activity remained significantly elevated in that group (1.6 ± 0.2 nmol Glu./min/cm/ μg DNA) compared to vehicle treated rats (0.4 ± 0.04 nmol Glu./min/cm/ μg DNA), Table 6.7. This suggests that administration of $5 \mu\text{g/g/day}$ LR³IGF-I peptides to older suckling rats accelerated the appearance of sucrase activity in.

Figure 6.5: Total lactase and sucrase activity in mucosal scrapings from 4 cm of jejunum in 19 day old rat pups treated with vehicle or IGF-I peptides.



Values are means \pm SEM with 5-9 rat per group. Statistically significance increase in lactase activity is indicated by **: $P < 0.01$. A significant reduction in lactase activity is indicated by ##: $P < 0.01$. Rats treated with $2 \mu\text{g/g/day}$ of LR³IGF-I (mLR³IGF-I) showed significantly lower sucrase activity (a): $P < 0.05$. Sucrase activity was increased in rats treated with $5 \mu\text{g/g/day}$ (hLR³IGF-I). All groups were compared to the vehicle rats by ANOVA. Rats were treated with doses of 2 (mIGF-I), 5 (hIGF-I) or 12.5 (sIGF-I) $\mu\text{g/g/day}$.

Table 6.7: Lactase and sucrase specific activities in jejunal tissue homogenates from 12 day old suckling rats treated for 6.5 days with vehicle or IGF-I peptides.

Treatment group	Wet tissue weight (mg)	Lactase (nmol	Sucrase (nmol
		Gluc./min/cm/ μ g DNA)	Gluc./min/cm/ μ g DNA)
Vehicle)	65 \pm 2	5.67 \pm 0.4	0.67 \pm 0.06
IGF-I 2 μ g/g/day	58 \pm 4	6.4 \pm 0.2	0.92 \pm 0.08
5 μ g/g/day	70 \pm 4	5.4 \pm 0.5	0.25\pm0.03^a
12.5 μ g/g/day	82\pm7*	6.1 \pm 0.9	0.54 \pm 0.10
LR ³ IGF-I 2 μ g/g/day	74 \pm 4	6.4 \pm 0.6	0.58 \pm 0.10
5 μ g/g/day	81\pm3*	3.4\pm0.4**	2.25\pm0.21^b

*Values are expressed as means \pm SEM. Each group comprised 5-9 rat pups. Statistically significant reduction in specific lactase activity is indicated by **:P<0.01. A significant increase in specific sucrase activity is shown by "b" :P<0.01. Rat pups treated with 5 μ g/g/day showed significantly reduced sucrase activity "a" :P<0.01, however, all measurements below 1nmol Glu./cm/min/ μ g DNA represent minimal enzyme activities.*

6.3.9 Histocytochemical detection of duodenal disaccharidases

The previous Chapter has shown that administration of IGF-I peptides increases lactase activities along the villus axis proportional to increases in villus length. At the same time, sucrase activity was not detectable in either vehicle or peptide treated rats. In the present study, lactase and sucrase activities were detected histocytochemically along the duodenal villus axis in rats treated with vehicle, 12.5 μ g/g/day of IGF-I and 5 μ g/g/day of LR³IGF-I in a manner similar to that described for the younger rats.

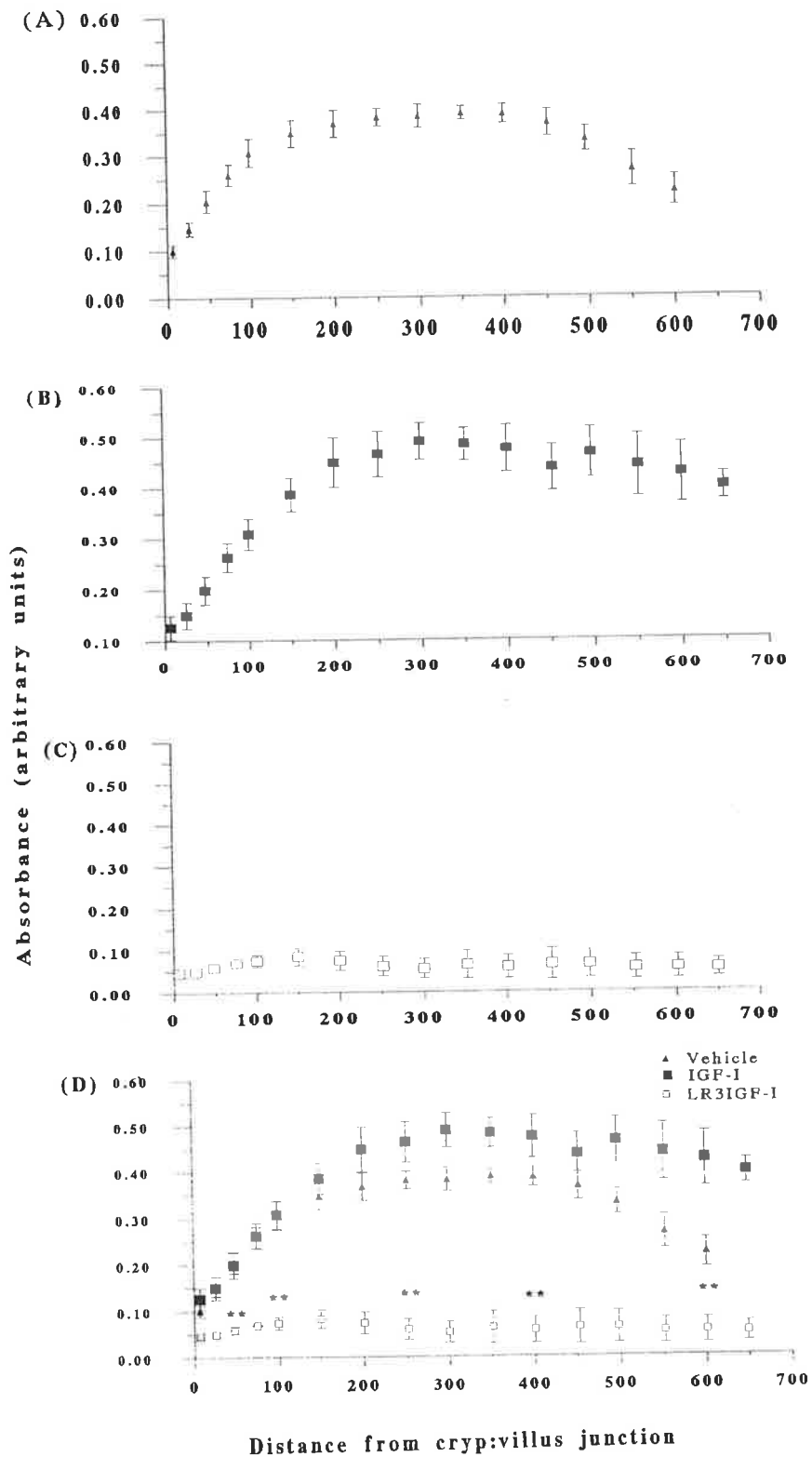
The enzyme distribution profiles for lactase and sucrase were comparable to the enzyme profiles obtained in the younger rats for the rats treated with either vehicle or IGF-I. For example, in both groups, lactase activity was low in enterocytes near the crypt:villus junction, increasing to maximal levels at approximately 200 μ m (Figure 6.6 A and B). No statistically significant difference in lactase activity (measured as the Absorbency (Ab) of the

enzyme colour reaction product) was detected between the vehicle and IGF-I treated rats at 50, 100, 250, 400, and 600 μ m along the villus axis. Most strikingly, however, in the rats treated with 5 μ g/g/day of LR³IGF-I a dramatic reduction in lactase activity was evident along the entire length of duodenal villi (Figure 6.6 C). Statistical significance was attained at 50, 100, 250, 400 and 600 μ m when the profiles was compared to those of the rats treated with either vehicle or 12.5 μ g/g/day of IGF-I (Figure 6.6 C). The marked reduction in lactase activity in this group is most clearly demonstrated in a photomicrograph comparing surface staining for lactase activity in cryostat sectioned duodenum from vehicle infused rats (Plate 6.2 A), with the staining pattern obtained for rat pups treated with 5 μ g/g/day of LR³IGF-I (Plate 6.2 B). As seen on the micrograph, lactase activity in vehicle treated pups covers the entire length of the duodenal villi, while in the LR³IGF-I treated rats, lactase activity appears in a patchy pattern, expressed chiefly in the brush border of enterocytes located in the mid-villus region.

