EFFECTS OF INSULIN-LIKE GROWTH FACTORS (IGFS) ON RECOVERY FROM GUT RESECTION IN RATS

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"He is not worthy of the honeycomb who shuns the hives because the bees have stings"

William Shakespeare

ABSTRACT

Circulating levels of insulin-like growth factor I (IGF-I) are depressed in conditions characterized by a catabolic state and/or inadequate nutrition. A gut resection model in which either 70% or 80% of the jejuno-ileum is removed from growing rats has been developed so that IGF-I therapies can be investigated in catabolic, nutritionally disadvantaged animals. In this study subcutaneous miniosmotic pumps which delivered either vehicle (0.1 M acetic acid), IGF-I, or one of its analogues, des(1-3)IGF-I or LR^3IGF-I were implanted into animals immediately following gut resection. The analogs, des(1-3)IGF-I and LR^3IGF-I, have a much reduced affinity for IGF binding proteins compared with intact IGF-I. Assessments of body weight, food and water intake, and collections of faeces and urine occurred daily throughout the experimental period. One week after surgery the animals were killed. At sacrifice internal organs were weighed and gut samples were taken for subsequent biochemical and histological analysis.

All groups lost 12 - 15g in the 24h following 80% resection, but over the next six days the peptide treated animals gained significantly more weight than the controls. The effects of surgery and peptide treatment on body weight were less profound in the 70% resection. Increased food intake could not account for the improved body weight gain of the IGF-treatment. Nitrogen balance measures and food conversion efficiency mirrored the trends for body weight gain with the peptide-treated rats performing significantly better than the controls. Measurement of 3-methylhistidine excretion indicated that the rate of muscle protein breakdown may have been reduced in the animals receiving IGF peptides. The two IGF-I analogs, des(1-3)IGF-I and LR^3IGF-I, were approximately 2.5 fold more potent than IGF-I in improving body weight, nitrogen retention and food conversion efficiency.

IGF peptide treatment produced increases in the relative weights (g/kg bw) of the spleen, kidneys and thymus, but not the liver, adrenals, heart, lungs, carcass or pelt. Nor were any effects on carcass composition observed. In contrast, substantial effects of IGF peptides were noted in the gastro-intestinal tract. Total gut weight (stomach to colon inclusive) was increased by up to 26% ($p < 0.001$) by IGF treatment, with growth responses being particularly apparent in the stomach, duodenum and remnant jejuno-ileum. Histological and biochemical analyses suggested that the growth observed in the gastro-intestinal tract occurred principally through proportional increases in the various cross-sectional layers of the gut, and via hyperplasia rather than hypertrophy. Concurrent with the accretion of greater gut mass, was a significant attenuation of post-resectional fat and nitrogen malabsorption in the IGF-treated animals. This effect was most apparent in the 70% resection experiment.

The present study has shown that IGF-I peptides are effective in diminishing post-surgical catabolism and enhancing adaptive gut hyperplasia in rats recovering from massive small bowel resection. These effects, together with the finding that IGF peptides improve compromised absorptive function, suggest that IGF-I or one of its analogs could be therapeutically valuable in treating gut disease.

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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 university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text. I consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan.

Dated at Adelaide: . . 31/12/92. . . . . .

Signed .

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ABBREVIATIONS

3-MH	3-methylhistidine
ANOVA	analysis of variance
bw	body weight
CCK	cholecystokinin
CCPR	crypt cell production rate
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
g	gravitational acceleration
GH	growth hormone
HDL	high density lipoprotein
IGF	insulin-like growth factor
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFBP	insulin-like growth factor binding protein
kDa	kilo dalton
$LR^3IGF\text{-}I$	$longR^3$ insulin-like growth factor-I
mRNA	messenger ribonucleic acid
NIDDM	non-insulin dependent diabetes mellitus
N-terminus	amino terminus
p	probability
r	Pearson correlation coefficient
RIA	radioimmunoassay
s.c.	subcutaneous
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGS	short gut syndrome
TPN	total parenteral nutrition

CHAPTER 1

General Introduction

Severe physical trauma, major surgery, infections, cancer, end-stage organ disease and burns are conditions characterized by an elevated metabolic rate. To satisfy the substrate requirements of a heightened energy demand, the body assumes a catabolic state in which muscle protein is preferentially degraded. This catabolism, while providing amino acids necessary for gluconeogenesis and the synthesis of proteins essential for wound healing, immunologic defense, and the maintenance of vital organ function, if prolonged can result in devastating muscle weakness leaving the patient vulnerable to death from eventual multisystem failure (Christou et al., 1980; McMenamy et al., 1981). Since dietary supplementation has generally been found to be counter-productive in ameliorating severely catabolic states (McMahon, 1985), the exogenous administration of anabolic agents is viewed as a preferable therapeutic strategy.

This tactic is especially appealing in situations in which catabolism is accompanied by major impairments to nutrient absorption. Such a condition exists following extensive resection of the small bowel, a procedure commonly performed in adult patients with inflammatory bowel disease, vascular insufficiency or intestinal neoplasms, and in infants with congenital abnormalities, necrotizing enterocolitis or vascular disruptions. After massive resection, it is necessary to provide the patient with nutritional support by total parenteral nutrition (TPN) until sufficient compensatory bowel growth has been achieved. However, in addition to being expensive and inconvenient, prolonged TPN is associated with the inhibition of post-resectional adaptive hyperplasia (Morin et al., 1978), as well as numerous potentially severe complications (see Section *1.1.2* and *1.2.1*).

It is apparent that any clinical treatment which would improve nitrogen balance, whilst also potentiating post-resectional gut adaptation, hence attenuating malabsorption and enabling as early a shift from TPN to oral feeding as possible, would be of considerable assistance to resection patients.

The aim of the work described in this thesis was to determine the potential therapeutic value of insulin-like growth factor-I (IGF-I) in controlling catabolic weight loss and stimulating gut growth following massive bowel resection. IGF-I was advanced as a possible therapy for catabolic conditions and in particular, gut-resection, on the basis of : 1) its *in vitro* stimulation of protein synthesis and inhibition of protein breakdown (Ballard and Gunn, 1985), and preliminary results which demonstrated comparable *in vivo* actions (Hizuka et al., 1986; Schoenle et al., 1982), 2) the knowledge that plasma IGF-I levels are reduced in catabolic conditions (Clemmons et al., 1985; Frayn et al., 1984; Froesch and Zapf, 1985; Van Wyk, 1984). Indeed plasma IGF-I concentrations are more faithful indicators of catabolism than circulating growth hormone levels (Froesch and Zapf, 1985; Van Wyk, 1984), and 3) following the identification of IGF receptors (Laburthe et al., 1988) and IGF-I production (D'Ercole et al., 1986; Han et al., 1988) in the gut, the putative role of IGF-I as a gut mitogen.

The topics covered in this historical review broadly pertain to: 1) the functional deficits that occur following intestinal resection and the adaptive responses made by the gut to overcome these, 2) how the body responds to nutritional and surgical trauma, 3) growth promotants, other than IGF-I, that may be useful in ameliorating stress induced catabolism and augmenting adaptive hyperplasia in the resected gut, and 4) IGF-I - its structure, production and action.

This review generally cites work published prior to April 1988, the commencement date of my Ph.D candidature. The exceptions to this are in the sections on "IGF binding proteins" (1.4.5) and "Variant forms of IGF-I" (1.4.7), where it was necessary to include more recent studies. Information relevant to this dissertation published since April 1988 is included in the appropriate discussion sections of each chapter.

1.1. ADAPTIVE REGROWTH and FUNCTIONAL RECOVERY FOLLOWING GUT RESECTION

The animal trauma model used in this study involves removal of either 70% or 80% of the jejuno-ileum in rats. This model provides both surgical and nutritional stress. In 1925, Wildegans (1925) concluded that the loss of 33% or less of the small intestine in man is consistent with maintenance of normal weight and strength, while removal of between 33% and 75% of the intestine results in losses in weight and strength, together with diarrhoea, and loss of greater than 75% is characterized by severe marasmus. Animal and human studies conducted since then have confirmed that while loss of up to half the small intestine is generally tolerated, provided the residual bowel is healthy, progressively greater losses increasingly threaten survival and quality of life. Removal of 50-75% of small intestine from growing rats generally results in rapid and immediate post-surgical weight loss. The severity of this procedure is indicated by reports of 10-33% mortality (Ford et al., 1984; Goodlad et al., 1988; Hart et al., 1988; Wilson et al., 1986).

1.1.1 Absorptive deficits following bowel resection

As well as the catabolic weight loss associated with any form of major surgery, extensive resection of the small bowel presents additional nutritional complications that further compromise the long-term survival and health of the patient. Malabsorption of food, especially fat and protein, vitamins, minerals, electroytes and water is present in resection patients until adequate compensatory adaptation has occurred in the gut (Nygaard, 1966; Urban and Weser, 1980). Coupled with a reduction in food intake, impaired absorption of nutrients usually results in malnutrition following resection (Urban and Weser, 1980).

The different regions of the small intestine have different absorptive roles, so that the nature of the clinical complications associated with post-resectional malabsorption are determined by the site and extent of resection. The duodenum is the

principal site for iron, calcium and folate absorption, and the jejunum the major site for the absorption of fat, protein, carbohydrates and most water-soluble vitamins (Williamson, 1978). Additionally, the duodenal and jejunal mucosa are the sites of cholecystokinin (CCK) and secretin synthesis and release, hence extensive loss of upper small bowel may impair hormonal stimuli of biliary and pancreatic secretions thereby exacerbating the malabsorption of fat and protein. The ileum is the selective site for absorption of conjugate bile salts (Tilson et al., 1975), vitamin B_{12} (Bohane et al., 1979) and 1-25-dihydroxy vitamin D (Wilson and Schedl, 1987). Since the ileum can adapt to absorb carbohydates, protein and water-soluble vitamins in the absence of the duodenum and jejunum, but the jejunum cannot adapt to absorb bile salts or vitamin B_{12} (Fleming and Remington, 1981) distal resection is more deleterious than proximal resection. Impaired reabsorption of conjugated bile salts decreases their enterohepatic circulation, thus reducing bile salt concentration in the lumen of the small intestine and further reducing fatty acid absorption. Additionally, unabsorbed bile salts, when they enter the large bowel, are deconjugated by colonic bacteria. In this form the bile salts impede colonic water uptake and if in sufficiently high concentrations can cause active secretion of water into the colon (Mekhjian et al., 1971). Contributing further to this problem, unabsorbed fatty acids and their colonic by-products, hydroxy fatty acids, also impair water absorption in the large bowel (Ammon and Phillips, 1973). These effects on water uptake by the colon account for the increased diarrhoea and steatorrhoea characteristically observed following resection. In human infants, steatorrhoea can persist for nine months or longer following resection (Bohane et al., 1979). Due to impaired colonic uptake of water, diarrhoea and steatorrhoea, and reduced water reabsorption by the shortened small intestine, hypovolaemia and dehydration are significant risks associated with small bowel resection.

Since the ileum is also the selective site for the absorption of Vitamin B_{12} (Bohane et al., 1979) and 1-25-dihydroxy vitamin D, a vitamin D metabolite (Wilson and Schedl, 1987), malabsorption of these usually occurs following distal resection

(Urban and Weser, 1980). Potential consequences of inadequate vitamin B_{12} absorption include megaloblastic anaemia, neurologic deficits and changes in intestinal mucosal cell morphology coupled with deteriorations in mucosal transport functions (Arvanitakis, 1978). As 1-25-dihydroxy vitamin D is required for calcium transport (Wilson and Schedl, 1987), distal resection can produce calcium deficiencies. Calcium absorption is further compromised following resection as luminal calcium is sequestered as insoluble calcium soaps by steatorrhoea. Consequently, resected patients, especially those who have lost much of the ileum, often present with symptoms of tetany, osteopenia and osteomalacia (Urban and Weser, 1980).

As well as vitamins B_{12} and D, several other vitamins may be inadequately absorbed following resection. Malabsorbtion of fat may result in deficiencies of the fat soluble vitamins (A,D,E,K) (Urban and Weser, 1980), while vitamin C absorption also appears to decline after resection (Althausen et al., 1949).

1.1.2 Adaptive changes after bowel resection

To counter the loss of absorptive surface area, compensatory structural and functional adaptations occur in the remnant gut. Principally these adaptive changes involve: 1) epithelial hyperplasia and 2) elongation of the gut, to increase absorptive surface area, and 3) functional changes such as increases in the secretion or specific activities of digestive enzymes.

Intestinal epithelial cell division only occurs in a specific region at the base of the crypts of Lieberkuhn (Cairnie et al., 1965). After cell division, the new cells move up the crypt and on to the villus. During this migration the enzymes responsible for DNA synthesis are lost, whilst those associated with nutrient breakdown and absorption are acquired. After climbing the villus, senescent cells are extruded from the villus tip into the lumen. Following extensive intestinal resection there is a dramatic increase in the crypt cell production rate (Besterman et al., 1982; Bohane et al., 1979;

Dorney et al., 1985; Ford et al., 1984; Grey and Morin, 1985; Luk alnd Baylin, 1983; Sagor et al., 1983; Savage et al., 1985; Weser and Hernandez, 1971). In rats, this compensatory increase in cell renewal is evident within 24h of surgery, maximal two days after resection (Luk and Baylin, 1983), and maintained for at least 48 days (Savage et al., 1985), with the consequence that the wet weight, and protein and DNA content per cm length of remnant small bowel is double that of unresected controls within two weeks of surgery (Luk and Baylin, 1983; Savage et al., 1985; Wilson et al., 1986). Adaptive hyperplasia is more apparent in the ileal remnant than the jejunal (Besterman et al., 1982; Sagor et al., 1983; Savage et al., 1985; Weser and Hernandez, 1971), with the most pronounced response occurring immediately distal to the anastomosis (Sagor et al., 1983). In a number of species including rats, humans, dogs and pigs, the manifested features of mucosal hyperplasia in the remnant small bowel are increased villus height and crypt depth (see Urban and Weser, 1980 for a review). In the rat, hyperplasia results in a decreased density of larger villi, yet the increase in villus size is such that the net mucosal surface area per unit serosal surface area increases (Menge et al., 1983).

The literature suggets that post-resectional adaptive hyperplasia is dependent on the intraluminal presence of nutrients since hyperplastic adaptations were absent in the intestinal mucosa of resected dogs (Feldman et al., 1976) and rats (Morin et al., 1978) maintained on TPN. Consequently, it is in the resected patient's interests that nutrition should be switched to the oral route as soon as is practicable.

Whether elongation of the remnant bowel occurs in response to resection seems to be dependent on species, maturity, nutrition, and the extent of the resection. In rats, elongation is stimulated following removal of 70% or more of the small bowel (Ford et al., 1984; Hanson et al., 1977; Sachder and Ackerman, 1979; Sagor et al., 1982), and occurs within a week when 90% of the small bowel is removed (Sachder and Ackerman, 1979). Elongation also occurs in children, provided the resection is

sufficiently severe (Kalifa et al., 1979). Notwithstanding the capacity for elongation in a number of species, mucosal hyperplasia appears to be the predominant mechanism for increasing absorptive surface area following gut resection (Clatworthy et al., 1952; Flint, 1912; Ford et al., 1984; Hanson et al., 1977; Kalifa et al., 1979; Sachder and Ackerman, 1979; Sagor et al., 1982; Wilmore et al., 1971).

A number of functional changes have been demonstrated following intestinal resection. In rats, specific disaccharidase activity (units per g of mucosa, or per cell) is generally found to be decreased (Urban and Haley, 1978; Weser and Hernandez, 1971; Wilson et al., 1986). Similarly, Weser and Hernandez (1971) found that the net transport of amino acids in remnant rat intestine, when determined as a function of cell number, was reduced relative to control values. These decrements in cellular function indicate an immaturity of transport processes in the hyperplastic mucosa (Urban and Weser, 1980). However, despite this cellular immaturity, segmental disaccharidase activity and amino acid transport levels are equivalent to, or even higher than, those of controls due to mucosal hyperplasia (Ford et al., 1984; Urban and Haley, 1978; Weser and Hernandez, 1971; Wilson et al., 1986). To compensate for the loss of absorptive sites for bile salts, supranormal absorption of bile salts by the remnant small bowel has been described following both distal (Tilson et al., 1975) and proximal resection (Tilson et al., 1977). Whether the increase in bile salt uptake is due to induction of active transport or increased diffusion across the hyperplastic villi is not known (Urban and Weser, 1980). Although conclusive data are not available, one study (Roger and Bochenek, 1970) reports an increase in specific activity of lipid re-esterifying enzymes of the remnant ileum.

In summary, massive intestinal resection results in a catabolic loss of protein, which, due to the loss of absorptive surface area from the gut, is exacerbated by malabsorption of nutrients, vitamins and water. A number of adaptive changes are made to compensate for the loss of gut, the most important of these being epithelial

hyperplasia. Dorney et al. (1985) in their review of bowel resection literature, suggest that adaptation to massive resection in humans takes at least a year to become fully established. Whether there is permanent malabsorption, they conclude, is dependent on the site and extent of the resection, whether the ileo-caecal valve is retained, the condition of the bowel remnant, and the degree to which the adaptive morphological and functional changes described are achieved.

1.2 CATABOLIC WEIGHT LOSS FOLLOWING GUT RESECTION

The effect of stress on humans or animals varies with age, sex, nutritional status, reproductive state, as well as the type and severity of stress (Cuthbertson, 1979). Characteristically, malnutrition and surgery, as well as other stresses such as disease or accidental injury produce catabolic states wherein protein breakdown and synthesis appear to be largely related to trauma severity and substrate availability, respectively (Clague et al., 1983; Wiersma and Kastelijn, 1985). Since skeletal muscle represents a large protein reserve, it is not surprising that stress induced catabolism primarily affects myofibrillar protein (Kinney, 1976).

1.2.1 Response to Nutritional Stress

As described in section *1.1.1*, extensive loss of small bowel leaves insufficient mucosal surface for adequate absorption of nutrients and fluid (Urban and Weser, 1980). Consequently, malnutrition due to malabsorption of nutrients, especially fat (Althausen et al., 1949; Nygaard, 1966; Wapnick et al., 1974), fat-soluble vitamins (Compston and Creamer, 1977; Wilson et al., 1987), protein (Althausen et al., 1949; Nygaard, 1966; Urban and Weser, 1980), and calcium (Wilson et al., 1987), will persist in post-resectional patients until adequate adaptation has occurred in the remnant gut. This section reviews the catabolic response to nutritional stress.

During prolonged calorie deprivation, protein synthesis, particularly muscle protein synthesis, is reduced as a means of decreasing energy expenditure and conserving nitrogen reserves (Carmichael et al., 1980; Clague et al., 1983; Golden et al., 1977; Sender et al., 1975; Rennie et al., 1982; Stein et al., 1980). This reduction is due to inadequate total substrate availability rather than the type of substrate available (Stein et al., 1980). In contrast, the response of protein breakdown to malnutrition is determined by the nature of the deprivation. During protein-calorie deprivation, muscle protein breakdown rates increase, whilst when the diet is protein deficient but energy sufficient, breakdown rates diminish to enable conservation of muscle protein (Haverberg et al., 1975). Since food intake is markedly reduced and nitrogen absorption is impaired during the initial phase of recovery following gut resection, this period is characterized by protein-calorie deprivation and, consequently, decreased muscle protein synthesis and increased breakdown (Urban and Weser, 1980).

The problem of inadequate nutrition in post-resectional patients is exacerbated when it is necessary to maintain these patients on TPN, as this route of nutrition is associated with mucosal hypoplasia (see Section *1.1.2*), as well as deficiencies in fatty acids (Caldwell et al., 1972), zinc (Woolfe et al., 1987; Jarnum and Ladefoged, 1981) and selenium (Baptista et al., 1984). Additionally, patients maintained on TPN are at an increased risk of developing sepsis (Cooper et al., 1984; Yokoyama et al., 1988), fatty liver (Rombeau and Rolandelli, 1987), cholestasis (Cooper et al., 1984) and venous thrombosis (Kenney et al., 1985; Sprocata et al., 1980).

Significant weight loss is the most obvious consequence of nutritional deprivation (Haverberg et al., 1975; Stein et al., 1980). However, repletion rapidly reverses this loss of weight (Clague et al., 1983; Stein et al., 1980) as feeding acts to stimulate protein synthesis (Clague et al., 1983; Rennie et al., 1982), thereby restoring the positive nitrogen balance necessary for the resumption of growth. The impact of refeeding on protein degradation is not as clear, as there is evidence for both unchanged

(Rennie et al., 1982) and decreased (Haverberg et al., 1975) degradation, based on measurement of urinary output of 3-methylhistidine (3-MH). Urinary 3-MH excretion provides an index of muscle catabolism in rats and humans (Tomas and Ballard, 1987) since it is derived exclusively from actin and myosin, which are predominantly in muscle tissue, occurs at a constant amount in these proteins, and is excreted quantitatively in urine following actin and myosin catabolism in rats and humans (Tomas and Ballard, 1987). Since food intake and absorption are diminished in resected patients, especially during the intial phase of recovery, alternative means for improving nitrogen balance immediately following surgery are required.

1.2.2 Response to major injury and/or surgery

Major surgery, such as gut resection, like other forms of massive trauma evokes a catabolic response.

Cuthbertson and Munro (1937) claimed that up to 80% of total weight loss following severe surgery could be attributed to decrements in muscle mass. The magnitude of this loss, like that during sepsis, is too great to be explained by immobilization and reduced dietary intake (Cuthbertson, 1979). The elevated protein turnover rates and negative nitrogen balances of patients following surgery point to the major cause of protein loss being adaptive responses in protein anabolism and catabolism. The nature and duration of this response seems to be dependent on the severity of the trauma with moderate stress, such as elective surgery, causing a fall in whole-body protein synthesis (Crane et al., 1977; Kien et al., 1978; O'Keefe et al., 1974; Rennie et al., 1984) with little or no change in whole-body protein breakdown (Clague, 1981), and severe surgery generally causing an increase in both synthesis and breakdown rates, with the rise in the latter being far more pronounced (Clowes et al., 1983; Cuthbertson, 1930; Neuhauser et al., 1980; Yamamori et al., 1987). Following surgery, including colon resection, protein degradation increases irrespective of post-operative diet, whereas protein synthesis increases linearly with protein intake (Clague

et al., 1983; Neuhauser et al., 1980). Thus, the effect of trauma, be it accidental injury or surgery, on protein breakdown is largely determined by the severity of stress, whereas protein synthesis is mostly dependent on nutritional status (Clague et al., 1983).

The increase in catabolism following major surgery or injury is, in part, a response to the requirement for additional substrate during the heightened metabolism of the recovery period. The basal metabolic rates of patients following major traumas are often 20-40% greater than normal, whilst those of severe burn victims may be doubled (Kinney, 1976; Wilmore et al., 1974). Frayn et al. (1984) found that the metabolic rates of accident victims were elevated for two weeks, in which time an average of 2kg of lean body mass was lost by each patient. Whether hyperenergetic feeding can counteract such losses is in doubt, since augmenting dietary calorie and nitrogen intake merely succeeds in further elevating patients' energy expenditure and needs (McMahon, 1986). Additionally, carbohydrate supplements provided to cats recovering from femur fractures, although exercising a nitrogen, potassium and creatine sparing effect, failed to ameliorate muscle atrophy (Cuthbertson et al., 1939).

In summary, the gut resected patient is compromised by the dual catabolic responses to nutritional deprivation and severe surgical stress. To counteract nitrogen loss, supplementary feeding by oral or TPN routes has been suggested. Due to its demonstrated ineffectiveness in the past, and the nature of this particular trauma, hyperenergetic oral feeding is not an appropriate tactic for reducing post-resectional nitrogen losses. While nutrition by TPN, as well as exacerbating malnutrition and impairing adaptive hyperplasia in the resected patient, is associated with numerous additional risks (see Section *1.2.1*). Therefore, another strategy, such as anabolic or growth promoting agents, is called for. In the following section, the potential roles of anabolic steroids and growth hormone are briefly reviewed.

1.3. GROWTH PROMOTING AGENTS

The ideal therapeutic agent for treating gut-resection patients would be one that prevented, or reduced, catabolic weight loss and concomitantly enhanced gut regeneration and adaption.

A net positive nitrogen balance is essential for growth, and vital in the treatment of and recovery from catabolic conditions such as nutritional deprivation, sepsis, muscle-wasting diseases and trauma. Several groups of agents have been shown to increase growth rates, prominent amongst these are anabolic steroids, growth hormone, and IGF-I. This section reviews anabolic steroids and growth hormone, while IGF-I is discussed in detail in Section *1.4*.

1.3.1 Anabolic Steroids

Anabolic steroids are synthetic versions of testosterone that stimulate protein synthesis in many tissues, especially skeletal muscle, as well as having anabolic actions on reproductive organs (Santidrian et al., 1982). Administration of large doses of anabolic steroids to normal humans generally produces increases in lean body mass with a reduction in body fat (Forbes et al., 1985; Hervey et al., 1976; Hervey et al., 1981). These responses are more widely documented in experimental animals, and are especially pronounced when female, castrated male, and hypophysectomized animals are used (Heitzmann, 1976; Kochakian, 1976).

Although relatively few investigators have looked at the effects of anabolic steroids in catabolic humans, improvements in nitrogen balance following steroid treatment have been reported in patients with head injuries (Hausmann et al., 1984) and following abdominal surgery (Tweedle et al., 1972), and in chronically ill women (Harris et al., 1961). Similarly, the gut actions of these agents have also received little attention. Stimulatory effects on enzyme reactions in the small intestine of adult female rats (Sandu et al., 1980), and increased mitotic activity in gastric mucosa, with

attendant improvement in symptoms, in patients with stomach ulcers (Korepanov and Gorbunov, 1987), are reported following anabolic steroid administration. In addition, nandrolone decanoate has been shown to improve post-operative splanchnic fuel metabolism in dogs (Souba et al., 1988).

Although the mechanism of anabolic steroid action is yet to be elucidated, Wangsness et al. (1981) hypothesised that these agents may act, at least in part, by modifying the operation of endogenous growth-affecting peptides, most probably growth hormone or the insulin-like growth factors (IGF-I and IGF-II). In support of this theory, increases in plasma IGFs and insulin have been observed following application of both trenbolone acetate and zeranol (Donaldson et al., 1981; Wangsness et al., 1981). IGF-I is a potent inhibitor of protein breakdown even at very low concentrations (see Section *1.4.2*) and insulin also has "anti-catabolic" properties (Ballard and Francis, 1983). Consequently, the growth stimulated by anabolic steroids may be partially mediated by factors such as IGF-I.

The usefulness of anabolic steroids is compromised by their various and well documented negative effects. Some of the potential side effects identified with chronic administration are: liver damage (Alen, 1985; Nadell and Kosek, 1977; Shephard et al., 1977; Johnson et al., 1975), liver carcinoma (Falk et al., 1981; Johnson et al., 1975), testicular atrophy (Aakvaaag and Stromme, 1974), diabetes (Shephard et al., 1977), muscle structure abnormalities (MacDougall et al., 1982), and depressed levels of HDL-cholesterol (Applebaum-Bowden et al., 1987; Kantor et al., 1985; Strauss et al., 1982) which may predispose long-term users to coronary heart disease (MacDougall, 1983). Furthermore, the premature closure of the epiphyseal plates induced by steroid use (Lamb, 1975) compromises growth in young steroid users. Some of the conditions described have been identified after just six months of steroid use. Since adaptation to massive gut resection, as evidenced by the need for TPN,

often requires twelve months or longer, the suitability of anabolic steroids as a post-resectional therapeutic agent is questionable.

1.3.2 Growth Hormone

Since the pioneering work of Lee and Schaffer (1934), the administration of growth hormone (GH) has been shown to augment growth and food conversion efficiency in a range of species. In normal man, GH promotes retention of nitrogen, as well as potassium and phosphate, mobilizes fat from adipose tissue, facilitates intracellular transport of amino acids, diminishes sensitivity to insulin, and stimulates collagen synthesis (Wilmore et al., 1974).

As well as these effects, GH is known to be essential for normal histological development of the gut (Cooke et al., 1986), reverses gut hypoplasia induced by hypophysectomy (Scow and Hagan, 1965) and has been shown to stimulate compensatory post-resection mucosal adaptation in the rat (LeBlond and Carriere, 1955; Taylor et al., 1975). Additionally, growth hormone releasing factor has been shown to stimulate crypt cell production rate (Lehy et al., 1986). Of further interest to this investigation are reports that treatment with GH improves nitrogen retention in burn victims (Prudden et al., 1956; Wilmore et al., 1974), patients recovering from major gut surgery (Ward et al., 1987), and normal volunteers receiving hypocaloric TPN (Ziegler et al., 1988). However, use of GH in doses sufficient to stimulate growth of intestinal mucosa has been shown to diminish deleteriously glucose tolerance within four days of commencing treatment (Hintz, 1984). Thus, the use of GH as a therapy for post-resectional patients is compromised by its intrinsic diabetogenic activity. From a clinical perspective, an agent having GH-like effects on body growth and mucosal hyperplasia but lacking its diabetogenicity would have clear advantages.

Contemporary theory proposes that most, if not all, of the anabolic effects of GH are not mediated directly, but via the endocrine/paracrine/autocrine action of the GH-dependent insulin-like growth factors (IGFs) (see Baxter, 1986; Daughaday, 1984; Herington et al., 1983, for reviews). GH regulation of IGF-I production has been demonstrated in a variety of *in vitro* systems (Adams et al., 1983; Atkinson et al., 1980; Clemmons et al., 1981b; Phillips et al., 1976; Schalch et al., 1979; Shapiro and Pimsone, 1978), as well as *in vivo* studies using rats and humans. When endogenous GH is eliminated in growing rats by anti-rat GH antiserum, plasma IGF levels are reduced within 3h, and growth is retarded within 24h (Gause et al., 1983). Conversely, circulating IGF-I levels, which are dramatically diminished following hypophysectomy, are largely restored in rats by exogenous GH (Schoenle et al., 1985; Schwander et al., 1983). In humans, improved growth rates of GH-deficient children (Schalch et al., 1982) and healthy adults (Copeland et al., 1980) given exogenous GH are significantly correlated with the GH-elevated serum total IGF and IGF-I levels. The concept of IGF-I mediated GH action is further supported by the discovery that IGFs inhibit pituitary GH secretion via a negative feedback mechanism (Van Wyk, 1984).

While IGF-I levels are regulated by GH under normal circumstances, this is not the case during certain stress conditions when supranormal levels of GH, but depressed IGF-I concentrations, co-exist. This situation has been identified in numerous circumstances including malnutrition (Fekete et al., 1983; Grant et al., 1973; Hintz et al., 1978), hypoglycaemia (Delitala et al., 1987), following surgery (Adashi et al., 1980; Delitala et al., 1987; Malatinsky et al., 1986; Pouttu et al., 1987; Ward et al., 1987) and venepuncture (Herdon et al., 1984), after a change in environment, and during painful stimuli (Meyer and Knobil, 1984). The disruption to the GH-IGF axis in these conditions, appears to be an adaptive response aimed at preserving nitrogen stores, since preference is given to the expression of the lipolytic action rather than the growth-promoting effects of GH (Van Wyk, 1984). The resistance to the action of

GH to stimulate IGF-I production during nutritional deprivation is well recognised in both rats and humans. In starving rats, liver receptors for GH are depleted (Baxter et al., 1981), thus retarding the synthesis of IGFs by the liver, while treatment with hGH fails to raise the diminished IGF-I levels in fasting adult humans (Merimee et al., 1982). The antagonism to the anabolic effects of GH in some catabolic conditions, particularly those which feature inadequate nutrition, would appear to limit its efficacy as a post-resectional therapy.

Although the effects of enhancing body weight gain and intestinal hyperplasia are highly desirable, the usefulness of GH as an aid to recovery following resection is compromised by its diabetogenic activity and the resistance to its growth promoting action that exists in certain catabolic conditions. Since IGF-I may have, in this context, the positive but not the negative qualities of GH, it offers theoretical advantages over GH as a therapeutic agent for treating post-resection patients.

1.4 INSULIN-LIKE GROWTH FACTORS

Circulating levels of IGF-I are depressed in catabolic conditions, particularly those associated with nutritional deprivation (Eigenmann et al., 1985). Conversely, administration of exogenous IGF-I stimulates growth in rats made catabolic by hypophesectomy (Schoenle et al., 1982) or induction of diabetes (Scheiwiller et al., 1986). Thus, it is appealing to speculate that treatment with IGF-I might attenuate catabolism, and in so doing, accelerate recovery following gut resection, a procedure that causes catabolism as a consequence of surgical trauma and nutritional deficiency.

1.4.1 Molecular Structure

Insulin-like growth factors (IGFs) are a group of polypeptides which have insulin-like metabolic effects and are mitogenic for a variety of cells. Known also as "somatomedins" because of their supposed role as mediators of GH (Daughaday et al.,

1972), the group is composed of two related but distinct factors: IGF-I and IGF-II. IGF-I is a basic molecule composed of 70 amino acids with 3 disulfide bridges and a molecular mass of 7649 Da (Rinderknecht and Humbel, 1978b). IGF-II is slightly acidic, has 67 amino acids and a molecular mass of 7471 Da (Rinderknecht and Humbel, 1978a). The two IGFs are chemically similar having a 62% amino acid sequence homology as well as related receptor specificity and *in vitro* biological actions (Adams et al., 1983). Both peptides also have a high structural homology (38-48%) with the A and B domains of human proinsulin (Adams et al., 1983). Comparison of human IGF-I (hIGF-I), bovine IGF-I (bIGF-I) (Francis et al., 1986), and, recently, porcine IGF-I (pIGF-I) (Tavakkol et al., 1988) reveals complete identity and hence biological equipotence.

1.4.2 Production Sites

IGF-I mRNA has been identified in virtually all body fluids and tissues (Atkinson et al., 1980, 1981; Baxter et al., 1984a,b; Binoux et al., 1981; Clemmons et al., 1981b; Hall et al., 1983; Murphy et al., 1987) including rat and human fetal stomachs (D'Ercole et al., 1986; Han et al., 1988) and postnatal rat intestine (Brown et al., 1986). The widespread presence of IGF-I mRNA suggests that IGF-I has important autocrine and/or paracrine actions (Baxter et al., 1986; Murphy et al., 1987; Rutanen et al., 1988).

Although the production of IGFs occurs throughout the body, the major source is the liver. Schwander et al. (1983) observed a continuous secretion rate of 50 μU IGF/g liver/h from perfused rat livers over a 4 h period. On the basis of earlier data (Cohen and Nissley, 1976; Kaufmann et al., 1978) which suggested that the normal half-life of human and rat IGF is 3h, these investigators determined that this secretion rate was sufficient to account for the rat serum IGF level of approximately 130 μU/ml (Kaufmann et al., 1978) and, as a consequence, concluded that the liver, at least in the rat, is the primary synthesis site of serum IGF. This role is supported by observations

that levels of circulating total IGF and IGF-I fall dramatically following hepatic failure (Schimpff et al., 1977; Takano et al., 1977; Zapf et al., 1978). The fact that IGF is not stored in measurable amounts in the liver indicates that it is rapidly secreted following synthesis (Schwander et al., 1983). Thus, IGFs clearly act in an endocrine as well as a paracrine and autocrine manner.

In contrast to GH, IGF-I is a good indicator of stress, with reductions in plasma levels of IGF-I reported for a number of stress situations in which plasma GH is elevated (see Section *1.3.2*), including gut surgery (Ward et al., 1987) and malnutrition (Clemmons et al., 1981a; Takano et al., 1978). The fasting-induced decline in IGF-I is rapid, with reports of an 80% decline in rats after one day of fasting (Takano et al., 1978), and falls of 40% for normal human subjects within 72h of commencing a fast (Takano and Shizume, 1978), and 75% for obese subjects fasted for ten days (Clemmons et al., 1981a). Restoration of normal IGF-I levels in fasted obese and non-obese subjects is dependent on nutrition (Clemmons et al., 1981a; Isley et al., 1983), particularly protein intake (Clemmons et al., 1981a; Isley et al., 1983; Phillips et al., 1978; Takano et al., 1980). As IGF-I levels are linearly related to protein intake, Van Wyk (1984) proposes that:

> "...measurement of immunoreactive Sm-C (IGF-I) provides a viable biochemical index of protein energy nutrition and that it may in fact be the most responsive objective biochemical parameter of changes in the nutritional state."

1.4.3 IGF Receptors

IGF actions are initiated following interaction with specific cell surface receptors. Three distinct receptor types bind insulin and IGF; the type-1 and type-2 IGF receptors and the insulin receptor. The type-1 receptor contains tyrosine-specific autophosphorylation acceptor sites and intrinsic substrate protein kinase activity (Czech et al., 1984), and has a 2-5 fold greater affinity for IGF-I than IGF-II (Bhaumick et al., 1981; Chernausek et al., 1981; Kasuga et al., 1981; Massague and Czech, 1982).

The type-2 receptor contains single polypeptide chains of molecular weight 260kDa under reducing conditions (Kasuga et al., 1981; Massague and Czech, 1982) and only binds IGF-II (Ballard et al., 1988). Type-1 and insulin receptors share considerable structural homology (Jones et al., 1982; Kasuga et al., 1983; Kull et al., 1983), whereas the type 2 receptor is unrelated. Recent work has shown the type-2 receptor to have distinct binding sites for IGF-II and mannose-6-phosphate (Braulke et al., 1988).

Specific receptors for IGFs have been located in a wide variety of fetal and adult tissues (Adams et al., 1983; August et al., 1983; Baskin et al., 1988; Kasuga et al., 1981; Lowe and Le Roith, 1986; Megyesi et al., 1975; Oonk and Grootegoed, 1988; Phillips and Orawski, 1977; Polychronakos et al., 1983; Rosenfield et al., 1982). The presence of IGF receptors, as well as IGF mRNA (see Section *1.4.2*), in the gut (Laburthe et al., 1988) and muscle (Pfeifle and Ditschuneit, 1983) suggests that these organs are responsive to IGFs and, therefore, that delivery of exogenous peptide may be effective in countering muscle catabolism and promoting gut growth in gut-resected animals.

1.4.4 IGF Binding Proteins

In the circulation, IGFs are usually associated with binding proteins (IGFBPs). Consequently, although the total IGF concentration in plasma or serum is high compared with other protein hormones, the amount of free IGF circulating is very low (Zapf et al., 1975; Cohen and Nissley., 1976; Chatelain et al., 1983). To date six distinct structural IGFBPs have been identified (Baxter, 1991), although when my research commenced IGFBPs-4, 5, and 6 were yet to be discovered. The functions of the binding proteins, and their effect on the delivery of IGFs to their target tissues, are poorly understood. Although most studies have demonstrated that the biologic response to IGF-I is diminished when bound to IGFBPs (Cascieri et al., 1988; Forbes et al., 1988; Hardouin et al., 1987; Knauer and Smith, 1980; Pekonen et al., 1988;

Ross et al., 1989; Rutanen et al., 1988; Szabo et al., 1988), Elgin et al. (1987) report that binding to IGFBP-1 enhances IGF-I action *in vitro*. Resolution of the role of binding proteins, and in particular whether they act to inhibit or enhance the biological effects of IGF-I, is fundamental to understanding the mechanism of IGF action.

Studies using radioimmunoassays (RIAs) have shown that the concentration of IGFBP-3 in adult human blood (approximately 5mg/l) (Baxter and Martin, 1989) is 20 times that of IGFBP-2 (approximately 150μg/l) (Clemmons et al., 1991), and 50 times that of IGFBP-1 (approximately 50μg/l) (Rutanen et al., 1982). Given its abundance and high affinity for both IGF-I and IGF-II (Martin and Baxter, 1986), it is hardly surprising that IGFBP-3 serves as the major carrier of circulating IGFs (Daughaday et al., 1982b; Hintz et al., 1981). Nearly all of the IGFBP-3 in the circulation is found in a ternary complex of approximately 140kDa, comprising an acid-labile or α-subunit of approximately 85kDa, IGFBP-3 (binding or ß-subunit), which electrophoreses on SDS-PAGE under reducing conditions at either 40 or 43kDa, and IGF-I or IGF-II (growth factor or γ-subunit) (Baxter and Martin, 1987; Baxter et al., 1987; Martin and Baxter, 1986). In the absence of IGFs, association between the α and ß subunits rarely if ever occurs (Baxter and Martin, 1987; Baxter et al., 1987).