The sucrase distribution profiles for vehicle and IGF-I treated rats was similar and no statistically significant difference in sucrase activity was detected at any distance along the duodenal villi between vehicle and IGF-I treated rats (Figure 6.7 A and B). However, a marked increase in sucrase activity along duodenal villi was observed in rat pups treated with 5 μ g/g/day of LR³IGF-I. Statistically significant differences in sucrase activity were detected at 50, 100, 250, 400 and 600 μ m, when compared to the sucrase activities measured in IGF-I treated rats (Figure 6.7 C). Comparing the enzyme profiles in LR³IGF-I treated rats with that from vehicle treated rats showed that enterocytes located at higher positions along the villus axis in LR³IGF-I rats expressed sucrase activity to a greater extent (Figure 6.7 C). In addition, because the length of duodenal villi in LR³IGF-I treated rats was significantly increased, sucrase was expressed over a greater distance in LR³IGF-I treated rat pups. Sucrase activity was significantly higher in the brush border of enterocytes positioned at 400 and 600 μ m,

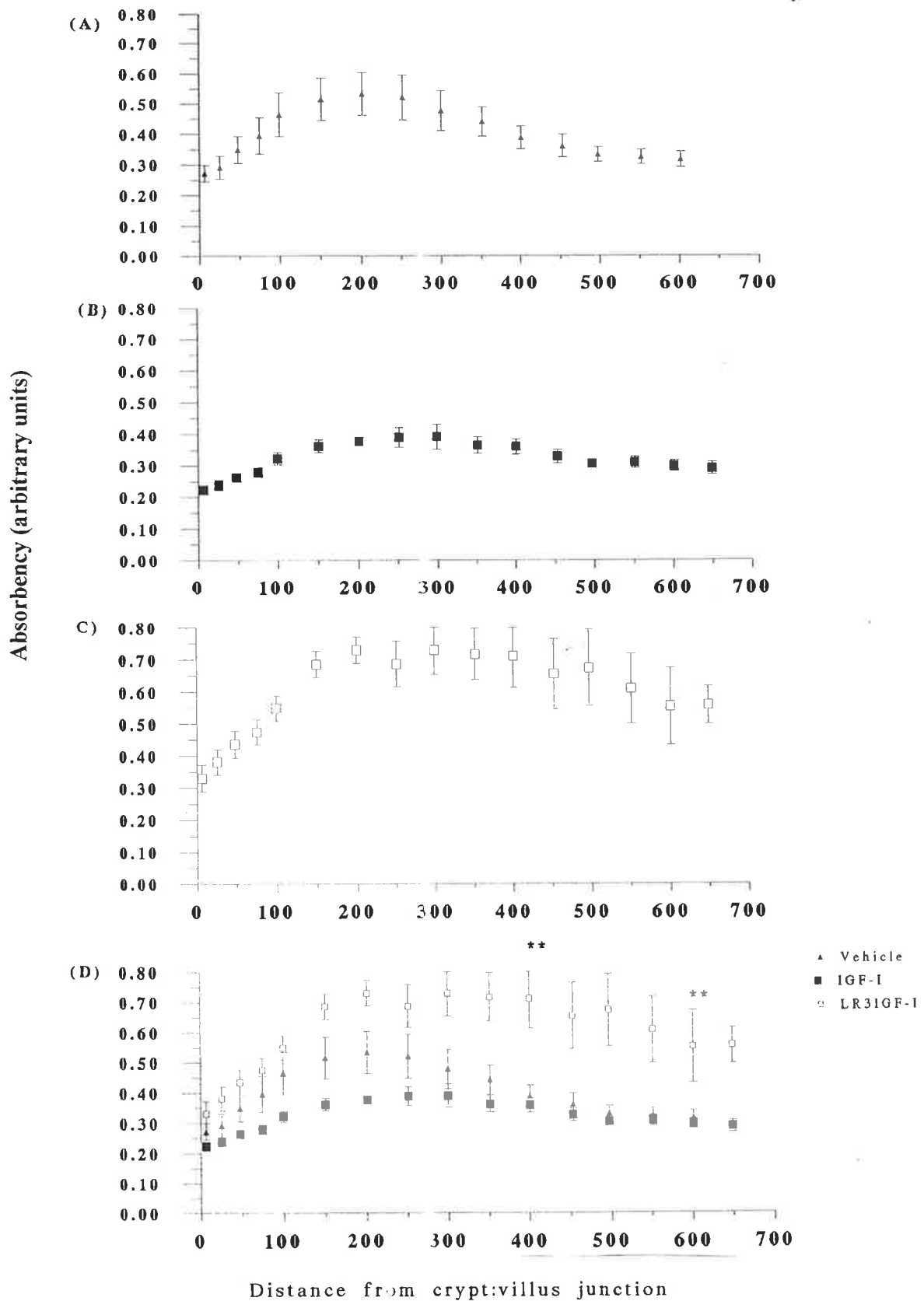
indicating that sucrase expression in the rat pups treated with 5µg/g/day of LR³IGF-I was more advanced compared to either the vehicle or IGF-I treated groups. Vehicle and IGF-I treated rats showed insignificant levels of sucrase activity in the upper third of the villus structure, I concluded that administration of LR³IGF-I precociously induced the expression of sucrase activity.

Figure 6.6: Distribution profile of lactase activity along duodenal villi in suckling rats treated with vehicle (A), 12.5 μ g/g/day of IGF-I (B) or 5 μ g/g/day of LR3IGF-I (C).



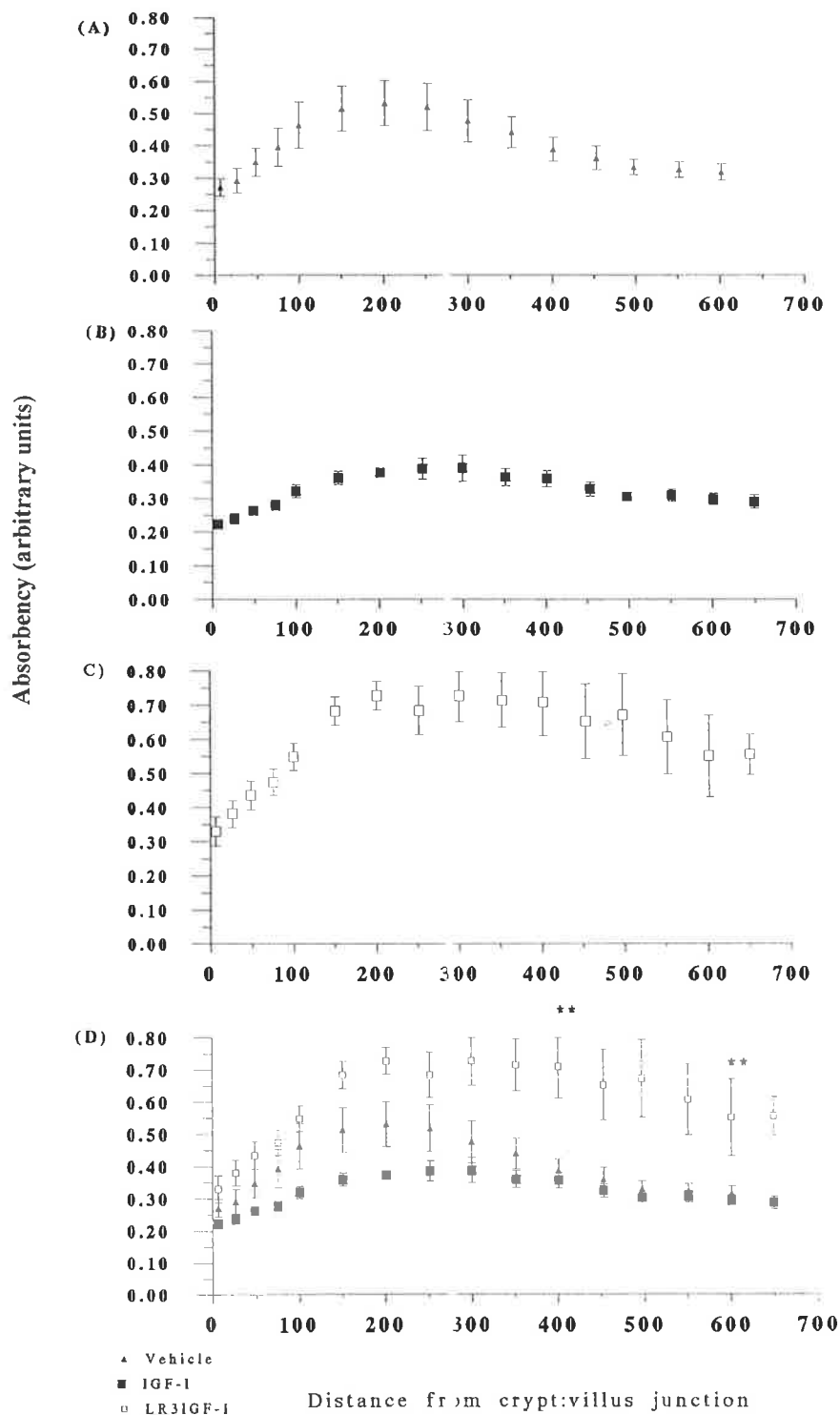
Values are means \pm SEM for 6-9 rats per group. The Absorbency of the enzyme reaction product was measured from the base of duodenal villi to the tip as described in Materials and Methods. Figure 6.6 D shows the lactase profiles superimposed for rats treated with vehicle (▲), IGF-I (■) or LR³IGF-I (□) for comparison. Statistically significance from the vehicle groups is indicated by **P: <0.01 (ANOVA).

Figure 6.7: Distribution profile for sucrase activity along duodenal villi in suckling rats treated with vehicle (A), 12.5 μ g/day of IGF-I (B) or 5 μ g/day of LR3IGF-I (C).



Values are means \pm SEM for 6-9 rats per group. The Absorbency of the enzyme reaction product was measured from the base of duodenal villi to the tip as described in Materials and Methods. Figure 6.7 D shows the sucrose profiles superimposed for rats treated with vehicle (▲), IGF-I (■) or LR³IGF-I (□) for comparison. Statistically significance from the vehicle groups is indicated by **P: <0.01 (ANOVA).

Figure 6.7: Distribution profile for sucrase activity along duodenal villi in suckling rats treated with vehicle (A), 12.5 μ g/g/day of IGF-I (B) or 5 μ g/g/day of LR3IGF-I (C).



Values are means \pm SEM for 6-9 rats per group. The Absorbency of the enzyme reaction product was measured from the base of duodenal villi to the tip as described in Materials and Methods. Figure 6.7 D shows the sucrase profiles superimposed for rats treated with vehicle (▲), IGF-I (■) or LR³IGF-I (□) for comparison. Statistically significance from the vehicle groups is indicated by **P: <0.01 (ANOVA).

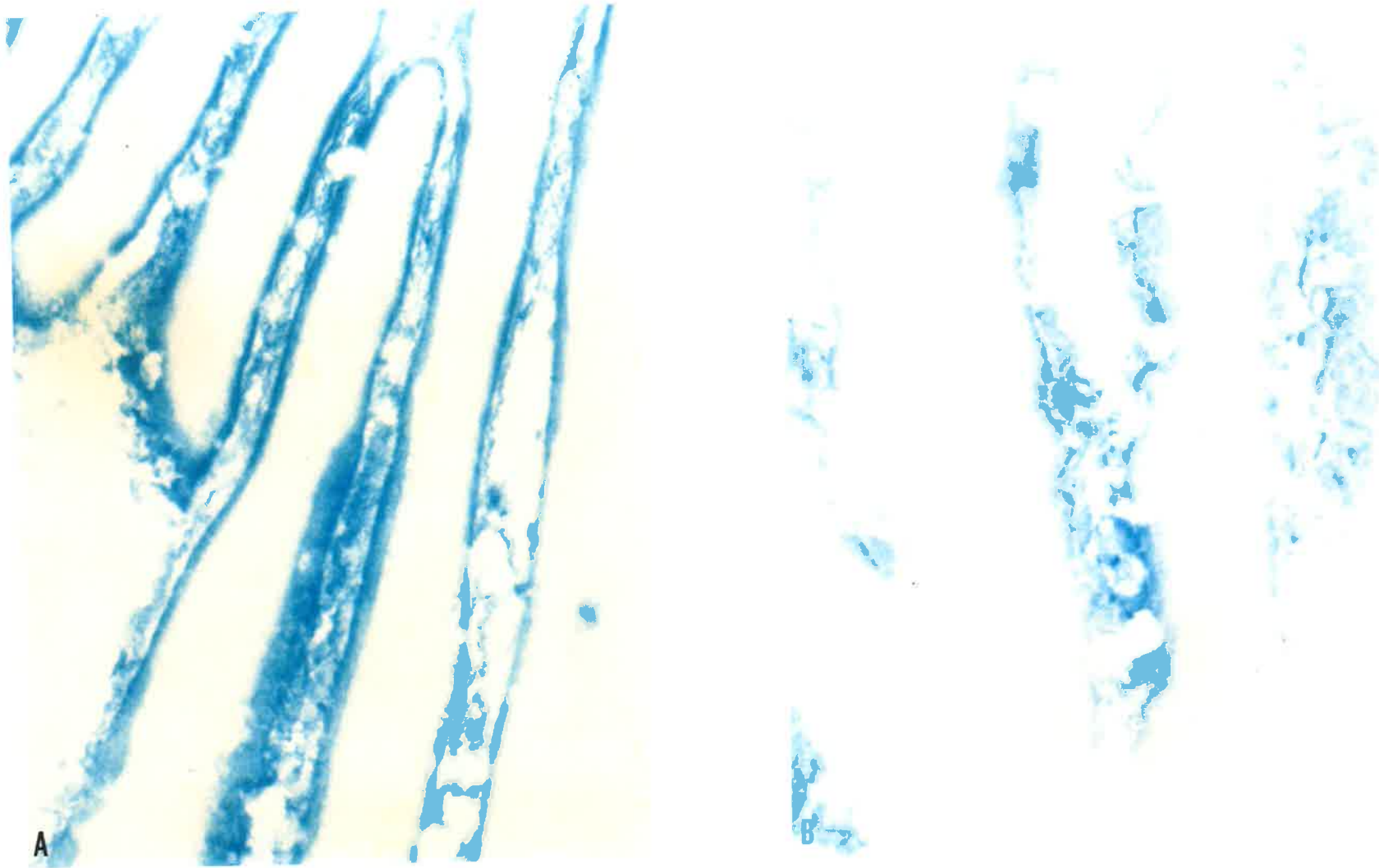


Plate 6.2: Histocytochemical detection of lactase along duodenal villi in 19 day old suckling rats treated for 6.5 days with either vehicle (**A**) or 5 μ g/g/day of LR³IGF-I (**B**). Cryostat sectioned duodenum was incubated with saturating concentrations of 5-Br-4-Cl-3-indolyl- α -frucopyranoside for 15 minutes. Lactase was localised in the brush border of villus enterocytes. The blue enzyme reaction colour product was measured from the base to the tip of the villi along the longitudinal villus axis. Lactase activity in rat pups treated with LR³IGF-I was markedly reduced and was irregularly distributed along the length of duodenal villi (**B**).