Since levels of the 140kDa ternary form are enhanced in acromegalics, diminished in hypopituitary patients, and restored following depletion by GH therapy (D'Ercole and Wilkins, 1984, Hossenlopp et al., 1987), it is generally believed that the 140kDa ternary form is GH-dependent (Baxter et al., 1986; Furnanetto, 1980; Wilkins and D'Ercole, 1985). GH-dependence probably exists for the α-subunit, since the α-subunit does not appear in the ternary form in the absence of GH, and potent GH stimulation of α-subunit production has recently been demonstrated in cultured rat hepatocytes (Scott and Baxter, 1991). However, it is not yet certain whether IGF-I or GH is the principal stimulus for IGFBP-3 production, since IGFBP-3 increases in

hypophysectomized rats following treatment with either IGF-I or GH (Zapf et al., 1989).

While the precise function of IGFBP-3 remains unclear, several roles have been suggested. When bound to IGFBP-3 the plasma half-life of IGF is extended from about 10min to 10-15h (Guler et al., 1989). Predictably, variant forms of IGF-I which feature much reduced affinities for IGFBP-3, have much shorter serum half lives than native IGF-I (Cascieri et al., 1988; Ballard et al., 1987; Francis et al., 1992). This suggests that IGF bound to IGFBP-3 is cleared more slowly than free peptide, and that IGFBP-3 provides a reservoir for IGFs. This would be an important function as IGFs are not stored in significant amounts in any tissues (D'Ercole et al., 1984). When bound to IGFBP-3 in the ternary complex it appears very likely that IGF action is inhibited. Such inactivation is most probably due to the inability of the complex to pass through the capillary barrier and interact with target tissues (Binoux and Hossenlopp, 1988). Since the plasma concentration of IGFs is relatively high, about 1000 times greater than that of insulin, it is presumed that the IGFBPs have a role in inhibiting indiscriminate IGF action on the peripheral tissues. The importance of this role is illustrated by pharmacological studies which have demonstrated that the acute hypoglycemic potency of free IGFs is about 5-10% that of insulin (Guler et al., 1987), and, therefore, the hypoglycemic potential of total circulating IGFs is some 50-100 times greater than that of insulin.

IGFBP-1 appears at approximately 28kDa on non-reduced SDS-PAGE and has about 20% the affinity for IGFs of IGFBP-3 (Baxter et al., 1987). In both children and adults, serum IGFBP-1 shows marked diurnal variation, with levels typically rising during sleep and declining after waking (Baxter and Cowell, 1987; Busby et al. 1988; Yeoh and Baxter, 1988). This pattern is independent of GH secretion (Baxter and Cowell, 1987), but can be disrupted by glucose status. Serum IGFBP-1 levels are increased during hypoglycemia and suppressed during hyperglycemia (Cotterill et al.,

1988; Yeoh and Baxter, 1988). These responses have been shown, in glucose "clamp" studies, to be dependent on insulin levels (Suikkari et al., 1988). As Baxter points out (Baxter, 1991), the behaviour of IGFBP-1 is characteristic of substances involved in glucose counter-regulation. Since it has been estimated that the IGFs not bound to either IGFBP-3 or IGFBP- 2 could exert a hypoglycemic effect similar in magnitude to that of insulin, it is thought that IGFBP-1 may play a backup role to insulin in the regulation of glucose homeostasis.

IGFBP-2, a polypeptide of approximately 31kDa on SDS-PAGE (Binkert et al., 1989), has been shown to have a marked preferential affinity for IGF-II (Forbes et al., 1988). Conditions characterized by reduced concentrations of the ternary complex, such as fasting or hypopituitarism (Clemmons et al., 1991), or conditions aimed at overwhelming the binding capacity of the ternary complex, such as infusion of IGF-I (Zapf et al., 1990a,b), all produce marked increases in IGFBP-2 levels. The common factor in each of these situations is increased "free" IGF-II, and since IGFBP-2 is essentially a binding protein for IGF-II, it seems likely that IGFBP-2 production increases in response to elevated IGF-II levels.

IGFBP-4 has a molecular mass around 25kDa on SDS-PAGE (Mohan et al., 1989) and also binds IGF-II with greater affinity than IGF-I (Mohan et al., 1989). Very little is known about the biological importance of IGFBP-5 and IGFBP-6.

Prior to this investigation, little was known about the effect of catabolic conditions, and nothing of the impact of major surgery, on the levels or functions of the IGFBPs. As mentioned previously, understanding the role of the IGFBPs is one of the keys to identifying the mechanism of action of IGFs *in vivo*.

1.4.5 Growth effects

Salmon and Daughaday (1957) were the first to describe a GH-dependent factor in serum that induced *in vitro* sulphate incorporation by cartilage. Since then this "sulfation factor" has been shown to stimulate *in vitro* cell proliferation and/or differentiation in a variety of cell types (see Van Wyk, 1984 for review). These anabolic effects result in part from the stimulation of protein synthesis and inhibition of protein breakdown (Ballard et al., 1981), with the response of the catabolic pathway, generally, being the more powerful (Ballard and Gunn, 1985). *In vitro*, the growth inducing potency of IGF-I has been found to be approximately 50-60 times that of IGF-II (Ballard et al., 1986; Zapf et al., 1978a).

Although the growth effects of infused IGFs had not been fully characterized when this study began, the relationship between endogenous levels of the peptides and body size were well established. In humans, acromegalics typically have IGF-I levels about 4-7 times normal adult values (Baxter et al., 1982; Zapf et al., 1978a, 1980), although no elevation is apparent for IGF-II levels (Daughaday et al., 1981; Hintz and Liu, 1982; Zapf et al., 1981). The clinical state of acromegaly is, in fact, more strongly correlated with circulating IGF-I than GH levels (Wass et al., 1982), since acromegalic symptoms attenuate when IGF-I levels, but not GH levels, fall. In GH-deficient and growth-retarded subjects, IGF-I values are typically found to be between 15-25% those of healthy normals (Baxter et al., 1982; Zapf et al., 1978a), with rapid and dramatic rises in circulating IGF-I levels and growth following GH administration (Draznin et al., 1980).

In human umbilical cord blood, IGF-I levels for both males and females (Kaplowitz et al., 1982) are only about half those of adults, and correlate with birth weight and gestational age (Bennett et al., 1983; Gluckman et al., 1983). During puberty, a marked rise in levels of IGF-I coincides with the growth spurt, with values 2-3 times those normally found in adults evident. IGF-II levels, in contrast, do not

rise during adolescence (Luna et al., 1983; Zapf et al., 1981). During adulthood, serum IGF-I concentrations typically subside, probably because of the age-related decline in GH secretion (Van Wyk, 1984).

When this study commenced, relatively few studies had investigated the *in vivo* actions of IGFs. Schoenle et al. (1982) had demonstrated that IGF-I injected into hypophysectomized rats increased weight gain, tibial epiphyseal widths, and DNA synthetic activity in costal cartilage in a dose-dependent manner. Infusion of IGF-I had subsequently been shown to increase body weight gain in Snell dwarf mice (Van Buul-Offers and Van der Brande, 1982), previously growth-arrested diabetic rats (Scheiwiller et al., 1986), and normal rats (Hizuka et al., 1986). The demonstration that IGFs increased somatic growth in growth-arrested or growth-retarded animals was particularly interesting, and suggested that IGF-I might be a valuable therapeutic agent in treating conditions characterized by growth inhibition and GH-resistance.

At this time, the specific organ effects of IGF-I administration *in vivo* had received little attention. In the few studies to investigate these effects, various results had been reported. Van Buul-Offers et al. (1986) observed increases in the absolute weight of the brain, salivary glands, heart, liver, kidneys, thymus and spleen in Snell dwarf mice, Hizuka et al. (1986) reported increased absolute kidney, liver, testes and pituitary weights in normal rats, and Guler et al. (1988) noted increases in the weights of the kidneys and spleen relative to body weight in hypophysectomized rats, following IGF-I infusion. Prior to this investigation no one had investigated the specific gut effects of IGF-I. Since IGF receptors had been located in fetal gut tissue (Laburthe et al., 1988), GH has known effects on the gut (Cooke et al., 1986; Scow and Hagan, 1965), and insulin produces dose-related precocious maturation of sucrase, maltase and lactase activities in suckling mouse small intestine (Menard et al., 1981), it was likely that IGF-I exercised some influence over gastro-intestinal growth.

1.4.6 Metabolic Action

The metabolic actions of IGFs are well characterised: they exert acute insulin-like effects on adipose tissue, skeletal muscle (Zapf et al., 1981a), cardiac muscle (Meuli and Froesch, 1975), chondrocytes (Froesch et al., 1976), and calvaria cells (Canalis, 1980, Schmid et al., 1983a). More specifically, their effects in adipose tissue include stimulation of glucose metabolism to carbon dioxide, lipids and glycogen, and inhibition of both glycogen breakdown and epinephrine-induced lipolysis (Oelz et al., 1970; Underwood et al., 1972; Van Wyk et al., 1974; Zapf et al., 1978b). In these actions, IGF-I is reported to have only about 1% and 40% the potencies of insulin and IGF-II, respectively (Zapf et al., 1978b). In muscle preparations, IGFs induce glucose incorporation into glycogen and glycolysis (Borland et al., 1984; Florini et al., 1977).

Of the two IGF forms, IGF-I is more likely to be successful in treating catabolic conditions, based on its enhanced anabolic potency compared to IGF-II, which would make it more likely to improve nitrogen retention, and its reduced insulin-like effects on glucose metabolism. The latter consideration is important due to the concern that exogenous IGF may overwhelm the circulating binding protein capacity, allowing the infused, or displaced endogenous, IGFs to provoke a potentially dangerous hypoglycaemic state.

1.4.7 Variant forms of IGF-I

It has been discovered that removing 1 to 5 of the amino acid residues from the N-terminus changes the biological potency of IGF-I *in vitro* (Francis et al., 1986), with IGF action being enhanced or reduced depending on which residues are removed. Bovine des(1-3)IGF-I, which has the last 3 N-terminal residues (gly, pro and glu) absent, is 4-50 times as effective *in vitro* as bIGF-I or hIGF-I in stimulating DNA and protein synthesis and inhibiting protein degradation depending on the cell type (Ballard et al., 1988; Francis et al., 1986; Szabo et al., 1988). Ballard et al. (1988) found, after

adding IGF-I and des(1-3)IGF-I to three cell lines (human lung and skin fibroblasts, and L6 myoblasts), that a greater proportion of des(1-3)IGF-I, than IGF-I, bound to type-1 receptors, and attributed this to the differences in affinity for IGFBPs. The truncated des(1-3)IGF-I form has a much reduced affinity for IGFBP-2 (Szabo et al., 1988) and IGFBP-1 (Ross et al., 1989) as well as marginal less affinity for IGFBP-3 (Forbes et al., 1988) than either IGF-I or IGF-II in competitive binding assays.

Another variant has been engineered that has Glu^3 replaced by Arg^3, thus changing the charge, at position 3 (Bagley et al., 1989). Since this variant also contains a 13-residue amino acid extension from porcine GH, it is referred to as longR^3IGF-I or LR^3IGF-I. Despite having only one third the affinity for the type-1 receptor, LR^3IGF-I has a similar *in vitro* potency to des(1-3)IGF-I by virtue of having even lower affinities for IGF binding proteins (Francis et al., 1992). Studies with des(1-3)IGF-I and LR^3IGF-I have established the importance of the N-terminus of the IGF-I molecule in determining the affinity of IGF-I for the IGFBPs.

When this study commenced, the variants, des(1-3)IGF-I and LR^3IGF-I, were yet to be used in animal trials. Consequently it was not known whether their enhanced potencies *in vitro* would also be evident *in vivo*. Resolution of their *in vivo* potencies was important for a number of reasons: 1) should the variants prove to be more potent than native IGF-I *in vivo*, and should infusion of a high-dose of IGF-I induce hypoglycaemia, then infusion of a lower dose of a variant with greater anabolic potency may offer an alternative and safer therapeutic strategy, 2) since des(1-3)IGF-I only differs from IGF-I in its affinity for the binding proteins, the comparative potencies of the two peptides would provide valuable information on the *in vivo* role of the IGFBPs, and 3) since des(1-3)IGF-I and LR^3IGF-I vary in their affinity for both binding proteins and the type-1 receptor, comparison of the potencies of these analogues would help to resolve the relative importance of binding protein and receptor

affinities in the expression of IGF-I action *in vivo*. Answers to points 2) and 3), in turn, would determine the direction taken in engineering further IGF-I variants.

1.5 STATEMENT OF THE PROBLEM

IGF-I is a powerful anabolic agent and mitogen which may be clinically useful in treating catabolic conditions, especially those in which therapy with alternative growth-promotants is inappropriate. Such a condition exists following massive small bowel resection, when both growth and nutrient absorption have been compromised. The aim of this study was to determine whether IGF-I has any therapeutic potential in the treatment of patients following gut-resection and, in the process, to further the understanding of IGF action *in vivo*.

The historical section has revealed that little was known of the actions of IGF peptides *in vivo* at the start of this work. In particular, nothing was known of the effect of IGF-I on post-surgical catabolism, or in the gastro-intestinal tract, nor of the *in vivo* potencies of the IGF-I analogues des(1-3)IGF-I and LR^3IGF-I. Moreover, the organ specific effects of IGF-I were not well characterized. Additionally, the role of the IGFBPs in modifying IGF action *in vivo* was unresolved, and the response of the binding proteins to the stress of resection unknown.

As such, the specific aims of this project were:

1) to establishing an appropriate animal resection model for examining the efficacy of IGFs as a post-resectional therapy.

2) to assess the response of plasma IGF-I and IGFBP levels to resection and treatment with exogenous IGF peptides.

3) to assess the ability of IGF-I peptide treatment to restore a positive nitrogen balance and whole body growth in catabolic animals, and if effective to investigate the mechanism of action.

4) to define the organ specific effects of IGF-I, thereby helping to determine whether IGF acts generally or preferentially with regard to body tissues, and in so doing, further clarify the action and potential therapeutic uses of IGF.

5) to determine whether IGF-I peptides increase gut regeneration following resection .

6) to discover whether IGF-I peptides enhance the absorptive capacity of the regenerating post-resectional gut.

7) to investigate the mechanism of IGF-I action on the gut.

8) to determine whether the greater *in vitro* potencies of the IGF-I variants, des(1-3)IGF-I and LR^3IGF-I, are also apparent *in vivo.*

In the experiments described in the following chapters, work done by others is acknowledged in the appropriate Methods sections. Note that the development of the animal model used in this investigation, all of the surgery, biochemical measurements, Western-blot analyses, statistical analyses, most of the daily collections and measurements, and much of the nitrogen balance measurements; and kill-day tissue collections and measurements, were performed by myself. In the case of the work performed exclusively by others, namely the assessment of urinary 3-MH excretion, carcass composition analysis, plasma IGF-I RIAs, and histological measurements, I was involved in the interpretation of results. Data from analyses performed by others on samples generated by this study, is included in this thesis as they assist in the understanding of the overall effects seen following IGF-I administration to gut-resected rats.

CHAPTER 2

Development of the resection model

2.1 INTRODUCTION

The aim of this thesis was to assess whether IGF-I would attenuate catabolism and enhance adaptive gut growth in intestinally-resected animals. Thus, the initial task was to develop a suitable animal model of resection. Ideally this model had to satisfy the following criteria: 1) the animal used should be inexpensive, 2) the animal should be small, so that peptides requirements would be minimized, but not so small as to complicate surgery, 3) the animal should be one that lends itself easily and inexpensively to metabolic studies, and 4) the surgery should be severe enough to produce weight loss and growth cessation, but not so severe that high mortality is incurred.

2.2 METHODS and MATERIALS

2.2.1 Species

The rat was chosen as the animal that best satisfied the requirements of the criteria. Rats had been shown to be ammenable to resection studies and their response to resection was well characterized (for a review, see Urban and Weser, 1980). Additionally, I had decided to assess nitrogen balance, protein turnover, food conversion and nutrient absorption. Since this requires measurement of food intake and collection of faeces and urine, it was necessary to house the animals in metabolism cages. Rats are appropriate for metabolic studies as a large number of animals can be individually housed in metabolic cages in a manner that is relatively inexpensive and not overly demanding of space.

Animals larger than rats, as well as being more expensive to purchase and maintain, would require considerably more peptide. This was a major consideration as IGF-I was only available in mg amounts when this study commenced. On the other hand, mice were considered unsuitable as their size could complicate surgery, and the technique chosen to assess muscle protein breakdown (urinary 3-methylhistidine, see

section *2.2.4*) is inappropriate for this species (Tomas and Ballard, 1987). Lastly, by using rats comparison could be made with the results of on-going collaborative studies investigating the effects of IGF-I using other rat models of catabolic stress (Martin et al., 1991; Tomas et al., 1991a,b,c).

The rats used for all the resection experiments were male Sprague-Dawley rats bred in a specific pathogen free environment at the CSIRO Division of Human Nutrition (Glenthorne, South Australia). The rats were four weeks old when received.

2.2.2 Procedure for resection

The surgical protocol adopted was based on the method of Ford et al. (1983). The animals were anaesthetised by an intraperitoneal injection of tribromoethanol in amylene hydrate ("Avertin", 1ml/100g bw). Using aseptic techniques the small intestine was exposed through a midline incision and its length from the ligament of Treitz to the caecum measured (Fig. 2.1). Following determination of the jejuno-ileal length, the length of jejunum and ileum to be retained was calculated. Points corresponding to these calculated lengths were located distal to the ligament of Treitz and proximal to the ileo-caecal valve. The parallel blood vessels immediately adjacent to these points were tied off with 6-0 silk thread, then 0-0 silk thread was used to tie off the blood vessels supplying the intestinal segment between the points. After vascular isolation was confirmed by colour change, this segment was excised. An end-to-end anastomosis was carried out with interrupted stitches using 6-0 silk sutures. The abdominal layer was closed with 3-0 silk sutures using over-under continuous stitches, with a locking stitch at the half way point. The pelt (skin plus fur) was then closed using surgical clips. Antibiotic cover was provided by intramuscular injection with 0.2ml of procaine penicillin (Vetspen; Glaxo, Nth. Ryde, NSW, Australia) immediately prior to surgery, and by a solution of gentamycin in sterile sodium lactate (20mg/2ml) which was used to bathe the gut and peritoneal cavity during the operation. To confirm

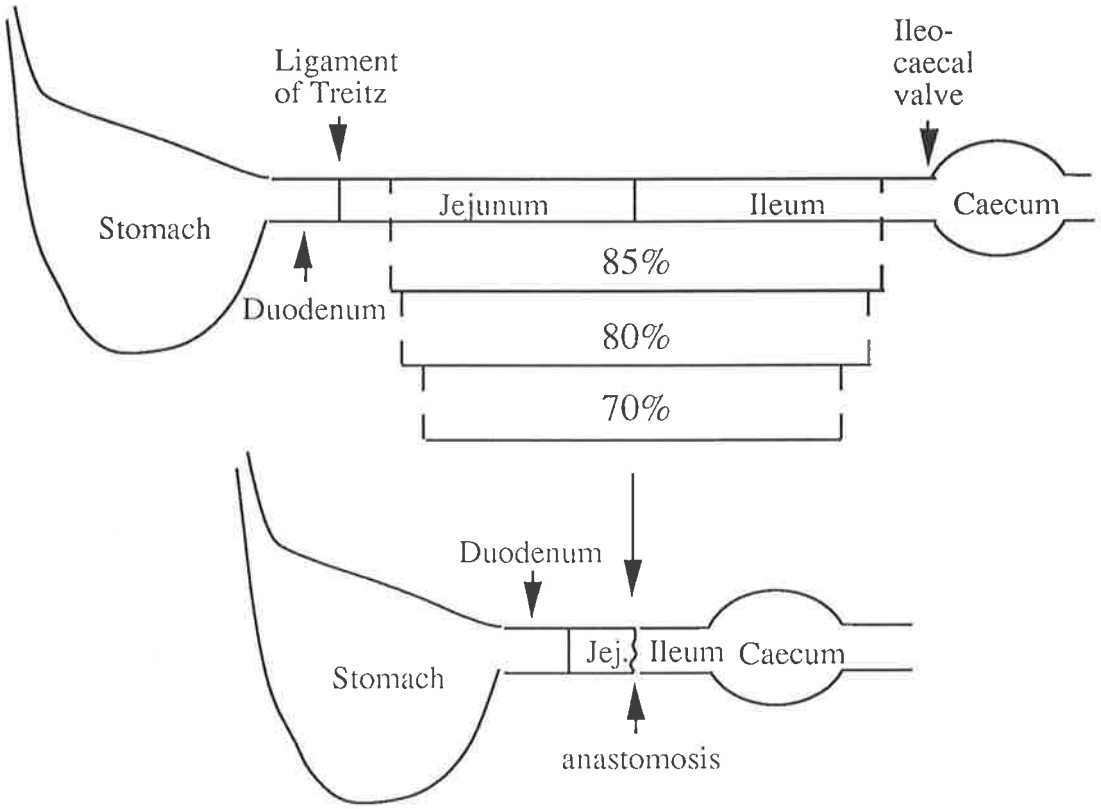

Fig. 2.1 Schematic representation of 70%, 80% and 85% jejuno-ileal resection.

the extent of resection, the excised gut was measured for weight and length before being discarded. Animals were returned to their cages once they were awake and had become mobile.

2.2.3 Determining the optimal extent of resection

After choosing gut-resection in rats as an appropriate model it was necessary to determine the extent of small bowel that needed to be removed to cause a cessation of growth in young rats for seven days, the projected period of growth factor administration. Consequently trials were conducted in which either the mid 70%, 80% or 85% of the small intestine between the ligament of Treitz and the caecum was removed from growing rats by the method outlined in section *2.2.2*.

2.2.4 Measurements and collections for metabolic studies

To enable metabolic studies of the rats before and after surgery a daily measurement and collection protocol was strictly adhered to. The rats were weighed when received, and then housed in individual metabolic cages (Tecniplast 1700 series, Italy) at 25°C with lighting controlled over a 12h light/dark cycle. Provided the animals were growing normally, daily measurement of body weight, food and water intake, and quantitative collection of urine and faeces commenced after a four day familiarization period (Fig. 2.2). To provide a baseline of normal functioning, daily collections and measurements were made for three days prior to surgery. This period is termed the "pretreatment" phase. These measurements and collections continued at precise 24h intervals for seven days following surgery. Thus the entire experimental period was ten days (Fig. 2.2). Food and water were available *ad libitum*. The diet consisted of a high carbohydrate, meat-free powder containing 180g casein plus 2.5g methionine per kg as the protein source (for diet recipe see appendix 2.1). It was necessary to eliminate meat from the diet as it contains 3-methylhistidine (3-MH). 3-MH direct from dietary sources would increase urinary 3-MH excretion and therefore compromise our determination of muscle protein breakdown.

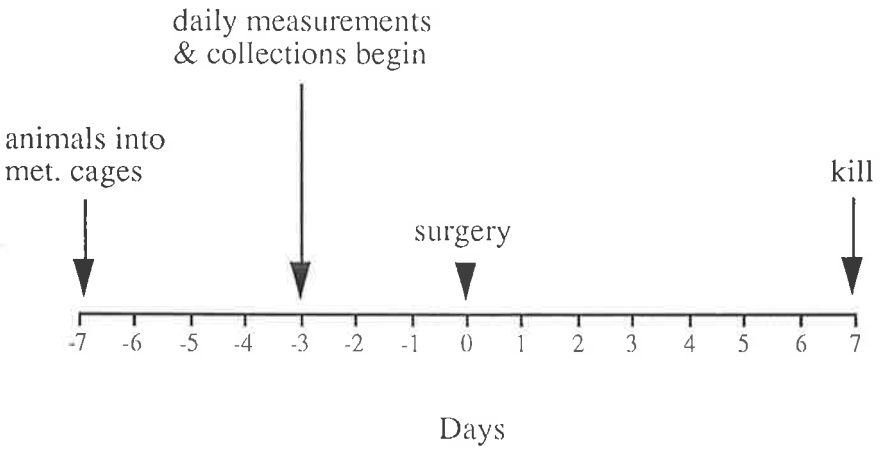

Fig. 2.2 Protocol for the 80% resection experiments

2.2.5 Kill procedure

After one week of treatment, the animals were killed by exsanguination via cardiac puncture under Avertin anaesthesia. To assess the effects of IGF-I treatment on the growth of gut and non-gut organs, the liver, spleen, gut, kidneys, thymus, adrenals, heart and lungs were sequentially removed and weighed. The gut from stomach to rectum was removed intact and whilst in iced saline solution separated into stomach, duodenum, jejunum, ileum, caecum and colon. The lengths of the duodenum, jejunum, ileum and colon were measured with the tissue lying unstretched on an iced glass slab. Following the clearance of luminal contents, each of the gut regions was weighed. After discarding the head, feet and tail, the pelt (skin plus fur) and carcass were weighed separately.

2.2.6 Exclusion criteria

So that the final results of experiments were not compromised by sick or dying animals, exclusion criteria were established whereby the data and samples collected from any animal which 1) died during the treatment period, or 2) survived the treatment period but (i) failed to regain its appetite, (ii) had diarrhoea for four or more days, (iii) did not drink for three or more days, (iv) showed evidence of anastamosal blockage at the kill, or (v) was clearly infected, were excluded from subsequent analysis.

2.2.7 Preliminary IGF-I trial

To evaluate experimental procedures, a preliminary experiment was conducted in which 80% of the jejuno-ileum was removed from nineteen rats. To assist in the determination of responsive IGF dose rates for future experiments, IGF-I was infused into nine of these animals during the seven day recovery period.

Surgery was as described in Section *2.2.2*, with the gut lying between points 10cm distal from the ligament of Treitz and 10cm proximal to the caecum excised. Immediately following abdominal closure, an osmotic mini-pump (Alzet Model 2001,

Alza Co, Palo Alto; CA., USA) was implanted subcutaneously within the scapular region. The hole made in the pelt for pump implantation was opened by blunt dissection and closed with surgical clips. The pumps were filled with either 0.1M acetic acid as vehicle or 7.6mg recombinant human IGF-I/ml (Genentech, San Francisco, CA., USA). Body weights at the time of surgery were (means±SEM) 157±3 and 151±4g for the ten vehicle-treated and the nine IGF-I-treated rats, respectively. At a delivery rate of 0.92µl/h, and based on the body weights at surgery, the pumps supplied 0 or 1.1mg IGF-I/kg bw/d.

Before this experiment began, the *in vivo* delivery rate of peptides from Alzet 2001 minipumps was verified by Dr. P. C. Owens (CSIRO Division of Human Nutrition, Adelaide, South Australia) using the manufacturer's recommendations. Pumps containing 7.6mg IGF-I/ml were implanted subcutaneously into seven rats in the manner outlined above. After seven days the pumps were recovered and the residual IGF-I measured by RIA (see section *3.2.4*). The average delivery rate determined was 98% of the manufacturer's specification.

Delivery of IGF-I via osmotic minipumps s.c. was chosen as the means of administration as this route had demonstrated no influence on blood glucose (Scheiwiller et al., 1986; Schoenle et al., 1982; Schoenle et al., 1985), whereas bolus injections of IGF-I had been shown to precipitate hypoglycaemia (Zapf et al., 1986) which could prove fatal.

2.3 RESULTS

2.3.1 The optimal extent of resection

In order to determine the optimal percentage of resection, survival and growth rates were compared in rats that were unresected, or subject to 70%, 80% or 85% resection of the jejuno-ileum (Table 2.1). The unresected rats (mean±SEM:

164.2±3.0g) grew at approximately 6-7g/d (Fig. 2.3). Removal of 70% of the jejuno-ileum had little or no effect on growth of nine rats weighing 168.0±2.7g at the time of surgery (Fig. 2.3). This procedure caused no deaths. On the other hand, the 80% jejuno-ileal resection performed on nine animals weighing 162.4±2.1g caused transient body weight loss and growth cessation in the eight survivors, with 5-8 days generally required before normal growth resumed (Fig. 2.3). None of the five animals (164.1±1.8g) subjected to an 85% resection lived beyond six days. On the basis of our criteria that the surgical intervention should impair growth but not incur high mortality the 80% jejuno-ileal resection was adopted as our model of surgical stress.

Table 2.1 Survival and growth over the first 7 post-surgical days, of rats following 70%, 80% or 85% resection of the jejuno-ileum.

% resection	70%	80%	85%
no. resected	9	9	5
no. survived	9	8	-
post-surgical growth (g, mean±SEM)	19.6±2.0	-1.2±1.5	

Due to the uniformity of jejuno-ileal lengths in the rats used in the preliminary trials (mean±SEM: 100±2cm), it was decided that rather than continuing to measure the jejuno-ileal length and leaving the calculated 10% of this length at the extremes, for subsequent experiments, 10cm of intestine should be left at each end (i.e. the proximal jejunum and distal ileum) (Fig. 2.4). This modification not only saved time but substantially reduced handling of the gut.

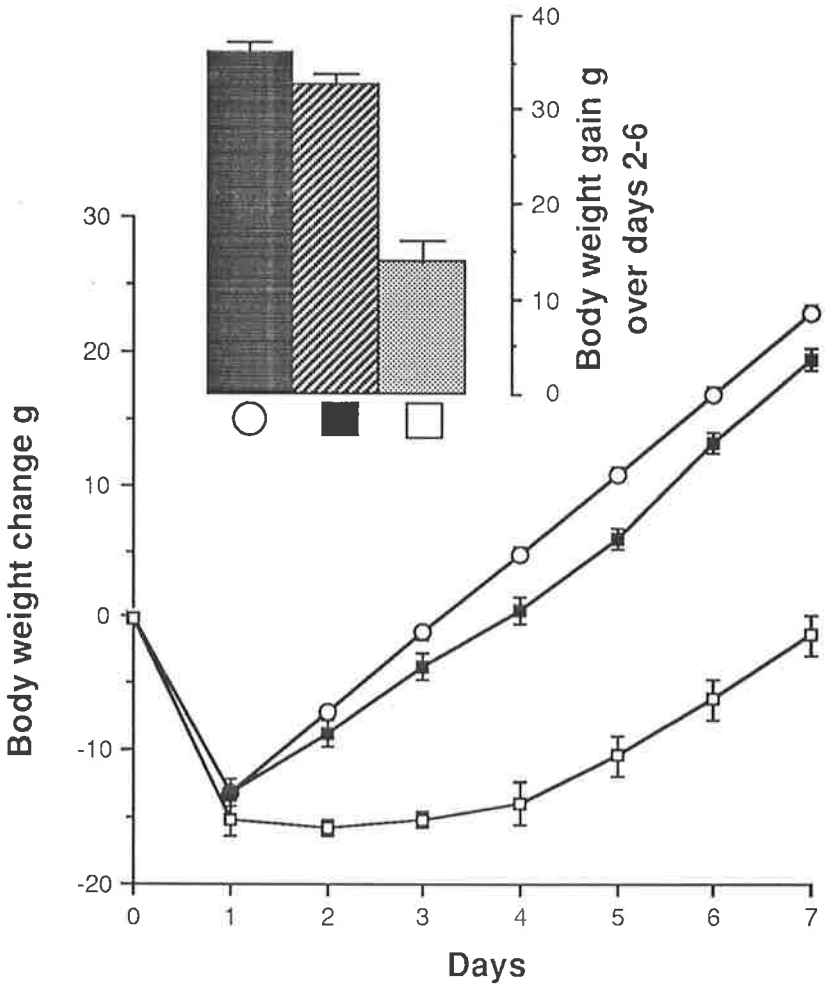

Fig. 2.3 Changes in body weight in rats following removal of 0%, 70% or 80% of the jejuno-ileum on day 0. Treatment groups were 70% resected (closed squares), 80% resected (open squares), and unresected (open circles) rats. Values are means±SEM for 8-9 animals in each group. Bar graph displays body weight gains±SEM over days 2-7.

2.3.2 A preliminary trial on the effect of IGF-I on growth in resected rats

Having chosen 80% resection as the experimental model, a second trial was conducted, firstly, to characterize in more detail the growth retardation induced by resection and, secondly, to obtain information on the effect of IGF-I on growth following resection. The dose of IGF-I (1.1 mg/kg bw/d) was chosen on the basis of the limited information available at the time on the *in vivo* response to chronic IGF-I

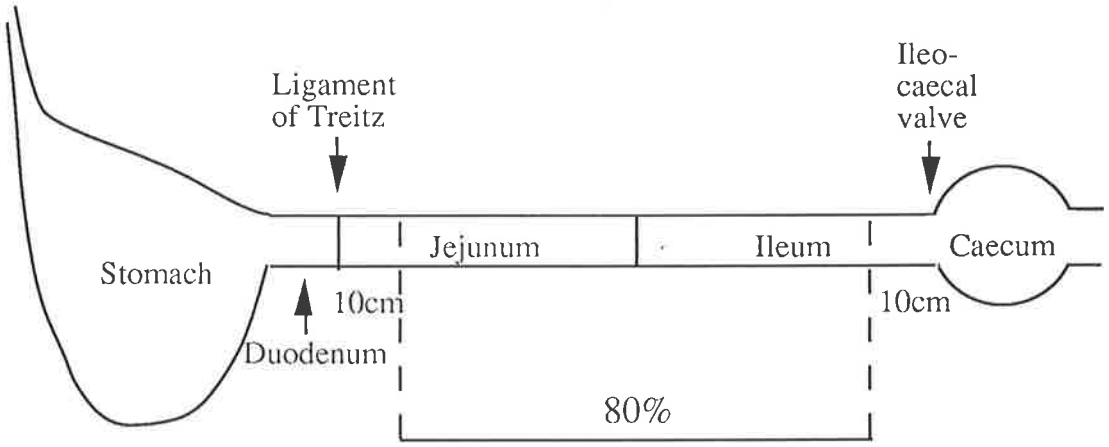

Fig. 2.4 Schematic representation of the 80% resection method adopted for IGF-I therapy experiments.

infusion. Data are reported as means±SEM.

Final group numbers were nine vehicle-treated controls and eight IGF-I treated rats as two animals died following surgery. The deaths were attributed to peritonitis (control), and anastomosal blockage (IGF-I treated) and appeared to be unrelated to treatment.

Prior to surgery the animals were generally growing at 6-7 g/d, with no differences in pretreatment growth existing between the groups (Fig. 2.5(a)). Removal of 80% of the jejuno-ileum resulted in a weight loss of 14-15g during the first 24h of recovery, of which only about 5g could be accounted for by the resected tissue. During days 2-4, the average daily body weight gain of the vehicle-treated controls was reduced to 0.5±0.6g (Fig. 2.5(a)). Growth rates for these animals were restored towards normal over days 5-7 with the rats gaining 4.1±0.5g per day. For the entire treatment period (days 1-7), the control group lost an average of 1.2±2.4g relative to its mean presurgical body weight.

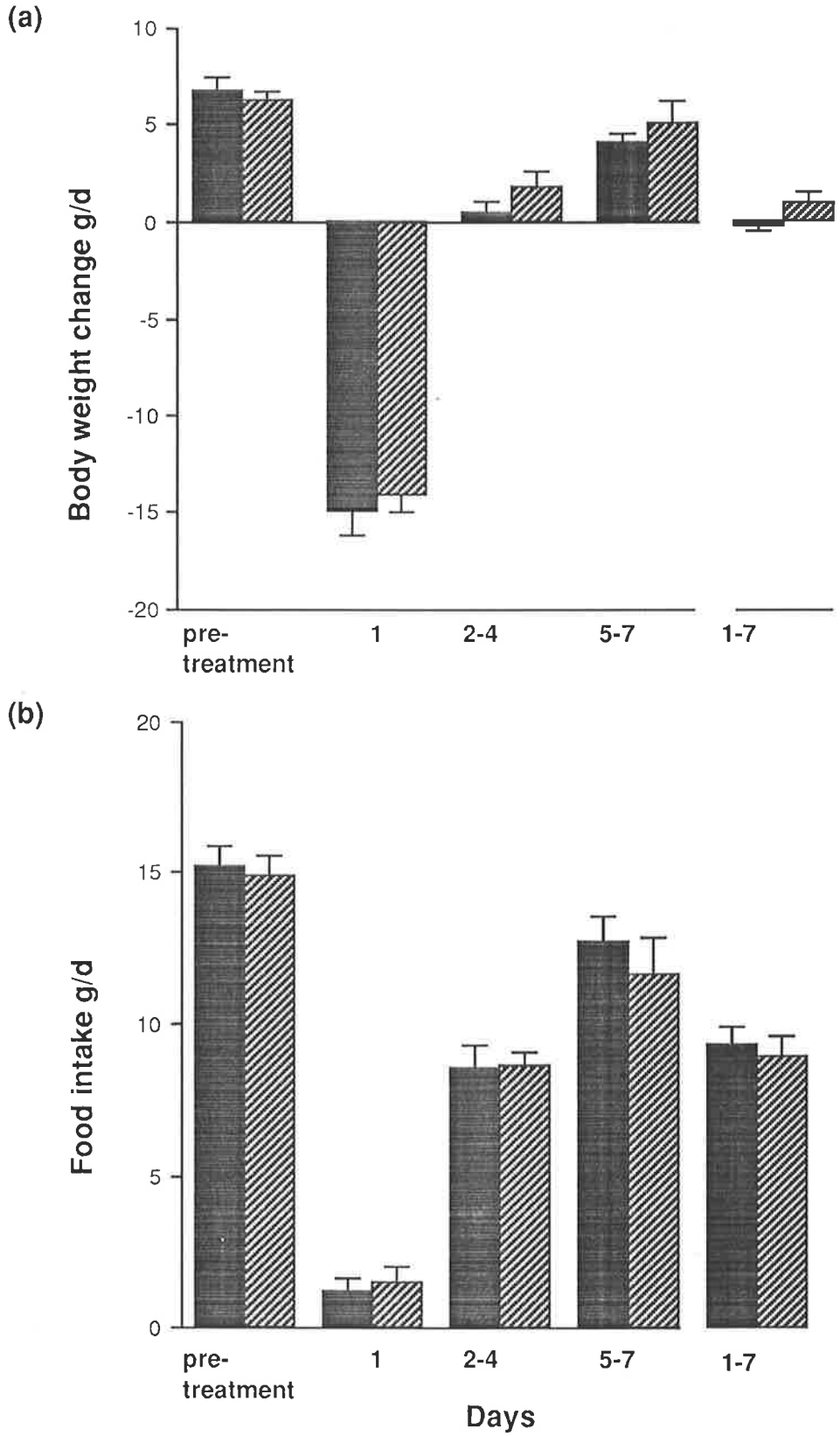

Fig. 2.5 Bodyweight changes (a) and average daily food intake (b) of rats calculated for the 3 pretreatment days prior to 80% resection of the jejuno-ileum, and for day 1, days 2-4, days 5-7, and days 1-7 following surgery. Treatment groups were vehicle (solid bars), and 1.1 mg IGF-I (hatched bars). Values are means±SEM for 8-9 rats in each group.

As anticipated, gut resection caused a marked reduction in food intake during the first days of recovery, with daily intakes gradually increasing toward normal by the end of the treatment week (Fig. 2.5(b)).

Food conversion efficiency, the ratio of body weight gain to food intake over the same period, was calculated to provide an indirect measure of food utilization (Fig. 2.6). Day 1 was ignored in this analysis as during the initial 24h the animals ate minimally or not at all, and some of the weight lost was directly attributable to excised tissue. The body weight gain and food consumed over all three pretreatment days was used to calculate the pretreatment food conversion efficiencies. No differences in food conversion efficiencies existed between the groups prior to surgery and peptide treatment. During days 2-4 following removal of 80% of the jejuno-ileum, food conversion efficiency in the controls fell markedly relative to pretreatment levels. Although partially restored, the food conversion efficiency of these animals remained significantly lower ($p<0.05$) than the pretreatment level over days 5-7.

Although the difference was not significant, the rats receiving 1.1 mg/kg bw/d IGF-I gained weight at a greater rate than the controls (Fig. 2.5(a)). Over the entire treatment period (days 1-7), the group receiving IGF-I gained 6.7±4.3 g relative to their presurgical weight, while the controls lost 1.2±2.4g. Restitution of presurgical weight, which was not achieved by the controls, took an average of five days for the IGF-I treated animals.

The tendency for improved body weight gain in gut resected rats following IGF-I administration relative to the controls could not be attributed to food intake since this did not differ between groups (Fig. 2.5(b)).