Plate 6.3: Histocytochemical detection of α -glucosidase (sucrase) along duodenal villi in 19 day old suckling rats treated for 6.5 days with either vehicle (**A**) or $5\mu\text{g}/\text{g}/\text{day}$ of LR³IGF-I (**B**). Duodenal sections were incubated with saturating concentrations of 2-naphthyl- α -D-glucopyranoside. Enzyme activity was located in the brush border of duodenal crypt enterocytes, visualised in orange by the coupling agent hexazonium-p-rosaniline. In rat treated with LR³IGF-I, sucrase activity was more intense and expressed in enterocytes located in higher positions towards the villus tip (**B**). Sections have been photographed with a blue-filter for background tissue contrast.

6.4 DISCUSSION

6.4.1 *IGF-I peptides stimulates growth and functional maturation of the gut*

The present study has clearly shown that continuous infusion of IGF-I peptides selectively stimulates growth and maturation of the gastrointestinal tract during the later suckling period. Highly significant increases in wet tissue weights were observed in all regions of the gastrointestinal tract. The responses were dose-dependent and as observed in previous studies, the proximal gut, in particular the stomach and duodenum, were the most growth factor sensitive regions. Histological analyses indicated that cross-sectional thickness of both mucosal and non-mucosal tissue components were increased. In addition, small and large intestinal length significantly contributed to the massive increase in total gut weight. As observed for the younger rat pups, the proximo-distal gradient was maintained, suggesting that IGF-I peptide administration did not perturb the intrinsic developmental pattern. However, the most significant observation of the present study was the marked stimulation of functional maturation of the intestine following growth factor administration.

6.4.2 *IGF-I peptide effects on lactase activity*

The present study showed that s.c. infusion of IGF-I peptide markedly stimulated the maturation of duodenal disaccharidases in older suckling rats. In the younger rat pups, infusion of the highest dose of IGF-I or LR³IGF-I enhanced expression of lactase activity in proportion to increases in villus enterocyte population. At the same time, IGF-I peptides did not induce a premature decline in lactase activity, nor was sucrase activity precociously induced. In the older rat pups, several interesting observations have emerged. Firstly, a bi-phasic response of IGF-I on the expression of lactase activity was observed. Second, sucrase activity was precociously induced.

Rat pups treated with the highest dose of IGF-I and the lowest dose of LR³IGF-I showed an increase in duodenal lactase activity. This response was comparable to that in the younger rats and indicated that administration of IGF peptides at these doses stimulated lactase activity proportional to increases in villus enterocyte population. On the other hand, administration of the highest dose of the more potent analogue LR³IGF-I (5µg/g/day) resulted in a marked reduction in lactase activity, despite the fact that villus enterocyte population was also vastly increased in this group. This suggested that administration of lower doses of IGF-I enhanced lactase activity in proportion to mucosal epithelial growth, while administration of 5µg/g/day of LR³IGF-I accelerated the premature decline in lactase activity that otherwise would have occurred a couple of days later (approximately 21-24 days post partum). These findings implicate IGF-I peptides and in particular the potent analogue LR³IGF-I in maturation of the intestinal epithelium in rats during the late suckling period.

As shown by others, the expression of intestinal lactase activity is regulated by intrinsic timing mechanisms, nutritional factors as well as endocrine and paracrine regulators (reviewed by Koldovsky, 1981b). For instance, while it is established that prolonged starvation significantly increases lactase activity (Nsi-Emvo and Raul, 1984), regulation of lactase activity by its substrate is less clear. Lebenthal et al. (1973) have shown that prolonged feeding with lactose initially increased lactase activity, however, the developmentally regulated decline in lactase activity was not altered. Several other studies also argue against dietary adaptation to lactose as the principle controlling factor in the decline of lactase activities at the time of weaning (Leichter, 1973, Bolin et al., 1979 and Goda et al., 1985). The design of the present study did not permit quantitation of food intake, hence dietary effects can not be discounted. It seems unlikely that variations in dietary inputs account for the altered pattern of lactase activity, because lactase activity increased with some doses while reduced lactase activity was observed in rat treated with the highest dose of the analogue.

Recent evidence suggests that the regulation of lactase occurs at the level of transcription of the lactase gene (Krasinski et al., 1993). Although, this may provide the ultimate control, regulation by endocrine or paracrine factors may determine the final amount of enzyme present in intestinal enterocytes (Freund et al., 1989, Tsuboi et al., 1985, Smith and James, 1987, Yeh et al., 1991a, Rings et al., 1992, and Boyle et al., 1980). A study by Yeh and Moog (1974) has shown that hypophysectomy and thyroidectomy retarded the natural decline in lactase activity that occurs at the end of the 3rd postnatal week, while thyroxine replacement therapy restored the normal lactase pattern. Administration of a single dose of cortisone to 6 day old suckling rats increased lactase activities, while thyroxine alone induced no changes at the early suckling period. In combination, lactase activities decreased, however, to a lesser extent as observed with thyroxine treatment alone (Yeh et al., 1991b). In the present study, thyroxine and glucocorticoids were not measured in plasma of treated and untreated rats. Thus, I can not exclude the possibility that administration of IGF-I peptides may have changed the secretory pattern of these hormones, resulting in altered expression of lactase activity. To exclude this possibility, the endocrine status may be monitored in future studies. Alternatively, to rule out stress-effects, continuous infusion of IGF-I peptides in adrenalectomised rats may be included in the experimental design.

It is likely that IGF-I peptides regulate lactase expression directly. As shown in the previous Chapters, IGF-I peptides stimulate proliferative events in the intestine presumably by binding to IGF receptors present on crypt enterocytes. Potentially, this may involve an increase in cell turn-over rates of enterocytes as they migrate up the villus, leading to accelerated replacement of villus enterocytes. As shown in Chapter 4, LR³IGF-I is not only more potent in stimulating cellular proliferation but also accelerates proliferative events. It is therefore possible that, in the present study, LR³IGF-I accelerated the normal maturational decline in lactase activity by stimulating cell turn-over rates and/or by inhibiting the rate at

which lactase activity is expressed in new enterocytes. In support, it has been shown that enterocyte turn-over rates regulate intestinal lactase activity (Tsuboi et al., 1985, Smith and James 1987). As suggested by Tsuboi et al. (1985), the decline in specific lactase activity was dependent on the change in cell turn-over rates. At the same time, lactase synthesis remained constant. Similarly, Smith and James (1987) suggested that between day 15 to 23 the decline in lactase activity is related to an increase in cell turn-over rates so that the time available for lactase expression is shorter. In the same study, the rate of lactase synthesis declined during the later postnatal period (between day 23 to 46), suggesting a two stage process.

6.4.3 *LR3IGF-I precociously induced sucrase activity*

The premature decline in lactase activity in rats treated with LR³IGF-I was accompanied by a more mature expression pattern of sucrase activity in these rats. In the rat, the spontaneous and irreversible maturation of sucrase at the time of weaning is dependent primarily on the intrinsic program that governs intestinal development (Ferguson, et al., 1973). At the same time it has been shown that dietary changes and the modification of the hormonal status that occurs at this time modulate the intrinsic program. (Freund et al., 1993 and Henning, 1987). For instance, administration of a solution containing sucrose or maltose increases intestinal sucrase activity (Lebenthal et al., 1972). On the other hand, starvation in pre-weaned rats causes the precocious induction of sucrase-isomaltase without altering significantly lactase expression (Duluc et al., 1992). Some of the responses observed in studies of nutritional manipulation may stress-related. In bypassed intestinal segments or in adrenalectomised rats, precocious appearance of sucrase in response to sucrose administration was not observed (Tsuboi et al., 1981 and Lebenthal et al., 1972).

In the present study, rat pups treated with LR³IGF-I were in general terms “more advanced”, thus it is possible that these rats started to ingest small amounts of the rat chow

that was freely available to the dam. In fact, I had noticed that in some animals non-mammary products were contained within the gut contents. This was the case for 3 out of the 6 rats treated with 5µg/g/day LR³IGF-I. Increased duodenal sucrase activity was, however, evident in the duodenum of all 6 animals so that this possibility seems unlikely.

Hormonal regulation of sucrase by glucocorticoids has been studied extensively (Yeh and Moog, 1977 and 1978, Henning, 1978, Doell and Kretchmer, 1964 and Nordström et al., 1968). Precocious appearance of sucrase activity can be accelerated after injection of hydrocortisone or cortisone (Doell et al., 1965 and Yeh et al., 1989) and can be abolished by the administration of the antiglucocorticoid RU-38486 (Galand, 1988). Some studies have suggested that the maturation effect of glucocorticoids on the intestinal epithelium is indirect, and may be mediated via mesenchymal interactions. For example, expression of intestinal brush border enzymes can be modulated by secretory basement membrane molecules, such as laminin and type IV collagen, which have been shown to be regulated by corticoids (Walsh et al., 1987 and Simo et al., 1992).

Glucocorticoid levels were not measured in the present study, but it appears unlikely that stress related effects were responsible for the increase in LR³IGF-I treated pups, because all rat pups were treated the same, and stimulation of sucrase activity was not observed in rats treated with vehicle. Furthermore, all animals were handled daily for approximately 2-4 minutes before and after surgical implantation of the pumps and no signs of stress were observed during handling in any of the treated or untreated pups. They appeared playful and seemed unaffected by the experimental procedures.

More likely, LR³IGF-I stimulated the expression of sucrase expression directly. As shown by Lund and Smith (1987), α-glucosidases, a mixture of sucrase, maltase, isomaltase

and trehalase disaccharidases, appear first in enterocytes at the base of intestinal villi. α -Glucosidase activity can be observed as early as 16 days *post partum* in the rat (Lund and Smith, 1987). During normal development, enzyme activity then increases and spreads along the whole villi during the next 4-5 days. In the present study, sucrase activity in rat pups treated with 5 μ g/g/day of LR³IGF-I was evident histochemically in the brush border membranes of villus enterocytes located further along the length of the crypt:villus axis compared to vehicle or IGF-I treated rat pups, supporting that enterocyte development was more mature. However, sucrase activity was not stimulated to adult-like levels as indicated by the low sucrase activities in enterocytes at the villus tip. As shown by Lund and Smith (1987), villus enterocytes express quite suddenly sucrase activity both during normal development and after injection of hydrocortisone. This indicates that villus enterocytes rather than crypt epithelium may respond to hormonal (growth factor) stimulation. In support, several other factors, including thyroxine, Vitamin D₃ as well as EGF have been shown to increase the capacity of villus enterocytes to express different proteins (Smith, 1985, Hewitt and Smith, 1986, James et al., 1987, Lund and Smith, 1987). It may be postulated that LR³IGF-I stimulated enhanced expression of sucrase activity via receptor ligand interaction on villus enterocytes rather than crypt enterocytes.

In recent study by Nsi-Emvo et al. (1994), the effects of starvation and refeeding on the developmental pattern of intestinal sucrase-isomaltase have been studied in pre-weaned rats. This group has shown that starvation on postnatal day 12 caused precocious expression of sucrase activity and mRNA. Immunocytochemical localisation showed that sucrase activity appeared first in enterocytes at the base of villi. In addition, the starvation-evoked appearance was preceded by a transient burst of expression of the protooncogene *c-fos*. At the same time this groups did not observe an increase in intestinal cell turn-over time (Nsi-Emvo et al., 1994). Thus, it appears that in pre-weaned rats, precocious appearance of sucrase evoked by

starvation is unrelated to an increase in cell turn-over rates. As for the present study, it remains hypothetical at this stage by which mechanism LR³IGF-I stimulated the intestinal sucrase.

Although intrinsic timing mechanism control the reciprocal shift in disaccharidase expression in the rat intestine (Kendall et al., 1979 and Montgomery et al., 1981) the timing and degree to which α -glucosidases are expressed prior to weaning may reflect a combination of factors including an increased sensitivity of villus and/or crypt enterocytes to hormonal regulators and possibly the removal of factors that may inhibit gene expression of adult-like enzymes Lund and Smith (1987). Likewise, it is quite possible that a combination of factors may have been responsible for the altered pattern of disaccharidase activity observed in the present investigation.

6.4.3 Suckling rats are highly responsive to IGF-I peptide administration during the pre-weaning period

Another interesting observation of the present study relates to the sensitivity of the suckling rat pups to IGF-I peptides during the later suckling period. In the present study, continuous infusion of IGF-I peptides to older suckling rats markedly stimulated body weight gain and selectively influences growth of visceral organs, most notably the gastrointestinal tract. Moreover, comparing the growth responses from previous Chapters with the findings from the present study suggests that suckling rats are more sensitive to IGF-I peptide administration during the later suckling period, compared to either the early suckling period or during adulthood. For instance, in the younger suckling rats, body weight gain was only stimulated following infusion of the highest dose of LR³IGF-I but not IGF-I. In the present study, a dose dependent increase in body weight gain was observed, so that even the lower dose of IGF-I significantly promoted body weight gain. As for the younger rats, the

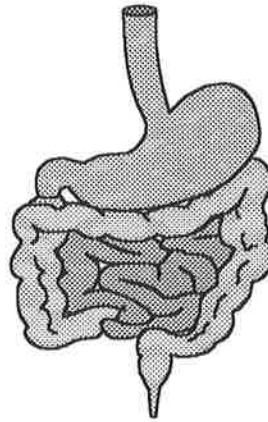
experimental design did not permit the quantitative assessment of food intake; nor can it be excluded that some rat pups may have started to “nibble” on the adult diet, supplied for the dam. Nevertheless, for reasons discussed above and in previous Chapters, it is not expected that an increased food intake accounts for the marked growth response observed following infusion of IGF-I peptides. In agreement with the present observations, Philipps et al. (1988) have shown that administration of IGF-I promotes body weight gain in pituitary intact rats. As discussed in Chapter 5, IGF-I effects were also more pronounced during the later suckling period compared to the growth curves obtained during the early suckling period.

The increased sensitivity of IGF-I peptides on the renal and splenic tissues during the late suckling period was one of the most surprising observations. For example, an increase in fractional kidney weight of 69% above control values in rat pups treated with 5µg/g/day of LR³IGF-I. In comparison, although the kidneys of the younger rats were also highly responsive to IGF-I peptide administration, the same peptide dose increased fractional kidney weight by 48% above control values in the younger rats. Selective growth responses on the kidneys are commonly observed by other researchers. The kidney and spleen are amongst the most commonly reported organs to increase following infusion of IGF-I peptides. For instance in normal adult male rats, fractional kidney increased by 9% above control values following s.c. infusion of 120µg of IGF-I day (Hizuka et al., 1986). In nitrogen restricted adult rats, a 16% increase in fractional kidney weight was observed following administration of 2.9µg/g/day of IGF-I (Tomas et al., 1990). Similar increases in kidney weight have also been reported in animal models of pathological conditions (Lemmey et al., 1991, Martin, 1991 and Guler et al., 1988) or during fasting (Asakawa, et al., 1992). Although these responses were significant, they do not compare with by the massive increases in kidney weight observed in the present study.