The decline in food conversion efficiency over days 2-4 was less pronounced for the IGF-I-treated rats than for the vehicle-treated controls (Fig. 2.6). During days

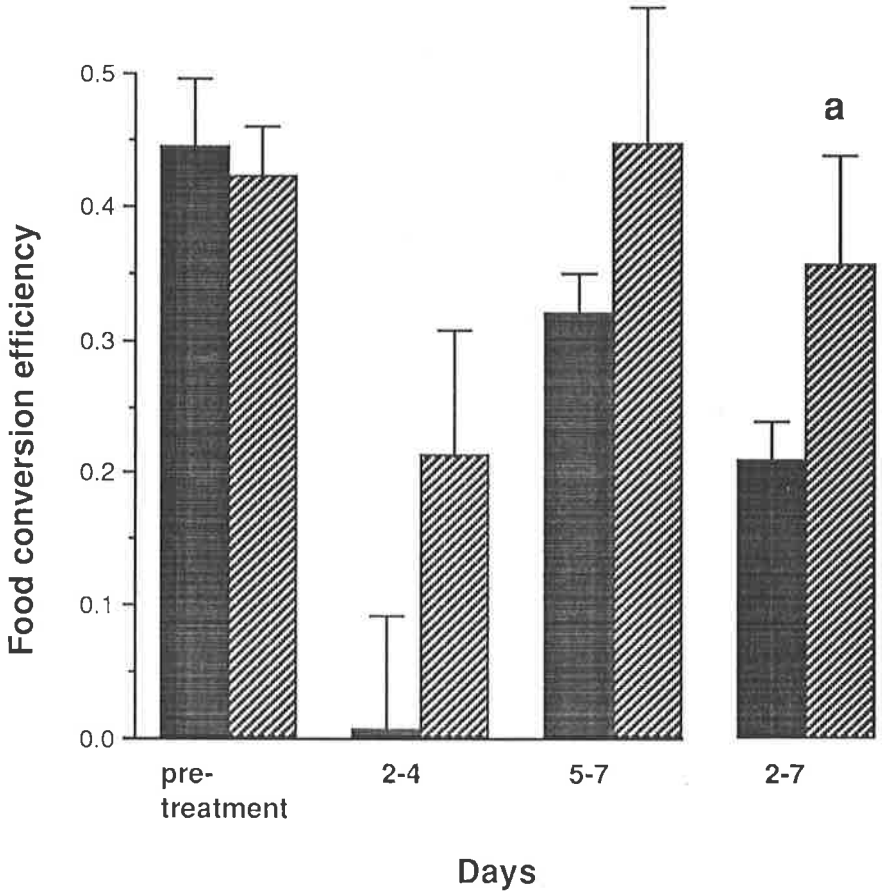

Fig. 2.6 Food conversion efficiency (g body weight gain/g food intake) for the 3 pretreatment days prior to 80% resection of the jejuno-ileum, and for days 2-4, days 5-7, and days 2-7 following surgery. Treatment groups were vehicle (solid bars), and 1.1 mg IGF-I (hatched bars). Values are means±S.E.M. for 8-9 rats in each group. **a**: $p<0.05$ versus vehicle-treated rats.

5-7, pretreatment food conversion levels were restored in the peptide-treated, but not vehicle-treated, rats. For the combined period (days 2-7), average food conversion efficiency in the rats receiving IGF-I was 70% higher than in the controls ($p<0.05$).

Non-gut and gut organ weights were measured to identify any tissue specific responses to IGF-I. Treatment with IGF-I had no effect on the absolute or relative

(g/kg bw/d) weights of any of the non-gut organs examined (Table 2.2). Although the effect was not significant, total gut weight (stomach to colon inclusive) was 15% higher in the IGF-I treated animals relative to the controls (Table 2.3), with sensitivity appearing to be more pronounced in the upper gastro-intestinal tract. Total intestinal length (small + large bowel) was 6% greater in the IGF-I group than the controls, with this difference also failing to achieve significance.

2.4 DISCUSSION

2.4.1 Performance of the 80% resection model

The 80% resection model features transient catabolism, with three distinct stages occurring during the first week of recovery: the initial 24h (day 1), when there is substantial weight loss due to negligible food intake, tissue removal and surgical stress; days 2-4 following surgery, which are characterised by an improving appetite and a consolidation of body weight; and the final three days (days 5-7), when near normal food intake and growth rates are restored.

Although the sustained stasis in growth which was originally sought could not be achieved, 80% resection did cause marked and reproducible disruptions to body growth and appetite in previously growing rats, particularly during the early stages of recovery. Additionally, the decline in food conversion efficiency and prevalence of diarrhoea, especially during the initial post-surgery days, suggested that post-resectional malabsorption was present. Consequently, it was concluded that the 80% gut resection model in rats provides transient stress from surgical trauma and nutritional deprivation, and as such may be representative of acute trauma in humans.

When this study commenced the anabolic effects of IGF-I had only been investigated in normal rats (Hizuka et al., 1986), growth-retarded rodents such as diabetic (Scheiwiller et al., 1986), fasted (Asakawa et al., 1988) and hypopituitary or

TABLE 2.2 Organ weights on day 7 after 80% jejuno-ileal resection and treatment with 1.1mg IGF-I/kg bwt/d.

Treatment	Final Bodyweight	Carcass	Pelt	Liver	Spleen	Kidneys	Adrenals	Thymus	Heart	Lung
					Absolute weight (g)					
Vehicle	156.2±3.1	61.3±2.2	18.8±0.6	9.42±0.50	1.02±0.18	1.63±0.04	0.028±0.003	0.355±0.035	0.828±0.045	1.47±0.23
IGF-I	158.0±5.6	60.0±3.3	19.3±1.0	8.59±0.64	0.97±0.10	1.69±0.10	0.029±0.003	0.359±0.051	0.760±0.031	1.28±0.07
					Relative weight (g/kg body weight)					
Vehicle	-	391±8	120.6±3.0	60.5±3.5	6.56±1.14	10.48±0.42	0.182±0.020	2.30±0.25	5.32±0.30	9.47±1.53
IGF-I	-	378±12	121.6±3.1	54.4±3.8	6.25±0.81	10.95±0.78	0.187±0.021	2.24±0.29	4.81±0.09	8.18±0.57

Values are means ± SEM for 8-9 rats in each group.

TABLE 2.3 Gut weights and lengths in 80% gut-resected rats following 7 days of peptide treatment

	Treatment group	
	Vehicle	IGF-I(1.1mg/kg bw/d)
Final bw (g)	156.2±3.1	158.0±5.6
Stomach wt (g)	0.987±0.042	1.155±0.058
Duodenum length (cm)	7.5±0.3	7.8±0.5
Duodenum weight (g)	1.209±0.100	1.411±0.148
Jejunum length (cm)	8.1±0.4	8.7±0.7
Jejunum weight (g)	1.089±0.114	1.350±0.143
Ileum length (cm)	8.4±0.4	9.1±0.5
Ileum weight (g)	1.141±0.086	1.279±0.128
Caecum weight (g)	1.160±0.129	1.359±0.151
Colon length (cm)	10.2±0.6	10.8±0.8
Colon weight (g)	1.131±0.130	1.176±0.196
Total gut weight (g)	6.716±0.404	7.729±0.646

Values are Means ± SEM.

otherwise GH-deficient (Guler et al., 1988; Schoenle et al., 1985; van Buul-Offers et al., 1986) rats, and mice expressing a human IGF-I transgene (Mathews et al., 1988). Thus, our resection model of acute stress offered a new perspective for evaluating the actions of IGF-I.

2.4.2 Effect of IGF-I in resected rats

A concurrent study by a collaborating group within the Cooperative Research Center had shown responses to 1.2mg IGF-I/kg bw/d in nitrogen-restricted rats (Tomas et al., 1991b). Accordingly, a similar dose, 1.1mg IGF-I/kg bw/d, was chosen as the starting point for a preliminary trial with IGF-I. This dose significantly

improved food conversion efficiency and tended to improve body weight gain following 80% jejuno-ileal resection compared to vehicle-treated controls. More pronounced gains in body weight had previously been demonstated in hypophysectomized (Guler er al., 1988; Schoenle et al., 1985), diabetic (Scheiwiller et al., 1986), fasted (Asakawa et al., 1988) and normal (Hizuka et al., 1986) rats, and pituitary-deficient dwarf mice (van Buul-Offers et al., 1986). This difference in body weight effect is probably attributable to the generally higher dose rates (2.3-3.7mg IGF-I/kg bw/d) administered in the other studies.

The lack of effect of IGF-I on the non-gut organs of our animals contrasts with the restoration of heart, liver, kidney, spleen and thymus weights demonstrated in hypophysectomized rats (Guler er al., 1988; Schoenle et al., 1985) and genetically GH-deficient Snell dwarf mice (van Buul-Offers et al., 1986), and the increase in the relative kidney, liver, testes and pituitary weights observed in normal rats (Hizuka et al., 1986) following IGF-I treatment. As with body weight gain, the lack of response of the non-gut organs to IGF-I in our rats is best accounted for by the substantially lower dose we administered.

Although the effects of IGF-I on the gastro-intestinal tract failed to achieve significance, they suggested that the gut, especially the upper gut, may be sensitive to IGF-I.

In summary, the trends noted toward improved body weight gain and compensatory gut growth in gut-resected animals following administration of a relatively low dose of IGF-I encouraged the view that significant effects would be achieved using either higher doses of IGF-I, or more potent forms of IGF-I.

CHAPTER 3

Experiment 1: IGF-1 or a lower dose of des(1-3)IGF-I improves weight gain and nitrogen balance in rats following 80% resection

3.1 INTRODUCTION

The aim of this experiment was to further the understanding of IGF-I action *in vivo*. In particular, 1) to determine whether the response to IGF-I treatment could be enhanced by increasing the dose infused, 2) to clarify the mechanism of IGF-I action on body weight gain by assessment of nitrogen balance and, as an index of muscle protein breakdown, urinary 3-methylhistidine (3-MH) excretion, 3) to resolve whether the gastro-intestinal tract is sensitive to IGF peptides, and 4) to obtain preliminary information on the *in vivo* potency of des(1-3)IGF-I, an analogue that binds to the type-1 receptor with equal affinity to IGF-I, but has much lower affinity for the IGFBPs.

In the preliminary experiment described in Chapter 2, administration of a relatively low dose of IGF-I (1.1mg/kg bw/d) to rats for seven days following 80% jejuno-ileal resection significantly improved food conversion efficiency, and tended to improve body weight gain and gut growth. This experiment, using the same animal model, repeated the low-dose (1.0mg/kg bw/d), but also included a 2.4 times higher dose of IGF-I. In addition, the response to 1.0mg/kg bw/d of des(1-3)IGF-I was evaluated. Des(1-3)IGF-I was known to be considerably more potent than IGF-I in stimulating DNA synthesis and protein accretion *in vitro* as a consequence of its much lower affinity for IGFBPs (Forbes et al., 1988; Szabo et al., 1988). However, its efficacy *in vivo* had not been determined. Accordingly, a single dose of des(1-3)IGF-I was included in this investigation to obtain preliminary information on the effect of binding proteins on the biological action of IGF-I *in vivo*.

3.2 MATERIALS and METHODS

3.2.1 Peptides and dose rates

Peptides or vehicle were delivered by osmotic minipumps in the manner described in section *2.2.7*. The pumps were filled with either 0.1M acetic acid as

vehicle, 7.6 or 18.9mg recombinant human IGF-I/ml, or 7.6mg recombinant human des(1-3)IGF-I/ml. Body weights at the time of surgery were (mean±SEM) 172±2, 174±4, 178±5 and 173±3g for the vehicle, low-dose IGF-I, high-dose IGF-I and des(1-3)IGF-I groups, respectively. Consequently, the pumps delivered 0, 1.0 or 2.4mg IGF-I/kg bw/d, or 1.0mg des(1-3)IGF-I/kg bw/d, based on the body weights at surgery. An IGF-I dose of 2.4mg /kg bw/d dose was chosen as collaborative studies (Tomas et al., 1991b,c) underway at this time in other rat models were indicating that des(1-3)IGF-I had a potency 2-3 times that of IGF-I *in vivo*. Recombinant human IGF-I and recombinant human des(1-3)IGF-I were provided by Genentech, San Francisco, CA., USA.

3.2.2 Animals and experimental design

The animals and their maintenance have been described in sections *2.2.1* and *2.2.4*. All faeces and urine collected were stored at -20°C. Surgery for the 80% resection of the jejunum plus ileum was as described in section *2.2.7*. Following anaesthetisation, and prior to surgery, approximately 200µl of tail vein blood was collected into a heparinized capillary tube. In the same manner, blood was collected from conscious rats on the fourth day of recovery (day 4). Blood was taken at sacrifice as detailed in section *2.2.5*. Once collected, blood was immediately transferred to an Eppendorf tube, centrifuged at 400*g* for 30sec in a benchtop centrifuge, and frozen in liquid nitrogen, before eventually being stored at -20°. The faeces was subsequently dried under vacuum, and then mechanically ground. The kill procedure and the collection of organs were the same as that outlined in section *2.2.5* . On the basis of the exclusion criteria described in Section *2.2.6*, 5 of 30 resected rats were excluded: one low-dose IGF-I and two high-dose IGF-I-treated animals died from infection, another high-dose IGF-I died following intestinal rupture, and a further high-dose IGF-I animal was excluded at kill when anastomosal blockage was revealed. Although only IGF-I treated animals were excluded, the cause of death or exclusion did not appear to be related to peptide treatment.

The protocol used for all experiments followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and was approved by the Animal Ethics Committee's of both the University of Adelaide and the Women's and Children's Hospital, Adelaide.

3.2.3 Analytical measurements

To determine nitrogen intake and excretion, the nitrogen content of dried food, faeces and urine was measured by a Carlo Erba NA 1500 Nitrogen Analyser (Milan, Italy) using the Dumas Procedure. In determining food and faecal nitrogen a weighed sample of approximately 5mg was sealed in a tin container and loaded into the automatic autosampler, with atropine (4.84% nitrogen) acting as the standard. Urine nitrogen was determined in the same manner following the addition of chromosorb (C-6514, 60-80 mesh, acid-washed; Sigma Chemical Co., St. Louis, MO., USA) to 30µl of urine, with urea (1% nitrogen) as the standard.

Urinary 3-methylhistidine (3-MH) excretion was measured as an index of muscle protein catabolism. 3-MH is derived exclusively from actin and myosin and is released from muscle during protein breakdown providing a valid index of muscle protein catabolism provided a) the subject/animal is on a 3-MH free diet, b) released 3-MH is not reincorporated into protein, and c) it is excreted quantitatively into urine soon after its catabolic release from muscle (Tomas and Ballard, 1987). On condition that the diet is free of 3-MH for at least several days prior to measurement, these criteria are satisfied in several species, including rats (Tomas and Ballard, 1987). In this series of experiments, the rats were fed a casein-based, muscle-free diet containing no 3-MH for four days prior to the start of any measurements and maintained on this diet for the entire treatment period. Urine was collected daily, thereby enabling calculation of the rate of 3-MH excretion per day. Urinary 3-MH was measured by an automated method (Murray et al., 1981) using a Technicon AutoAnalyser I (Technicon Corp., Tarrytown, NY, USA). Hydrolysis of acetyl-3-MH in 5ml of urine was achieved using 10% (v/v)

HCl in a boiling-water bath for 1h, before the urine was loaded on to columns containing 4ml of ion-exchange resin (Tomas et al., 1979). The resin was sequentially washed with 50ml of 0.15M citrate/phosphate buffer (pH 4.5) and 50ml of water. The 3-MH was then eluted with 9ml of 0.2M sodium phosphate buffer (pH 8.3).

Urinary creatinine concentrations were also measured as the daily excretion of creatinine provides an index of muscle mass (Muldowney et al., 1957), and, in the event of a treatment effect on muscle mass, the molar ratio of 3-MH to creatinine excretion provides a sensitive measure of muscle protein catabolism (Tomas and Ballard, 1987). Creatinine concentrations in urine were determined using the Skalar continuous flow analyser method no. 07907001 (Skalar Analytical, Breda, Netherlands).

Measurement of 3-methylhistidine and creatinine were performed by Mrs. J. Burgoyne, Mrs. S. Madden, Ms. A. Collins and Mr. I. Skene under the direction of Dr. F. Tomas at the CSIRO, Division of Human Nutrition, Adelaide, South Australia.

3.2.4 Plasma IGF-I radioimmunoassays

To determine the effect of IGF infusion on plasma IGF-I levels, IGF-I was measured in acid-ethanol extracts of plasma collected prior to surgery (day 0), on day 4 of treatment, and at sacrifice (day 7) by a modification (Johnson et al., 1990) of the original procedure described by Daughaday et al. (1982a). Acid-ethanol extraction was performed to reduce interference by binding proteins in the RIA. Briefly, 100µl sodium phosphate-buffered saline (0.04M , pH 7.4) containing 0.003M NaN_3 and 0.05M Tween 20 was added to 25µl plasma. After adding an acid-ethanol solution (500µl; 87.5% ethanol and 12.5% 2M HCl), the mixture was vortexed and left to stand for 30min at 25°C. Following addition of Tris base (250µl; 0.855M), the tubes were centrifuged at 2500*g* for 10min at 4°C. After standing for 30min at 25°C, a 50µl aliquot of the extract was taken for the RIA.

In addition to acid-ethanol extraction of all plasma samples, plasma collected on day 7 was also chromatographed under acid gel-permeation conditions to separate IGFBPs from the growth factors. While this procedure is considerably more labour intensive, it results in a more complete separation of IGFs from the binding proteins (Owens et al., 1989). In this procedure, 70µl plasma sample was diluted with running buffer to obtain 350µl of solution (pH 2.8) containing acetic acid (200mM), trimethylamine (50mM) and Tween (0.05%). Each solution was then mixed 1:1 by volume with Freon (1, 1, 2-trichloro -1, 2, 2-trifluoroethane), AR grade (Mallinckrodt, Paris, KY., USA) to extract lipids. After centrifugation at 10000*g* for 10min the supernatant was collected, and a 200µl aliquot of this defatted solution was applied to a Protein-Pak 125 molecular sieve chromatography column (Waters-Millipore, Lane Cove, NSW, Australia) using a calibrated autoinjector. The column was equilibrated with the running buffer described above. A fraction collector was used to collect 0.5ml fractions, and absorbance at 280nm was used to measure protein elution. Dr. P. C. Owens (CSIRO Division of Human Nutrition, Adelaide, South Australia) in preliminary RIAs of all fractions determined that IGFBPs elute at 6-8ml, and IGF-I at 9-12.5ml. Consequently, pools eluting between 6 and 8ml (binding protein region), 8 and 9ml (intermediate fraction), 9 and 12.5ml (IGF-I region) and were routinely analysed in all RIAs conducted during this investigation.

The RIA used recombinant human IGF-I as its standard and radioligand. The IGF-I was radiolabelled by iodination with chloramine T to a specific activity of 63-84Ci/g, and purified by chromatography through Sephadex G-50 in 50mM sodium phosphate (pH 6.5) containing 1mg/ml bovine serum albumin. When analysing the acid-ethanol extracts, a blank solution of buffer, acid-ethanol and Tris base (1:4:2, by volume) was added to the non-specific binding and standard tubes to achieve the same final concentration of acid, ethanol and Tris as in sample tubes. For RIA of samples from the acid-column pooled fractions, a volume of running buffer equivalent to the fraction to be assayed was added to the non-specific binding and standard tubes. All

samples, standards and blank tubes were assayed in triplicate. To each tube containing 50µl of sample or standard, 50µl rabbit anti-bovine IGF-I (1:30,000) was added. The antiserum was prepared by immunizing rabbits with a conjugate of bovine IGF-I and ovalbumin. After addition of antiserum, ^{125}I-labelled IGF-I ($20x10^3$cpm) was added to each tube, with the mixture incubated for 16h at 4°C. 10µl of normal rabbit serum (1:50 dilution) and 50µl of sheep anti-rabbit immunoglobulin (1:20 dilution) were then mixed and incubated for 30min at 4°C. Following the addition of 1.5ml of chilled 5.5% polyetheylene glycol (PEG 6000; Fluka Chemic AG, Buchs, Switzerland) in 0.9% NaCl, the tubes were vortexed and then centrifuged at 2500*g* for 20min at 4°C. After the supernatant was aspirated, radioactivity in the pellet was counted.

Rabbit anti-bovine IGF-I has equal cross-reactivity with bovine and human IGF-I since the two peptides are structurally the same, but only cross-reacts 70% as well with rat IGF-I or recombinant human des(1-3)IGF-I (P. C. Owens, personal communication).

Analysis of plasma IGF-I peptide levels by acid-ethanol extraction and RIA for human IGF-I, and acid gel-premeation chromatography were performed by Ms. M. Pearce under the supervision of Dr. P. C. Owens, CSIRO Division of Human Nutrition, Adelaide, South Australia.

3.2.5 Statistical Analysis

Data are reported as means and SEM. The treatment groups were initially compared by a one way analysis of variance (ANOVA). When significant difference ($p<0.05$) was revealed, Fisher's Protected Least Significant Difference *post hoc* test was employed to identify the source. Statistical analysis was performed using the SuperANOVA (Abacus Concepts, CA., USA) program.

3.3 RESULTS

3.3.1 Plasma IGF-I concentrations

IGF-I concentrations were measured in plasma taken immediately before gut resection and on days 4 and 7 after surgery by RIA after an acid-ethanol extraction (Fig. 3.1). The purpose of these measurements was twofold: 1) to describe changes in endogenous IGF-I in response to removal of 80% of the jejuno-ileum, and 2) to determine the extent by which exogenously administered growth factors could influence plasma IGF-I levels.

There were no between-groups differences in endogenous plasma IGF-I concentrations prior to surgery and peptide administration. In the vehicle-treated reference group, plasma IGF-I fell immediately following surgery (day 4) before recovering to exceed pretreatment levels by day 7. A very similar pattern was evident in the groups treated with 1.0mg/kg bw/d of either IGF-I or des(1-3)IGF-I, indicating that at this dose infusion of IGF-I peptides failed to raise circulating IGF-I levels above those of the vehicle-treated controls. On the other hand, the higher dose of IGF-I progressively increased plasma IGF-I above pretreatment levels, so that by day 7, the high-dose IGF-I group had double the circulating IGF-I concentration of the control animals. Even when the relative cross-reactivities of the assay were accounted for, the plasma IGF-I concentrations of the high-dose IGF-I infused animals were considerably higher than the levels of circulating IGF-I in the other groups. This point is discussed in greater detail in section *4.3.1*.

Removal of the binding proteins from day 7 plasma by gel-permeation chromatography under acid conditions resulted in 30-50% higher estimations of plasma IGF-I than obtained in acid-ethanol extracts of the same samples (Fig. 3.1, inset). This presumably reflects the residual binding protein interference in the acid-ethanol extracted samples. Nevertheless, the two methods were strongly correlated (r=0.82), suggesting that acid-ethanol extraction provided a valid measure of the time course of

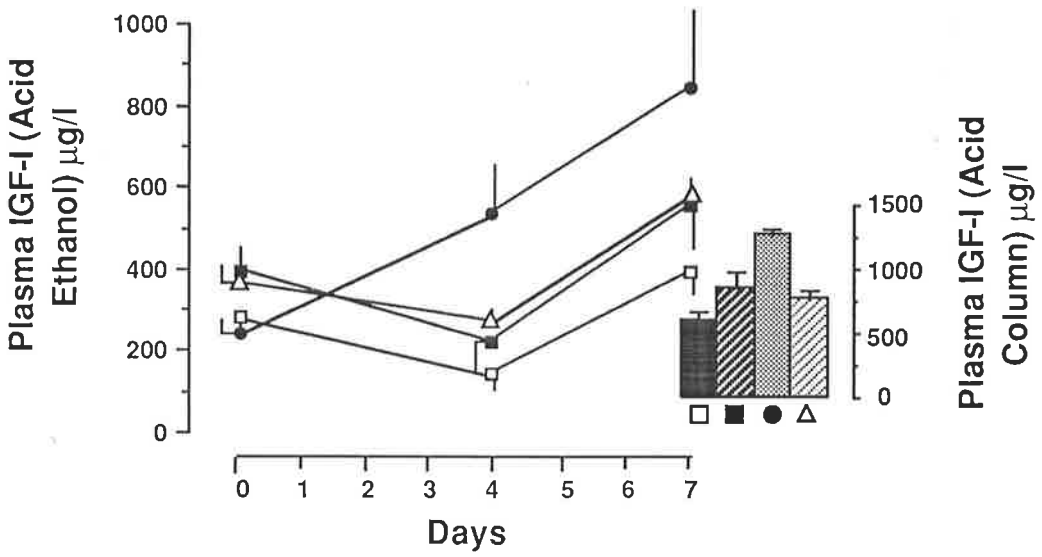

Fig. 3.1 Plasma IGF-I concentrations immediately before, and on the 4th and 7th days after, gut resection and insertion of osmotic pumps. Treatment groups were vehicle (open squares), 1.0mg IGF-I/kg bw/d (closed squares), 2.4mg IGF-I/kg bw/d (closed circle), and 1.0 mg des(1-3)IGF-I (open triangle). Values are means±SEM for 5-7 rats in each group, and are expressed as µg/l measured in acid-ethanol extracts. Bar graphs display mean IGF-I concentrations (±SEM) in plasma on day 7 after removal of binding proteins by acid gel-permeation chromatography.

changes in plasma IGF-I following resection.

The gel-permeation chromatography also provided estimates of IGFBP activity. IGFBP levels were estimated by the apparent IGF-I concentration in the 6-8ml pooled fractions. These levels were similar in all treatment groups (mean±SEM, units of IGF-I equivalents: 257±54, 219±43, 260±29 and 205±37 in the vehicle, low-dose IGF-I, high-dose IGF-I and des(1-3)IGF-I groups, respectively), suggesting no difference in total plasma binding protein activity.

3.3.2 Body weight changes

The effects of resection on body weight were similar to those described for the first experiment in Chapter 2. Prior to surgery, the animals were growing at about 8-10g/d with no differences existing between the groups (Fig. 3.2(a)). In the 24h immediately following resection, the vehicle-treated controls lost approximately 14g. Over days 2-4, body weight was stabilized but not gained by these animals. Although the controls resumed growing during days 5-7, the rate of body weight gain remained less than that prior to surgery.

Administration of 1.0 mg IGF-I/kg bw/d failed to modify growth performance, relative to the controls, in any of the periods examined (Fig. 3.2(a)). In contrast, treatment with des(1-3)IGF-I or the high-dose IGF-I (2.4mg/kg bw/d), although having no effect on weight loss over the initial 24h following surgery, improved body weight gain thereafter relative to the controls and low-dose IGF-I group. Thus, over days 2-4, the des(1-3)IGF-I and high-dose IGF-I groups gained weight while the other groups experienced marginal net weight losses, and during days 5-7 the weight gained by the des(1-3)IGF-I (20.8±1.0g) or high-dose IGF-I (21.9±1.7g) was significantly greater ($p<0.05$) than that gained by the low-dose IGF-I (14.4±2.9) or control (14.0±1.7) groups. Furthermore, presurgical growth rates were fully restored in the des(1-3)IGF-I and high-dose IGF-I groups during days 5-7, while growth remained significantly depressed for the controls and low-dose IGF-I animals during the same period. Presurgery body weight , which was not fully recovered by the animals infused with vehicle or the low-dose of IGF-I, was regained within 5 or 6 days in the des(1-3)IGF-I or the high-dose of IGF-I treated rats.

As in the preliminary experiment, food intake was not significantly affected by peptide infusion (Fig. 3.2(b)).

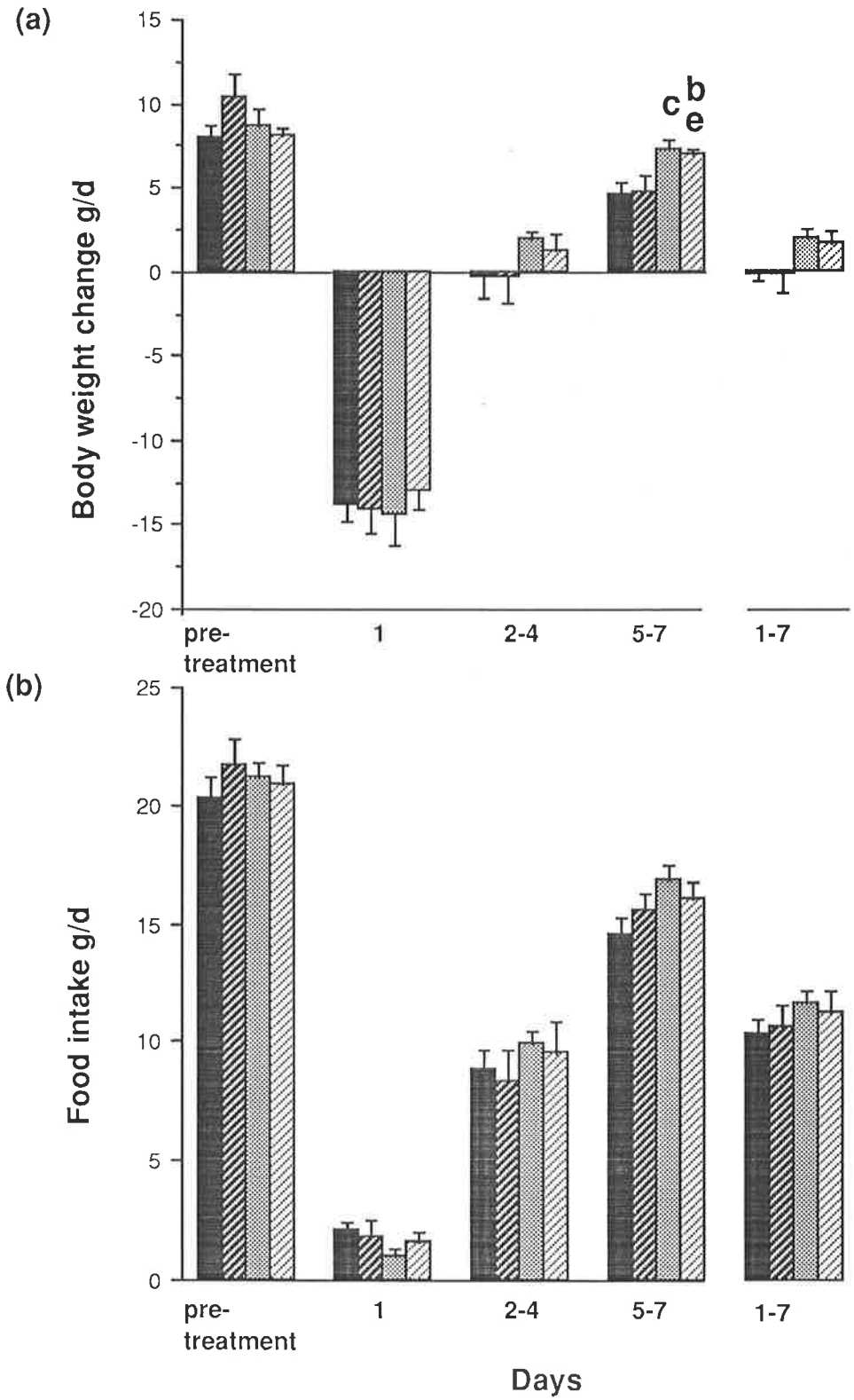

Fig. 3.2 Bodyweight changes (a) and average daily food intake (b) of rats calculated for the 3 pretreatment days prior to 80% resection of the jejuno-ileum, and for day 1, days 2-4, days 5-7, and days 1-7 following surgery. Treatment groups were vehicle (■), 1.0mg IGF-I/kg bw/d (▨), 2.4 mg IGF-I/kg bw/d (▩), and 1.0 mg des(1-3)IGF-I (▨). Values are means±SEM for 5-7 rats in each group. **a**: $p<0.05$; **b**: $p<0.01$; **c**: $p<0.001$ versus vehicle-treated rats; **e**: $p<0.01$ versus IGF-I at the same dose.

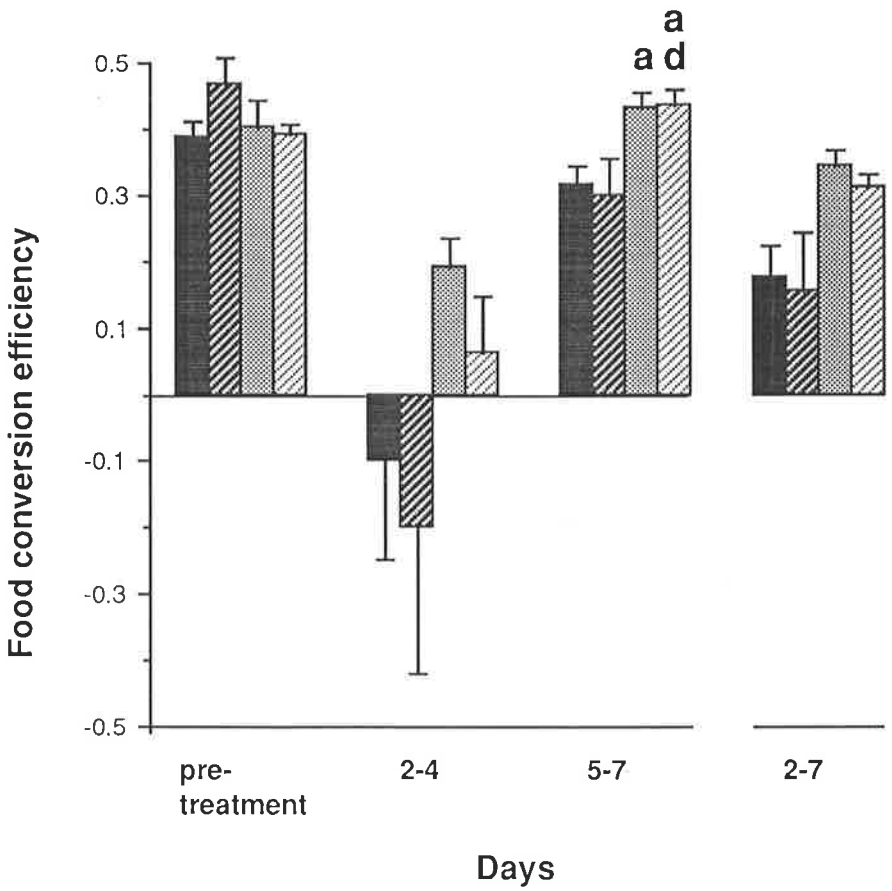

Fig. 3.3 Food conversion efficiency (g body weight gain/g food intake) for the 3 pretreatment days prior to 80% resection of the jejuno-ileum, and for days 2-4, days 5-7, and days 2-7 following surgery. Values are means±SEM for 5-7 rats in each group. Treatment groups were as in Fig. 3.2. **a:** $p< 0.05$; **b**: $p< 0.01$; **c**: $p< 0.001$ versus the vehicle group; **d**: $p<0.05$ versus IGF-I at the same dose.

Food conversion efficiency was calculated as described in section *2.3.2*. The food conversion efficiencies of all animals, and especially those in the low-dose IGF-I and control groups, fell during days 2-4 (Fig. 3.3). By days 5-7, the food conversion efficiencies of the des(1-3)IGF-I and high-dose IGF-I groups had been restored to pretreatment levels, while the low-dose IGF-I and control groups had significantly lower food conversion efficiencies. As with body weight gain, the values for food conversion efficiency of the controls in this experiment replicated those of the controls in the preliminary experiment.

3.3.3 Nitrogen balance measures

Nitrogen balance was calculated in this experiment to determine whether improvements in body growth following treatment with IGF-I peptides reflected accretion of protein or fluid retention. Nitrogen balance is a reliable measure of protein accretion since virtually all of the nitrogen in the body occurs in protein. Nitrogen balance was calculated as the difference between dietary nitrogen intake and nitrogen excreted in the faeces and urine.

Due to the stress of surgery, intake of nitrogen during the initial 24h of recovery was very low for all groups. Although remaining below pretreatment levels, nitrogen intake in each group recovered steadily thereafter (Fig. 3.4(a)).

Relative to pre-treatment levels, nitrogen excretion in the control animals was not significantly altered by surgery (Fig. 3.4(b)). No differences in the excretion of nitrogen existed between the controls and the low-dose IGF-I group. In the des(1-3)IGF-I and high-dose IGF-I groups, nitrogen excretion on day 1 tended to be less than for the controls. This difference became significant over days 2-4 with the des(1-3)IGF-I or high-dose IGF-I groups excreting about 40% less nitrogen than the controls. During days 5-7, the controls continued to lose more nitrogen than the animals treated with either des(1-3)IGF-I or the high-dose of IGF-I, although the differences were not significant. For the entire treatment period, approximately 20% less nitrogen was excreted by the des(1-3)IGF-I and high-dose IGF-I rats than by the controls.

Since the nitrogen intakes of the groups did not vary significantly, it is not surprising that the changes in nitrogen balance (Fig. 3.4(c)) broadly reflected those described for nitrogen excretion. After net losses of nitrogen on day 1, nitrogen retention in each group progressively improved during the recovery week. However, even by days 5-7, daily nitrogen retention in the control animals was only half what it

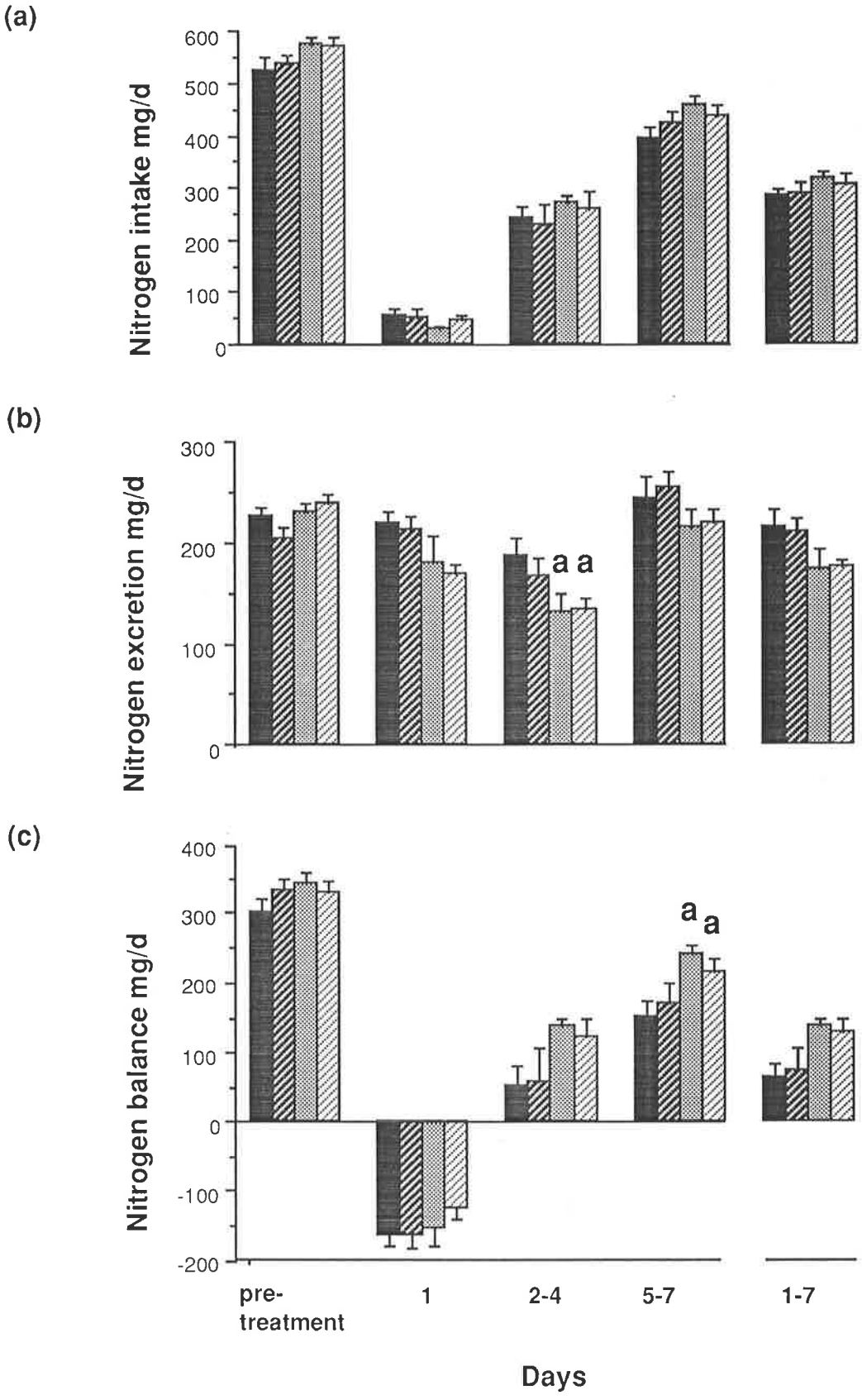

Fig. 3.4 Nitrogen intake (a), excretion (b), and balance (c) for the 3 pretreatment days prior to 80% resection of the jejuno-ileum, and for day 1, days 2-4, days 5-7, and days 1-7 following surgery. Treatment groups were as in Fig. 3.2. Values are means±SEM for 5-7 rats in each group, and are expressed in mg N/day: **a** $p < 0.05$ versus the vehicle group.

had been before resection. Throughout the treatment period, nitrogen balances were more positive in the des(1-3)IGF-I and high-dose IGF-I groups than the controls. Although not achieving statistical significance, the average daily balances for days 2-4 were 137% and 163% higher, respectively, in the des(1-3)IGF-I and high-dose IGF-I animals than the controls. During days 5-7, the difference between the groups treated with either des(1-3)IGF-I or the high-dose of IGF-I and those receiving vehicle achieved significance ($p< 0.05$). The improved nitrogen status of the des(1-3)IGF-I and high-dose IGF-I animals can also be seen from the time required to achieve a positive nitrogen balance (Fig. 3.5). For the des(1-3)IGF-I and high-dose IGF-I groups this occurred first on day 2. For the low-dose IGF-I and control groups, this was not achieved until days 4 and 5, respectively.