Without further examination of the renal tissues it remains speculative to what extent IGF-I or LR³IGF-I may have stimulated cortical and/or medullary tissue components. IGF-I immunoreactivity has been localised in the collecting ducts and the thin Loop of Henle (Anderson et al., 1988 and Bortz et al., 1988). In addition, IGF receptors have also been identified on cortical structures (Hammermann et al., 1987) and in glomeruli (Conti et al., 1989) in normal adult rat kidney. Thus, it is tempting to speculate that infusion of IGF-I peptides results in either up-regulation of renal IGF receptors and/or paracrine or autocrine stimulation of renal tissues. This hypothesis is supported by observations showing that during compensatory renal growth, an increased level of IGF-I and its messenger RNA are present in several sites in the kidney (Stiles et al., 1985 and Flyvbjerg et al., 1988). This suggests a paracrine action of IGF-I on renal tissues. Observations that these sites are quite distant from the location of the majority of IGF-I immunoreactivity may contradict a paracrine action of IGF-I in the kidneys, but as suggested by Anderson et al. (1990), it is possible that IGF-I is secreted by the kidney to act on sites some distance away. From the observations in the present study, it remains hypothetical whether IGF-I may have acted directly on the renal tissues and if so, whether an increased renal mass equates to improved renal function in IGF-I treated rat suckling pups. In adult nephrectomised rats improved renal function has been shown following administration of IGF-I (Martin et al., 1991).

CHAPTER 7:

CONCLUDING REMARKS AND FUTURE DIRECTIONS



Concluding remarks and future directions

Gastrointestinal growth and function is similar in all mammals and is determined by a complex interactions between intrinsic timing mechanisms, endogenous regulators and nutritive and environmental factors. In recent years, research directed towards identifying drugs suitable for the treatment of gastrointestinal disease or malfunction extended to “naturally occurring” peptide growth factors that enhance gastrointestinal growth and function. However, the mechanisms by which growth factors stimulate growth needs to be explicitly determined and growth responses recognised before clinical trials commence. The studies presented in this thesis have characterised the effects of s.c. administered IGF-I peptides on the growth and function of the mature and immature intestine in rats. IGF-I gut effects were described for normal animals rather than under pathological conditions to identify the general mechanisms of IGF-I peptides on the gut.

7.1 The mature and immature gut respond differently to subcutaneously administered IGF-I peptides.

In this thesis, I have shown that IGF-I and the IGF-I analogue LR³IGF-I selectively stimulate growth of both the mature and immature intestine. However, there is differential sensitivity to IGF-I in different regions of the gastrointestinal tract, as well as developmental changes in response to IGF-I peptides. Moreover, the gastrointestinal tract also responds differently to the IGF analogue during the suckling period compared to the adult period.

Regional differences:

In both, the immature and mature gastrointestinal tract, the proximal gut region, namely the stomach and the duodenum, were the most peptide sensitive gut regions. In the mature intestine, only the mucosal compartment seemed to be responsive to IGF-I infusion,

while the musculature appeared relatively unaffected by the growth factors. In contrast, IGF-I peptide infusion markedly stimulated mucosal and non-mucosal tissue layers in the rats treated during the early and late suckling period. This suggests that during the suckling period IGF-I peptides act as a more general growth promoter, influencing cell of endothelial and mesenchymal origin, while in the mature intestine IGF-I action may be restricted to the epithelium.

Although small intestinal length increased following a challenge with IGF-I peptides in the adult model, the large intestine appeared relatively unresponsive. Conversely, in the suckling rats, a lengthening of the large intestine was observed in both age groups. This indicates that in suckling rats the large intestine responds in a manner similar to the small intestine. These findings are not surprising because the large intestine resembles functionally the small intestine during foetal life and during the early postnatal period. For example enzymes generally found in the small intestine and associated with digestion and absorption of carbohydrates and proteins during adult life are found in the colonic epithelium in the foetal and neonatal period (reviewed by Potter, 1989).

Developmental changes in sensitivity to IGF-I:

Administration of the native IGF-I was most effective in stimulating gastrointestinal growth in the adult rats. In contrast, administration of IGF-I during the suckling period, and particularly the early suckling period resulted in relatively flat growth response curves. Thus, the optimal dose of IGF-I that would result in maximal growth responses of the gastrointestinal tract was not identified and is at present not known. Although the gastrointestinal tract was more sensitive to the native IGF-I during the later suckling period, growth responses were not as marked compared to the adult rats for similar doses of the growth factor.

The neonatal period (first 2-3 weeks post partum) in the rat represents a time of transition between pituitary independence as seen during foetal growth, and total pituitary dependent growth as seen in juvenile and adult rats (Glasscock et al., 1990). Studies by Glasscock and Nicoll (1981) have shown that despite similar reductions in IGF-I and IGF-II levels following hypophysectomy of suckling or juvenile rats, somatic growth retardation is less severe in neonatal rats. These authors concluded that mechanisms other than circulating IGF-I levels may be responsible for the persistent growth in the neonatal but not juvenile rats. While I did not compare the same developmental stages, the results of the present study suggest that rats are more sensitive to IGF-I during the late suckling period compared to the early suckling period. It seems possible that the tissue sensitivity is bi-phasic, reaching maximum just prior to weaning. Alternatively, other factors enhance the sensitivity of the gastrointestinal tract to IGF-I.

Because at weaning the internal environment exhibits substantial changes, several other factors may explain the different in responsiveness to IGF-I observed in the present study. For instance, it is noteworthy that changes of free and total thyroxine parallel each other and increase markedly at the time of weaning in the rat (Koldovsky, 1985). Similarly, free and total corticosterone increase at the end of the 3rd postnatal week in rats (Koldovsky, 1985). It is therefore possible that the increased sensitivity of the older suckling rats to IGF-I infusion represents a combination of effects involving IGF-I and GH as well as synergistic interaction of corticosteroids and thyroxine. This issue needs further clarification.

Sensitivity to LR³IGF-I:

In contrast to the flat growth response curves obtained with IGF-I, LR³IGF-I markedly stimulated gastrointestinal growth. Maximal growth responses were observed when LR³IGF-I was administered to 12 day old suckling rats, terminating peptide treatment just prior to

weaning. Thus, LR³IGF-I was more effective during the late suckling period compared to either the early suckling period or during adulthood. However, despite the marked proliferative effect of the gut tissue by the more potent IGF-I analogue, the delicate balance between cell production and cell loss was maintained following growth factor treatment. This indicates that the normal intrinsic pattern was not over-ruled by LR³IGF-I peptides. However, it remains to be established if intestinal growth and proliferation continues at an accelerated rate following withdrawal of the growth factor.

As summarised in Chapter 1, the increased potency of LR³IGF-I relates to the fact that the analogue shows greatly reduced affinity to several of the IGF-BPs. Conversely, the reduced sensitivity of the gastrointestinal tract to the native IGF-I during the suckling period may also be determined by binding proteins produced locally by tissues of the gastrointestinal tract. It is well established that circulating IGF-BPs are developmentally regulated. In addition, during the foetal and early neonatal period, IGF-BPs are also abundant in gastrointestinal tissues. It is therefore likely that locally produced IGF-BPs play a major role in the regulation of IGF-I bioavailability to gastrointestinal tissues.

7.2 IGF-I responses under different physiological conditions

It seems that IGF-I gut effects (weight and length) differs also under different physiological conditions. Following resection of 70% of the small intestine in adult rats, a similar dose of LR³IGF-I as used in the present study administered for a 7 day period failed to increase small and large intestinal length, but maintained a stimulatory effect on the weight of small and large intestine (Lemmey et al., 1991, Vanderhoof et al., 1992). As such intestinal resection represents a situation where intestinal proliferation is at its maximum to compensate for intestinal tissue loss and IGF-I effects were moderate. More substantial gains in gut weight have been observed in streptozocin-induced diabetic rats and in dexamethasone treated rats

(Tomas et al., 1991 and Read et al., 1992a), representing models of mild intestinal hyperplasia and hypoplasia, respectively. In contrast, intestinal proliferation in normal neonatal rats is lower than that for normal adult rats. Thus, it seems that the differences in responsiveness of the gastrointestinal tract to IGF-I peptides is also determined by the proliferative base line at the time of peptide treatment.

7.3 Changes in IGFs: Role of IGF-II in growth during neonatal development?

In the present study plasma IGF-II levels were not measured, but it may be expected that infusion of exogenous IGF-I may have displaced some exogenous IGF-II from their IGFBPs. As such, IGF-I growth response in the present study may be the result of a combination of IGF-I/IGF-II mediated responses. However, this may only apply to the rats pups treated with LR³IGF-I.

Developmental changes in circulating IGF concentrations are most prominent during the immediate perinatal period. While in humans, circulating IGF-I levels are low during foetal life (Daughaday and Rotwein, 1989), IGF-II is the predominant circulating IGF species in the foetal rat (Moses et al., 1980). In the rat, IGF-II declines rapidly after approximately 2 weeks *post partum* and IGF-I takes over as the major IGF species (Glasscock et al., 1991). Similar observations have been made for sheep (Mesiano et al., 1980).

Although IGF-II is thought to be primarily a foetal growth factor (DeChiara et al., 1990, Sara and Hall 1990 and Humbel, 1990), several studies suggest that IGF-II is also an important growth factor during postnatal life. Like IGF-I, IGF-II promotes a variety of growth promoting and metabolic actions that are mediated mainly through the IGF-I (type 1) receptor (Nissley et al., 1991 and Kiess et al., 1989b). It has been shown that administration of IGF-II increases body weight gain in hypophysectomised rats (Shaar et al., 1989). Similarly,

Van Buul Offers et al. (1988) demonstrated that administration of IGF-II to Snell dwarf mice significantly promoted body weight gain and other growth indices. Thus, the possibility that IGF-II may also have contribute significantly to promote gastrointestinal growth in this study can not be disregarded.

7.4 Therapeutic applications

Difference in responsiveness throughout postnatal development as well as during adulthood need to be carefully examined when considering therapeutic application of IGFs. However, the data presented in this thesis suggests that IGF-I may be useful in the treatment of gut disease such as short bowel syndrome. At present, there are no drugs available that adequately overcome the adverse effects of short bowel syndrome. For instance in short bowel syndrome, the catabolic weight loss associated with surgery and reduced absorption of water and nutrients greatly compromise the well being and survival of patients. Consequently, short bowel syndrome patients need to be maintained on TPN which further compromises their recovery. Because TPN is marked by a mucosal atrophy malabsorption of water and nutrients is further enhanced.

In adult rats, administration of IGF-I peptides to gut resected rats has been shown to markedly improve body weight gain, food conversion efficiency, nitrogen balance, gut growth, and fat and nitrogen absorption (Lemmey et al., 1991 and Vanderhoof, et al., 1992). In a recent communication, Zhang et al. (1995) have shown that administration of 2.4µg/g/day of IGF-I by s.c. implanted mini osmotic pump significantly increased crypt cell production rates, villus height, crypt depth and villus surface area as well as glucose and water absorption. Together, these studies indicated that IGF-I peptide administration in adult, gut resected animals significantly improve the mucosal structure and function of the remaining gut and diminish the adverse catabolic conditions associated with short bowel

syndrome. Because IGF-I peptides significantly stimulated intestinal length in suckling rats, it is tempting to speculate that administration of IGF-I peptides would improve gut regrowth and intestinal function in neonatal resected animals. In analogy, EGF has been shown to significantly improve intestinal regrowth in weanling rats (Read et al., 1986). However unlike IGF-I, EGF fails to stimulate somatic growth (Oak et al., 1983). As such, IGF-I may be a suitable candidate for treatment of short bowel syndrome in neonates.

In the future, improved intestinal function, in particular stimulation of intestinal disaccharidases by IGF-I peptides may also represent a target of research. For instance, in conditions of hypolactasia (very low levels of lactase in jejunal mucosa), or lactose deficiency/intolerance and lactose malabsorption occurring secondary during infections or infestation of the small intestine in coeliac disease (Sahi, 1972 and Sahi, 1994) or in conditions of protein malnutrition (Bolin, et al., 1971), IGF-I may improve intestinal function. However, it needs to be clearly established if the histological appearance of the mucosa and activities of other disaccharidases are normal and additionally, if lactose deficiency is genetic or acquired.

7.5 *Future directions*

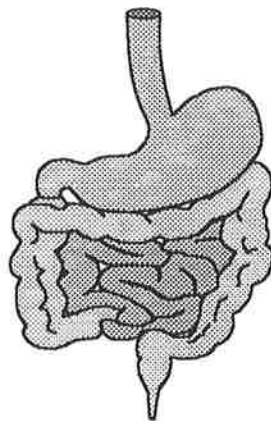
One of the most interesting aspects of the present study was the marked stimulation of gastrointestinal tissue and non-gut organs following subcutaneously administered IGF-I peptides. From a clinical point of view, challenges for the future are to determine the efficiency of IGF-I peptides on gastrointestinal growth and function when administered via the oral route. Specifically, are IGF-I peptides more effective when administered orally, given that mammary secretions contain a significant amount of IGF-I and proteolytic activity in the neonatal suckling rat is greatly reduced compared to the adult? Secondly, are oral IGF-I effects restricted to the mucosal compartment? Marked stimulation of non-gut organs may

be undesirable in situations where treatment is aimed specifically at gut function rather than growth. Thus, applications directed at the mucosal compartment rather than underlying tissues and non-gut organs provides a great advantages. Thirdly, does IGF-I cross the intestinal epithelium intact in the suckling intestine and if so, is IGF-I transport via paracellular or intracellular pathways *in vivo*. Studies by Koldovsky et al. (1992) suggest that that degradation of IGF-I, IGF-II and other growth factors including EGF and TGF α is much lower in the suckling intestine compared to the adult intestine, suggesting that orally derived IGFs may influence intestinal growth and function. This issue represents an interesting topic by itself and relates to the importance of drug delivery across the intestinal epithelium. These questions are of paramount interest especially for the development treatment strategies.

From a more general point of view, the role of locally produced IGFbps (in the intestinal mucosa) and their function in determining the bioavailability of IGF-I peptides needs further attention. At present, it is well established that the IGFbps in serum change markedly throughout development, however, the role of tissue derived IGFbp in different parts of the immature intestine is less clear. For instance, does continuous infusion of IGF-I peptides alter the expression pattern of IGFbp in the intestinal mucosa, and if so, does this correlate with the responsiveness of the intestine in the suckling and adult rats?

Finally, while this study has elucidated the effects of IGF-I peptides on gastrointestinal growth and function in the mature and immature intestine, the mechanisms by which intestinal growth has been achieved at the cellular level need further addressing. For example, does IGF-I stimulate intestinal proliferation directly or via synergistic interaction with other factors? Furthermore, is priming by other growth factors such as EGF required for IGF-I mediated proliferation *in vivo*. These questions represent a challenge in the future and will help piecing together a complex picture of how IGF-I peptides stimulate gastrointestinal growth.

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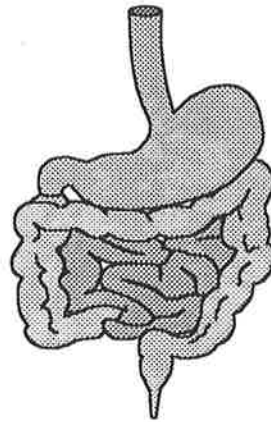
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APPENDICES



APPENDIX 2.1

Histological fixatives:

1) Bouin's fluid:	75	ml aqueous. solution of picric acid
	25	ml Formalin
	5	ml glacial acetic acid
2) Methacarn fixative:	60	ml methanol
	30	ml chloroform
	10	ml glacial acetic acid
3) Fomal calcium	10	ml 40% Formaldehyde
	90	ml distilled water
	1.1g	calcium chloride

Gelatine coated Slides

- 1) Add 5g gelatin in 1 litre of hot distilled water.
- 2) Add 0.25g of chromic potassium sulphate.
- 3) Dip cover-slips in hot distilled water, then immerse cover-slips in gelatin solution for 2-3 sec.
- 4) Dry in oven at 37°C for 1 hour.