3.3.4 Muscle protein breakdown

To assist in the interpretation of the body weight and nitrogen balance data, muscle protein breakdown was assessed by the urinary 3-methylhistidine (3-MH) excretion rate to determine if the improvement in nitrogen retention reflected a reduction in muscle protein breakdown. The experimental design allowed quantitative measurement of daily 3-MH excretion, thereby permitting a continuous assessment of muscle protein degradation during the treatment period. In addition to measuring 24h urinary 3-MH excretion, the ratio of 3-MH to creatinine was also measured to normalize the 3-MH excretion data against any changes in muscle mass that might have occurred with peptide treatment. Urinary creatinine excretion was measured as an index of muscle mass.

Creatinine excretion rates (μm/kg bw/d) were unaffected by resection or peptide treatment (average daily creatinine excretion rates during the treatment period: 206.9±5.0, 198.6±11.4, 196.1±5.6 and 205.6±5.6 μmol/kg bw/d for the control, 1.0mg IGF-I/kg bw/d, 2.4mg IGF-I/kg bw/d, and 1.0mg des(1-3)IGF-I/kg bw/d,

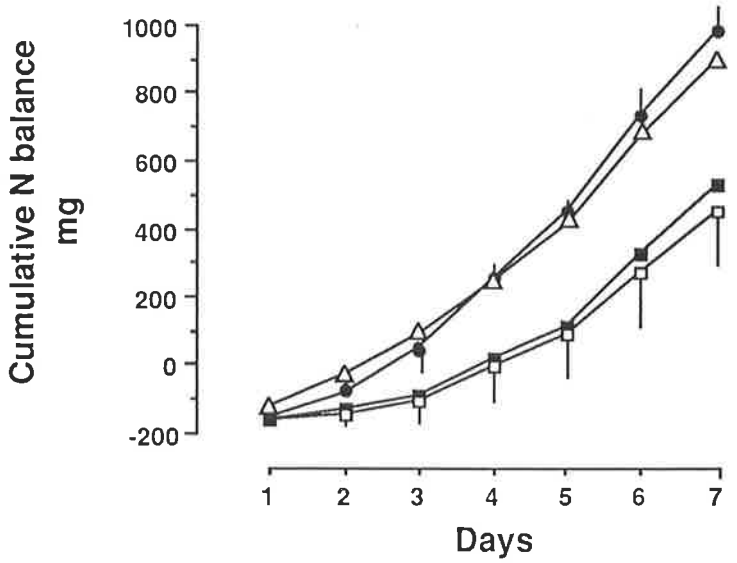

Fig. 3.5 Cumulative nitrogen balance over 7 days following gut resection and insertion of osmotic pumps. Treatment groups were vehicle (open squares), 1.0mg IGF-I/kg bw/d (closed squares), 2.4mg IGF-I/kg bw/d (closed circle), and des(1-3)IGF-I open triangle. Values are means±SEM for 5-7 rats in each group, and are expressed in mg N/day. SEM bars are only plotted for vehicle and the high dose IGF-I groups.

respectively) indicating that infusion of IGFs did not markedly affect muscle mass. Due to the similarities in creatinine excretion between the groups, the 3-MH to creatinine excretion ratio mirrored total 3-MH excretion, consequently, only the data from the direct measure of 3-MH excretion are presented (Fig. 3.6). A substantial increase in 3-MH excretion for the controls over the initial 24h recovery period showed that resection had induced a catabolic state. Thereafter, muscle protein catabolism in these animals steadily diminished with pretreatment levels restored over days 5-7. Despite the fact that the increase in 3-MH excretion was transient, the average daily 3-MH excretion over the 7 day treatment period was significantly higher ($p< 0.05$) in the control group than it had been prior to surgery.

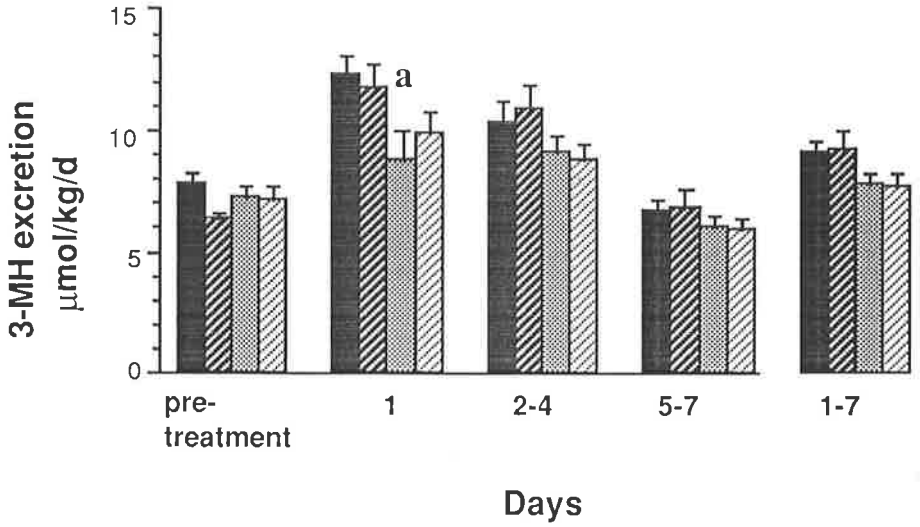

Fig. 3.6 Urinary 3-methylhistidine (3-MH) excretion of rats calculated for the 3 pretreatment days prior to 80% resection of the jejuno-ileum, and for day 1, days 2-4, days 5-7, and days 1-7 following surgery. Treatment groups were as in Fig. 3.2. Values are means±SEM for 5-7 rats in each group. **a**: $p<0.05$ versus the vehicle-treated rats.

In the first 24h after surgery, when 3-MH excretion was maximal, des(1-3)IGF-I but not IGF-I at the high or low dose, significantly attenuated the rise in 3-MH ($p< 0.05$). During the remainder of the recovery week, administration of des(1-3)IGF-I or IGF-I at the high-dose tended to reduce 3-MH excretion compared to the controls, although these effects were not significant. The lack of significant effects on 3-MH excretion during this period is not surprising given the transient stress of the model. Nevertheless, the reduction in 3-MH excretion for the des(1-3)IGF-I and high-dose IGF-I animals is exemplified by the fact that, in contrast to the control animals, the average daily 3-MH excretion rate over the treatment period was not elevated in these rats relative to their pretreatment levels. For days 5-7, 3-MH excretion for the des(1-3)IGF-I and high-dose IGF-I animals was reduced ($p<0.05$) relative to pretreatment levels.

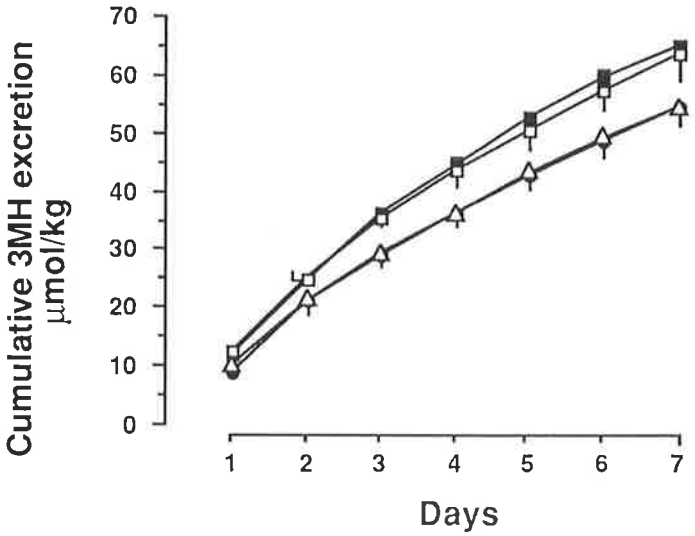

Fig. 3.7 Cumulative 3-methylhistidine excretion over 7 days following gut resection and insertion of osmotic pumps. Treatment groups were vehicle (open squares), 1.0mg IGF-I/kg bw/d (closed squares), 2.4 mg IGF-I/kg bw/d (closed circle), and 1.0mg des(1-3)IGF-I (open triangle). Values are means±SEM for 5-7 rats in each group, and are expressed in mg N/day. SEM bars are only plotted for vehicle and the high dose IGF-I groups.

Although the trend for the animals receiving des(1-3)IGF-I or IGF-I at the higher dose to excrete less 3-MH was generally not significant, expression of the data on a cumulative basis shows that the trend was constant and progressive throughout the treatment period (Fig. 3.7).

3.3.5 Organ effects

Despite the differences in nitrogen balance and the trends in muscle protein breakdown, treatment with IGF-I peptides had no effect on the relative carcass (g/kg bw) or pelt weights (g/kg bw) (Table 3.1). It therefore appears that IGF-I induced proportional growth, with no selective increase in carcass mass.

Organ weights have been compared between treatment groups both as absolute weights (g) and as relative weights corrected for body weight (g/kg bw) (Table 3.2).

TABLE 3.1 **Relative carcass and pelt weights of rats following 80% jejuno-ileal resection and 7 days treatment with IGF peptides.**

Treatment group	Carcass (g/kg bw)	Pelt (g/kg bw)
Vehicle	423.7±7.5	123.4±3.8
IGF-I (1.0 mg/kg bw/d)	406.1±13.5	126.2±3.5
IGF-I (2.4 mg/kg bw/d)	422.4±3.2	128.7±3.5
des(1-3)IGF-I (1.0 mg/kg bw/d)	416.6±5.5	130.5±2.0

Values are means ± SEM for 5-7 rats in each group.

The absolute weights of the liver and heart were significantly greater ($p<0.01$) in the high-dose IGF-I treated animals than in the controls. In the des(1-3)IGF-I group, absolute thymus weight was markedly increased ($p<0.001$) relative to both the low-dose IGF-I and control groups, and liver weight was also significantly heavier relative to the controls. Absolute kidney weights were significantly heavier in each of the treated groups, with the difference from the controls being particularly pronounced ($p<0.001$) in the des(1-3)IGF-I and high-dose IGF-I groups. Some of the effects on organ growth were lost when correction was made for body weight. However, the kidney weights of the treated animals remained significantly heavier than the controls, as did the thymus weights of the des(1-3)IGF-I-treated animals ($p<0.001$). The fractional weight of the thymus in the des(1-3)IGF-I-treated animals was also heavier than that of animals treated with an equivalent dose of IGF-I. Although the trend toward heavier absolute and relative spleen weights in the peptide-treated animals was not significant, it is consistent with the significant effect observed in Experiment 2 (see section *4.3.5*).

TABLE 3.2 Organ weights on day 7 after 80% jejuno-ileal resection and treatment with IGF-I peptides.

Absolute weight (g)

Treatment group	Liver	Spleen	Kidneys	Adrenals	Thymus	Heart	Lung
Vehicle	8.60±0.47	0.406±0.024	1.60±0.04	0.080±0.002	0.361±0.034	0.760±0.024	1.09±0.04
IGF-I (1.0mg/kg bw/d)	9.18±0.24	0.483±0.043	1.85±0.05[a]	0.032±0.002	0.345±0.074	0.773±0.032	1.19±0.04
IGF-I (2.4mg/kg bw/d)	10.60±0.59[b,d]	0.456±0.028	1.99±0.06[c]	0.026±0.002	0.490±0.020	0.894±0.053[b,d]	1.25±0.07
des(1-3)IGF-I(1.0mg/kg bw/d)	9.89±0.35[a]	0.512±0.022	1.96±0.08[c]	0.031±0.002	0.600±0.045[c,e]	0.840±0.028	1.22±0.10
			Relative weight (g/kg bodyweight)				
Vehicle	50.0±2.2	2.37±0.16	9.33±0.19	0.175±0.010	2.00±0.20	4.44±0.15	6.36±0.24
IGF-I (1.0mg/kg bw/d)	53.2±1.9	2.82±0.31	10.68±0.29[c]	0.185±0.020	1.94±0.39	4.47±0.11	6.89±0.34
IGF-I (2.4mg/kg bw/d)	55.5±2.7	2.38±0.11	10.44±0.29[b]	0.136±0.010	2.57±0.10	4.71±0.36	6.55±0.34
des(1-3)IGF-I(1.0mg/kg bw/d)	53.8±1.8	2.79±0.10	10.64±0.22[c]	0.168±0.010	3.30±0.21[c,e]	4.57±0.14	6.60±0.40

Values are means ± SEM for 5-7 rats in each group: [a] $P < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs vehicle-treated rats, [d] $p < 0.05$, [e] $p < 0.001$ vs IGF-I at the same dose.

3.3.6 Gut effects

Substantial effects of IGF-I peptides were apparent on the growth of the gastro-intestinal tract (Table 3.3). Relative to vehicle-treated controls, total gut weight (stomach to colon inclusive) was increased by 22%, 13% and 26%, following treatment with des(1-3)IGF-I, and the low and high doses of IGF-I, respectively. The changes in total gut weight were mainly due to striking effects in the upper gastro-intestinal tract, with stomach weight, and especially duodenum weight, significantly increased by administration of des(1-3)IGF-I or the high-dose of IGF-I. As in the preliminary experiment, treatment with a low-dose of IGF-I produced non-significant increases in stomach and duodenum weight. Significant increases in the weight of the combined jejuno-ileal remnant, compared with the controls, were observed for all the treatment groups, with responsiveness more apparent in the remnant ileum. No significant effects on caecum or colon weight were observed, although both tended to be heavier in the peptide-treated groups.

IGF treatment had no significant effect on small or large bowel lengths, although the duodenum, jejunum, ileum and colon tended to be longer in the treated animals (Table 3.3). Therefore, it appears that while treatment with IGF-I peptides increased the overall mass of the gut, linear regrowth was not enhanced in gut-resected rats.

3.4 DISCUSSION

In this experiment, administration of IGF-I and particularly the analogue des(1-3)IGF-I was shown to significantly ameliorate post-surgical catabolism and enhance gut regeneration in rats following removal of 80% of the jejuno-ileum. The improved body weight gain was associated with a more positive nitrogen balance which in turn was attributed, at least in part, to a reduction in muscle protein degradation.

TABLE 3.3 Gut weights and lengths in 80% gut-resected rats following 7 days of peptide treatment

	Treatment groups			
	Vehicle	IGF-I(1.0mg)	IGF-I(2.4mg)	des(1-3)IGF-I(1.0mg)
Final body weight (g)	171.6±4.5	173.4±5.9	190.9±4.2	184.1±5.6
Stomach weight (g)	0.980±0.073	1.089±0.047	1.236±0.026[b]	1.137±0.052[a]
Duodenum length (cm)	8.6±0.5	9.2±0.6	9.2±0.5	9.4±0.5
Duodenum weight (g)	1.067±0.075	1.179±0.088	1.572±0.081[c]	1.542±0.102[c]
Jejunum length (cm)	8.6±0.5	8.9±0.4	10.6±0.5	12.0±0.3
Jejunum weight (g)	1.123±0.121	1.174±0.180	1.161±0.081	1.234±0.161
Ileum length (cm)	9.7±0.8	9.8±0.8	11.2±0.5	9.6±0.2
Ileum weight (g)	0.862±0.041	1.044±0.095[a]	1.127±0.022[b]	1.025±0.057
Caecum weight (g)	1.163±0.083	1.259±0.196	1.456±0.120	1.443±0.127
Colon length (cm)	11.3±0.3	11.7±0.6	12.4±0.2	12.3±0.4
Colon weight (g)	1.067±0.162	1.305±0.164	1.330±0.065	1.250±0.077
Total gut weight (g)	6.262±0.319	7.048±0.476	7.881±0.156[b]	7.630±0.185[b]

Values are Means ± SEM: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ versus vehicle-treated animals.
Doses are per kg body weight per day.

3.4.1 Effect of a high-dose of IGF-I in rats following gut resection

Body weight, food conversion efficiency, nitrogen balance and muscle protein breakdown in gut-resected rats were improved following seven days infusion of 2.4mg IGF-I/kg bw/d, but not 1.0mg IGF-I/kg bw/d, clearly demonstrating that IGF-I can stimulate body growth when administered at a sufficiently high dose. Measurement of plasma IGF-I concentrations during the treatment period revealed that the biological efficacy of IGF-I correlated with the increment in circulating IGF-I levels, as only the high dose of IGF-I increased plasma IGF-I and induced significant biological responses. This finding agrees with other catabolic animal studies where circulating IGF-I concentration has reflected biological activity. Thus, it has been shown that the reduction in circulating IGF-I, and coincident growth retardation, characteristic of various catabolic states can be overcome by infusion of sufficient IGF-I (Ballard et al., 1991b; Binz et al., 1990; Guler et al., 1988; Maes et al., 1983; Martin et al., 1991; Schoenle et al., 1985; Scheiwiller er al., 1986; Tomas et al., 1991a,c). The IGF-I dose required to increase plasma levels and, consequently, produce a biological response would appear to vary according to the nature of the growth impairment, as doses similar to the low dose used in this experiment have been shown to increase circulating IGF-I and enhance growth in other catabolic or growth-deficient rat models (Guler et al., 1988; Skottner et al., 1989). On the other hand, rats which are growth-retarded through dietary protein restriction alone (Tomas et al., 1991b), appear less responsive than the gut-resected rats in the current investigation, which are subjected to both nutritional and surgical stress.

The improvement in body weight gain without concomitant increases in food intake in the high-dose IGF-I group, imply that IGF treatment improved food utilization, a finding that is consistent with the effect seen when 1.1mg IGF-I/kg bw/d was administered in the preliminary experiment. A similar effect on food conversion efficiency to that presented here has been reported in a separate study (Vanderhoof et al., 1991) following delivery of 1.5mg IGF-I/kg bw/d to 80% jejuno-ileum resected

rats. However, these data are only published in abstract form, and although the comment is made that food intake was not affected by peptide treatment, actual data are not presented. While the effects of IGF treatment on food conversion efficiency data have only been described in a limited number of catabolic studies, significant improvements are reported in each (Martin et al., 1991; Tomas et al., 1991a,b,c).

3.4.2 Mechanisms of IGF stimulation of weight gain

To determine the mechanism underlying the significant weight gains of the animals treated with IGF-I peptides, nitrogen balance and muscle protein breakdown rates were investigated.

Since the effect of treatment on nitrogen retention mirrored that of body weight change in the animals receiving the high-dose of IGF-I it is unlikely that the weight gained by these animals reflected an increase in water retention. However, as analysis of carcass composition was not performed in this experiment it is not possible to exclude an increased fat mass, as occurs following administration of insulin to diabetic rats (Tomas et al., 1991a), as the source of weight gain. As carcass composition was determined in the next experiment, this point will be discussed in Chapter 4. The more positive nitrogen balance of the high-dose IGF-I animals was due to significant reductions in nitrogen excretion, since nitrogen intake did not differ between groups. Before I started this investigation no reports of nitrogen balance measurements had been made in animals treated with IGF peptides, but recently, improved nitrogen balance, by virtue of diminished nitrogen excretion, has been demonstrated in partially nephrectomized (Martin et al., 1991), diabetic (Tomas et al., 1991a), nitrogen-restricted (Tomas et al., 1991b) and dexamethasone-treated (Tomas et al., 1991c) rats following the infusion of IGF peptides.

The principal mechanism for improving nitrogen retention following IGF treatment could be a stimulus of protein synthesis, an inhibition of protein breakdown,

or a combination of the two. To provide a daily index of muscle protein catabolism in resected rats over the treatment period, urinary 3-MH excretion was measured. In the control animals, 3-MH rose markedly immediately following resection and thereafter declined towards normal. This initial elevation following resection confirms that post-surgical catabolism is a feature of this animal model, since during purely nutritional deprivation 3-MH excretion falls immediately and progressively (Tomas et al., 1991b). The rise in 3-MH during the initial recovery period tended to be lower in the IGF-treated animals relative to the controls, suggesting an attenuation of muscle protein breakdown by IGFs which could have contributed to the improved nitrogen balance of the treated animals. Significant falls in 3-MH excretion following IGF-I peptide administration have been observed in dexamethasone-treated rats (Tomas et al., 1991c) and partially nephrectomized rats (Martin et al., 1991), while a non-significant decline is reported for fasted rats (Asakawa et al., 1992). Conversely, no change in 3-MH excretion was seen following IGF treatment to diabetic (Tomas et al., 1991a) and nitrogen-restricted (Tomas et al., 1991b) rats, suggesting that the effect of IGFs on muscle protein degradation may be dependent on the catabolic condition.

Although muscle protein synthesis rates were not measured in this investigation, increases in muscle protein synthesis following treatment with IGF-I have been demonstrated in a number of catabolic rat models (Tomas et al., 1991a,b,c). Protein synthesis rates were not determined in resected rats because, within the confines of the treatment protocol, assessment could only have occurred on day 7. I considered that these measurements would have been unlikely to yield useful information because by day 7, due to the transient stress of this model, the controls have achieved similar levels of nitrogen retention to the IGF-treated animals. Therefore, even if enhanced protein synthesis rates had contributed to the improved nitrogen balance of the treated animals earlier in the recovery period, this effect would have been obscured by day 7. Assessment of 3-MH excretion, on the other hand, was

appropriate as it could be measured daily and, consequently, gives a better reflection of the time course of IGF effects on protein metabolism.

Despite improving nitrogen retention, tending to reduce muscle protein breakdown, and increasing absolute carcass weight, administration of IGF peptides had no effect on fractional carcass weight in the animals used in this experiment. This lack of effect agrees with similar non-responses in diabetic (Tomas et al., 1991a), partially nephrectomized (Martin et al., 1991) and dexamethasone-treated (Tomas et al., 1991c) rats, and suggests that the somatic gains stimulated by IGFs are due to proportional growth.

3.4.3 Tissue-specific effects of IGF-I treatment

In addition to increased body weight gain, IGF-I infusion selectively increased the weight of several tissues. At both the low and high-dose, IGF-I enhanced fractional kidney weight, an effect which appears to represent a consistent response to IGF-I administration *in vivo* under a range of conditions (Asakawa et al., 1992; Guler et al., 1988; Martin et al., 1991; Skottner et al., 1989; Tomas et al., 1991b,c; van Buul-Offers et al., 1988). Following uninephrectomy, renal IGF-I levels in the remnant kidney increase and correlate with compensatory renal hyperplasia (Andersson et al., 1988). It has been speculated that these anabolic responses may be related to the IGF-I mediated increases in glomerular filtration and renal plasma flow observed in normal rats and humans (Guler et al., 1989a,b; Hirschberg and Kopple, 1989). For the surgical-nutritional stress model used in this investigation, renal hypertrophy may also be facilitated by the increased IGF-I receptor mRNA and receptor levels evident in fasted rat kidneys (Lowe et al., 1989).

Infusion of IGF-I peptides increased thymus weight in gut-resected animals, with the effect being significant in the des(1-3)IGF-I treated group. Stimulation in thymus growth has also been observed following administration of des(1-3)IGF-I in

diabetic rats (Ballard et al.,1991b) and following treatment with IGF-I in diabetic (Binz et al., 1990), hypophysectomized (Guler et al., 1988) and dwarf (van Buul-Offers et al., 1988) rats. Binz et al. (1990) demonstrated that treatment with IGF-I increased the weight and partially restored the histology of a rat thymus severely atrophied by insulin deficiency. Possibly accounting for this effect was an IGF-I induced increase in [^{3}H] thymidine incorporation by thymocytes, which they suggested indicated the reappearance of replicating immature thymocytes. Although the effect on the spleen in this experiment failed to achieve statistical significance, the trend to increased splenic weight following IGF-I treatment agrees with the effect widely reported in a number of rat models (Asakawa et al., 1992; Binz et al., 1990; Guler et al., 1988; Tomas et al., 1991c; van Buul-Offers et al., 1988), as well as the response observed in Experiment 2 (see section *4.3.5*).

Significant increases in the absolute weights of a range of non-gut organs, including liver (van Buul-Offers et al., 1988), adrenals (Asakawa et al., 1992), heart, brain, salivary glands (van Buul-Offers et al., 1986), testes and pituitary (Hizuka et al., 1986), have been described. However, these effects were usually lost when fractional organ weights were calculated. The organs most regularly reported as being responsive to IGF-I are those that were most affected in this study; the kidney, thymus and spleen.

This investigation was the first to report the *in vivo* effect of IGF peptides on the gut in rats (Lemmey et al., 1991). Following 80% jejuno-ileal resection on rats, and 7 days treatment with either a high-dose of IGF-I, maximal increases of 26% for the stomach, 47% for the duodenum, 15% for the combined jejunum and ileum, 25% for the caecum, 25% for the colon, and 26% for the total gut were observed. The enhanced gains in gut weights appear to be due to increases in cross-sectional dimensions since intestinal lengths were not significantly affected by treatment. The marked sensitivity of the gut to IGF peptides contrasts with the lack of effect on the

fractional weights of the carcass, pelt and most non-gut organs, indicating that the gut is a selective target tissue for IGF-I.

Gastro-intestinal responsiveness to IGF peptides is not unexpected as type-1 and type-2 IGF receptors are located throughout the gut in several species (Rouyerfessard et al., 1990; Termanini et al., 1990), including the adult rat (Lowe et al., 1989), and both IGF-I and IGF-II are produced by the gut postnatally (Adamo et al., 1989; Brown et al., 1986). In addition, a recent pharmacokinetic study suggests that the gut may be favoured by a selective transfer of exogenous IGF-I from the circulation to the upper gastro-intestinal tract (Ballard et al., 1991a). The sensitivity of the stomach in resected rats to IGF-I may also be related to a reduction in endogenous IGF-I production and increase in gastric type-1 receptor number observed in fasting rats (Lowe et al., 1989), since food intake falls dramatically following resection.

The responsiveness of the gut, especially the upper gastro-intestinal tract, to IGF peptides is not confined to the resection model, since stomach, duodenum, combined jejunum and ileum, and total gut weight in dexamethasone-treated rats (Tomas et al., 1991c), total gut weight in partially nephrectomized (Martin et al., 1991) and diabetic (Read et al., 1991) rats, stomach weight in the lit/lit strain of GH-deficient mice (Gillespie et al., 1990), and duodenum weight in transgenic dwarf mice (Behringer et al., 1990), are all increased by IGF treatment.

3.4.4 Effects of the analogue, des(1-3)IGF-I

Similar effects on body weight, food conversion efficiency, nitrogen balance, muscle protein breakdown and gut growth were observed following infusion of 1.0 mg/kg bw/d of the analogue des(1-3)IGF-I as were seen after administration of a 2.4 fold higher dose of IGF-I. In addition, des(1-3)IGF-I stimulated thymus growth to a much greater extent than IGF-I. These observations are consistent with the increased *in*

vitro anabolic potency of des(1-3)IGF-I relative to IGF-I in cultured cells (Ross et al., 1989; Walton et al., 1990).

In contrast to IGF-I, the efficacy of des(1-3)IGF-I does not appear to be related to immunoreactive IGF-I plasma levels. Thus, infusion of 1.0mg/kg bw/d of des(1-3)IGF-I failed to raise RIA IGF-I activity, yet unlike IGF-I at the same dose, elicited a range of biological responses. Even taking into account the reduced immunoreactivity of des(1-3)IGF-I with the anti-IGF-I antiserum used in the RIA, it is clear that the circulating rat IGF-I/des(1-3)IGF-I activity in these animals was not significantly higher than the IGF-I level of the controls. Since des(1-3)IGF-I, in a functional context, apparently only differs from IGF-I in its affinity for IGFBPs (Forbes et al., 1988; Walton et al., 1990), it is likely that the superior potency of the analogue is a consequence of enhanced access to tissue receptors. As such, the enhanced potency of des(1-3)IGF-I provides evidence favourable to the argument that IGFBPs inhibit the action of IGF peptides *in vivo* (this point will be discussed in greater detail in Chapter 4).

This investigation was the first to demonstrate that IGF-I, and especially its variant des(1-3)IGF-I, are effective in attenuating post-surgical catabolism. It also, for the first time, showed the widespread *in vivo* sensitivity of the gut to IGF-I peptides.

CHAPTER 4

Experiment 2: Dose-responsive effects of IGF-I, des(1-3)IGF-I, and LR^{3}IGF-I on weight gain and gut growth in rats following 80% resection

4.1 INTRODUCTION

The aims of Experiment 2 were 1) to compare the *in vivo* potencies of another IGF-I analogue, LR^3IGF-I, with des(1-3)IGF-I, 2) to determine whether IGF-I peptides stimulate growth in a dose-responsive manner, 3) to characterize the nature of IGF-I action on the gut and assess whether these changes affect gut function, and 4) to identify the effects of resection and delivery of exogenous IGF-I peptides on the IGFBP profile.

Following the demonstration that des(1-3)IGF-I was generally more potent than IGF-I in gut-resected rats, it was decided that the effects of a further analogue, LR^3IGF-I, should be investigated. Although LR^3IGF-I and des(1-3)IGF-I have similar *in vitro* potencies there are several differences between the two analogues (Francis et al., 1992). Compared with des(1-3)IGF-I, LR^3IGF-I has only one third the affinity for the type-1 receptor and binds even less readily to the IGFBPs (Francis et al., 1992). Thus, comparison of the growth responses of LR^3IGF-I and des(1-3)IGF-I would further understanding of the respective importance of receptor and binding protein affinity in determining *in vivo* potency.

For comparative purposes, and also to determine whether IGF-I peptides induce growth in a dose-responsive manner, IGF-I, des(1-3)IGF-I and LR^3IGF-I in this experiment were each administered in three different doses.

Although Experiment 1 had revealed that the gut was particularly sensitive to IGF-I peptides, the mechanisms underlying these effects remained unknown. Consequently, the analyses of gut tissues in Experiment 2 were extended to include biochemical and histological measures of intestinal growth. In addition, nitrogen and fat excretion were assessed to determine if the accretion of gut mass which followed IGF treatment conveyed the benefit of improved nutrient absorption, since the ability to

enhance both adaptive hyperplasia and functional capacity is highly desired of any proposed treatment for short-gut syndrome.

4.2 MATERIALS and METHODS

4.2.1 Peptides and dose rates

Peptides or vehicle were delivered by osmotic minipumps as described in section *2.2.7*. The pumps were filled with either 0.1M acetic acid as vehicle, or for the IGF-I treated rats, 5.0, 12.6 or 31.5mg recombinant human IGF-I/ml. In recognition of its increased potency in Experiment 1, des(1-3)IGF-I was infused at lower concentrations, with the pumps filled with 2.0, 5.0 or 12.6mg recombinant human des(1-3)IGF-I/ml. Recombinant human LR^3IGF-I/ml, which has a similar *in vitro* potency to des(1-3)IGF-I, was infused at the same concentrations as des(1-3)IGF-I. The mean body weights of the groups at the time of surgery were between 155-158g. Consequently, the pumps delivered 0, 0.7, 1.8 or 4.5mg IGF-I/kg bw/d, and 0.3, 0.7 or 1.8mg des(1-3)IGF-I/kg bw/d or LR^3IGF-I/kg bw/d based on the body weights at surgery. Recombinant human IGF-I and recombinant human des(1-3)IGF-I were provided by Genentech, San Francisco, CA., USA. Recombinant human LR^3IGF-I was supplied by GroPep Pty Ltd., Adelaide, South Australia, 5000.

4.2.2 Animals and experimental design

The animals and their maintenance are described in sections *2.2.1* and *2.2.4*. To ensure that groups had similar starting weights, animals were allocated to treatment regimes on the basis of their body weight on day 0. Blood was collected on days 0, 4 and 7 as described in sections *3.2.2* and *2.2.1*. Surgery for removal of 80% of the jejuno-ileum was performed as for Experiment 1 and is described in section *2.2.7*. Faeces and urine were stored and treated as detailed in *3.2.2* except that prior to analysis, the faeces collected on the three pretreatment days from each animal were pooled. The faeces collected for days 2 through 4, and 5 through 7 were similarly

pooled, while that collected on day 1 was kept separate. Urine was pooled in the same fashion. This procedure of faeces and urine pooling was adopted since the resection model is one of transient catabolism featuring three distinct stages of recovery: the initial 24h. (day 1) during which there is marked weight loss due to negligable food intake, tissue removal and surgical stress; days 2-4 following surgery when weight stabilizes; and the final three days (days 5-7) when growth resumes. Faeces and carcass were dried under vacuum and mechanically ground for subsequent analyses of fat and nitrogen, and determination of carcass water.

4.2.3 Gut measurements

Since an IGF-I responsiveness in the gut was seen in Experiment 1 (see section *3.3.6*) an effort was made to determine more precisely the nature and origin of this effect. Consequently, after weighing the duodenum, a 1cm segment located 1cm distal to the pylorus was taken for histology. This segment was opened longitudinally by cutting along the mesenteric border, placed serosal side down onto filter paper and fixed in Bouin's fixative. In addition, after weighing the jejunum, ileum and colon, the mucosa and submucosa of these segments were scraped from the muscularis externa layers using a glass slide. The mucosal+submucosal scrapings were weighed and stored frozen in 1ml/g tissue of 10mM sodium phosphate buffer containing 0.002% Triton X -100 (pH 6.1). The muscularis layers were weighed and frozen in 1.5ml/g tissue of 2M NaOH. Both the mucosal+submucosal and muscularis samples were kept at -80°C until required for biochemical analyses. All other procedures at sacrifice were as described in section *2.2.1*. The experimental procedure was co-ordinated by myself, with technical assistance required for the collection and weighing of samples at the kill.

Prior to analysis, the mucosal+submucosal scrapings were thawed, homogenized at 4°C for 20sec at 15000*g* using an ultraturex, combined with an equivalent volume of 1M NaOH, and digested at 4°C for 48h. The muscularis scrapings in 2M NaOH were thawed, homogenized and digested as described above.

The protein content of homogenates was measured by Dulley and Grieve's modification of the Lowry method (Dulley and Grieve, 1975). DNA was measured by the method of Burton (Burton, 1956) in samples of homogenate extracted with 0.3M perchloric acid and finally dissolved in 0.5M perchloric acid.

Sucrase activity was determined in the jejunal mucosa plus submucosa by a modification of the Dahlqvist method (Dahlqvist, 1968). Briefly, this method measures the sucrase activity of a sample by a colorimetric estimation of the rate of glucose liberation from sucrose using a Tris-buffered glucose oxidase reagent. In a preliminary series of experiments, I determined the optimal sample treatments, incubation times and substrate concentrations for assaying sucrase in the mucosal+submucosal scrapings of gut-resected rats. These experiments revealed that sucrase could be measured quantitatively provided the incubation time did not exceed 60min, and the samples had not been previously thawed. The conditions finally adopted for assaying sucrase activity in mucosal+submucosal scrapings from Experiments 2 and 3 involved incubating the sample with an equivalent volume of 0.2M sucrose for 30min at 37°C, before adding a Tris-buffered glucose oxidase reagent (Dahlqvist, 1968) to achieve a final sample concentration of 0.17M, and incubating for a further 30min at 37°C.

The methods for measurement of protein, DNA and sucrase were adapted to microplates, and optical density was determined in a Titertek Multiscan MCC microplate reader (Flow Laboratories, North Ryde, Australia). Optimal conditions for the protein and DNA asssays had been determined previously by others in the laboratory. The assay protocols for protein, DNA and sucrase determination appear in appendices 4.1, 4.2 and 4.3.

On the basis of the exclusion criteria described in Section *2.2.6*, 11 of 86 resected rats were excluded. The excluded animals were evenly distributed amongst the groups, and treatment did not appear to be a causative factor for death or exclusion.

4.2.4 Analytical measurements

The nitrogen content of food, faeces and dried carcass was measured by the Dumas procedure using a Carlo Erba NA 1500 Nitrogen Analyser (Milan, Italy) as described in section *3.2.3*. In determining carcass nitrogen, a weighed sample of approximately 5mg of dried, powdered carcass was sealed in a tin container and loaded into the autosampler, with acetanilide (10.36% nitrogen) as the standard. The fat content of food, faeces and carcass was determined gravimetrically following chloroform/methanol extraction of the dried samples. Briefly, 0.5g of sample was added to chloroform:methanol (1:1 vol/vol) and agitated for 3h. After being centrifuged for 10min at 800*g*, the solvent was removed by filtration. Chloroform:methanol was then added to the pellet and the process repeated. The combined filtrates were dried overnight by a Savant Speed Vac Concentrator system allowing the weight of the fat residue in the preweighed tube to be determined. Carcass water was calculated from the difference between the wet and dry carcass weights.

Urinary 3-methylhistidine was determined by an automated method (Murray et al., 1981) after hydrolysis and ion-exchange chromatography steps (Tomas et al., 1984) as outlined in section *3.2.3*. Urinary creatinine was measured using the Skalar continuous flow analyser method no. 07907001 (Skalar Analytical, Breda, Netherlands).

Measurement of 3-methylhistidine, creatinine and carcass composition were performed by Mrs. J. Burgoyne, Mrs. S. Madden, Ms. A. Collins and Mr. I. Skene under the direction of Dr. F. Tomas at the CSIRO, Division of Human Nutrition, Adelaide, South Australia.

4.2.5 Histology

The duodenal samples taken for histological analysis were removed from Bouin's fixative after 18h, dehydrated by passage through graded alcohols, and cleared

in chloroform, before embedding in paraffin wax in transverse orientation. From each sample, 4μm sections were cut at four levels, each separated by 100μm. Sections were then stained with haematoxylin and eosin and examined with a light microscope. Quantitative analyses were conducted on images acquired with a color video camera, and digitized by a PRISM Image Analysis Software System (Dapple Systems Inc., Sunnyvale, CA., USA) coupled to an Apple Macintosh IICX computer. Duodenal villus height and crypt depth for each animal were determined from the mean of measurements made from ten well-orientated villi and crypts, at each of three levels. Mucosal circumference was taken as the maximum length of the muscularis mucosae measured from four sections. Mucosal, submucosal and muscularis externa thickness were calcuated by measuring two areas of each zone at each of four levels, dividing the area measurements by the appropriate muscularis mucosae length, and then obtaining a mean of these measures. Cross-sectional area was defined as the product of thickness and circumference.

The sectioning and staining of samples was carried out by Mrs. Kathryn Davey of the Child Health Research Institute, North Adelaide, South Australia. The histological analysis was performed by Mr. G. Howarth, also of the Child Health Research Institute.

4.2.6 Plasma IGF-I radioimmunoassays

IGF-I was measured in acid-ethanol extracts of plasma collected prior to surgery (day 0), on day 4 of treatment, and at kill (day 7) in the manner described in section *3.2.4*. With the polyclonal antiserum used, cross-reactions of 70%, 70% and 10% were obtained for rat IGF-I, des(1-3)IGF-I and LR^3IGF-I, respectively, relative to human IGF-I (Dr. P. C. Owens, personal communication).

Acid-ethanol extraction and IGF-I RIAs were performed by Ms. M. Pearce under the supervision of Dr. P. C. Owens, CSIRO Division of Human Nutrition, Adelaide, South Australia.