APPENDIX 5.1

Body weight change in 6 day old rat pups prior to pump implantation and assignment to treatment groups

Lot no.	Litter no.	"0" no. pups at birth	day 2 pp no. pups culled	day 3 pp animal number	day 3 pp body wt. (g)	day 4 pp body wt. (g)	day 5 pp body wt. (g)	day 6 pp body wt. (g)	treatment code	treatment
Lot 1	1	10	1	1	10.16	12.03	13.83	15.79	D	sIGF-I
				2	9.82	11.73	14.34	15.52	C	hIGF-I
				3	9.98	11.50	13.72	15.26	A	vehicle
				4	9.89	11.01	12.93	14.70	E	mLR3
				5	9.91	11.69	13.69	15.07	SP	con
				6	9.45	11.08	13.08	14.75	B	mIGF-I
				7	9.80	11.49	12.65	14.25	F	hLr3
				8	9.97	11.84	13.47	14.80	SP	con
				9	9.64	11.05	12.94	14.79	SP	con
Lot 1	2	13	4	1	9.05	10.57	12.41	13.59	D	sIGF-I
				2	9.25	10.60	12.34	13.84	C	hIGF-I
				3	9.30	10.58	12.46	13.72	A	vehicle
				4	9.68	11.98	12.95	14.38	E	mLR3
				5	9.29	10.42	12.35	13.85	F	hLR3
				6	9.16	10.48	12.25	13.80	SP	con
				7	9.00	10.08	11.54	13.30	SP	con
				8	9.55	10.82	12.78	14.16	B	mIGF-I
				9	9.17	10.43	12.16	13.62	SP	con
Lot 1	3	9	0	1	10.11	11.78	14.10	16.13	SP	con
				2	10.98	12.67	14.29	16.52	A	vehicle
				3	10.26	12.13	14.11	16.01	D	sIGF-I
				4	9.99	12.01	13.67	15.57	C	hIGF-I
				5	10.58	12.51	14.72	15.44	SP	con
				6	10.42	12.02	13.59	15.34	F	hLR3
				7	11.04	12.77	15.34	16.51	B	mIGF-I
				8	10.62	12.13	14.62	16.31	SP	con
				9	10.45	12.06	14.15	15.81	E	mLR3

Code:

- A vehicle (0.1M acetic acid)
- B mIGF-I (2mg/kg/day)
- C hIGF-I (5mg/kg/day)
- D sIGF-I (12.5mg/kg/day)
- E mLR3 (2mg/kg/day)
- F hLR3 (5mg/kg/day)
- SP spare animals

APPENDIX 5.2

Protein Assay Protocol (mucosal scrapings) Method by Dahlqvist (1965)

- 1) Thaw tissue sample and add 1.5ml of 1M NaOH
- 2) homogenise for 30 seconds
- 3) create 2 sub-samples
 - I) 600µl homogenate & 400µl 1M NaOH
 - II) 600µl homogenate and 200µl 1M NaOH
- 4) leave sub-samples at 4°C for 48 hours
- 5) vortex samples and from each of I and II, take 100µl and add 900µl 0.5M NaOH.
- 6) place 50µl into microplate (4 replicates).
- 7) add 150µl of Reagent A every 15 seconds with pipette. Exactly 10 minutes later add 50µl of 25% Folin Reagent. Mix well, leave for 1 hour at room temperature. Read at filter 8&2.

Standard: Stock solution 4mg BSA/ml distilled water.

Stock soln.	Water	1M NaOH	µl/well
0	400	400	0
10	390	400	2.5
25	375	400	6.25
50	350	400	12.5
75	325	400	18.5
100	300	400	25
125	275	400	31.25

add 50µl of standard to each well. Replicate 4 times.

Reagent A: 75ml 2.56% NaCO₃ and 750µl 2.56% Na tartrate and 750µl of 1.28% CuSO₄.5H₂O.

25% Folin: 5ml Folin and 15 ml of water

DNA assay protocol (mucosal scrapings)

- 1) Thaw sample (same as for proteins)
- 2) homogenise sample for 30 seconds
- 3) create 2 sub-samples
 - I) 600µl sample and 400µl 1M NaOH
 - II) 600µl sample and 200µl 1M NaOH
- 4) leave sub-samples for 48 hours at 4°C.

continued Appendix 5.2

- 5) vortex digested homogenates and for each of the sub-samples place 500µl of the homogenate into Eppendorfs.
- 6) add 50µl 5M HCl and 2.5ml cold 0.36M perchloric acid (PCA).
- 7) extraction procedure: vortex and place on ice for 30 minutes
centrifuge at 2500rpm for 20 minutes
remove and discard supernatant, add 1ml of 0.3M cold PCA to pellet.
vortex and place on ice for 30 minutes
centrifuge as before and prepare standard curve
remove and discard supernatant, add 2ml of cold 0.5M PCA to pellet.
vortex and heat at 70°C for 20 minutes, cool at room temperature (10min.).
place on ice for 30 minutes
centrifuge as before. Remove supernatant and place 200µl of supernatant into each well on microplate (4 replicates).
- 8) add 200µl diphenylamine reaction solution to each well , mix well and leave for 17 hours at 30°C in covered water bath. On the next day read samples at filter 7.

Standard: Stock solution, 200µl DNA/ml water

Stock soln.	water	1M PCA	µg DNA/well
0	500	500	0
100	400	500	2
200	300	500	4
300	200	500	6
400	100	500	8
500	0	500	10

heat at 70°C for 20 minutes, cool and place on ice for 30 minutes. Add 100µl/well.

Reagents: A) 1.5g diphenylamine (DPA) and 100ml acetic acid and 1.5ml concentrated H₂SO₄.

B) 200µl acetylaldehyde and 9.5ml cold water= aqueous aldehyde

Add 55µl of reagent B to 100ml of reagent A (DPA reaction solution).

continue Appendix 5.3

TGO solution

3.75ml glucose oxidase

15mg horseradish peroxide

2.5ml O-dianisidine solution (0.1g/ml 95% EtOH)

400ml of 0.25M Tris buffer pH 7.0

TGO is only stable for approximately 2 weeks, photosensitive, store wrapped in alfoil

50mM phosphate buffer

50mM acidic and 50mM basic phosphate buffer

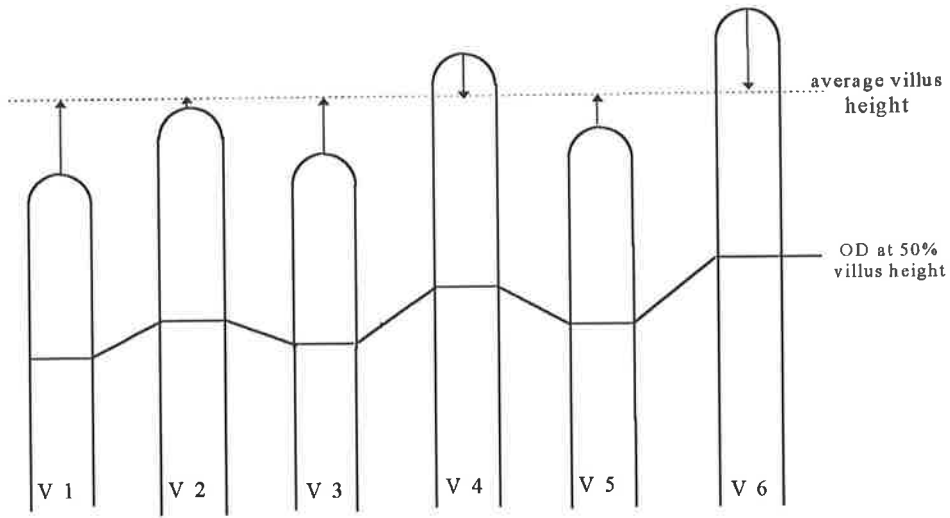
acidic: NaH_2PO_4 7.8g/l water

basic: Na_2HPO_4 anhydrous 7.1g/water

titrate acidic PO_4 with small amount of basic PO_4 to pH 6.1, add 200 μl triton/l buffer.

APPENDIX 5.5

Correction of villus height for OD measurements of lactase and sucrase enzyme products along the villus axis.



V1-V6 indicated 6 individual villus structures measured for histochemical detection of sucrase and lactase activity.