4.2.7 Western ligand blot analysis

The presence of IGFBPs in plasma was determined by Western ligand blot analysis. The plasma collected at kill, after being extracted with Freon as detailed in section *3.2.4*, was pooled according to treatment group. Additionally, the Freon-extracted pretreatment plasma of all animals was pooled. A 10µl sample from each of the extracted plasma pools was incubated with 50µl of concentrated sodium dodecylsulphate (SDS)-loading buffer at 65°C for 15min before 20µl of this mixture was loaded onto a 1.5mm thick, discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% separating gel atop a 4% stacking gel (Laemmli, 1970). Running in lanes either side of the samples were ^{14}C-labelled Rainbow markers (Amersham International, Amersham, Bucks., UK) which had been incubated, as above, with loading buffer. Following electrophoresis at 10mA for 20h, proteins were transferred onto nitrocellulose sheets (0.45µ; Schleicher and Schuell, Dassel, Germany) at 300mA for 3h by a Hoefer Transphor TE 42 apparatus (Hoefer Scientific, San Fransisco, CA., USA). After being dried overnight, the nitrocellulose sheets were washed in 500ml of buffer (35ml 2M NaCl, 10ml 0.5M Tris at pH 7.4) containing 1% Triton X-100 (v/v) for 30min, in buffer with 1% bovine serum albumin (w/v) for 90min, and then buffer containing 0.1% Tween 20 (v/v) for 10min. The washed nitrocellulose was then incubated for 2h with 1% bovine serum albumin buffer containing 0.1% Tween 20 and ^{125}IGF-I (5.5×10^6 cpm). Immediately following the 24h incubation with radiolabelled IGF-I, the sheets were washed every 20min for 2h with fresh 0.1% Tween 20 buffer. All washing and probing steps were conducted in a shaking water-bath. Once the sheets were dry they were exposed to X-ray film (A8323; Konica, Tokyo, Japan) and stored at -70^{O}C. Development of the film occurred 15 days later.

4.2.8 Statistical analysis

Means and SEM are reported for all measures. The treatment groups were initially compared by a one-way analysis of variance (ANOVA). When significance ($p<0.05$) was attained, Fisher's Protected Least Significant Difference *post hoc* was used to identify between-group differences. Statistical analysis was performed using the SuperANOVA (Abacus Concepts, CA., USA) program.

4.3 RESULTS

4.3.1 Plasma IGF-I concentrations

IGF-I concentrations in plasma collected immediately before resection and pump implantation (day 0), on day 4 following surgery, and at the end of treatment (day 7) were determined by RIA following acid-ethanol extraction (Table 4.1). Prior to gut resection and growth factor treatment, there were no differences between the groups in the level of circulating IGF-I. As in Experiment 1, plasma IGF-I levels in the vehicle-treated animals fell following resection (day 4), and thereafter recovered slightly, although in this experiment they remained below the pretreatment level (day 7). Infusion of IGF-I progressively increased plasma levels in a dose-responsive manner, so that by day 7, plasma concentrations in the animals receiving the higher doses of IGF-I were more than double that of the controls ($p< 0.001$), and nearly 90% higher than the respective pretreatment values. Despite the fact that a higher dose of des(1-3)IGF-I was infused in this experiment, administration of des(1-3)IGF-I, as in the experiment 1, failed to raise measured IGF-I levels above those of the vehicle-treated rats on either day 4 or day 7. For the animals receiving LR^3IGF-I, measured plasma IGF-I concentrations were inversely related to dose, with the RIA-detectable IGF-I circulating in the rats treated with the highest dose of LR^3IGF-I being about 50% less than the controls.

TABLE 4.1 Plasma IGF-I concentrations immediately before (Day 0), and on the 4th and 7th days after, 80% jejuno-ileal resection and IGF-I treatment.

Treatment group	Plasma IGF-I µg/l Acid Ethanol Extraction		
	day 0 (pretreatment)	day 4	days 7
Vehicle	402±40	290±23	312±41
IGF-I (0.7mg)	462±40	406±34[a]	546±27[c]
IGF-I (1.8 mg)	376±22	499±43[c]	638±36[c]
IGF-I (4.5 mg)	387±27	477±60[c]	728±90[c]
des(1-3)IGF-I (0.3 mg)	427±22	225±22	345±44
des(1-3)IGF-I (0.7 mg)	402±20	265±32	318±22
des(1-3)IGF-I (1.8 mg)	339±28	249±35	339±46
LR^3IGF-I (0.3 mg)	375±31	236±29	268±37
LR^3IGF-I (0.7 mg)	395±30	134±30[b]	244±35
LR^3IGF-I (1.8 mg)	401±31	112±19[c]	161±20[a]

Values are means ± SEM for 7-8 animals in each group: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs vehicle-treated rats.
Doses are per kg body weight per day.

In interpreting the IGF-I levels presented in Table 4.1, the relative cross-reactivities of the RIA need to be considered. Since rat IGF-I is only recognised 70% as well as human IGF-I by the assay, the actual IGF-I levels in the vehicle group are closer to 570µg/l (day 0), 410µg/l (day 4) and 450µg/l (day 7) (data not shown). The same adjustment can be made for the groups receiving des(1-3)IGF-I, as des(1-3)IGF-I and rat IGF-I (the two IGF-I forms in these animals) have the same cross-reactivity. Recalculating the data in this manner confirms that administration of des(1-3)IGF-I failed to raise IGF-I levels above those described for the vehicle-treated rats. Similar calculations cannot be made for the animals receiving LR^3IGF-I, since cross-reactivity for LR^3IGF-I (10% compared to human IGF-I) and endogenous rat IGF-I (70%) are markedly different, and the relative amounts of these peptides in the animal's circulation

were unknown. Nevertheless, it is clear that administration of LR^{3}IGF-I induced a marked suppresion of measured IGF-I. Since the relative amounts of infused peptide and rat IGF-I could not be determined in the animals infused with human IGF-I, a precise estimation of total plasma IGF-I levels in these rats (which carried a mixture of human IGF-I and rat IGF-I) was not possible either. However, even after consideration of the relative cross-reactivities, the IGF-I-treated groups still had higher plasma IGF-I concentrations than the other groups.

4.3.2 Effect of treatment on IGFBPs

To determine any change in the levels of specific forms of IGFBPs following resection and IGF treatment, the binding proteins in day 7 rat plasma were quantified by Western ligand blot analysis (Fig. 4.1). By this analysis, bands were detected that correspond with the molecular weights of four classes of binding proteins: a double band eluting at 42-48 kDa, another doublet appearing at about 28-32 kDa, and a 24-25 kDa band. The apparent intensities of these bands, when compared to the pre-resection plasma pool, were clearly diminished in the plasma of the vehicle-treated animals. Treatment with des(1-3)IGF-I or the low dose of LR^{3}IGF-I (0.3 mg/kg bw/d) appeared to restore the intensity of the 42-48kDa and 28-32kDa bands, whereas LR^{3}IGF-I at the intermediate (0.7 mg/kg bw/d) or high (1.8 mg/kg bw/d) dose only partially restored these bands, relative to the pretreatment pool. On the other hand, IGF-I at all doses administered markedly elevated the intensities of the 42-48kDa and 28-32kDa bands. The 24-25kDa band appeared to be incompletely restored to the pretreatment level by peptide therapy.

4.3.3 Body weight changes

As demonstrated in Experiment 1 (see Section *3.3.2*), treatment with IGF-I peptides improved weight gain following resection of 80% of the jejuno-ileum (Table 4.2). Also evident were the characteristic growth responses of the vehicle-treated rats, with body weight stable over days 2-4 following surgery, and normal growth

Figure 4.1 Western ligand blot analysis of IGFBPs in plasma from gut-resected rats treated with IGF peptides

Plasma pooled from Experiment 2 rats were incubated in SDS-loading buffer, electrophoresed, transferred to nitrocellulose and probed with ^{125}I-labelled insulin-like growth factor-I (^{125}I-IGF-I). Lanes 1 & 14, radio-labelled molecular mass markers; lane 2, pooled plasma (3.33µl) from rats immediately prior to 80% jejuno-ileal resection and peptide treatment (i.e. day 0, n=84); lane 3, day 7 plasma (3.33µl) from vehicle-treated gut-resected rats (n=8); lanes 4,5 & 6, day 7 plasma (3.33µl) from gut-resected rats (n=8 in each group) infused with 0.7 (medium=m), 1.8 (high=h) and 4.5 (very high=v.h) mg IGF-I/kg bwt/d, respectively; lanes 7, 8 & 9, day 7 plasma (3.33µl) from gut-resected rats (n=7, 7 & 8, respectively) infused with 0.3 (low=l), 0.7 (m) and 1.8 (h) mg des(1-3)IGF-I/kg bwt/d, respectively; lanes 10, 11 & 12, day 7 plasma (3.33µl) from gut-resected rats (n=7, 8 & 8, respectively) infused with 0.3 (l), 0.7 (m) and 1.8 (h) mg LR^{3}IGF-I, respectively; lane 13, day 7 plasma (3.33µl) from gut-resected rats (n=7) infused with 0.2mg/kg bwt/d of epidermal growth factor (EGF) (from a separate experiment which was not a part of this study). The autoradiograph was exposed for 15 days.

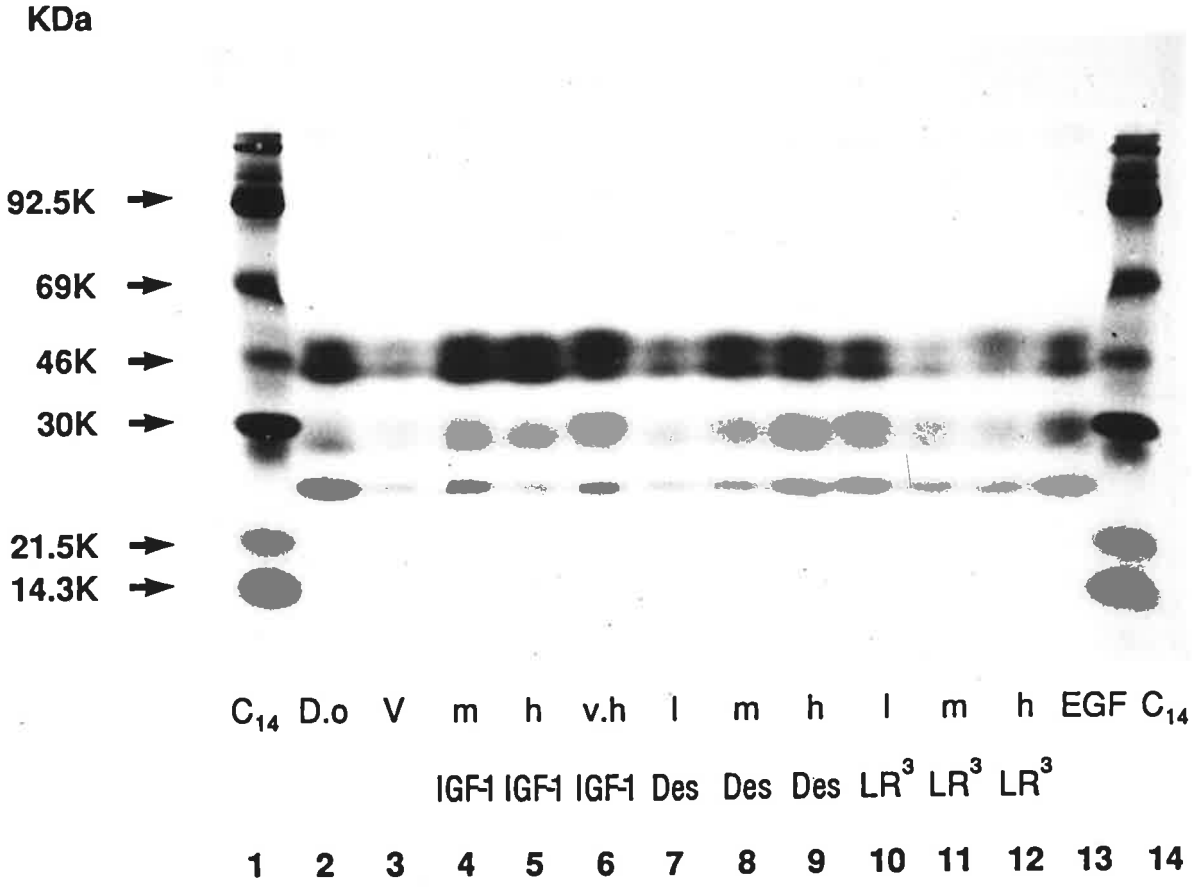
KDa
92.5K
69K
46K
30K
21.5K
14.3K
C14 D.o V m h v.h l m h l m h EGF C14
IGF-1 IGF-1 IGF-1 Des Des Des LR3 LR3 LR3
1 2 3 4 5 6 7 8 9 10 11 12 13 14

TABLE 4.2 Body weight changes (g/d) before and after 80% jejuno-ileal resection 7 days treatment with IGF-I peptides.

Treatment group	pretreatment	day 1	days 2-4	days 5-7
Vehicle	8.0±0.4	-13.2±0.9	0.9±0.7	4.3±0.8
IGF-I (0.7mg)	7.7±0.3	-12.5±1.1	1.2±0.8	5.4±0.4
IGF-I (1.8 mg)	7.0±0.4	-12.8±0.6	2.2±0.6	5.0±0.7
IGF-I (4.5 mg)	7.9±0.3	-12.9±0.4	1.5±1.0	6.2±0.7
des(1-3)IGF-I (0.3 mg)	8.4±0.3	-14.2±0.6	1.1±1.0	5.7±0.5
des(1-3)IGF-I (0.7 mg)	8.2±0.6	-14.1±0.9	1.5±0.5	4.5±0.6
des(1-3)IGF-I (1.8 mg)	7.8±0.3	-12.6±0.5	3.1±0.4	5.8±0.5
LR^3IGF-I (0.3 mg)	7.9±0.3	-11.8±0.7	2.1±0.5	5.7±0.6
LR^3IGF-I (0.7 mg)	8.4±0.5	-11.9±1.5	2.3±0.8	5.7±0.4
LR^3IGF-I (1.8 mg)	7.4±0.6	-11.4±0.9	2.4±0.8	6.3±0.4

Values are means ± SEM for 7-8 rats in each group.

Doses are per kg body weight per day.

incompletely restored during days 5-7 (Table 4.2).

Pretreatment growth for all groups averaged about 8g/d. In the 24h following removal of 80% of the jejuno-ileum the control group lost 13.2±0.9g (mean±SEM). Similar losses occurred in the groups treated with IGF-I or des(1-3)IGF-I. Although less weight (range: 11.4-11.9g) was lost by the groups receiving LR^3IGF-I treatment relative to the controls, the difference was not significant. Over the entire treatment period, IGF-I peptides stimulated body weight gain in a dose-responsive manner, with significant increases observed for the animals receiving IGF-I at 4.5 mg/kg bw/d, des(1-3)IGF-I at 1.8 mg/kg bw/d, and all of the LR^3IGF-I doses (0.3, 0.7, 1.8 mg/kg bw/d) (Fig. 4.2). Des(1-3)IGF-I at the two lower doses had no impact on body growth, but at the highest dose was as effective as LR^3IGF-I.

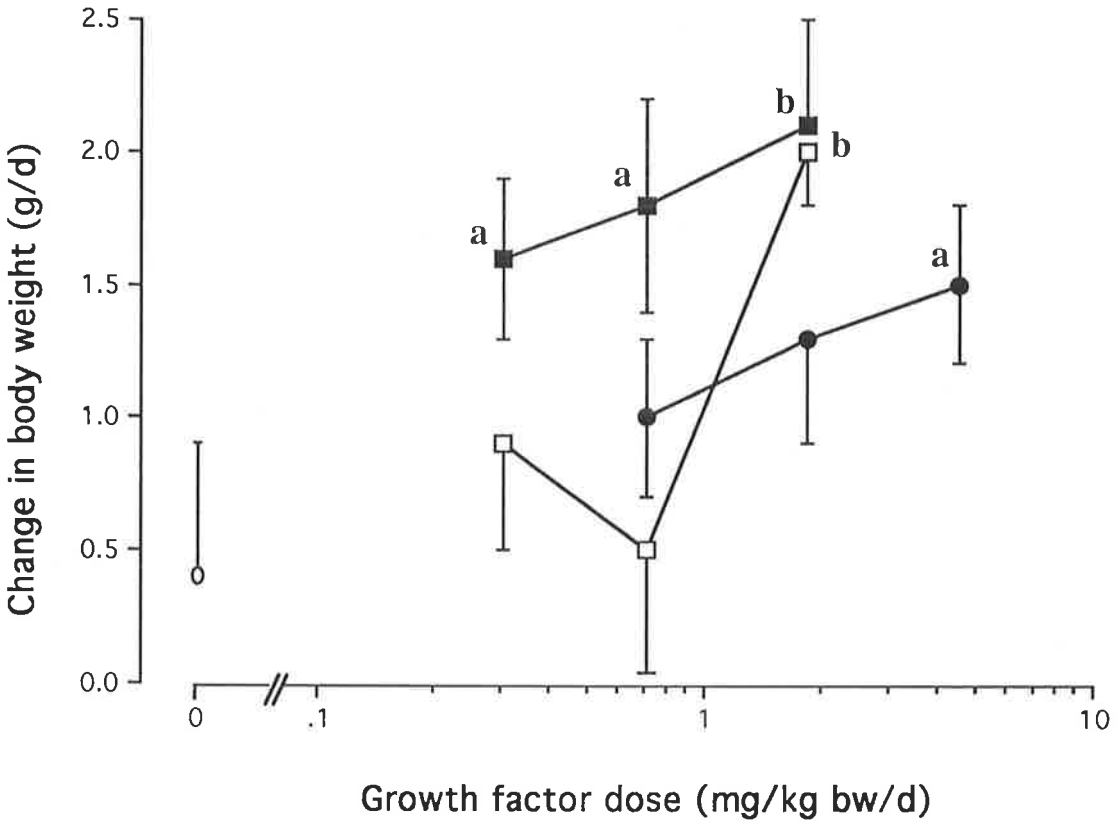

Fig. 4.2 Dose response curves for body weight change over 7 days following removal of 80% of the jejuno-ileum. Treatment groups were vehicle (open circles), IGF-I (closed circles), des(1-3)IGF-I (open squares), and LR3IGF-I (closed squares). Values are means±SEM for 7-8 rats in each group: **a** $p< 0.05$, **b** $p< 0.01$, **c** $p< 0.001$ versus the vehicle-treated animals.

As demonstrated previously (see sections *2.3.2* and *3.3.2*) peptide treatment had no effect on food consumption (Table 4.3). Food conversion efficiency was calculated according to the method outlined in section *2.2.2*. As food consumption was unaffected by peptide treatment , the effects on body weight were also apparent in the food conversion efficiency results (Table 4.4). For the combined period (days 2-7), the groups receiving 4.5mg IGF-I/kg bw/d, 1.8mg des(1-3)IGF-I/kg bw/d, and all three doses of LR^3IGF-I had significantly better food conversion efficiencies than the controls. Each of the other treated groups tended to have improved efficiencies relative to the controls. By days 5-7, the controls were the only group not to have retained their pretreatment level of food conversion efficiency.

TABLE 4.3 Food intake (g/d) before and after 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.

Treatment group	pretreatment	day 1	days 2-4	days 5-7	days 2-7
Vehicle	18.0±0.5	1.4±0.5	8.1±0.7	12.6±0.9	9.1±0.6
IGF-I (0.7mg)	17.7±0.3	0.6±0.3	7.4±0.9	13.4±0.5	9.0±0.6
IGF-I (1.8 mg)	17.2±0.6	0.5±0.1	8.1±0.4	12.7±1.0	9.0±0.5
IGF-I (4.5 mg)	18.0±0.5	1.2±0.2	7.5±0.7	12.9±0.6	8.9±0.6
des(1-3)IGF-I (0.3 mg)	18.3±0.5	0.8±0.2	7.8±0.5	13.7±0.7	9.3±0.5
des(1-3)IGF-I (0.7 mg)	17.8±0.7	0.2±0.1	8.3±0.6	12.8±0.6	9.1±0.5
des(1-3)IGF-I (1.8 mg)	17.7±0.3	0.8±0.2	7.9±0.6	13.2±0.8	9.2±0.4
LR^3IGF-I (0.3 mg)	18.0±1.0	1.3±0.2	9.0±0.3	13.3±0.4	9.7±0.4
LR^3IGF-I (0.7 mg)	18.0±0.6	0.5±0.1	8.4±0.8	13.1±0.8	9.3±0.8
LR^3IGF-I (1.8 m)	17.8±0.8	0.8±0.2	7.3±0.6	13.1±0.3	8.9±0.5

Values are means ± SEM for 7-8 rats in each group.

Doses are per kg body weight per day.

TABLE 4.4 Food conversion efficiency (g body weight gain/g food intake) before and after 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.

Treatment group	pretreatment	days 2-4	days 5-7	days 2-7
Vehicle	0.442±0.018	0.083±0.072	0.327±0.041	0.245±0.037
IGF-I (0.7mg)	0.437±0.016	0.080±0.101	0.408±0.040	0.314±0.023
IGF-I (1.8 mg)	0.404±0.019	0.260±0.065	0.385±0.038	0.343±0.036
IGF-I (4.5 mg)	0.440±0.007	0.090±0.174	0.481±0.056	0.373±0.023[a]
des(1-3)IGF-I (0.3 mg)	0.459±0.009	0.112±0.125	0.413±0.025	0.314±0.037
des(1-3)IGF-I (0.7 mg)	0.469±0.032	0.216±0.036	0.378±0.022	0.316±0.025
des(1-3)IGF-I (1.8 mg)	0.438±0.012	0.394±0.052	0.437±0.021	0.423±0.019[a]
LR^3IGF-I (0.3 mg)	0.443±0.020	0.231±0.058	0.424±0.039	0.349±0.031[a]
LR^3IGF-I (0.7 mg)	0.467±0.015	0.226±0.096	0.440±0.026	0.365±0.034[a]
LR^3IGF-I (1.8 mg)	0.413±0.015	0.286±0.121	0.475±0.022	0.420±0.032[a]

Values are means ± SEM for 7-8 rats in each group: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs vehicle-treated rats.

Doses are per kg body weight per day.

4.3.4 Nitrogen balance measures

As in Experiment 1, nitrogen balance was measured to determine if body weight gain reflected protein accretion (Table 4.5). After grouping all animals together, the overall pretreatment levels for nitrogen intake, excretion and balance were (mean±SEM) 470.3±15.6, 183.7±9.7, and 286.6±13.6mg/d, respectively. The effect of resection on nitrogen intake, excretion and balance in the vehicle-treated animals closely adhered to the patterns observed in Experiment 1. Administration of IGF peptides had no effect on nitrogen intake, but, in a generally dose-responsive manner, reduced nitrogen excretion (Fig 4.3(a)(b)). The enhanced potencies of the analogues, des(1-3)IGF-I and LR^3IGF-I, relative to IGF-I in attenuating nitrogen excretion are evident in this experiment, as they were for des(1-3)IGF-I in Experiment 1, with the animals receiving 1.8mg/kg bw/d of either des(1-3)IGF-I or LR^3IGF-I excreting less nitrogen over the 7 day treatment period than the rats receiving a 2.4 fold higher dose of IGF-I (Fig 4.3(b)).

Nitrogen balance, which was calculated from the difference between intake and excretion, was more positive for all the IGF-treated groups in each of the treatment periods investigated than the vehicle-treated controls (Table 4.5). Due to large variability, however, these differences were not significant. The trend to improved nitrogen retention with IGF treatment was accounted for by significant reductions in the nitrogen excreted by most of the treated groups (Table 4.5). The increased potency of the analogues is again apparent with the groups treated with 1.8mg des(1-3)IGF-I/kg bw/d or LR^3IGF-I at any dose (0.3, 0.7 and 1.8mg/kg bw/d) all having numerically superior nitrogen balances for the entire treatment period compared to the group receiving 4.5mg/kg bw/d of IGF-I (Fig 4.3(c)).

TABLE 4.5 Nitrogen balance measures before and after 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.

Treatment group	Nitrogen (mg/d) day 1	days 2-4	days 5-7
Nitrogen Intake			
Vehicle	36.4±12.1	207.0±20.8	332.5±25.2
IGF-I (1.7 mg	15.3±7.4	196.1±23.4	353.0±12.2
IGF-I (1.8 mg)	11.8±2.5	213.6±10.4	336.2±25.5
IGF-I (4.5 mg)	32.5±5.2	197.5±19.7	339.5±15.0
des(1-3)IGF-I (0.3 mg)	24.4±5.5	195.5±13.1	345.4±27.0
des(1-3)IGF-I (0.7 mg)	6.4±2.4	219.2±15.2	337.6±19.7
des(1-3)IGF-I (1.8 mg)	20.2±6.4	209.3±16.1	348.2±21.6
LR^3IGF-I (0.3 mg)	33.6±5.7	237.2±7.9	349.4±9.9
LR^3IGF-I (0.7 mg)	13.6±3.8	221.2±20.2	345.8±20.9
LR^3IGF-I (1.8 mg)	20.8±4.3	193.2±17.3	346.5±8.5
Nitrogen Excreted			
Vehicle	122.4±8.5	133.2±9.2	168.5±9.3
IGF-I (0.7 mg)	92.3±7.9	109.8±9.2	163.0±5.4
IGF-I (1.8 mg)	123.5±8.1	98.5±6.4^b	143.5±11.9^a
IGF-I (4.5 mg)	118.6±7.2	81.4±7.6^c	136.2±8.4^a
des(1-3)IGF-I (0.3 mg)	118.9±4.7	101.9±4.2^a	165.3±8.6
des(1-3)IGF-I (0.7 mg)	126.2±8.3	113.3±9.9	143.6±8.4
des(1-3)IGF-I (1.8 mg)	109.8±7.3	81.6±9.4^c	129.5±10.5^b
LR^3IGF-I (0.3 mg)	116.4±7.9	118.3±8.9	156.3±6.4
LR^3IGF-I (0.7 mg)	123.7±6.2	94.3±9.2^b	133.6±10.5^b
LR^3IGF-I (1.8 mg)	112.9±7.1	86.8±10.2^c	129.7±5.3^b
Nitrogen Balance			
Vehicle	-86.0±10.0	86.4±18.2	164.0±28.7
IGF-I (0.7 mg)	-75.1±10.1	103.3±23.7	190.0±10.2
IGF-I (1.8 mg)	-111.7±8.1	113.8±15.1	192.6±18.3
IGF-I (4.5 mg)	-86.1±7.3	111.7±180	203.3±13.9
des(1-3)IGF-I (0.3 mg)	-94.5±6.1	101.1±14.8	180.0±23.4
des(1-3)IGF-I (0.7 mg)	-119.8±10.3	106.9±8.0	194.0±12.2
des(1-3)IGF-I (1.8 mg)	-89.6±11.1	125.7±17.7	218.6±17.0
LR^3IGF-I (0.3 mg)	-82.9±9.1	128.3±8.3	193.1±10.8
LR^3IGF-I (0.7 mg)	-110.1±7.5	122.6±14.3	212.2±18.1
LR^3IGF-I (1.8 mg)	-92.2±10.1	104.3±22.9	216.8±8.3

Values are means ± SEM for 7-8 rats in each group: ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$ vs vehicle-treated controls.
Doses are per kg body weight per day.

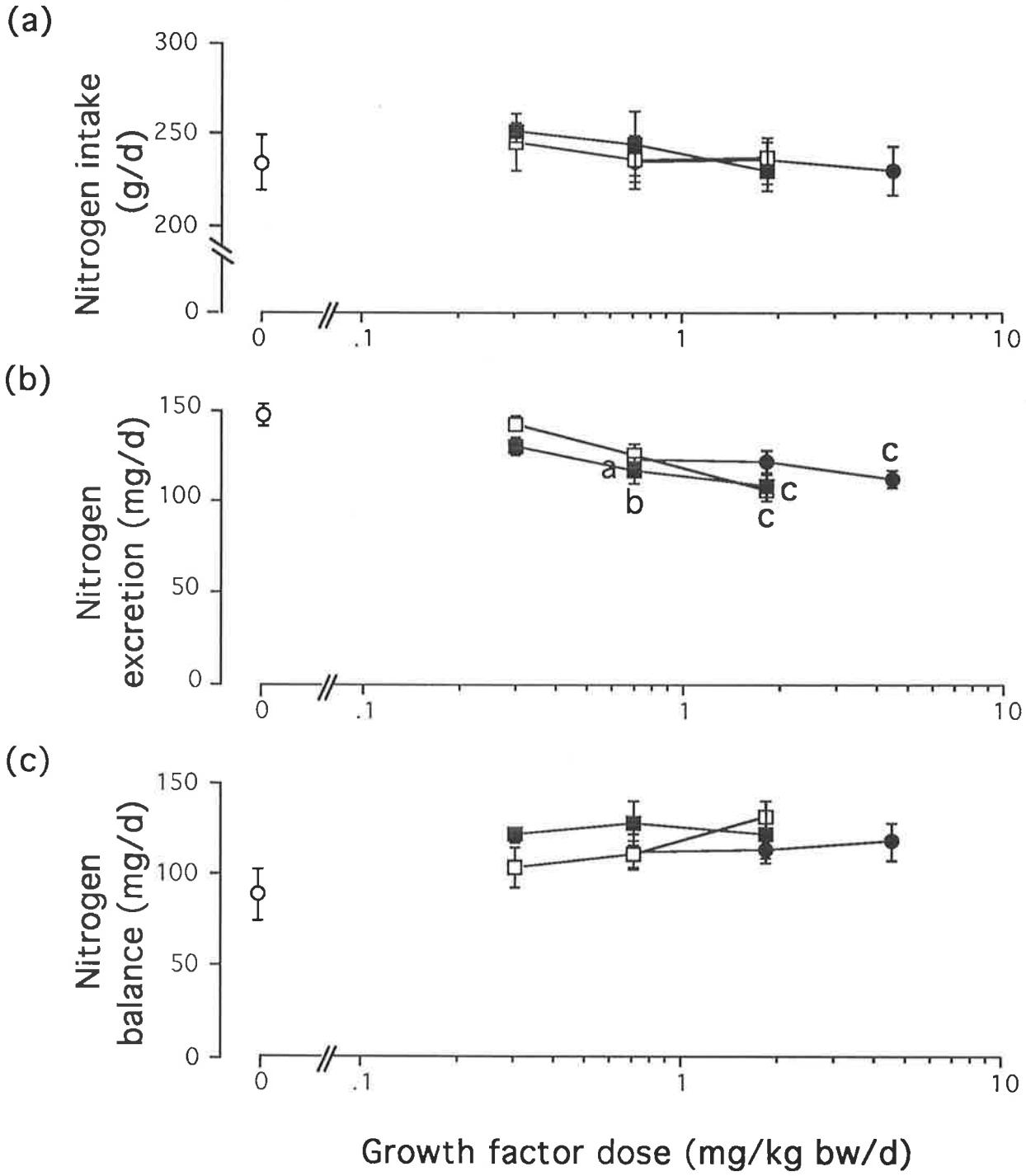

Fig. 4.3 Dose response curves for average nitrogen (a) intake, (b) excretion, and (c) balance over 7 days following removal of 80% of the jejuno-ileum. Treatment groups were vehicle (open circles), IGF-I (closed circles), des(1-3)IGF-I (open squares), and LR^3IGF-I (closed squares). Values are means±SEM for 7-8 rats in each group: **a** $p< 0.05$, **b** $p< 0.01$, **c** $p< 0.001$ versus the vehicle-treated animals.

4.3.5 Muscle protein breakdown

To further clarify the improvements in body weight gain and nitrogen balance brought about by IGF-I peptide administration, muscle protein breakdown was measured by determining the urinary 3-methylhistidine (3-MH) excretion. As in Experiment 1, the urinary creatinine excretion rates were unaffected by peptide treatment (data not shown). The urinary 3-MH excretion (Table 4.6) of the control rats during the first four post-surgical days was significantly increased relative to the pretreatment rate. By days 5-7, the 3-MH excretion rate of these animals had returned to the pretreatment level, again demonstrating the transient nature of the stress associated with removal of 80% of the jejuno-ileum. Throughout the treatment period, all the IGF-treated groups tended to excrete less 3-MH than the controls, albeit these

TABLE 4.6 Urinary 3-methylhistidine (3-MH) excretion rates before and after 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.

Treatment groups	pretreatment	day 1	days 2-4	days 5-7	days 1-7
Urinary 3-MH excretion (μmol/kg bw/d)					
Vehicle	7.9±0.3	9.6±0.7↑	9.9±0.5↑	6.7±1.1	8.5±0.5
IGF-I (0.7 mg)	7.4±0.2	7.7±0.7	8.2±0.7	6.0±0.3*	7.2±0.3
IGF-I (1.8 mg)	7.8±0.3	9.2±0.6	8.5±0.7	6.2±0.3*	7.6±0.4
IGF-I (4.5 mg)	7.2±0.3	8.6±0.5	8.4±0.5	6.5±0.3	7.6±0.3
des(1-3)IGF-I (0.3 mg)	7.0±0.2	8.5±0.3↑	8.4±0.4	6.0±0.3*	7.4±0.2
des(1-3)IGF-I (0.7 mg)	7.3±0.3	9.9±0.5↑	7.1±0.6	5.6±0.3*	8.0±0.4
des(1-3)IGF-I (1.8 mg)	7.2±0.3	8.6±0.6	8.4±0.8	5.8±0.3*	7.3±0.4
LR^3IGF-I (0.3 mg)	7.6±0.3	9.0±0.5	8.5±0.7	6.2±0.3*	7.6±0.5
LR^3IGF-I (0.7 mg)	7.7±0.4	8.6±0.6	9.0±0.4	6.1±0.3*	7.7±0.3
LR^3IGF-I (1.8 mg)	7.6±0.2	7.8±0.7	9.5±1.0	6.3±0.3*	7.9±0.4

Values are means ± SEM for 7-8 rats in each group: ↑ 3-MH excretion significantly increased ($p < 0.05$) vs respective pretreatment value; * 3-MH excretion significantly decreased ($p < 0.05$) vs. respective pretreatment value.

Doses are per kg body weight per day.

differences failed to achieve significance. In contrast to the controls, all the peptide-treated animals, except for those treated with IGF-I at the highest dose (4.5mg/kg bw/d), had significantly reduced 3-MH excretion over days 5-7 relative to their respective pretreatment levels.

4.3.6 Organ effects

Administration of IGF-I peptides, as in Experiment 1, had no effect on the relative carcass or pelt weights (Table 4.7). To further clarify the nature of body weight gain following IGF-I peptide administration, carcass composition analysis was performed. As there were no differences in carcass water, protein or fat between the groups (Table 4.8), it appears that the effect of IGF peptides to increase body weight, as suggested in Chapter 3, is due to proportional growth.

TABLE 4.7 Relative carcass and pelt weights of rats following 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.

Treatment group	Carcass (g/kg bw)	Pelt (g/kg bw)
Vehicle	401.4±9.1	141.7±2.2
IGF-I (0.7 mg/kg bw/d)	401.8±7.6	142.7±4.2
IGF-I (1.8 mg/kg bw/d)	400.9±8.1	138.6±2.6
IGF-I (4.5 mg/kd bw/d)	400.7±4.4	140.9±3.7
des(1-3)IGF-I (0.3 mg/kg bw/d)	407.3±5.8	139.1±3.7
des(1-3)IGF-I (0.7 mg/kg bw/d)	404.8±7.2	140.5±4.7
des(1-3)IGF-I (1.8 mg/kg bw/d)	404.0±6.6	141.4±3.6
LR^3IGF-I (0.3 mg/kg bw/d)	403.2±7.9	141.7±2.7
LR^3IGF-I (0.7 mg/kg bw/d)	396.1±7.7	137.4±2.8
LR^3IGF-I (1.8 mg/kg bw/d)	388.1±6.9	145.2±3.2

Values are means ± SEM for 7-8 rats in each group.

TABLE 4.8 Carcass composition of rats following 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.

	Percentage of carcass as			
Treatment groups	Water	Protein	Fat	Residue
Vehicle	73.14±0.25	19.73±0.18	3.47±0.39	3.67±0.21
IGF-I (0.7 mg/kg bw/d)	72.86±0.24	19.74±0.13	3.68±0.37	3.73±0.13
IGF-I (1.8 mg/kg bw/d)	72.87±0.29	19.58±0.22	3.96±0.52	3.58±0.30
IGF-I (4.5 mg/kg bw/d)	73.23±0.33	19.79±0.36	3.02±0.34	3.44±0.13
des(1-3)IGF-I (0.3 mg)	72.42±0.28	19.59±0.21	4.24±0.40	3.75±0.14
des(1-3)IGF-I (0.7 mg)	72.53±0.52	19.83±0.26	3.44±0.40	4.20±0.22
des(1-3)IGF-I (1.8 mg)	73.01±0.21	19.55±0.11	3.77±0.36	3.66±0.24
LR^3IGF-I (0.3 mg)	72.95±0.48	19.48±0.25	4.04±0.54	3.53±0.25
LR^3IGF-I (0.7 mg)	73.25±0.21	19.52±0.27	3.35±0.25	3.88±0.23
LR^3IGF-I (1.8 mg)	73.71±0.41	19.46±0.26	3.10±0.34	3.73±0.15

Values are means ± SEM for 7-8 rats in each group.

Doses are per kg body weight per day.

Thymus weights, expressed relative to body weight (g/kg body weight), were increased in the groups administered des(1-3)IGF-I at the highest dose or LR^3IGF-I at any dose, relative to the controls (Fig. 4.4(a)). Whereas the effect of des(1-3)IGF-I appeared to be dose-responsive, the magnitude of response by the thymus to LR^3IGF-I was independent of dose rate with the largest effect observed for the lowest dose. The sensitivity of the thymus to LR^3IGF-I is evident in that the smallest response to LR^3IGF-I was equal to or greater than the effect of the other peptides at any dose. Despite administration of IGF-I at a dose 2.4 times that of the highest dose for the variants, IGF-I did not significantly increase thymus weight. To further emphasise the enhanced potency of the analogues, both des(1-3)IGF-I and LR^3IGF-I produced significantly higher thymus weights than IGF-I at an equivalent dose.

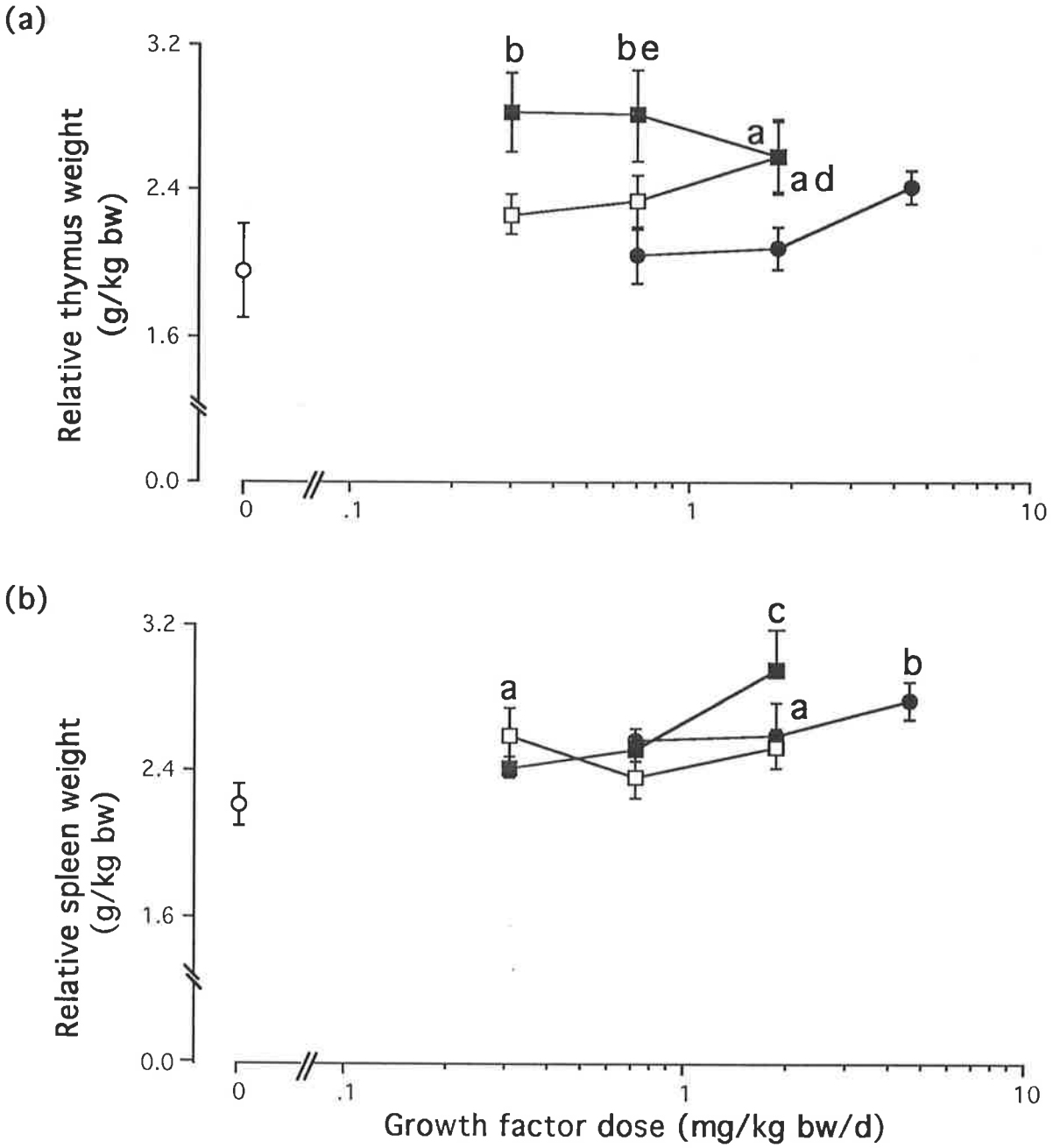

Fig. 4.4 Dose response curves for relative weights (g/kg bw) of the (a) thymus and (b) spleen following removal of 80% of the jejuno-ileum and 7 days treatment with IGF peptides. Treatment groups were vehicle (open circles), IGF-I (closed circles), des(1-3)IGF-I (open squares), and LR^3IGF-I (closed squares). Values are means±SEM for 7-8 rats in each group: **a** $p < 0.05$, **b** $p < 0.01$, **c** $p < 0.001$ versus the vehicle-treated animals, **d** $p < 0.05$, **e** $p < 0.01$ versus IGF-I at the same dose.

Relative spleen weights were also significantly increased by IGF peptide administration (Fig. 4.4(b)). Dose-responsiveness was demonstrated for IGF-I and LR^3IGF-I, but not for des(1-3)IGF-I, and there were no obvious differences in the potencies of the peptides. None of the weights of the other organs examined were affected by peptide treatment (appendix 4.4).

4.3.7 Gut effects

As noted previously (see section *3.3.6*), widespread effects of IGF-I peptide treatment were apparent in the weights of gastro-intestinal organs (Table 4.9(a)). Significant increases in stomach weight, relative to the controls, were observed in each of the peptide treated groups, with the largest increase being 34% in the group receiving 1.8mg LR^3IGF-I/kg bw/d. Increases of up to 28%, 41%, 23% and 21%, respectively, were noted for duodenum, jejunum, colon and total gut weight in the treated groups relative to the controls. No effects were observed in the ileum or caecum. While administration of IGF peptides had no significant effects on small or large bowel lengths, the duodenum, jejunum and ileum tended to be longer in the treated groups (Table 4.9(b)). It is interesting to note that in contrast to the greater potency of des(1-3)IGF-I and LR^3IGF-I compared to IGF-I on body growth, an enhanced potency for the analogues was not apparent in the gut.

Increases in intestinal weight, without significant increments in length suggested that administration of IGF-I peptides to gut-resected rats increased cross-sectional area in the intestine. This possibility was tested by biochemical and histological analyses. The mucosa+submucosa and muscularis externa weights (mg/cm) of the jejunum, ileum and colon give an indication of the source of the increased weight:length ratio (Table 4.10). Increasing doses of IGF-I peptides tended to augment both the mucosa+submucosa and the muscularis layer weights, although none of the changes achieved statistical significance. The increments in the mucosa+submucosa and muscularis layers were proportional, as evidenced by the

TABLE 4.9(a) Gut weights in 80% jejuno-ileal resected rats following 7 days treatment with IGF-I peptides.

Treatment group	Final body weight	Absolute weight (g) Stomach	Duodenum	Jejunum	Ileum	Caecum	Colon	Total gut weight
Vehicle	159.2±3.4	0.81±0.02	1.13±0.05	0.98±0.07	0.92±0.06	0.93±0.06	1.06±0.04	5.82±0.17
IGF-I (0.7mg)	163.6±4.0	0.90±0.02[a]	1.32±0.07	1.35±0.17[a]	1.11±0.07	0.97±0.10	1.15±0.07	6.81±0.32[b]
IGF-I (1.8mg)	165.6±5.0	0.96±0.03[c]	1.44±0.07[b]	1.39±0.12[b]	1.00±0.06	0.90±0.07	1.24±0.07	6.92±0.17[b]
IGF-I (4.5mg)	166.0±4.9	1.06±0.04[c]	1.43±0.07[b]	1.38±0.15[b]	1.00±0.04	0.90±0.08	1.30±0.10	7.07±0.34[c]
des(1-3)IGF-I (0.3mg)	168.3±4.7	0.93±0.03[b]	1.25±0.08	1.05±0.09	1.14±0.11	0.96±0.13	1.05±0.07	6.37±0.32
des(1-3)IGF-I (0.7mg)	163.9±5.1	0.96±0.02[b]	1.32±0.07	1.08±0.04	0.92±0.05	0.86±0.07	1.18±0.06	6.32±0.06
des(1-3)IGF-I (1.8mg)	169.4±4.2	1.09±0.02[c]	1.38±0.10[a]	1.21±0.09	1.03±0.07	0.78±0.05	1.27±0.08	6.59±0.25[a]
LR^3IGF-I (0.3mg)	166.0±8.6	0.91±0.03[a]	1.25±0.06	1.11±0.05	0.91±0.07	0.92±0.09	1.07±0.10	6.16±0.24
LR^3IGF-I (0.7mg)	167.8±5.0	1.02±0.04[c]	1.40±0.10[b]	1.18±0.06	0.92±0.07	0.99±0.05	1.17±0.11	6.71±0.29[a]
LR^3IGF-I (1.8mg)	172.1±4.0	1.09±0.05[c]	1.33±0.03[a]	1.31±0.12[a]	1.04±0.06	0.93±0.07	1.15±0.08	6.85±0.16[b]

Values are means ± SEM for 5-7 rats in each group: [a] $P < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs vehicle-treated rats.

Doses are per kg body weight per day.

TABLE 4.9(b) **Gut lengths in 80% jejuno-ileal resected rats following 7 days treatment with IGF-I peptides.**

Treatment group	Final body weight	Gut length (cm)				
		Duodenum	Jejunum	Ileum	Colon	Jejuno-ileum
Vehicle	159.2±3.4	8.7±0.5	8.4±0.3	8.6±0.3	12.0±0.5	17.0±0.5
IGF-I (0.7mg)	163.6±4.0	10.1±0.3	9.0±0.5	9.8±0.8	12.2±0.6	18.8±1.2
IGF-I (1.8mg)	165.6±5.0	10.0±0.1	9.7±0.9	9.2±0.7	12.4±0.4	18.9±1.5
IGF-I (4.5mg)	166.0±4.9	9.9±0.4	9.1±0.2	9.1±0.3	13.2±0.3	18.2±0.5
des(1-3)IGF-I (0.3mg)	168.3±4.7	9.9±0.5	9.5±0.6	11.0±1.1	11.7±0.5	20.5±1.7
des(1-3)IGF-I (0.7mg)	163.9±5.1	9.5±0.2	9.2±0.4	9.2±0.7	12.1±0.5	18.4±0.9
des(1-3)IGF-I (1.8mg)	169.4±4.2	9.6±0.4	10.3±0.5	9.7±0.5	12.4±0.2	20.0±0.6
LR^3IGF-I (0.3mg)	166.0±8.6	9.5±0.3	8.9±0.5	8.8±0.3	12.2±0.8	17.7±0.8
LR^3IGF-I (0.7mg)	167.8±5.0	9.8±0.3	9.6±0.4	9.3±0.8	13.0±0.5	18.9±1.1
LR^3IGF-I (1.8mg)	172.1±4.0	9.7±0.4	9.0±0.5	9.5±0.3	12.9±0.6	18.6±0.5

Values are means ± SEM for 5-7 rats in each group.

Doses are per kg body weight per day.

TABLE 4.10 **Mucosa+submucosa and muscularis externa weights in rats following 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.**

Treatment group	mucosa+sub-mucosa weight mg/cm	muscularis externa weight mg/cm	mucosa+sub-mucosa weight as % of total weight
		Jejunum	
Vehicle	63.9±4.3	45.9±4.5	58.4±2.6
IGF-I (0.7 mg)	85.2±13.7	69.4±12.1	55.8±2.5
IGF-I (1.8 mg)	96.7±14.3	47.2±5.4	66.5±3.3
IGF-I (4.5 mg/kg bw/d)	94.2±12.0	61.7±14.8	62.8±4.2
des(1-3)IGF-I (0.3 mg)	68.7±5.2	33.8±4.8	67.1±3.3
des(1-3)IGF-I (0.7 mg)	73.7±7.2	39.0±4.9	65.1±3.3
des(1-3)IGF-I (1.8 mg)	65.5±6.5	41.8±5.3	60.9±2.4
LR^3IGF-I (0.3 mg)	71.7±11.9	52.6±8.8	57.7±2.8
LR^3IGF-I (0.7 mg)	65.6±6.5	47.3±5.5	58.0±3.1
LR^3IGF-I (1.8 mg)	84.1±12.2	61.3±11.6	57.9±4.8
		Ileum	
Vehicle	58.8±4.9	42.1±2.8	57.8±3.1
IGF-I (0.7 mg)	64.9±5.2	43.6±5.3	60.6±1.8
IGF-I (1.8 mg)	73.5±7.5	44.4±5.9	62.7±2.4
IGF-I (4.5 mg)	70.4±5.0	43.4±3.5	61.6±3.2
des(1-3)IGF-I (0.3 mg)	73.0±11.3	39.1±5.8	64.6±3.6
des(1-3)IGF-I (0.7 mg)	61.4±9.3	40.6±3.8	59.1±3.1
des(1-3)IGF-I (1.8 mg)	77.4±8.3	50.8±4.3	59.8±2.2
LR^3IGF-I (0.3 mg)	61.8±9.7	43.1±3.5	56.9±4.5
LR^3IGF-I (0.7 mg)	56.4±9.2	44.0±6.0	55.5±2.5
LR^3IGF-I (1.8 mg)	62.3±8.2	50.6±3.2	53.9±3.2
		Colon	
Vehicle	42.1±3.1	46.1±2.0	47.5±2.0
IGF-I (0.7 mg)	46.5±4.1	57.3±6.6	45.4±3.1
IGF-I (1.8 mg)	47.9±3.1	55.8±4.9	46.5±2.1
IGF-I (4.5 mg)	51.0±2.9	48.6±4.3	51.7±3.2
des(1-3)IGF-I (0.3 mg)	39.6±3.4	49.9±2.8	44.1±2.5
des(1-3)IGF-I (0.7 mg)	51.1±5.8	57.3±5.9	47.0±3.5
des(1-3)IGF-I (1.8 mg)	57.4±7.6	61.2±5.3	47.7±3.7
LR^3IGF-I (0.3 mg)	42.3±3.4	43.3±3.1	49.4±3.3
LR^3IGF-I (0.7 mg)	47.6±1.9	54.4±4.6	47.2±2.6
LR^3IGF-I (1.8 mg)	46.4±4.1	58.1±3.3	44.1±3.1

Values are means ± SEM for 9 animals in each group.
Doses are per kg body weight per day.

similarities, irrespective of gut region or treatment group, in the mucosa+submucosa weight as a percentage of total weight.

The increases in mucosa+submucosa and muscularis wet weights were mirrored by small increments in protein and DNA content in both layers of each of the intestinal segments examined (Tables 4.11 and 4.12). None of these responses achieved statistical significance, and none of the gut regions were more responsive to IGF-I peptides than the others. Thus, it appears that IGF-I peptides produced proportional changes in the mass of the mucosa+submucosa and muscularis externa layers throughout the gut which, while not being individually statistically significant, combine to produce substantial increments in intestinal weights. Furthermore, since the protein to DNA ratio was not altered by peptide treatment, the accretion of intestinal mass is more likely to be due to hyperplasia rather than hypertrophy of gut cells.

A generalized growth response was supported by the histological measurements (Table 4.13). Due to the time-consuming nature of histological analyses, these measures were only determined in duodenal sections from the animals treated with either vehicle or the same 1.8 mg/kg bw/d dose of IGF-I, des(1-3)IGF-I or LR^{3}IGF-I (Table 4.13). The duodenum was chosen for analysis as it was the intestinal segment which showed the greatest increase in wet weight in response to IGF treatment. The peptide dose was chosen as it was the highest dose, and hence the most likely to show an effect, at which IGF-I and the analogues, des(1-3)IGF-I and LR^{3}IGF-I, could be directly compared. Treatment with IGF-I, des(1-3)IGF-I or LR^{3}IGF-I produced small, non-significant, increases in duodenal villus height (up to 3.2% higher than the controls), crypt depth (up to 13.8% greater), and mucosal, submucosal and muscularis externa thicknesses (up to 10.9%, and 16.8% and 18.2% higher, respectively). Consequently, the stimulation of duodenal weight by IGF treatment can be attributed to small, proportional, increases in the cross-sectional areas of each of the duodenal layers as well as a similarly small increment in duodenal length. Indeed, when the duodenal

TABLE 4.11 DNA and protein contents in intestinal mucosa+submucosa of rats following 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.

Treatment group	Protein mg/cm	DNA µg/cm	Protein:DNA µg/µg
		Jejunum	
Vehicle	11.11±1.13	400.5±29.1	27.9±2.3
IGF-I (0.7 mg)	9.93±0.91	482.4±70.6	22.4±2.2
IGF-I (1.8 mg)	13.04±2.08	496.8±32.7	25.7±2.6
IGF-I (4.5 mg)	12.48±1.19	529.1±40.3	23.5±1.1
des(1-3)IGF-I (0.3 mg)	10.56±0.76	391.0±24.5	27.3±1.3
des(1-3)IGF-I (0.7 mg)	10.50±0.78	426.4±28.7	24.7±0.7
des(1-3)IGF-I (1.8 mg)	10.65±0.80	406.4±27.0	26.6±1.8
LR^3IGF-I (0.3 mg)	9.51±1.54	380.9±56.4	24.8±1.1
LR^3IGF-I (0.7 mg)	10.11±0.93	379.9±27.2	26.6±1.8
LR^3IGF-I (1.8 mg)	12.14±1.64	476.5±47.5	25.2±1.6
		Ileum	
Vehicle	3.97±0.34	189.7±15.4	21.0±1.1
IGF-I (0.7 mg)	4.00±0.28	186.0±13.6	21.5±0.8
IGF-I (1.8 mg)	4.59±0.35	226.5±13.8	20.2±0.7
IGF-I (4.5 mg)	4.60±0.41	205.4±12.2	22.4±0.9
des(1-3)IGF-I (0.3 mg)	4.28±0.32	205.8±15.8	20.8±1.2
des(1-3)IGF-I (0.7 mg)	4.60±0.29	208.1±13.7	22.1±1.0
des(1-3)IGF-I (1.8 mg)	4.81±0.36	234.8±14.1	20.5±1.2
LR^3IGF-I (0.3 mg)	4.07±0.40	179.3±8.6	22.7±1.1
LR^3IGF-I (0.7 mg)	4.31±0.37	203.3±15.4	21.2±0.8
LR^3IGF-I (1.8 mg)	4.09±0.50	172.5±18.5	23.6±0.5
		Colon	
Vehicle	5.02±0.30	137.5±6.5	36.9±2.7
IGF-I (0.7 mg)	5.24±0.39	151.8±18.6	36.2±2.5
IGF-I (1.8 mg)	5.61±0.45	128.5±9.9	44.5±3.8
IGF-I (4.5 mg)	6.28±0.42	166.5±14.1	39.0±3.6
des(1-3)IGF-I (0.3 mg)	4.91±0.49	126.6±7.6	38.8±3.6
des(1-3)IGF-I (0.7 mg)	5.67±0.58	145.9±20.1	40.9±3.2
des(1-3)IGF-I (1.8 mg)	6.37±0.64	164.6±22.5	41.3±3.9
LR^3IGF-I (0.3 mg)	4.92±0.44	153.5±17.9	33.6±3.7
LR^3IGF-I (0.7 mg)	6.07±0.43	158.5±8.7	38.7±2.8
LR^3IGF-I (1.8 mg)	5.25±0.49	143.9±13.5	37.5±3.5

Values are means ± SEM for 9 animals in each group.
Doses are per kg body weight per day.

TABLE 4.12 DNA and protein contents in intestinal muscularis externa of rats following 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.

Treatment group	Protein mg/cm	DNA µg/cm	Protein:DNA µg/µg
		Jejunum	
Vehicle	6.90±0.74	189.3±14.9	37.6±4.0
IGF-I (0.7 mg)	9.66±1.45	242.7±46.7	45.1±7.3
IGF-I (1.8 mg)	7.26±0.67	167.2±19.3	45.3±4.1
IGF-I (4.5 mg)	8.33±1.43	228.0±41.3	37.7±3.5
des(1-3)IGF-I (0.3 mg)	6.26±0.60	136.0±24.1	50.0±4.0
des(1-3)IGF-I (0.7 mg)	7.45±1.21	196.6±28.6	37.5±3.3
des(1-3)IGF-I (1.8 mg)	7.31±1.40	167.8±27.4	43.4±3.4
LR^3IGF-I (0.3 mg)	8.46±1.49	214.6±23.3	38.5±3.2
LR^3IGF-I (0.7 mg)	8.59±1.09	217.9±38.2	43.3±5.1
LR^3IGF-I (1.8 mg)	9.98±1.80	228.2±34.6	44.1±4.0
		Ileum	
Vehicle	5.33±0.40	181.1±13.7	30.6±3.2
IGF-I (0.7 mg)	5.40±0.70	170.3±16.2	31.7±2.6
IGF-I (1.8 mg)	5.85±0.65	161.7±8.3	36.1±3.3
IGF-I (4.5 mg)	6.21±0.63	187.6±21.8	33.1±2.9
des(1-3)IGF-I (0.3 mg)	5.82±0.57	196.0±12.7	29.7±3.0
des(1-3)IGF-I (0.7 mg)	5.97±0.90	178.7±14.6	33.4±2.9
des(1-3)IGF-I (1.8 mg)	6.56±0.44	222.9±20.1	30.5±2.5
LR^3IGF-I (0.3 mg)	5.63±0.50	177.6±9.7	31.7±2.4
LR^3IGF-I (0.7 mg)	6.14±0.28	189.5±11.6	32.4±2.9
LR^3IGF-I (1.8 mg)	6.00±0.44	217.5±24.2	28.9±2.8
		Colon	
Vehicle	5.00±0.31	127.3±10.4	42.0±5.4
IGF-I (0.7 mg)	5.86±0.62	132.2±7.4	44.4±4.1
IGF-I (1.8 mg)	5.52±0.49	105.4±10.4	57.2±7.6
IGF-I (4.5 mg)	4.86±0.45	122.2±21.2	53.7±14.7
des(1-3)IGF-I (0.3 mg)	5.15±0.34	108.8±7.2	48.0±2.9
des(1-3)IGF-I (0.7 mg)	4.84±0.51	115.0±18.7	47.7±7.8
des(1-3)IGF-I (1.8 mg)	6.31±0.55	130.0±7.9	49.5±4.6
LR^3IGF-I (0.3 mg)	4.34±0.38	127.0±17.4	38.8±6.3
LR^3IGF-I (0.7 mg)	5.38±0.43	121.3±10.0	45.8±4.0
LR^3IGF-I (1.8 mg)	5.52±0.34	149.8±12.3	39.3±4.7

Values are means ± SEM for 9 animals in each group.
Doses are per kg body weight per day.

TABLE 4.13 **Duodenal histology of rats following 80% jejuno-ileal resection and 7 days treatment with IGF-I peptides.**

	Treatment groups			
	Control	**IGF-I** (1.8 mg)	**Des(1-3)IGF-I** (1.8 mg)	**LR^{3}IGF-I** (1.8 mg)
Circumference, mm	10.8±0.6	11.5±0.5	11.3±0.3	12.0±0.4
Mucosa				
Villus height, mm	0.827±0.047	0.853±0.047	0.854±0.059	0.833±0.027
Crypt depth, mm	0.241±0.013	0.248±0.014	0.253±0.013	0.274±0.007
Thickness, mm	0.955±0.052	1.060±0.058	1.041±0.076	1.035±0.038
Cross-sectional area, mm^2	10.5±1.0	12.2±0.9	11.6±0.7	12.3±0.5
Submucosa				
Thickness, mm	0.058±0.005	0.660±0.004	0.065±0.004	0.068±0.004
Cross-sectional area, mm^2	0.638±0.066	0.742±0.038	0.740±0.059	0.817±0.053
Muscularis externa				
Thickness, mm	0.153±0.011	0.160±0.018	0.181±0.013	0.172±0.006
Cross-sectional area, mm^2	1.70±0.20	1.79±0.16	2.06±0.02	2.06±0.11
Total				
Thickness, mm	1.17±0.06	1.29±0.07	1.29±0.07	1.28±0.04
Cross-sectional area, mm^2	12.8±1.3	14.7±0.9	14.4±0.7	15.2±0.6

Values are means ± SEM of 8 animals in each group.

Doses are per kg body weight per day.

length was multiplied by the total cross-sectional areas, the calculated duodenal volumes of the IGF-I, des(1-3)IGF-I and LR^{3}IGF-I groups were 36%, 28% and 37%, respectively, greater than the controls. Comparison with Table 4.9 shows that these increments in volume estimates correspond well with the observed increases in duodenal weight. Since histological analysis was not performed on samples from all IGF doses, no information on dose-responsiveness can be derived from this part of the experiment.

TABLE 4.14 **Sucrase activity in jejunal mucosa+submucosa of 80% jejuno-ileal resected rats following 7 days treatment with IGF-I peptides.**

Treatment group	Sucrase activity		
	total activity	activity/cm	activity/mg DNA
Vehicle	3.00±0.39	0.367±0.053	0.93±0.12
IGF-I (0.7mg)	4.43±1.30	0.530±0.165	1.01±0.22
IGF-I (1.8 mg)	5.59±1.43	0.596±0.164	1.16±0.24
IGF-I (4.5 mg)	4.90±0.80	0.548±0.097	0.99±0.12
des(1-3)IGF-I (0.3 mg)	4.58±0.50	0.483±0.043	1.23±0.08
des(1-3)IGF-I (0.7 mg)	3.61±0.39	0.396±0.047	0.97±0.13
des(1-3)IGF-I (1.8 mg)	3.06±0.47	0.306±0.055	0.75±0.11
LR^3IGF-I (0.3 mg)	2.75±0.69	0.292±0.066	0.91±0.26
LR^3IGF-I (0.7 mg)	3.88±0.78	0.415±0.085	1.03±0.16
LR^3IGF-I (1.8 mg)	4.55±1.13	0.565±0.115	0.99±0.20

Values are means ± SEM for 7-8 animals in each group.
Sucrase activity is expressed as moles of glucose generated/min, "total activity" is the product of jejunum length (cm) x "activity/cm".

Doses are per kg body weight per day.

Sucrase activity was measured in mucosa+submucosa scrapings obtained from the jejunum (Table 4.14) to provide an indication of the functional maturity of this tissue. Following removal of 80% of the jejuno-ileum, IGF peptide treatment had no effect on jejunal sucrase activity per mg DNA, although sucrase activity per cm and total sucrase activity (sucrase activity per cm x jejunal length) tended to be higher in the IGF-treated animals, possibly reflecting small increases in mucosal mass. Thus, there is no indication that treatment with IGF-I peptides accelerates the rate of cell maturation in the jejunal mucosa+submucosa.

4.3.8 Fat and nitrogen absorption

To assess the effect of IGF treatment on the efficiency of absorption in the recovering gut, faecal excretion as a percent of dietary intake was determined for fat and nitrogen in the control and 1.8 mg growth factor/kg bw/d groups (Fig. 4.5(a)(b)). Comparison on this basis was valid since food intakes were similar in each of these groups. The reason for conducting a limited study, and the rationale for selecting these groups, were the same as those outlined for the histological analysis. Prior to surgery, the pooled mean for faecal fat excretion as a percent of fat intake calculated from all the animals used in this experiment was 4.48±0.10% (Fig. 4.3(a)). For days 2-4 following surgery, the percentage fat excretion increased 5-7 fold with malabsorption being marginally higher in the vehicle-treated controls. A marked improvement was evident for all groups, but especially those treated with IGF peptides, over days 5-7. When related to the control level, the percentage fat excretion over days 2-7 was reduced 24%, 22% and 37%, respectively, by treatment with IGF-I, des(1-3)IGF-I and LR^3IGF-I.

The trends observed for faecal nitrogen as a percent of nitrogen intake following removal of 80% of the jejuno-ileum parallelled those of the percentage fat excretion (Fig. 4.3(b)). For days 2-4, the percentage excretion rose from the pooled pretreatment mean (5.13±0.12%) by 319%, 238%, 248% and 235% for the control, IGF-I, des(1-3)IGF-I and LR^3IGF-I groups respectively. Over days 5-7, the percentage excretion fell in all groups, with the improvement in the LR^3IGF-I treated animals being significantly better than that in the vehicle or IGF-I groups. For the combined period (days 2-7), the percentage nitrogen excretion for the controls was 18%, 28% and 47% higher than for the IGF-I, des(1-3)IGF-I and LR^3IGF-I groups, respectively, indicating that these animals absorbed nitrogen less efficiently than those treated with IGF peptides.

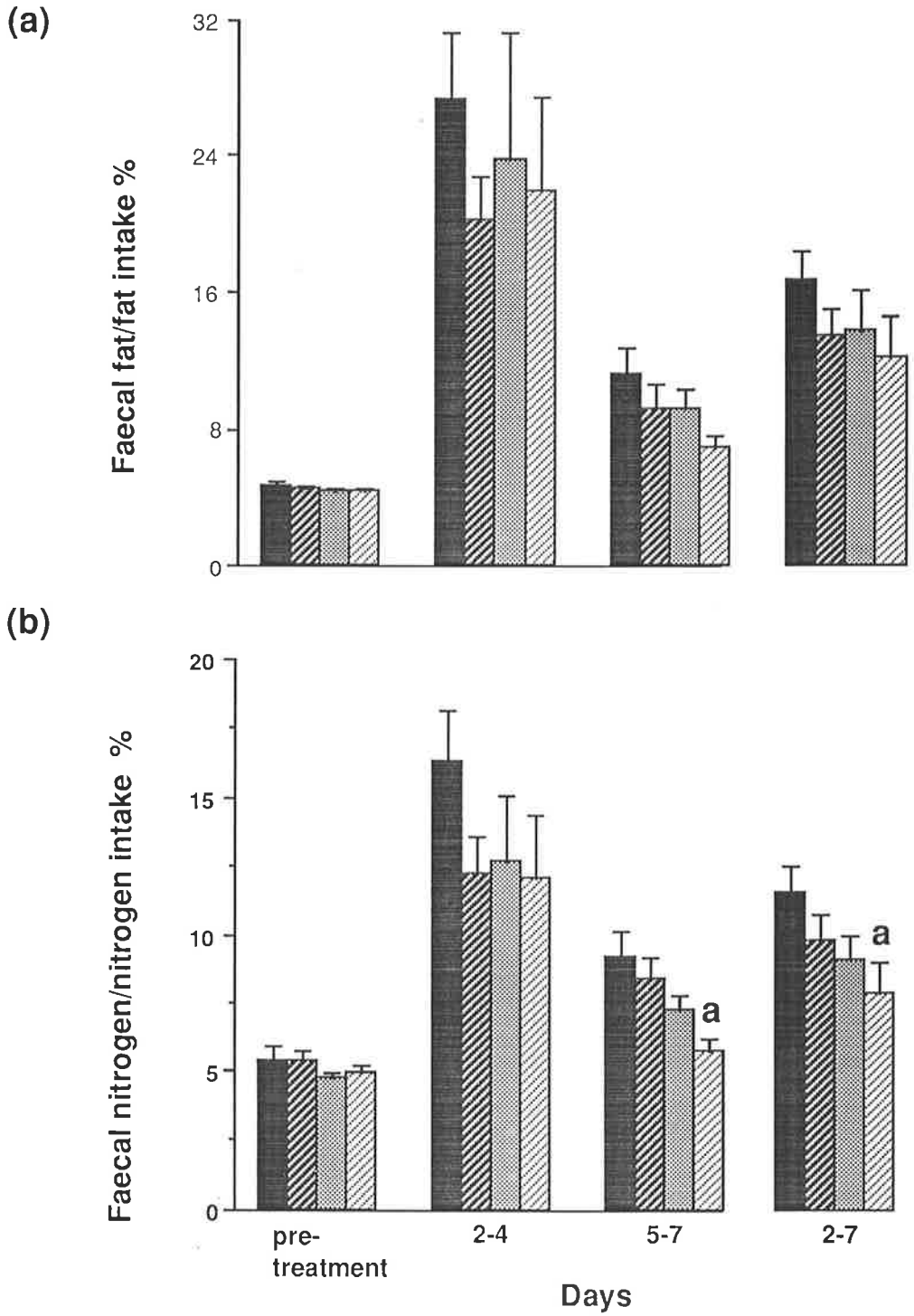

Fig. 4.5 Faecal fat (a) and faecal nitrogen (b) expressed as a percent of fat and nitrogen intake, respectively. Treatment groups were vehicle (■), 1.8mg IGF-I/kg bw/d (▨), 1.8mg des(1-3)IGF-I/kg bw/d (▩), and 1.8 mg LR3IGF-I/kg bw/d (▨). The % excretion for fat and nitrogen is shown for the 3 pretreatment days and for days 2 through 4 (2-4), days 5 through 7 (5-7), and days 2 through 7 (2-7) following 80% jejuno-ileal resection. Values are means±SEM for 8 rats in each group. **a**: $p < 0.05$ versus the vehicle group.

4.4 DISCUSSION

4.4.1 Effects of treatment on plasma IGF-I and IGFBP levels

Interpretation of IGF-I levels as determined by the radioimmunoassay used in this experiment is complex because four types of IGF-I (endogenous rat IGF-I, human IGF-I, des(1-3)IGF-I, and LR^3IGF-I), each with different cross-reactivities, were present in combinations that varied between groups, and proportions that differed with dose rates due to supression of endogenous IGF-I by the infused peptide. As a consequence of these considerations, it was not possible to determine precisely the total amount of circulating bio-active IGF-I in all groups. Even so, it is clear that infusion of IGF-I raised plasma levels in a dose-dependent manner. Since des(1-3)IGF-I has the same cross-reactivity in the assay as rat IGF-I, it was possible to calculate total plasma IGF-I levels, in the experiments where this analogue was administered. It is apparent that infusion of des(1-3)IGF-I failed to elevate circulating levels of IGF-I. While the level of plasma IGFs cannot be determined in the animals infused with LR^3IGF-I, it is highly unlikely that total IGF-I is increased. Also evident in these animals is a dose-dependent suppresion of endogenous IGF-I.

The inability of des(1-3)IGF-I to raise plasma IGF-I levels may be due to its enhanced clearance from the circulation. Indeed, a recent pharmacokinetic study showed that des(1-3)IGF-I is removed from the blood at a faster rate than IGF-I (Ballard et al., 1991a). The rapid removal of the analogues from the blood was attributed to their reduced affinity for binding proteins (Francis et al., 1992; Forbes et al., 1988). Since LR^3IGF-I has similar *in vitro* and *in vivo* potencies to des(1-3)IGF-I (Walton et al., 1990; Tomas et al., 1991c) despite a three-fold lower affinity for the type-1 IGF receptor (Walton et al., 1990), it would appear that the reduced receptor affinity is compensated for by an even lower affinity for IGFBPs and, thus, a more rapid transfer from the circulation to the tissues compared with des(1-3)IGF-I (Walton et al. 1990). Another factor which may be contributing to the inability of the analogues to raise circulating IGF-I levels above those of the vehicle-treated animals, is negative

feedback by the infused peptide on GH release from the pituitary gland with subsequent reductions in endogenous IGF-I production. The most convincing indications of this are the inverse relationship between infusion dose and plasma IGF-I levels of the LR^3IGF-I treated animals, and the failure of increasing doses of des(1-3)IGF-I to elevate IGF-I levels, even when enhanced clearance is accounted for. However, whether or not infusion of native IGF-I has a negative-feedback effect on the production of endogenous peptide, cannot be assumed with certainty because the relative contributions of rat and human IGF-I to the plasma levels could not be determined.

Changes in plasma binding protein profile following resection and peptide treatment were identified by Western ligand blot analysis. The decline in intensity of the bands corresponding to the positions of IGFBP-3 (42-48kDa), IGFBP-1 and IGFBP-2 (28-32kDa), and IGFBP-4 (24-25kDa) that followed gut resection, and the subsequent restitution or enhancement of the 42-48kDa and 28-32kDa bands following IGF-I or des(1-3)IGF-I treatment that we observed, has also been demonstrated by Vanderhoof et al. (1992b) in their resection study. The post-resectional fall in intensity of the band corresponding to the molecular weight of IGFBP-3 in our control rats may be due to the reduced plasma IGF-I concentration in these animals, since IGFBP-3 in rats is IGF-I dependent (Clemmons et al., 1989; Zapf et al., 1989). Such reasoning is supported by the coincident increases in 42-48kDa band and plasma IGF-I of the IGF-I treated animals. Vanderhoof et al. (1992b) suggest that the post-resectional fall in IGFBP-3 and IGFBP-4 may, in part, be due to decreased food intake or absorption as levels of these binding proteins have been shown to decline in fasted rats (McCusker et al., 1989). This is consistent with the increase in IGFBP-3 and IGFBP-4 levels following peptide infusion observed in this experiment for although treatment had no effect on food consumption it did improve nutrient uptake. The increased intensity of the bands corresponding to the positions of IGFBP-1 and IGFBP-2 in the IGF-I treated animals is also probably due to the increased concentration of IGF peptides since the

levels of these binding proteins increase after IGF-I infusion (Zapf et al., 1990a,b). IGFBP-1 levels would also be expected to increase if the infused IGF peptides provoked hypoglycaemia (Cotterill et al., 1989; Suikkari et al., 1988; Yeoh and Baxter, 1988). This possibility cannot be discounted since Vanderhoof et al. (1992b), using rats of the same strain, sex and size, similar peptide doses and identical delivery methods, found that the post-resectional administration of 1.5 mg IGF-I/kg bw/d induced hypoglycaemia.

Although restoration of band intensities was less apparent for the analogue-treated animals, especially those receiving LR^3IGF-I, compared to those administered IGF-I, this experiment provides evidence that des(1-3)IGF-I and LR^3IGF-I are able to induce increases in binding proteins that bind very poorly to the analogues. This effect, which was also seen by Vanderhoof et al. (1992b) following infusion of des(1-3)IGF-I, suggests that the mechanism for increasing circulating levels of IGFBPs does not require binding between IGFs and binding proteins.

4.4.2 Dose-responsiveness of IGF-I and des(1-3)IGF-I on non-gut growth

To determine whether IGF-I and des(1-3)IGF-I act in a dose-responsive manner each was infused at three different concentrations. In view of the demonstration of enhanced potency *in vivo* in the previous experiment (see Chapter 3), des(1-3)IGF-I was administered at dose rates 2.4 times lower than IGF-I.

In this investigation, as in Experiment 1 (see Section *3.3.2*), IGF-I and des(1-3)IGF-I significantly increased body weight gain and food conversion efficiency, with des(1-3)IGF-I displaying greater potency than native IGF-I. The greater potency of des(1-3)IGF-I compared to IGF-I in increasing body weight and food conversion has been reported elsewhere (Tomas et al., 1991a,c; Vanderhoof et al., 1991).

In accordance with the result from Experiment 1 (see Section *3.3.3*), nitrogen retention in the IGF-treated rats tended to be improved by virtue of significant reductions in nitrogen excretion. Although des(1-3)IGF-I at 1.8 mg/kg/d had a substantially greater effect on nitrogen excretion and balance than an equivalent dose of IGF-I, and a slightly greater effect than a 2.4 fold higher dose of IGF-I, the enhanced potency of des(1-3)IGF-I was not evident at the lower doses. A superior potency for des(1-3)IGF-I in increasing nitrogen retention, compared to IGF-I, has been demonstrated in other rat models (Ballard et al., 1992; Tomas et al., 1991b,c). The effect of IGF-I or des(1-3)IGF-I treatment on nitrogen balance in resected rats was less than that seen for dexamethasone-treated (Tomas et al., 1991c) and diabetic (Tomas et al., 1991a) rats, but similar to that observed for partially-nephrectomized (Martin et al., 1991) or nitrogen-restricted (Tomas et al., 1991b) rats. As such, improvement in nitrogen balance following IGF-I administration appears to be greatest in those catabolic conditions that produce the most substantial reductions in nitrogen retention.

Responses in body weight gain, food conversion efficiency, and nitrogen excretion to IGF-I and des(1-3)IGF-I were apparent at the lowest doses employed, and substantially greater at the highest rates infused, thus demonstrating sensitivity and dose-responsiveness to these peptides. Progressive increases in body weight gain and nitrogen retention with increasing doses of IGF-I and des(1-3)IGF-I have also been demonstrated in dexamethasone-treated rats (Tomas et al., 1991c). It is important to note that no adverse effects were observed following administration of high doses of IGF-I or des(1-3)IGF-I in the current experiment, or that of Tomas et al. (1992).

As noted in Experiment 1 (see Section *3.3.4*), our indices of muscle protein breakdown, urinary 3-MH excretion tended to be reduced by IGF treatment. In contrast to the effect described for 3-MH excretion in dexamethasone-treated rats (Tomas et al., 1991c), their was no indication that either IGF-I or des(1-3)IGF-I had a dose-responsive action, nor was an enhanced potency for des(1-3)IGF-I compared to

IGF-I apparent. The response in muscle protein degradation to treatment with IGF-I seems to be dependent on the animal's metabolic state, since IGFs have reduced 3-MH excretion in catabolic rat models which feature elevated breakdown rates (Martin et al., 1991; Tomas et al., 1991c), but not in nitrogen-restricted rats which already have a depressed rate of breakdown (Tomas et al., 1991b) or diabetic rats in which degradation rates are increased following IGF infusion (Tomas et al., 1991a). In the gut-resection model, muscle protein breakdown is transiently elevated, but then marginally depressed relative to pretreatment levels. Interestingly, in animals in which synthesis rates have been reduced by the experimental treatment (Tomas et al., 1991a,b,c), infusion of IGF peptides increases muscle protein synthesis (which was not measured in this study), indicating that the effect of IGF peptides on muscle protein anabolism may also be determined by the existing protein synthesis conditions.

Despite the improvements in nitrogen retention and the trend towards decreased muscle protein breakdown, treatment with either IGF-I or des(1-3)IGF-I, even at the highest doses, had no effect on the fractional carcass weight. This result agrees with that observed in Experiment 1 (see Section *3.3.5*), and is consistent with the absence of any peptide effect on body composition in this investigation. Since IGF-I peptides did not alter fractional carcass weight or body composition in gut-resected rats, the increased weight gain of the treated animals is due to proportional growth. Relative carcass weight and composition of diabetic (Tomas et al., 1991a) and partially-nephrectomized (Martin et al., 1991) rats are also unchanged by IGF treatment. In contrast, reduced body fat has been noted for nitrogen-restricted (Tomas et al., 1991b) and hypophysectomized (Guler et al., 1988) rats following infusion of IGF peptides. These results suggest, not surprisingly, that the effect of IGFs on carcass composition, like that on protein turnover, may vary according to the metabolic state of the animal.

The increase in thymus weight following des(1-3)IGF infusion, and enhanced potency of the analogue compared to IGF-I in eliciting thymus growth, agrees with our

previous results and the effect Ballard et al. (1991b) describe for diabetic rats. In contrast, des(1-3)IGF-I administered in the same dose range as that employed here, was unable to restore thymus weigh in dexamethasone-treated rats (Tomas et al., 1991c). From this the authors concluded that IGFs cannot prevent the immunosuppressive effects of dexamethasone that cause thymus atrophy. Although the effect IGF-I or des(1-3)IGF-I had on thymus weight was only significant when the highest dose of des(1-3)IGF-I was administered, there was a trend toward progressive increases in thymus weight with higher doses of either peptide. There are no other reports of IGF-I or des(1-3)IGF-I having a dose-responsive effect on thymus growth.

Treatment with both IGF-I and des(1-3)IGF-I increased spleen weight. Dose-responsiveness was apparent for IGF-I, but not for des(1-3)IGF-I which had its biggest effect at its lowest dose. Neither peptide appeared to be more potent than the other in its effects on the spleen. Augmented spleen weight following IGF-I treatment has also been reported in dexamethasone-treated (Tomas et al., 1991c), diabetic (Ballard et al., 1991b; Binz et al., 1990), hypophysectomized (Guler et al., 1988), pituitary GH-deficient dwarf (Skottner et al., 1989) and normal (F.J.Ballard, personal communication) rats, and IGF-I transgenic mice (Mathews et al., 1988). Mathews et al. (1988) concluded that since increases in splenic weight occur without an elevation in splenic IGF-I concentrations, and because systemic administration of IGF-I evokes an anabolic response from the spleen, the change in spleen size is likely to be due to endocrine, rather than paracrine or autocrine, IGF-I stimulation. The sensitivity of both the thymus and the spleen to IGF-I peptides suggests a general response to IGF-I in lymphoid organs, which Kurtz et al. (1982) believe may be connected to IGF-I's known haemopoietic action.

4.4.3 Potency of LR^{3}IGF-I on non-gut growth

This investigation introduced an IGF-I analogue, LR^{3}IGF-I, which, like des(1-3)IGF-I, had displayed enhanced potency *in vitro* compared to IGF-I (Francis et al.,

1992). Although featuring even less affinity for IGFBPs than des(1-3)IGF-I, LR^{3}IGF-I has only a third the affinity to the type-1 receptor of either IGF-I or des(1-3)IGF-I (Francis et al., 1992). As a consequence of these differences, one which could be expected to aid its action *in vivo* the other to hinder it, the relative potency of LR^{3}IGF-I to IGF-I and des(1-3)IGF-I *in vivo* could not be anticipated with any certainty.

Administration of LR^{3}IGF-I to rats following 80% gut-resection enhanced body weight gain, food conversion efficiency and nitrogen balance measures relative to the vehicle-treated animals. In regard to these measures, LR^{3}IGF-I generally showed a greater potency than IGF-I. The enhanced potencies of LR^{3}IGF-I, relative to IGF-I, in improving these measures is most obvious when comparisons are made between the effects of the analogue at any of the doses administered, and the effect of IGF-I at a 2.4 fold or higher dose. To illustrate this, nitrogen retention in the animals receiving the highest dose of IGF-I (4.5mg/kg bw/d) was 118.0±10.3 mg/d (mean±SEM), virtually identical to the 121.1±4.0 mg N/d retained by the animals receiving the lowest dose of LR^{3}IGF-I (0.3mg/kg bw/d). The same comparison can be drawn from the weight gain or food conversion data. Relative to des(1-3)IGF-I, LR^{3}IGF-I tended to be equipotent at the highest dose, but more potent at the lower doses in improving body weight gain, food conversion efficiency and nitrogen balance measures. Like IGF-I and des(1-3)IGF-I, the effect of LR^{3}IGF-I on body weight gain, food conversion efficiency and nitrogen excretion increased according to dose rate. However, this relationship was not apparent for nitrogen balance, since administration of 0.3 or 1.8 mg/kg bw/d produced similar improvements. Accounting for the similarities in nitrogen balance despite reduced excretion of nitrogen with higher doses of LR^{3}IGF-I, was an inverse (albeit nonsignificant) relationship between nitrogen intake and LR^{3}IGF-I dose. Such a relationship, however tenuous, was not observed following administration of the other IGF forms.

LR^{3}IGF-I has been reported as being more efficacious than IGF-I in promoting body weight gain and nitrogen retention in dexamethasone-treated (Tomas et al., 1991c), diabetic and normal rats (Ballard et al., 1992). Compared to an equivalent dose of des(1-3)IGF-I, LR^{3}IGF-I has tended to produce greater effects on body growth and nitrogen retention in normal and diabetic rats (Ballard et al., 1992) and similar responses in dexamethasone-treated rats (Tomas et al., 1991c).

LR^{3}IGF-I had no greater effect on 3-MH excretion, relative carcass weight, or body composition, or liver, kidney, adrenal, heart, or lung weights, than either IGF-I or des(1-3)IGF-I. These responses to LR^{3}IGF-I contrast with the enhanced effect for 3-MH excretion observed in dexamethasone-treated rats, but agree with the lack of additional effect on body composition and organ weights of the same animals (Tomas et al., 1991c). At the highest dose administered (1.8 mg/kg bw/d), LR^{3}IGF-I produced a greater increase in splenic weight than either IGF-I or des(1-3)IGF-I, but this superiority was not evident at lower dose rates. On the other hand, the thymus appeared to be particularly sensitive to LR^{3}IGF-I, especially at the lower doses.

LR^{3}IGF-I like des(1-3)IGF-I, another IGF-I analogue that binds poorly to IGFBPs, is more effective in improving body weight gain and food conversion efficiency, reducing nitrogen loss, and stimulating thymus growth than IGF-I in gut-resected rats (see Sections *3.3* and *4.3)*. The superior potency of the variants compared with IGF-I in this model is consistent with numerous *in vitro* and *in vivo* findings (see Sections *1.4.11*, *3.4* and *4.4*) and is ascribed to their reduced affinity for binding proteins (Forbes et al., 1988; Walton et al., 1990), which may enhance delivery from the blood to the tissues (Ballard et al., 1991a) and, consequently, improve access to receptors. As such, the enhanced potency of these analogues *in vivo* provides strong evidence that binding to IGFBPs inhibits the biological action of IGF peptides. LR^{3}IGF-I, in the measures made in this and other studies (Ballard et al., 1992; Tomas et al., 1991c), has generally been shown to be as potent, if not more potent, than des(1-

3)IGF-I. Since these peptides differ in that LR^{3}IGF-I has less affinity for both the IGFBPs and type-1 receptor, the advantage of reduced binding to the binding proteins must compensate for the disadvantage of poorer receptor affinity, to account for the similar potencies of the analogues. Thus, the enhanced action of LR^{3}IGF-I *in vivo* further indicates that binding proteins inhibit IGF-I activity *in vivo*.

4.4.4 Characterization of gut effects

Our earlier conclusion that the gut is particularly responsive to the anabolic effects of IGF-I peptides is reaffirmed by the current investigation. Even at the lower dose rates, significant effects on gut weights were observed for each of the IGF-I peptides used, with the effects tending to increase with higher doses. Despite the marked effects we observed, the gut responses to IGF peptides following resection are less than those seen in two other models of catabolic weight loss. More substantial gains in total gut weight have been demonstrated for streptozotocin-induced diabetic rats (Tomas et al., 1991a), and especially dexamethasone-treated rats (Tomas et al., 1991c). Since glucocorticoids induce slight hypoplasia in the gut, diabetes produces a condition of mild gut growth due to hyperphagia, and intestinal resection provides a powerful stimulus for gut growth, it appears that the degree of gut responsiveness to IGF-I treatment may be determined by the initial proliferative state of the gut, with effects diminishing in the face of increased gut stimulation. However, the fact that IGF-I treatment can still have significant additive effects on gut growth in the conditions of marked stimulation which follow resection, emphasizes the sensitivity of these tissues to IGFs.

Biochemical analyses demonstrated that the accretion of intestinal mass in response to IGF treatment following resection was attributable to a generalized growth response, wherein each of the cross-sectional layers and occasionally the lengths of the intestinal segments were marginally increased. Maximal increases in mucosal+submucosal weight of 51% and 32% were observed in the jejunum and

ileum, respectively, of the IGF-treated animals compared to the controls. Similar effects were noted by Vanderhoof et al. (1992b) following infusion of 1.5mg/kg bw/d of IGF-I or des(1-3)IGF-I to 80% jejuno-ileal resected rats. It is notable that their mucosal+submucosal weights per cm, in both the duodeno-jejunum and ileum and in all resected groups including the vehicle-treated controls, were 50-125% heavier than those observed in this study. Interestingly, their duodeno-jejunal DNA (μg/cm) values were 50-100% lower than those observed for the jejunum in this study, and their protein levels (mg/cm) from the same gut region, although elevated relative to those of this study (18-70%), were increased, proportionally, far less than tissue wet weight. This may indicate that some of the increase in mucosal weight per cm in their samples is due to water and, secondly, that since their protein to DNA ratio is markedly higher, a less pronounced hyperplastic response may have been present in their animals.

Consistent with the trend to proportional increases in the wet weights of the mucosa+submucosa and the muscularis externa layers throughout the intestinal tracts of the animals in Experiment 2, were small, proportional increments in protein and DNA content within both these layers. As with the changes to mucosal+submucosal and muscularis wet weights, the increases in DNA and protein content within these layers failed to achieve significance. Since gut growth occurred in the absence of changes to the protein: DNA ratio, hyperplasia rather than cellular hypertrophy is indicated. Hyperplasia in the mucosa+submucosa and muscularis layers was also proposed as the mechanism underlying gut growth induced by IGF-I, des(1-3)IGF-I and LR^3IGF-I in dexamethasone-treated rats (Read et al., 1992). The development of mucosal hyperplasia following massive small bowel resection is well documented (Nygaard, 1967; Vanderhoof et al., 1984), and the augmentation of mucosal hyperplasia in gut-resected rats following IGF treatment has been confirmed by Vanderhoof et al. (1992b).

Results from the histological analysis of the duodena support the biochemical finding of marginal, but generalized and proportional, growth of the cross-sectional layers of the intestinal tract in response to IGF treatment. Vanderhoof et al. (1992b), in the only other investigation of IGF effects in gut-resected rats, reported significant increases in duodeno-jejunal villus height, and ileal crypt depth. The greater effect of IGF administration that they observed is consistent with the gut of their animals being in a less proliferative state than the rats in this study as a consequence of differences in resection procedure. Increases in villus height and the cross-sectional areas of the mucosa, submucosa and muscularis externa in duodenal samples from dexamethasone-treated rats have also been reported following IGF treatment (Read et al., 1992).

Jejunal mucosal sucrase activity per cm tended to increase in IGF treated rats, compared to controls, following 80% resection. However, when sucrase activity was determined per mg DNA no treatment trends were observed. Similar IGF responses were found in duodeno-jejunal mucosal sucrase and maltase activity by Vanderhoof et al. (1992b) using the same animal model. These results suggest that IGF-I peptides , whilst capable of stimulating hyperplasia in the gut, have no effect on the rate of epithelial cell maturation.

Although trends in the anticipated direction are evident in the biochemical and histological results, the effect of IGF treatment failed to achieve significance in any of these measures. On the other hand, Vanderhoof et al. (1992b) making similar measurements in the same animal model reports a number of significant effects. Largely accounting for their ability to achieve significance is comparatively smaller variability in mucosal wet weights, DNA and protein content, and the histological measures. The tighter nature of their data may have been due to a relatively reduced proliferative state in the gut of their animals, or to differences in experimental procedure (see Section *5.2.2*).

The apparent increase in intestinal mucosal mass following IGF treatment suggests an enhanced absorptive surface area which may be physiologically significant under conditions of compromised absorptive capacity. To determine whether the gut growth following administration of IGF was associated with an effect on gut function, and to resolve whether the improvement in food conversion of the IGF-treated rats was in part due to an enhanced absorptive capacity, absorption of nutrients was investigated.

An improvement in the digestive and absorptive capacities of the IGF-treated animals is suggested by way of diminished percentage fat and nitrogen excretions. Relative to the control animals, treatment with IGF peptides reduced fat malabsorption by up to 26% over days 2-4, up to 38% over days 5-7 and up to 37% for the combined period (days 2-7). Virtually identical effects were noted for nitrogen malabsorption following peptide infusion, with maximal reductions of 26% over days 2-4, 38% over days 5-7 and 32% for the combined period relative to the controls. Since weight loss in gut-resected animals may in part be due to impaired food absorption (Sigalet et al., 1990), the ability of IGF peptides to improve food absorption and food conversion efficiency under these conditions suggests that IGF-I and its analogues may be therapeutically useful in circumstances of impaired gut function.

In contrast with their greater effect in improving body weight gain, food conversion efficiency and nitrogen balance measures, and stimulating thymus, spleen and stomach growth relative to IGF-I (see Sections *3.3* and *4.3*), the two analogues used in this experiment des(1-3)IGF-I and LR^3IGF-I do not appear to have enhanced actions in the gut compared to the native peptide. This point will be discussed in detail in the following chapter (see Section *5.4.4*)

This investigation demonstrated that in 80% gut-resected rats, IGF-I peptides affect body weight gain, food conversion efficiency, nitrogen excretion, and gut

growth in a dose-responsive manner. Additionally, it was the first to demonstrate the effect of the IGF-I analogue, LR^3IGF-I, on body weight, nitrogen balance and muscle protein breakdown in rats in which a transient catabolic condition had been produced by partial gut resection, and the first to show the *in vivo* sensitivity of the thymus, spleen and gut to LR^3IGF-I. Importantly, it also revealed that IGF treatment enhances the absorptive capabilities of recently gut-resected animals.

CHAPTER 5

Experiment 3: IGF-I peptides enhance gut function in rats following 70% resection

5.1 INTRODUCTION

While measurements of intestinal mucosal+submucosal and muscularis wet weights, DNA and protein content, duodenal histology, and jejunal sucrase activity made in Experiment 2 (Chapter 4) suggested that IGF-mediated increases of gut organ weights in resected rats were due to the combined effect of numerous small, proportional increments throughout the gut, none of these biochemical or histological reponses on its own achieved statistical significance. In contrast, Vanderhoof et al. (1992b) reported significant increases in duodeno-jejunal mucosal wet weight, DNA and protein content, and villus height, and ileal crypt depth, following administration of 1.5 mg/ kg bw/d of IGF-I or des(1-3)IGF-I to 80% gut-resected rats. Although they reported marginally greater peptide effects for most of these measures, the most notable difference between the data sets was the comparatively small variability in their results. In an effort to achieve similarly low levels of measurement variability, two modifications in methodology were adopted from their study. These changes involved the use of a different anaesthetic, and the introduction of fasting immediately before and after surgery. An additional change was the inclusion of a "day 0 control" group, comprising animals that were killed on day 0 rather than resected, which enabled assessment of tissue adaptation in the gut following resection and treatment.

The surgical protocol of removing all but the most proximal and distal 10cm of the jejuno-ileum, which was used in the previous experiments, was retained for this investigation. However, since the animals supplied for this experiment were lighter (although the same age) than those used previously, resection resulted in removal of 70% rather than 80% of the jejuno-ileum. This afforded the opportunity of comparing the effects of IGF administration on gut growth and function following 70% resection with those previously seen after 80% resection.

5.2 MATERIALS and METHODS

5.2.1 Peptides and dose rates

Peptides or vehicle were delivered by osmotic minipumps as previously detailed in section *2.2.7*. The pumps were filled with either 0.1M acetic acid as vehicle, or 9.5mg recombinant human IGF-I, des(1-3)IGF-I or LR^{3}IGF-I/ml. This growth factor concentration was chosen as it was equivalent to the highest dose of des(1-3)IGF-I and LR^{3}IGF-I employed in Experiment 2 and, as such, enabled comparison of the effects of IGF-I peptide treatment in 70% and 80% gut-resected animals. Body weights at the time of surgery were (mean±SEM) 114±2, 113±4, 114±2 and 113±2g for the vehicle, IGF-I, des(1-3)IGF-I and LR^{3}IGF-I groups, respectively. At a delivery rate of 1.01µl/h, and based on the body weights at surgery, the pumps infused growth factors at 2.0mg/kg bw/d. Recombinant human IGF-I and recombinant human des(1-3)IGF-I were supplied by Genentech, San Francisco, CA., USA. Recombinant human LR^{3}IGF-I was provided by GroPep Pty Ltd., Adelaide, South Australia.

5.2.2 Animals and experimental design

The animals and their maintenance were as described in sections *2.2.1* and *2.2.4*, with the following exceptions: 1) to minimize possible irritation to the jejuno-ileal anastomosis by luminal contents, the animals were fasted for 24h before and 24h after surgery (Vanderhoof et al., 1992b) (Fig. 5.1) rather than allowed to eat *ad libitum* as in previous experiments. Water was available *ad libitum* during the fasting periods; 2) due to a concern that the acidity of Avertin may be increasing adhesion formation within the peritoneal cavity, anaesthesia in this investigation was by an intraperitoneal injection of sodium methohexital (45mg/kg) and sodium pentobarbital (30mg/kg) (Vanderhoof et al., 1992b); 3) when initially received, the rats used for this experiment, although the same age (4 weeks), were approximately 20g lighter than those used previously. Thus, despite adhering to the same surgical protocol used in Experiments 1 and 2 (see Section *2.2.7*) and removing the gut lying beween the proximal 10cm of jejunum and the distal 10cm of ileum, the extent of resection was reduced by using

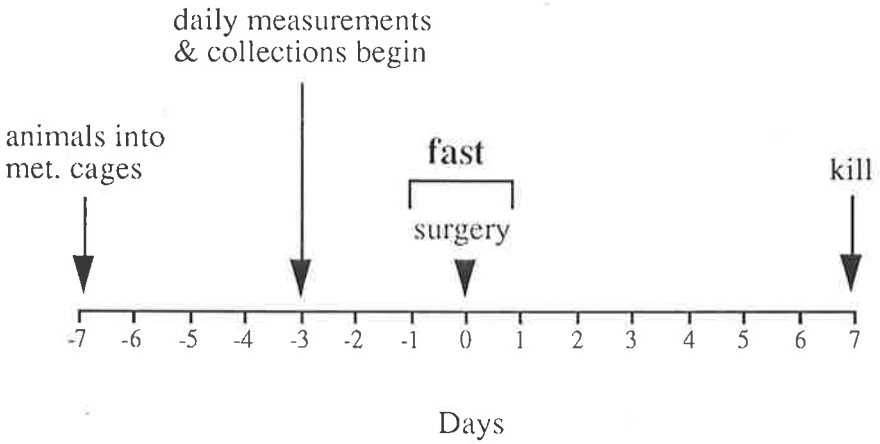

Fig. 5.1 Protocol for the 70% resection experiments

comparatively smaller rats (Fig. 5.2). Subsequent measurement of the excised segment of jejuno-ileum confirmed that, as a consequence of the smaller size and reduced gut length of these animals, only 70% of the jejuno-ileum had been removed; 4) in addition to the resected animals, nine rats served as "day 0 controls". On day 0, instead of undergoing surgery, these animals were killed. The function of the day 0 controls was to provide baseline information, thereby enabling quantification of the effects of resection and seven days of treatment. The pretreatment, sacrifice and sample processing procedures applied to these animals were identical to those of the resected rats. Animals were randomized between groups, including "day 0 controls", on day 0 to ensure that the groups had the same mean starting weight.

Faeces was stored and treated as in section *3.2.2*, and pooled as described in section *4.2.2*. Fecal fat and nitrogen excretion were subsequently determined to assess the effect of resection and IGF treatment on nutrient absorption by the gut. Since the focus of this experiment was the effect of IGFs on gut growth and function, and not post-resectional catabolism, no measures of nitrogen balance or 3-MH excretion were made.

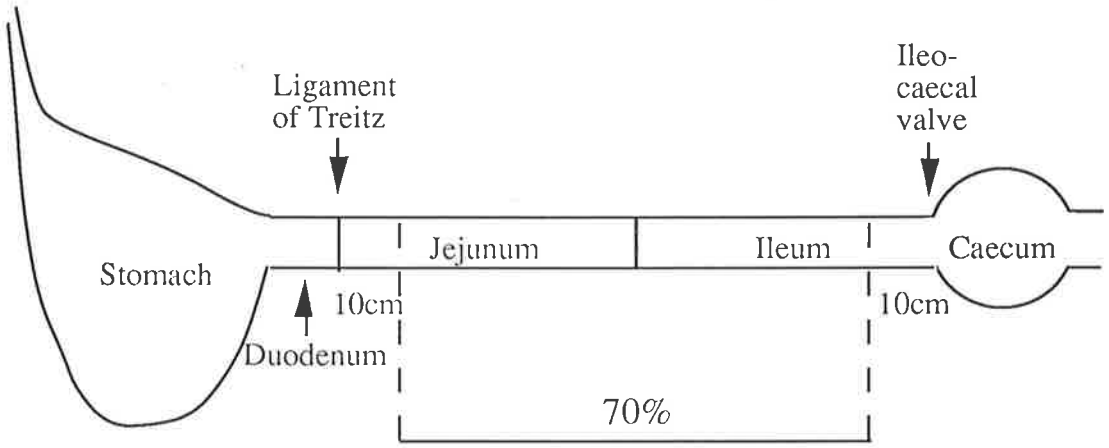

Fig. 5.2 Schematic representation of the 70% resection method adopted for Experiment 3.

At sacrifice, the combined mucosa and submucosa (mucosa+submucosa) of the duodenum, as well as that from the jejunum, ileum and colon, was scraped from the muscularis externa layers. This process, and the subsequent treatment of these samples is described in section *4.2.3*. No histology specimens were taken in this experiment. The collection and weighing of gut samples at sacrifice, although coordinated by myself, required technical assistance.

On the basis of the exclusion criteria described in Section *2.2.6*, 5 of 41 resected rats were excluded (two des(1-3)IGF-I-treated animals, and one from each of the other treatment groups).

5.2.3 Analytical measurements

The nitrogen content of faeces and food was measured by a Carlo Erba NA 1500 Nitrogen Analyser (Milan, Italy) according to the Dumas proceedure, as previously outlined (section *3.2.2*). The fat content of faeces and food were determined gravimetrically following chloroform/methanol extraction of the dried samples as detailed in section *4.2.4*.

Estimates of the net absorption of fat and nitrogen following resection were calculated as described previously (see section *4.3.7*). The faeces for the three pretreatment days were pooled. However, due to the presurgical fast, food was only consumed on two of these days, thus pretreatment fat and nitrogen excretion as a percentage of intake could not be directly determined. To enable an estimation of percentage excretions for the pretreatment period, food intake from the initial two pretreatment days was extrapolated to give three days feed. This calculation gave values virtually identical to those observed in the corresponding period of Experiment 2 (see section *5.3.3*).

The protein and DNA contents of mucosal+submucosal and muscularis scrapings were measured as described in section *4.2.3*. Sucrase activity in the duodenal, jejunal and ileal mucosal+submucosal scrapings was determined by a modification of the Dahlqvist method (Dahlqvist, 1968) adapted to microplates which is described in section *4.2.3*.

5.2.4 Statistical analysis.

Means and SEM are reported for all measures. An analysis of variance (ANOVA) was used to compare the treatment groups. When significant difference ($p < 0.05$) was achieved, the source was identified by Fisher's Protected Least Significant Difference *post hoc* test. Statistical analysis was performed using the SuperANOVA (Abacus Concepts, CA, USA) program.

5.3 RESULTS

5.3.1 Body weight changes

Prior to the presurgical fast the animals were gaining weight at about 6-7g/d (Fig. 5.3(a)), a growth rate similar to that observed during the pretreatment period of previous experiments. As a consequence of the presurgical fast, an absolute loss of

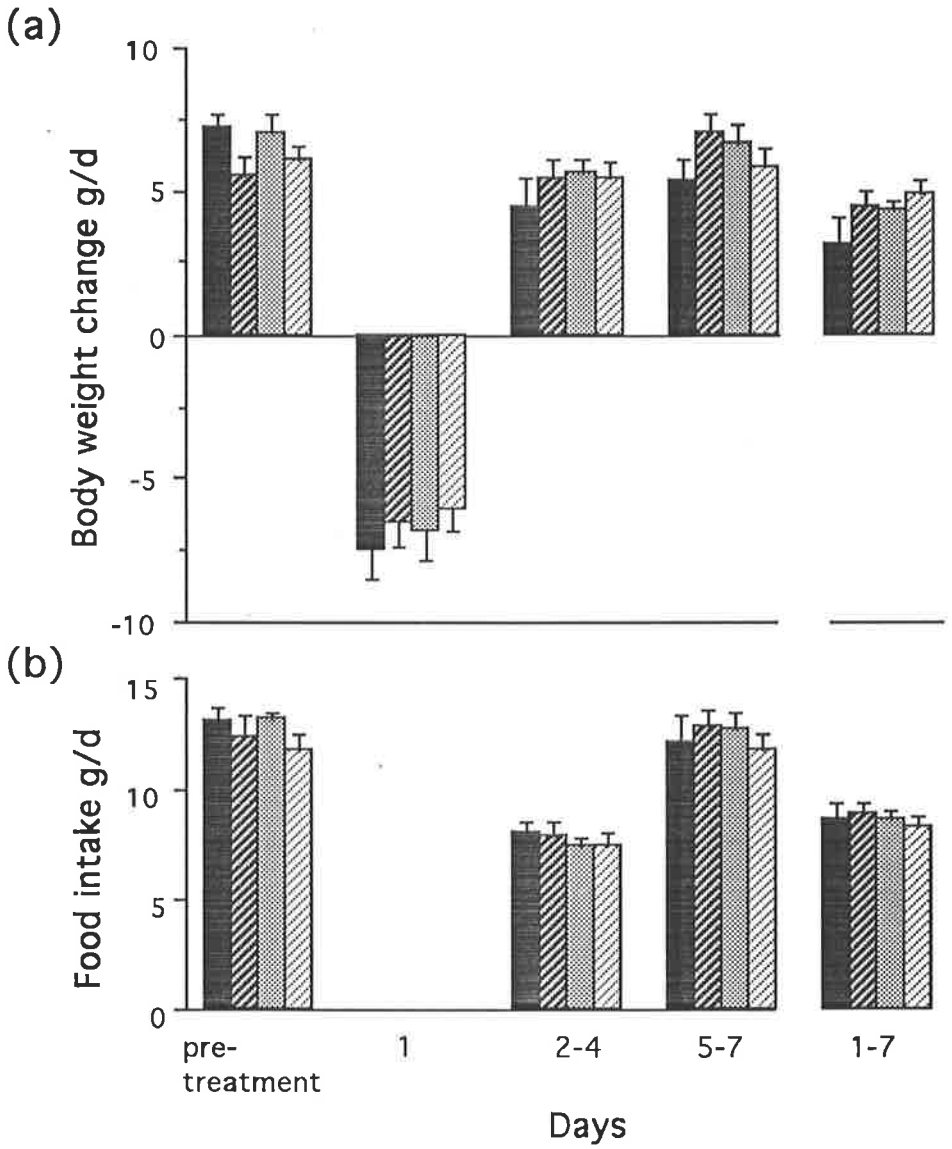

Fig. 5.3 Body weight changes (a) and average daily food intake (b) of rats calculated for the 2 pretreatment days prior to the presurgical fast, and for day 1, days 2-4, days 5-7, and days 1-7 following removal of 70% of the jejuno-ileum. Animals were fasted on days 0 and 1. Values are means±S.E.M. for 9 rats in each group. Treatment groups were vehicle (■), 2.0 mg IGF-I/kg bw/d (▨), 2.0 mg des(1-3)IGF-I/kg bw/d (▩), and 2.0 mg LR3IGF-I/kg bw/d (▨).

approximately 10g body weight was incurred during the 24h prior to resection. Removal of 70% of the jejuno-ileum resulted in the control animals losing a further 7.5±0 8g (mean±SEM) over the first recovery day (Fig. 5.3(a)), of which

approximately 4.5g could be accounted for by the resected tissue. In comparison, the 80% resection control groups of the previous experiments, lost 13.2-15.0g over the same period, of which about 5g was excised gut (see Sections *2.3.2, 3.3.2* and *4.3.2*). During days 2-4 following 70% resection the vehicle-treated controls gained weight at 4.5g/d, approximately two-thirds their growth rate prior to fasting or surgery (Fig. 5.3(a)). This contrasts with the stasis in growth observed in the vehicle-treated animals after 80% resection. Growth for the control animals over days 5-7 following 70% resection was also greater than that previously observed for 80% resected controls, so that over the entire treatment period, the controls in this experiment gained 22.2g relative to their presurgical weights (day 0), whereas the 80% resection controls barely restored their presurgical weight by day 7.

There was a trend towards increased body weight gain with IGF-I peptide treatment, but in contrast to the 80% resection studies this effect failed to achieve statistical significance in any of the recovery periods (Fig. 5.3(a)). Restoration of presurgical (day 0) and prefasting (day -1) body weights, which occurred on days 3 and 5, respectively, in the controls, were both achieved a day earlier in the animals receiving IGF peptides.

Prior to the presurgical fast the animals used in this experiment were consuming less food (g/d) than those of previous experiments. This difference was a consequence of their relatively smaller body size, since pretreatment food intake expressed relative to body weight was similar to that of the other experiments. Although the amount of food consumed (g/d) in each of the experiments following surgery was virtually identical, irrespective of whether 70% or 80% of the jejuno-ileum had been removed (see Sections *2.3.2, 3.3.2* and *4.3.2*), food intake relative to pretreatment intake was greater following 70% resection, than after 80% resection. Thus, pretreatment levels of food intake were restored during days 5-7 after 70%, but not 80%, resection. As with the

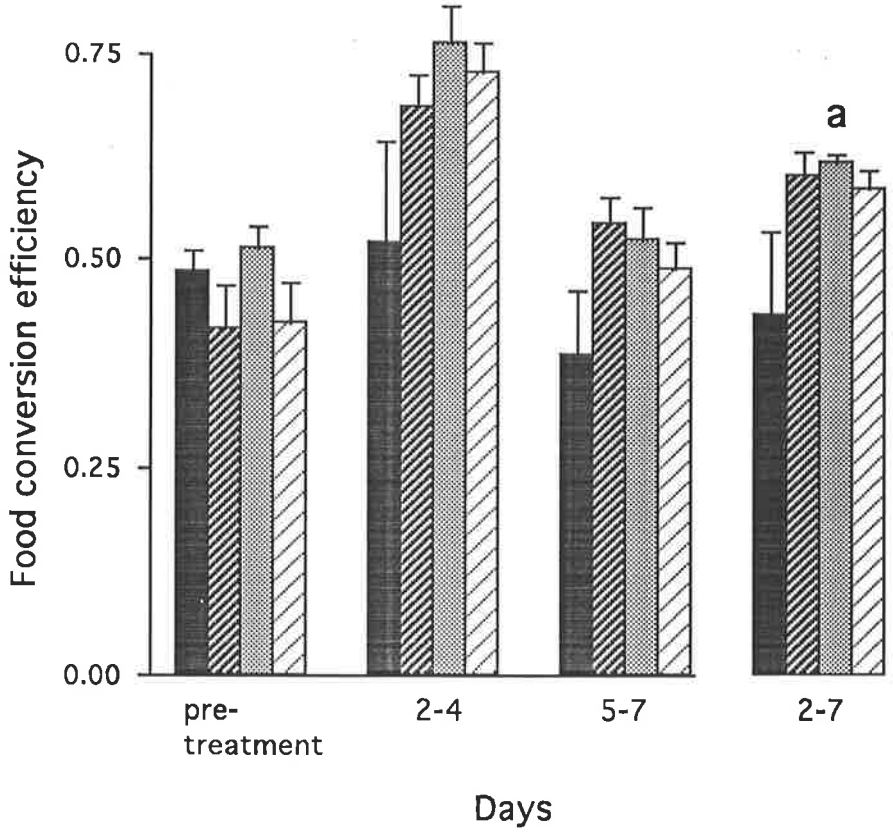

Fig. 5.4 Food conversion efficiency (g body weight gain/g food intake) for the 2 pretreatment days prior to the presurgical fast, and for days 2-4, days 5-7, and days 2-7 following removal of 70% of the jejuno-ileum. Values are means±SEM for 9 rats in each group. Treatment groups were as in Fig. 5.3. **a**: $p< 0.05$; **b**: $p< 0.01$; **c**: $p< 0.001$ versus the vehicle group.

previous investigations, peptide administration had no effect on food intake (Fig 5.3(b)).

Food conversion efficiency during the treatment period was calculated as described in section *2.2.2* (Fig. 5.4), and for the pretreatment period using only the two days when food was provided (days -2 and -1). No differences in the efficiency of food conversion existed between groups prior to surgery. Whereas food conversion efficiency had been markedly reduced, especially in the vehicle-treated rats, during days

2-4 following 80% resection (see Sections *2.3.2, 3.3.2* and *4.3.2*), food conversion efficiency over the same period actually increased after 70% resection. This seemingly unlikely result was due to removal of 70% of the jejuno-ileum initially having less effect on body weight gain than food intake. Over days 5-7, when food intake was restored to pretreatment levels, food conversion efficiency in the vehicle-treated controls returned to pretreatment values. Food conversion efficiency, like body weight gain, was higher throughout the recovery period for the 70% rececte rats compared to the 80% resection animals, such that for days 2-7, food conversion efficiency in the 70% resection controls was approximately double that of the 80% resection control groups.

Treatment with IGF peptides following 70% resection tended to improve food conversion efficiency throughout the recovery period, with the effect being significant over days 2-7 for the animals receiving des(1-3)IGF-I. Over the combined treatment period (days 2-7), food conversion efficiency, relative to the vehicle-treated controls, was improved approximately 40% by the average of all peptide treatments. This tendency for enhanced food conversion efficiency in the IGF-treated animals appeared to be consistent over the days 2-4 and 5-7 phases.

5.3.2 Gut effects: adaptive responses to intestinal resection

In this experiment, nine rats, the "day 0 controls", were killed on day 0 without intestinal resection to provide baseline information on gut weights and function. Total gut weight relative to body weight on day 0 for these animals was 36.1±1.0 g/kg bw (mean±SEM). The magnitude of the adaptive response to intestinal resection itself is indicated by the fact that despite removal of 70% of the jejuno-ileum, a week earlier, the relative total gut weight for the vehicle-treated controls at kill (36.4±3.2 g/kg bw) was virtually identical to that of the unresected day 0 controls.

Compensatory regrowth in the resected rats was most obvious in the segmental weights (g/cm) of the duodenum, jejunum and ileum (Tables 5.1 and 5.2), which were 2-3 times heavier in the vehicle-treated controls than the day 0 controls, and in the relative weights of the caecum (g/kg bw), in which 2 fold increases were noted (6.93±0.76 versus 3.37±0.13 g/kg bw for the vehicle-treated and day 0 controls, respectively). Adaptive growth in the duodenum occurred preferentially in the mucosa+submucosa layers, since the mucosa+submucosa weight as a percentage of total weight was 83.8% in the vehicle-treated resected controls compared to 68.7% in the day 0 controls (Table 5.2). In contrast to the duodenum, the ratio of mucosa+submucosa weight to muscularis weight in the jejunum, ileum and colon was not affected by resection (Table 5.2), indicating proportional responses in the layers of these segments.

Post-resectional mucosal hyperplasia in the duodenum, jejunum and ileum is revealed by the markedly increased DNA content of these regions in the resected controls compared to the day 0 controls (Table 5.3). In contrast, hypertrophy of colonic mucosal cells seems to account for the increase in colon weight of the resected controls as higher protein, but not DNA, content per cm relative to the day 0 controls is evident (Table 5.3).

In the muscularis externa layer of both the small and large intestine, both protein and DNA content per cm are increased in the resected animals relative to the day 0 controls (Table 5.4). Since the increase in protein was proportionally greater than that of DNA in these regions, a combination of hyperplasia and hypertrophy seems to be the mechanism underlying post-resectional increments in jejunum, ileum and colon muscularis externa weights.

Some of the increase in duodenum weight following resection can be accounted for by intestinal elongation since duodenal length was 43% greater in the resected

TABLE 5.1 Gut weights and lengths in 70% gut-resected rats following 7 days of peptide treatment

	Treatment groups				
	Vehicle	IGF-I (2.0 mg)	des(1-3)IGF-I (2.0 mg)	LR^3IGF-I (2.0 mg)	Day 0 Controls§
Final body weight (g)	135.7±4.0	144.1±4.3	144.0±2.6	140.5±4.1	114.8±1.5
Stomach weight (g)	0.691±0.015	0.875±0.031[c]	0.916±0.027[c]	0.853±0.016[c]	0.647±0.012
Duodenum length (cm)	10.0±0.2	10.3±0.2	10.3±0.2	10.2±0.2	7.0±0.3
Duodenum weight (g)	1.061±0.076	1.168±0.069	1.213±0.035[a]	1.247±0.036[a]	0.440±0.011
Jejunum length (cm)	9.5±0.7	10.9±1.1	9.9±0.7	10.0±0.5	34.7±1.1
Jejunum weight (g)	1.072±0.103	1.227±0.103	1.328±0.133	1.210±0.051	1.384±0.066
Ileum length (cm)	12.8±0.9	12.8±0.9	14.7±1.1	13.7±0.9	34.7±1.1
Ileum weight (g)	1.061±0.084	1.114±0.089	1.307±0.075	1.196±0.094	1.386±0.077
Caecum weight (g)	0.922±0.074	0.969±0.088	0.967±0.075	0.888±0.048	0.386±0.012
Colon length (cm)	9.5±0.5	9.8±0.6	9.5±0.4	9.6±0.6	8.6±0.2
Colon weight (g)	0.726±0.052	0.782±0.038	0.852±0.043	0.817±0.044	0.570±0.009
Total gut weight (g)	5.533±0.231	6.135±0.243[a]	6.583±0.178[c]	6.211±0.193[a]	4.144±0.135

Values are Means ± SEM: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ versus vehicle-treated animals.
§ animals killed, instead of gut resected, on day 0.
Doses are per kg body weight per day.

TABLE 5.2 Mucosa+submucosa and muscularis externa weights in rats following 70% jejuno-ileal resection and 7 days of IGF-I treatment.

Treatment group	mucosa+sub-mucosa weight mg/cm	muscularis externa weight mg/cm	mucosa+sub-mucosa weight as % of total weight
	Duodenum		
Vehicle	85.0±4.6	16.8±2.2	83.8±1.6
IGF-I (2.0 mg/kg bw/d)	90.6±3.5	16.1±1.9	85.1±1.4
des(1-3)IGF-I (2.0 mg/kg bw/d)	91.4±5.0	19.9±2.1	81.8±2.2
LR^3IGF-I (2.0 mg/kg bw/d)	91.3±2.9	20.8±3.0	81.8±2.1
Day 0 controls§	39.9±1.6	18.8±2.0	68.7±2.2
	Jejunum		
Vehicle	75.2±10.4	38.4±7.7	68.1±2.5
IGF-I (2.0 mg/kg bw/d)	75.7±4.9	34.5±3.6	68.9±1.7
des(1-3)IGF-I (2.0 mg/kg bw/d)	85.2±6.4	41.5±7.4	68.5±3.1
LR^3IGF-I (2.0 mg/kg bw/d)	80.4±5.4	34.2±2.5	70.1±1.2
Day 0 controls§	26.2±1.4	11.0±0.9	70.7±2.3
	Ileum		
Vehicle	50.7±4.7	28.4±1.9	64.3±2.8
IGF-I (2.0 mg/kg bw/d)	51.2±2.9	32.3±4.3	62.2±2.8
des(1-3)IGF-I (2.0 mg/kg bw/d)	52.8±3.9	30.4±3.2	63.8±2.4
LR^3IGF-I (2.0 mg/kg bw/d)	53.7±3.1	30.8±1.6	63.2±2.3
Day 0 controls§	23.7±1.1	13.5±1.2	63.9±2.8
	Colon		
Vehicle	19.7±1.7	52.2±2.7	27.2±1.4
IGF-I (2.0 mg/kg bw/d)	20.7±3.3	56.8±4.6	25.5±2.8
des(1-3)IGF-I (2.0 mg/kg bw/d)	22.9±1.7	59.9±4.4	28.0±2.1
LR^3IGF-I (2.0 mg/kg bw/d)	21.0±1.8	57.0±4.1	27.1±1.6
Day 0 controls§	15.9±0.9	41.0±1.4	28.0±1.2

Values are means ± SEM for 9 animals in each group.
§ animals killed, instead of gut resected, on day 0.

TABLE 5.3 DNA and protein contents in intestinal mucosa+submucosa of rats following 70% jejuno-ileal resection and 7 days of IGF-I treatment.

Treatment group	Protein mg/cm	DNA µg/cm	Protein:DNA µg/µg
	Duodenum		
Vehicle	7.96±0.66	375.5±35.5	22.1±2.0
IGF-I (2.0 mg/kg bw/d)	8.87±0.50	437.0±39.0	20.9±1.2
des(1-3)IGF-I (2.0 mg/kg bw/d)	8.72±0.61	441.2±35.6	20.1±0.8
LR^3IGF-I (2.0 mg/kg bw/d)	8.73±0.48	427.6±29.4	20.7±0.8
Day 0 controls§	2.22±0.23	137.2±12.6	18.1±2.3
	Jejunum		
Vehicle	6.48±1.01	315.4±36.2	20.8±2.0
IGF-I (2.0 mg/kg bw/d)	7.27±0.60	385.2±25.7	19.2±1.5
des(1-3)IGF-I (2.0 mg/kg bw/d)	8.20±0.81	452.3±46.2	18.5±1.1
LR^3IGF-I (2.0 mg/kg bw/d)	7.47±0.38	361.6±17.2	20.9±1.3
Day 0 controls§	2.23±0.15	75.14±6.8	29.7±2.0
	Ileum		
Vehicle	5.04±0.55	214.8±25.8	24.5±1.9
IGF-I (2.0 mg/kg bw/d)	4.75±0.25	237.7±18.8	20.7±1.4
des(1-3)IGF-I (2.0 mg/kg bw/d)	5.52±0.39	239.4±14.0	23.3±1.3
LR^3IGF-I (2.0 mg/kg bw/d)	5.41±0.30	239.7±17.5	23.2±1.6
Day 0 controls§	2.03±0.13	66.9±10.9	37.7±6.2
	Colon		
Vehicle	1.63±0.12	91.5±7.0	18.1±1.1
IGF-I (2.0 mg/kg bw/d)	1.79±0.28	98.8±17.4	18.8±1.4
des(1-3)IGF-I (2.0 mg/kg bw/d)	2.07±0.14	95.2±8.1	22.8±3.0
LR^3IGF-I (2.0 mg/kg bw/d)	1.85±0.14	101.8±6.7	18.5±1.4
Day 0 controls§	1.01±0.08	91.0±7.2	11.3±0.6

Values are means ± SEM for 9 animals in each group.
§ animals killed, instead of gut resected, on day 0.

TABLE 5.4 DNA and protein contents in intestinal muscularis externa of rats following 70% jejuno-ileal resection and 7 days of IGF-I treatment.

Treatment group	Protein mg/cm	DNA µg/cm	Protein:DNA µg/µg
	Duodenum		
Vehicle	2.03±0.43	56.4±16.6	39.1±4.4
IGF-I (2.0 mg/kg bw/d)	1.64±0.29	52.1±8.1	32.5±3.0
des(1-3)IGF-I (2.0 mg/kg bw/d)	2.44±0.43	73.3±16.7	35.3±2.6
LR^3IGF-I (2.0 mg/kg bw/d)	2.65±0.47	85.2±21.8	35.2±2.9
Day 0 controls§	2.41±0.28	92.7±17.3	30.7±5.8
	Jejunum		
Vehicle	4.84±0.98	134.3±40.0	38.6±3.4
IGF-I (2.0 mg/kg bw/d)	4.86±0.48	113.1±16.5	40.0±4.5
des(1-3)IGF-I (2.0 mg/kg bw/d)	4.88±0.87	139.5±18.3	37.2±2.4
LR^3IGF-I (2.0 mg/kg bw/d)	4.33±0.30	118.5±7.9	37.0±2.2
Day 0 controls§	1.70±0.10	79.7±5.4	21.6±1.1
	Ileum		
Vehicle	3.41±0.34	106.4±8.8	32.1±1.9
IGF-I (2.0 mg/kg bw/d)	3.72±0.36	124.6±14.4	30.6±2.0
des(1-3)IGF-I (2.0 mg/kg bw/d)	3.77±0.37	134.0±13.3	28.6±2.0
LR^3IGF-I (2.0 mg/kg bw/d)	3.75±0.33	119.6±10.8	31.7±1.2
Day 0 controls§	1.74±0.14	91.7±8.1	19.2±0.7
	Colon		
Vehicle	6.26±0.46	238.6±10.4	26.3±1.8
IGF-I (2.0 mg/kg bw/d)	6.85±0.54	251.2±23.0	28.1±2.0
des(1-3)IGF-I (2.0 mg/kg bw/d)	6.89±0.71	262.1±22.8	27.4±3.7
LR^3IGF-I (2.0 mg/kg bw/d)	6.61±0.58	258.4±21.1	26.4±2.5
Day 0 controls§	4.90±0.17	218.9±9.5	22.6±0.9

Values are means ± SEM for 9 animals in each group.
§ animals killed, instead of gut resected, on day 0.

controls than the day 0 controls (Table 5.1). However, as duodenal weight was 240% heavier in the resected controls, the contribution of elongation to the overall increase in duodenal mass subsequent to resection is clearly less substantial than the increase in cross-sectional area (Table 5.1 and 5.2). At kill, the jejuno-ileal length of the resected controls was 22.3cm (Table 5.1), only marginally more than the 20cm left at surgery. Thus, elongation of the jejuno-ileum did not appear to be a major compensatory mechanism in the post-resectional adaptation of these animals. Similarly, only minor, non-significant increases in colonic length occurred following resection (Table 5.1).

Sucrase activity was measured in the mucosa+submucosa from the duodenum, jejunum and ileum of the resected and day 0 control animals (Table 5.5). Functional immaturity of the hyperplastic tissue is suggested by the marked reductions in sucrase activity per cell (per mg DNA) in the jejunum and ileum of the resected animals relative to the unresected day 0 control rats. However, as a consequence of hyperplasia, sucrase activity per cm in the jejunum and ileum of the resected animals was equivalent to that of the day 0 control rats. In the duodenum, sucrase activity in the resected rats was similar to the day 0 controls when expressed per mg DNA, but much higher when expressed per cm.

5.3.3 Effects of IGF-I peptides on gut growth

Administration of IGF-I peptides following 70% jejuno-ileum resection increased total gut weights (Table 5.1), with the magnitude of the treatment effect being virtually identical to that observed following 80% resection (see sections *3.3.6* and *4.3.6*). Thus, relative to the respective vehicle-treated controls, total gut weights were increased 11-19% following 70% resection and 13-19% following 80% resection after treatment with comparable doses of the same peptides. Although treament with IGF-I peptides did not significantly increase jejunum and ileum weights relative to the vehicle-treated animals, augmentation of compensatory regrowth by these peptides is apparent, since only the weights of the remnant jejunum and remnant ileum in the vehicle-treated

animals were significantly less ($p<0.05$) than the intact jejuno-ileal weight of the day 0 controls (Table 5.1). No IGF effects on intestinal lengths were apparent (Table 5.1).

Although infusion of IGF-I peptides following 70% resection tended to increase mucosa+submucosa and muscularis externa weights (g/cm) (Table 5.2) and correspondingly DNA and protein content (Tables 5.3 and 5.4) in each of the intestinal segments, as in the 80% experiment (see Section *4.3.6*) none of these changes were statistically significant. As was the case following 80% jejuno-ileal resection (see Section *4.3.6*), increases in intestinal weights relative to the vehicle-treated controls were observed without changes in the protein to DNA ratio in either the mucosa+submucosa or muscularis layers.

In this experiment the treated groups, especially those administered IGF-I, tended to have lower levels of specific sucrase activity (per mg DNA, and per cm) in the duodenum, jejunum and ileum than the vehicle group (Table 5.5). However, as a consequence of the increased mucosal mass in the treated animals, the total sucrase activity of the intestinal segments revealed no differences to the controls. The general trends seen for sucrase activity in this experiment agree with those described for jejunal levels following 80% resection (see Section *4.3.7*), although the level of jejunal sucrase activity/mg DNA observed for the 70% resection animals was generally double that of the 80% resection rats, perhaps due to the lesser severity of the 70% resection procedure.

In this experiment, the IGF-I analogues, des(1-3)IGF-I and LR^3IGF-I, failed to demonstrate an enhanced potency relative to IGF-I in any of the measurements made of gut regeneration.

Changes in experimental protocol were introduced for this investigation with the express aim of reducing variability in assessments of post-resection gut adaptation.

TABLE 5.5 Sucrase activity in intestinal mucosa+submucosa of rats following 70% jejuno-ileal resection and 7 days of IGF-I treatment.

Treatment group	Sucrase activity: total activity	activity/cm	activity/mg DNA
	Duodenum		
Vehicle	7.49±0.70	0.755±0.076	2.17±0.30
IGF-I (2.0 mg/kg)	6.48±0.68	0.624±0.062	1.53±0.23
des(1-3)IGF-I (2.0 mg)	6.84±0.68	0.670±0.071	1.69±0.32
LR^3IGF-I (2.0 mg/kg)	7.78±0.68	0.767±0.072	1.92±0.27
Day 0 controls§	1.41±0.23	0.215±0.030	1.71±0.29
	Jejunum		
Vehicle	7.01±1.13	0.826±0.200	2.51±0.42
IGF-I (2.0 mg)	6.83±1.26	0.611±0.074	1.59±0.18
des(1-3)IGF-I (2.0 mg)	8.19±1.30	0.817±0.104	1.83±0.22
LR^3IGF-I (2.0 mg)	7.39±0.98	0.757±0.102	2.19±0.38
Day 0 controls§	24.08±1.19	0.708±0.037	9.42±0.49
	Ileum		
Vehicle	6.90±1.23	0.538±0.082	2.82±0.62
IGF-I (2.0 mg)	5.72±1.21	0.426±0.063	1.82±0.24
des(1-3)IGF-I (2.0 mg)	7.38±1.53	0.484±0.087	2.15±0.44
LR^3IGF-I (2.0 mg)	7.31±1.75	0.500±0.105	2.21±0.56
Day 0 controls§	18.70±2.18	0.546±0.060	9.43±1.18

Values are means ± SEM for 9 animals in each group.
Doses are per kg body weight per day.
§ animals killed, instead of gut resected, on day 0.
Sucrase activity is expressed as moles of glucose generated/min, "total activity" is the product the intestinal length (cm) x "activity/cm".

However, since the variance in gut wet weights and biochemical assessments of growth, as a proportion of mean values, was similar in this experiment to that observed in Experiment 2 (see Chapter 4), the modifications in methodology were not successful in this respect.

5.3.4 Fat and nitrogen absorption

Since the faeces for the three pretreatment days was pooled, but food was only available on two of these days, pretreatment means for fat excretion as a percent of intake were determined by extrapolating the food intakes from the initial two pretreatment days to give three days feed. The derived pooled pretreatment mean for percentage fat excretion was 4.38±0.14%, virtually identical to that calculated from all the animals used in Experiment 2 (4.48±0.10%). The less severe nature of a 70% jejuno-ileal resection, compared to an 80% resection, was evident in the lower levels of fat malabsorption during post-surgery days 2-4 (see section *4.3.7*) (Fig. 5.5(a)). Relative to their pretreatment levels, six fold increases in percentage fat excretion were observed for the controls after an 80% resection. Following a 70% resection, the percentage fat excretion for the controls only increased 3.6 times during days 2-4.

Although the decrements in fat absorption were less following a 70% jejuno-ileal resection than an 80% resection, the effect of IGF treatment on restoring net absorption was greater. For days 2-4, percentage fat excretion for the treated groups was significantly less than for the controls, with the percent excretion levels of the IGF-I and des(1-3)IGF-I groups less than half that of the vehicle-treated animals. By days 5-7, fat absorption in the des(1-3)IGF-I treated animals had been restored to pretreatment levels. For the combined period (days 2-7), each of the treated groups had significantly lower percentage fat excretion values than the controls.

The less severe nature of a 70%, compared to an 80%, resection is also reflected in the differing degrees of post-resectional nitrogen malabsorption. Comparing the respective control groups, percentage nitrogen excretion throughout the recovery phases following 70% jejuno-ileal resection was approximately half that observed after resecting 80% (see section *4.3.7*) (Fig. 5.5(b)).

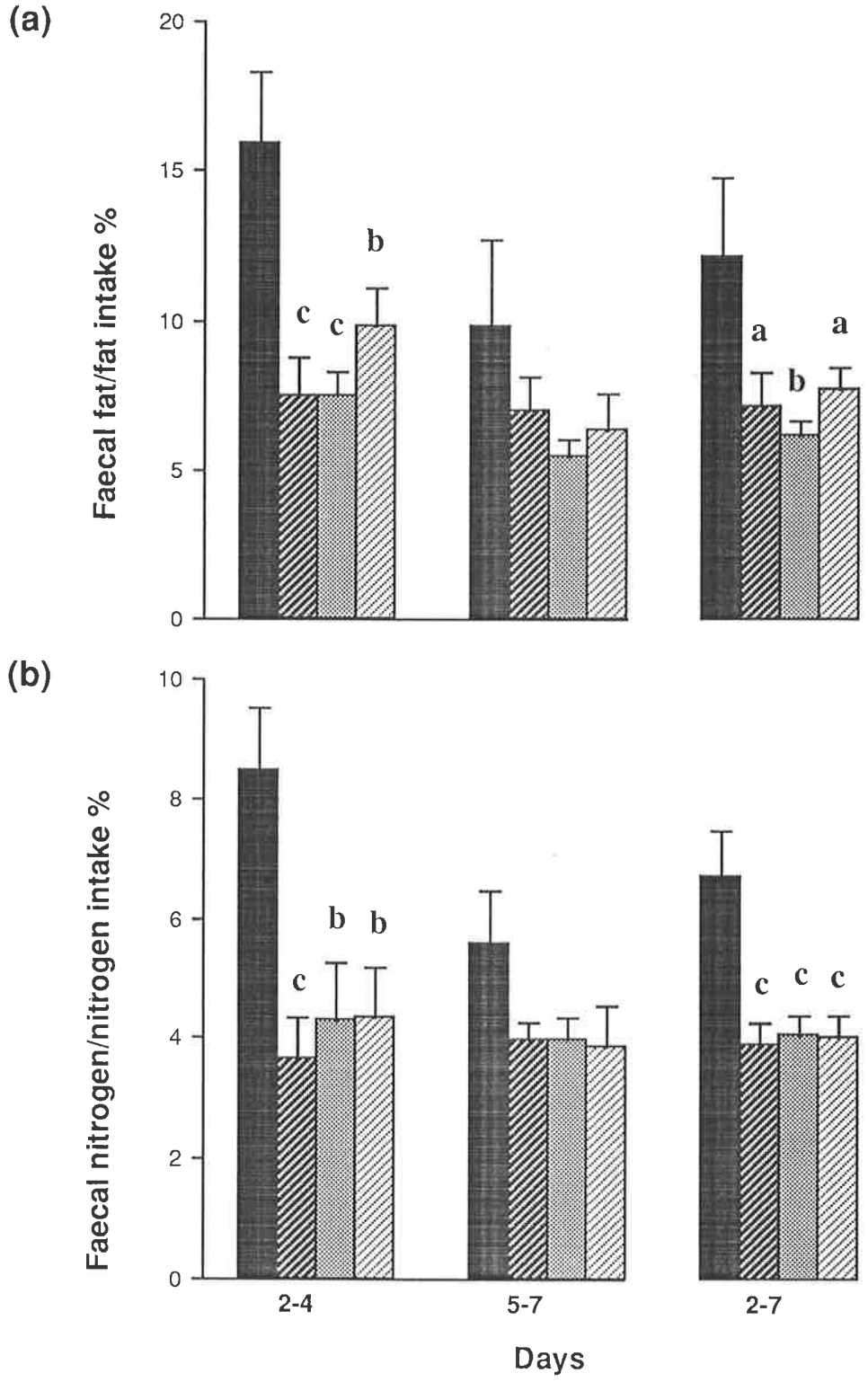

Fig. 5.5 Faecal fat (a) and faecal nitrogen (b) expressed as a percent of fat and nitrogen intake, respectively. The % excretion for fat and nitrogen is shown for days 2 through 4 (2-4), days 5 through 7 (5-7), and days 2 through 7 (2-7) following 70% jejuno-ileal resection. Values are means±SEM for 9 rats in each group. Treatment groups were as in Fig. 5.3. **a**: $p< 0.05$; **b**: $p< 0.01$; **c**: $p<0.001$ versus the vehicle group.

The effects of peptide-treatment on restoring nitrogen absorption, as with fat absorption, were more apparent in the 70% resection model than the 80% model. Administration of IGF-I peptides significantly improved net nitrogen absorption during the initial recovery phase (days 2-4) following 70% resection, with the percentage excretion of nitrogen in the IGF-treated groups being approximately half that of the vehicle-treated animals. During days 5-7, nitrogen excretion values for the treated-groups were about 30% lower than for the controls, although these differences were not statistically significant. However, for the combined period (days 2-7) the percentage excretion of nitrogen of the treated groups was significantly less ($p< 0.001$) than that of the controls. At no stage following 70% resection was post-resectional nitrogen malabsorption indicated in the animals administered IGFs. In contrast, the average percentage nitrogen excretion for the controls over the combined period (days 2-7) was significantly elevated compared to the derived pretreatment value ($4.80\pm0.36\%$).

As with the measures of gut growth, no improvements in variability relative to the previous experiment (see Section *4.3.8*) were apparent for the measures of absorption made in this investigation.

5.4 DISCUSSION

5.4.1 Measurement variability

Features of the protocol of Vanderhoof et al. (1992b) were adapted to this experiment in a bid to replicate their very low measurement variability. Since the variance for measures of gut growth and function obtained in this experiment were proportionally similar to that observed in Experiment 2 (see Section *4.4.4*), the modifications were unsuccessful in this regard. However, the magnitude of variability observed in Experiments 2 and 3 is comparable to, or better than, that reported elsewhere for measurements of mucosal wet weight (Erdman et al., 1989; Hart et al.,

1987, 1988; Grey and Morin, 1985; Vanderhoof et al., 1988), mucosal DNA content (Erdman et al., 1989; Ford et al., 1984; Hart et al., 1987, 1988; Park et al., 1989; Schulman et al., 1992; Vanderhoof et al., 1988), mucosal protein content (Erdman et al., 1989; Ford et al., 1984; Hart et al., 1987, 1988; Schulman et al., 1992; Vanderhoof et al., 1984; Weser and Hernandez, 1971), and mucosal sucrase activity (Ford et al., 1984; Hart et al., 1987, 1988; Park et al., 1989; Schulman et al., 1992; Vanderhoof et al., 1988; Weser and Hernandez, 1971) in intestinal samples from gut-resected rats. The magnitude of variability recently reported by Vanderhoof et al. (1992a,b) is considerably less than that reported elsewhere, and the reasons for this are unclear.

Due to the transient catabolic state and the hyperplastic condition of the gut, it is expected that gut-resected rats would demonstrate more variability in gut parameters than normal rats. Indeed, this is evident in the current study, with the variance for gut measures in the day 0 controls being considerably less than that for otherwise identical rats that have undergone gut resection. The inherent variability of catabolic models, such as the gut-resection model, may account for the statistical non-significance of some of the apparent effects of IGF-I peptide treatment. For example, administration of IGF-I peptides to rats in this study increased body weight up to 41% over days 1-7, compared to gains of 25% following 7 days infusion to normal rats (Ballard et al; 1992). However, due to the contribution of variance, the effect was only significant in the normal rat study (Ballard et al., 1992).

5.4.2 70% versus 80% gut-resection: The effects of IGF treatment

As described in Section *2.1*, removal of 80% of the jejuno-ileum in rats caused transient body weight loss with growth being retarded for a week or longer. On the other hand, 70% jejuno-ileal resection had little effect on somatic growth, apart from losses over the initial 24h. From this it is logical to assume that a 70% resection imposes a more moderate stress on the animal than an 80% resection. Therefore,

Experiment 3 allows us to review the effects of IGF-I peptide treatment in a model of moderate, as opposed to severe, stress.

Although trends were noted, growth rates were not significantly increased by administration of 2.0 mg/kg bw/d of IGF-I, des(1-3)IGF-I or LR^3IGF-I following 70% jejuno-ileal resection. In contrast, significant effects were noted when comparable doses (2.4 mg/kg bw/d in Experiment 1, 1.8 mg/kg bw/d in Experiment 2) were administered after 80% resection. Thus it appears that the effects of IGF treatment on body growth are more pronounced in catabolic conditions.

The effect of IGF peptides to improve food conversion efficiency following 70% jejuno-ileal resection agrees with the response observed in our 80% resected rats (see sections *3.3.2* and *4.3.2*), as well as a number of other catabolic or growth-retarded models (Martin et al., 1991; Tomas et al., 1991a,b,c; Skottner et al., 1989). In each of these other models, including 80% resection, peptide treatment helped to restore food conversion efficiency back to normal levels. In the current study we have demonstrated that administration of IGF peptides can elevate normal conversion efficiencies to supranormal levels.

The responsiveness of the gut, especially the upper gastro-intestinal tract, to IGF peptides is again evident in this investigation. As mentioned previously (see section *3.4.3*), the exceptional sensitivity to IGFs shown by the stomach of resected rats may in part be due to a fasting-induced reduction in local IGF-I production and increase in gastric type-1 receptor number (Lowe et al., 1989). If this effect were operating following resection, the stimulation of gastric growth by IGFs should be more pronounced in the 70% resection model due to the imposition of a presurgical fast, and indeed this appears to be the case.

The magnitude of effect of IGF treatment on total gut weight was very similar following either 70% or 80% gut-resection. Comparison with other studies in which IGFs were administered to catabolic rats (Tomas et al., 1991a,c) indicated that the responsiveness of the gut to IGFs was inversely related to the proliferative state of the gut (see Section 4.4.4). Therefore, it may have been anticipated that the gut would be more sensitive to IGFs following 70% resection than 80% resection. However, comparison of the gut organ weights, intestinal mucosa+submucosa and muscularis weights, and intestinal protein and DNA contents, of the control animals of Experiments 2 and 3 fails to identify clear differences between the proliferative states provoked by 70% or 80% resection. Thus, although an 80% resection clearly invokes a more catabolic condition than a 70% resection, the magnitude of effect on the gut of the two procedures appears to be the same. As such, the similarity in gut sensitivity to IGFs in Experiments 2 and 3, as determined by organ weights and gut biochemistry, is consistent with the belief that responsiveness to IGFs is determined by the proliferative state of the gut.

The biochemical analyses suggested that the accretion of intestinal mass in response to IGF treatment following 70% resection was due to a generalized growth response, wherein each of the cross-sectional layers was marginally increased. As the protein:DNA ratio, throughout the gut, was not affected by peptide treatment, it appears that IGF-I promotes growth in the regenerating gut by further elevating a heightened hyperplastic condition. The conclusion of generalized, proportional growth and augmented hyperplasia are consistent with indications given by our previous biochemical and histological findings, as well as those of Vanderhoof et al. (1992b).

Sucrase activity per mg DNA in the mucosal scrapings taken from the duodenum, jejunum and ileum of the peptide-treated animals tended to be lower than that in mucosa from the control group. This result supports our previous conclusion (see section *4.4.4*) that IGF-I peptides do not accelerate the rate of cellular maturation.

Contrary evidence, however, is provided by Vanderhoof et al. (1992b), who found ileal sucrase and maltase activity per mg DNA to be increased in 80% jejuno-ileal resected rats following IGF-I and des(1-3)IGF-I infusion. As these investigators only left 4cm of the proximal jejunum, whilst retaining more of the ileum than I did, the increased specific ileal sucrase activity of their animals may be due to greater luminal stimulation or an adaptive response to diminished total disaccharidase activity in the jejunum. However, in considering these results it is important to keep in mind that the DNA content used to provide an estimation of sucrase activity per cell, is derived from the combined mucosa plus submucosa layers. As such, this measure may give values that do not accurately reflect the sucrase activity of the epithelial cells.

The enhancement of fat and nitrogen absorption in 70% jejuno-ileal resected rats after IGF-I treatment is consistent with the improvements observed following 80% resection (see Section *4.3.8*), as well as the trend toward increased mucosal weight in the animals receiving IGF peptides.

In the animals subjected to an 80% jejuno-ileal resection, restoration of absorptive capacity by IGF treatment was more pronounced in the latter recovery period (days 5-7). By contrast, treatment with IGF-I peptides conferred most of its benefit following 70% resection during days 2-4. This difference in the timing of maximal IGF effect is best accounted for by the differing severities of catabolism induced by the two experimental procedures. Since removal of 70% of the jejuno-ileum, in contrast to 80%, has little effect on growth in rats, it is likely that catabolism is considerably less in the 70% resection model. Due to the reduced stress associated with this model, it may be argued that the rats having only 70% of the jejuno-ileum removed were better placed to make an earlier recovery. Supporting this are the similarities between the percentage excretion of nitrogen and fat values of the 70% resection rats over days 2-4, and those of the 80% resection rats during days 5-7.

The overall effect of IGF treatment on improving absorption was greater in the 70% resection animals than in the 80% resection animals. Relative to their respective control values, the net absorption of fat and nitrogen of the treated groups over days 2-7 improved 42% and 40% respectively in the 70% resection experiment, and 21% and 23% respectively in the 80% resection study. The greater response noted following 70% resection, may again be related to the more moderate catabolic stress involved with this model.

5.4.3 Action of the IGF-I analogues in the gut

The two analogues used in the 70% and 80% resection experiments, des(1-3)IGF-I and LR^{3}IGF-I were generally more effective in improving body weight gain, nitrogen retention, food conversion efficiency and stimulating thymus, spleen and stomach growth than IGF-I (see Sections *3.3*, *4.3* and *5.3*). These results are consistent with numerous *in vitro* and *in vivo* findings (see Sections *1.4.11*, *3.4* and *4.4*), including an enhanced effect of des(1-3)IGF-I and LR^{3}IGF-I, relative to IGF-I, in increasing total gut weight in dexamethasone-treated rats (Read et al., 1992). The superior potency of the variants compared with IGF-I is ascribed to their reduced affinity for the binding proteins (Forbes et al., 1988; Walton et al., 1990) which may facilitate delivery from the blood to the tissues and, consequently, improve peptide access to the receptors. Indeed, des(1-3)IGF-I, relative to IGF-I, has been shown to be preferentially transferred to the gut following bolus intravenous infusion into rats (Ballard et al., 1991a). With their generally superior potency in mind, it was surprising that the analogues were not more effective than IGF-I in improving gut growth and nutrient absorption in the 70% or 80% gut resected animals. An explanation for this paradox may lie in a recent finding that ileal IGFBP-3 mRNA in rats is reduced by approximately 70% following intestinal resection (Albiston et al., 1992). This effect occurs almost immediately after surgery and persists for at least seven days. If the decrease in IGFBP-3 synthesis were common to other intestinal segments, the inherent advantage of the analogues over IGF-I could be lost in these regions. Given such a

situation, one could anticipate that IGF-I, des(1-3)IGF-I and LR^3IGF-I would be equipotent in their specific gut effects.

This investigation presents effects or trends following administration of IGF-I peptides to 70% jejuno-ileal resected rats that are consistent with the improved body weight gain, food conversion efficiency, gut growth and enhanced nutrient absorption observed in 80% resected rats following IGF treatment. Thus, IGF-I therapy appears to be effective in conditions characterized by either severe and moderate catabolism.

CHAPTER 6

General Discussion

6.1 SUITABILITY OF IGF-I PEPTIDES AS A POST-RESECTIONAL THERAPY

The clinical problem of providing adequate nutritional support to short gut syndrome (SGS) patients is increasingly encountered due to improvements in surgical procedures and intensive care which have increased the survival rates of those undergoing extensive small bowel resection (Pigot et al., 1990). However, despite intensive investigation there are no drugs of treatments currently available that adequately support these patients. As a consequence, SGS patients often need to be maintained by TPN for long periods. In addition to being expensive, inconvenient and traumatic, TPN is associated with various secondary conditions (Caldwell et al., 1972; Cooper et al., 1984; Rombeau and Rolandelli, 1987; Sprocata et al., 1980), and results in mucosal atrophy (Morin et al., 1978), thereby exacerbating malabsorption and increasing the dependence on parenteral nutrition.

As explained in Chapter 1, the ideal therapeutic agent for massive gut resection would be one which improved nitrogen balance, enhanced compensatory gut growth and attenuated nutrient malabsorption. In this study, I have shown that administration of IGF-I, or one of its analogues, des(1-3)IGF-I or LR^3IGF-I, significantly improves body weight gain, food conversion efficiency, nitrogen balance, gut growth, and fat and nitrogen absorption in rats following extensive jejuno-ileal resection. Additionally, reproducible trends toward decreased muscle protein breakdown as indicated by urinary 3-methylhistidine excretion, and accretion of mucosal mass by augmentation of adaptive hyperplasia, have been demonstrated. As such, administration of IGF-I peptides has achieved each of the outcomes required for an optimal post-resectional therapy. Although a number of other drugs, hormones or nutritional strategies have been assessed in animal trials as potential treatments for SGS patients, none of these has demonstrated the ability to improve both intestinal adaptation and body weight gain, or proved to have any advantage over IGF-I as an agent for enhancing recovery from gut-resection.

Administration of epidermal growth factor (EGF) has been shown to increase intestinal regrowth in weanling (Read et al., 1986) and suckling (Read et al., unpublished) rats, as well as adult rats maintained on TPN (Goodlad et al., 1988), following small bowel resection. In contrast, EGF failed to enhance intestinal regeneration in gut-resected rabbits (Thompson et al., 1990). Thus, although EGF is known to have trophic effects in the normal gut (Chalot et al., 1983; Dembrinski et al., 1982; Goodlad et al., 1987; Puccio and Lehy, 1988), its responses in the resected gut are not consistent (Read et al., 1986, unpublished; Goodlad et al., 1988; Thompson et al., 1990). A major drawback of EGF as a post-resectional therapy is that it failed to improve body weight gain in any of the resection trials mentioned, and may even impair somatic growth (Heimberg et al., 1965; Oka et al., 1983).

Various nutritional strategies including polymeric diets (Lai et al., 1989) and dietary supplementation of linoleic acid (Park et al., 1989), short-chain triglycerides (Kripke et al., 1991) and medium--chain triglycerides (Smallridge et al., 1990) have been shown to enhance mucosal adaptation in resected rats, or reduce malabsorption in human patients (Smallridge et al., 1990). However, as with EGF, there has been no benefit on body weight gain with any of these treatments. Similarly, 16,16-dimethyl-prostaglandin-E_2 when administered to gut-resected rats increases mucosal hyperplasia, but not body weight (Vanderhoof et al., 1988). To compensate for the reduction in enterohepatic bile acid circulation, cholylsarcosine, a synthetic conjugated bile acid analog, has been administered orally to resected dogs (Longmire-Cook et al., 1992). Although increasing fat absorption 5-30 fold, this therapeutic tactic also failed to improve post-surgical body weight gain. It may be that nutritional strategies such as these would be useful adjuncts to IGF-I therapy in treating post-resectional patients, since administration of IGF-I would provide nitrogen sparing and somatic growth effects following resection, whilst also perhaps augmenting the enhanced gut adaptation achieved by the nutritional approaches described.

Glutamine has attracted recent interest as a potential therapy for SGS patients after studies had shown it to be important in the maintenance of intestinal metabolism, structure, and function. Notably, glutamine-supplemented elemental diets have been shown to stimulate intestinal mucosal growth following starvation (Salloum et al., 1989). Additionally, glutamine supplementation of TPN solutions are reported to increase jejunal mucosal weight and DNA content (Hwang et al., 1987) and significantly decreases the villus atrophy associated with standard TPN (Grant, 1988; Hwang et al., 1987). To date, TPN supplemented with glutamine has not been administered to gut-resected animals or patients, thus, its effects in this situation are unknown. However, Vanderhoof et al. (1992a) showed that oral glutamine supplementation reduced mucosal weight, and mucosal protein and DNA content in the small bowel of gut-resected rats, and from this concluded that supplemental glutamine impaired post-resectional intestinal adaptation.

Administration of the anabolic steroid, stanozolol, has been shown to improve post-operative nitrogen balance in patients receiving amino acids alone, but not those given a mixed diet, following colorectal surgery (Hansell et al., 1989). No other recent research interest has been shown in anabolic steroids as a means of reducing catabolism after major gut surgery, presumably because of their well documented side-effects. In addition to the association with liver damage, liver cancer, testicular atrophy, diabetes, muscle structure abnormalities, depressed levels of HDL-cholesterol and premature closure of epiphyseal plates described in section *1.3.1*, chronic use of anabolic steriods has now been linked to myocardial infarction and stroke (Ferenchick et al., 1992), hypertension (Cheever and House, 1992), increased tumour growth (Dauvois and Labrie, 1989), and bleeding oesophageal varices (Winwood et al., 1990). Since post-resectional therapies often need to be administered for up to two years, occasionally even longer (Dudrick et al., 1992), the use of anabolic steroids in such circumstances would appear to be injudicious.

Not surprisingly, of the therapeutic agents tried in resection studies, the one that comes closest to achieving the effects seen with IGF-I is GH. Shulman et al. (1992) observed that rats administered recombinant human GH (hGH) following 75% jejuno-ileal resection tended to gain more weight than the controls, although this effect was not statistically significant. The failure of hGH to significantly improve body weight gain in these animals can probably be attributed to the fact that serum IGF-I levels were not significantly increased by the doses of GH used, possibly reflecting a state of resistance to the anabolic actions of GH. Significant increases in ileal weight/cm and ileal mucosal height were observed in the GH-treated animals relative to the controls. However, since there was no indication of an increase in ileal mucosal DNA or protein content, the investigators concluded that the improvement in mucosal dimensions may have been due to water retention. In contrast, the increase in small bowel mucosal weight in resected rats following IGF treatment is probably due to augmented compensatory hyperplasia, since reproducible trends towards increased DNA and protein content have been shown in this study, whilst the same response achieved significance in the study of Vanderhoof et al. (1992).

When plerocercoid growth factor (PGF), an analog of hGH, was continuously infused into rats following 70% proximal jejuno-ileal resection, increases in the weight, and DNA content of the mucosa of the small bowel were noted (Hart et al., 1987). Body weight gain, however, was not improved by this treatment. In fact, the resected rats given PGF tended to gain less weight over the 14 day treatment period than the resected control rats (mean±SEM: 38.3±5.9g versus 53.0±6.3g). This may reflect a state of resistance to PGF following gut resection, since the same study showed that PGF administered to non-resected, sham operated rats induced a small improvement in body weight gain. As pointed out in section *1.3.2*, resistance to the anabolic action of GH in certain stress conditions is a major disadvantage in the use of GH as a means of attenuating stress-induced catabolism. Thus far, no indication of a stress-related antagonism to the anabolic effects of IGF-I or its analogues has been noted in the wide

variety of catabolic animal models in which these peptides have been tried (Asakawa et al., 1992; Guler et al., 1988; Lemmey et al., 1991; Martin et al., 1991; Skottner et al., 1989; Tomas et al., 1991a,b,c).

Although GH resistance is usually evident following surgery or during nutritional deprivation (see Section *1.3.2*), treatment with GH has, on occasions, been shown to be effective in improving nitrogen balance in these circumstances (Douglas et al., 1990; Ward et al., 1987; Ziegler et al., 1988). Not surprisingly, each of the studies which show an anti-catabolic effect of GH in conditions usually characterized by GH-resistance, observed an increase in the previously depressed plasma levels of IGF-I following GH administration. As anticipated, nitrogen balance in these studies displayed a closer relationship to the circulating levels of IGF-I than GH. In summary, treatment with IGF peptides offers two obvious advantages to GH in the treatment of patients following resection: 1) the anabolic effects of IGF-I, but not those of GH, appear to operate irrespective of the nature of the catabolic conditions, and 2) IGF-I does not feature the diabetogenic action of GH.

A number of gut peptides, including pancreaticobiliary secretions (Altmann, 1971; Deschner and Reicht, 1979; Williamson et al., 1978), gastrin (Johnson and Guthrie, 1974; Lichtenberger et al., 1973) and enteroglucagon (Besterman et al., 1982; Sagor et al., 1982; Savage et al., 1985) have been advanced as possible mediators of the adaptive changes that occur in the resected gut and, consequently, as potential therapeutic agents for resection patients. The belief that pancreaticobiliary sectetions play a part in post-resectional mucosal hyperplasia is prompted by the observation that the concentrations of these secretions in the intestinal lumen are markedly elevated following gut resection (Besterman et al., 1982; Sagor et al., 1982; Savage et al., 1985), and reports that ileal hyperplasia is stimulated when pancreaticobiliary secretions are redirected to the ileum of intestinally intact rats (Altmann, 1971; Williamson et al., 1978). However, a major role for luminal peptides in adaptive regrowth is unlikely as

hyperplasia following resection occurs despite ligation of the pancreaticobiliary duct. Furthermore, Grey and Morin (1985) showed that acid extracts of the pancreas and bile have no effect on mucosal DNA synthesis.

Gastrin has received considerable attention as a possible gut mitogen, largely because hypergastrinaemia due to Zollinger-Ellison syndrome and SGS is associated with gastric mucosal hyperplasia (Straus et al., 1974). Gastrin, though, is unlikely to be instrumental in adaptive hyperplasia as pharmacological doses of pentagastrin do not induce jejunal or ileal hyperplasia in rats (Mayston et al., 1975), and post-resectional increases in mucosal crypt cell production rate are not related to plasma gastrin levels (Sagor et al., 1982).

The role of enteroglucagon as a gut mitogen is suggested by the fact that its levels are elevated in a number of situations, such as certain malabsorption syndromes (Ghatei et al., 1983), following jejuno-ileal bypass (Holst et al., 1979), and after extensive small bowel resection (Besterman et al., 1982; Sagor et al., 1982; Savage et al., 1985), which are characterized by hyperplasia. However, Goodlad et al. (1988) reported that post-resectional intestinal proliferation can be stimulated without an increase in enteroglucagon. Furthermore, exogenous glucagon administration has been shown to reduce villus height and cell migration (Faloona and Unger, 1974), and continuous intravenous infusion for 7 days in parenterally nourished rats fails to prevent the mucosal hypoplasia associated with TPN (Bell and Weser, 1979).

Somatostatin has a multitude of actions in the gut, including inhibition of exocrine secretion, peptide secretion, motility and splanchnic blood flow (Mulvihill et al., 1986; Patel et al., 1981). As a consequence, octreotide, a somatostatin analogue, has been used to reduce fluid and electrolyte losses associated with rapid transit and malabsorption in the post-operative period of gut adaptation. Although successful in reducing diarrheoa in SGS patients (Dharmsathaphorn et al., 1982; Ladefoged et al.,

1989; Meier et al., 1992; Nightingale et al., 1989; Rodrigues et al., 1989), it does not improve energy absorption (Nightingale et al., 1989), and has been shown to inhibit mucosal hyperplasia in resected rats (Bass et al., 1991; Holmes et al., 1982). As such, perioperative treatment with somatostatin analogues may interfere with post-resectional adaptation and, therefore, delay the switch from parenteral to enteral nutrition (Bass et al., 1991). Thus, although the use of somatostatin is thought to be of advantage in some circumstances following massive gut resection, this agent on its own does not constitute an ideal SGS therapy.

Even if the gut peptides discussed, or any other gut peptides, proved to be potent gastro-intestinal mitogens, it is unlikely that they would have a direct effect on somatic growth. Consequently, the value of these peptides as post-resectional therapies would be limited to their specific gut effects and any secondary, and consequently delayed, somatic effects that might ensue from stimulated intestinal growth. The value of post-resectional therapeutic agents that have direct effects on nitrogen balance is emphasized by the evidence presented in this thesis that catabolism following resection is worst during the initial recovery periods, when intestinal adaptation is incomplete.

Compared to the therapies currently in use for gut-resection patients, or potential therapies that have been examined in animal trials, IGF-I clearly emerges as one of great promise. Underlining this promise are the outcomes of recent clinical trials which used IGF-I. These include a report showing that administration of a single, low dose of IGF-I to patients following major abdominal surgery normalised plasma IGF-I levels, but had no effect on blood glucose, and was safe (Miell et al., 1992). In normal volunteers, IGF-I has been shown to have similar qualitative effects on glucose, amino acid and lipid metabolism as insulin, whilst suppressing endogenous insulin levels, with no deleterious effects noted (Turkalj et al., 1991). Similar effects have also been noted in fasted subjects (Clemmons et al., 1992). In patients with non-insulin dependent diabetes mellitus (NIDDM), infusion of IGF-I normalizes blood glucose,

and may have a prophylactic effect in reducing the severity of vascular disease (Schalch et al., 1991; Zenobi et al., 1991). Thus, administration of IGF-I peptides have been shown to be safe in pituitary sufficient man. Since nutritional deprivation and surgery usually result in GH resistance, reports that exogenous IGF-I has anabolic effects in GH and pituitary-deficient subjects (Rosenfeld et al., 1992; Thoren et al., 1992) are also relevant in assessing the potential of IGF-I as a therapeutic agent. Of particular relevance to my study is a recent report that infusion of IGF-I reversed catabolism following dietary restriction in normal humans (Clemmons et al., 1992). Clearly, many more clinical trials have to be conducted before the value of IGF-I is clarified but thus far it has been shown to be anabolic as well as safe under a limited variety of conditions.

In conclusion, this study has shown that IGF-I, and especially the variants des(1-3)IGF-I and LR^3IGF-I, are effective in attenuating post-surgical catabolism. This result, together with those of concurrent studies at the Child Health Research Institute and the CSIRO Division of Human Nutrition using other catabolic animal models (Martin et al., 1991; Tomas et al., 1991a,b,c), strongly suggests that IGF-I peptides could be therapeutically important in treating a variety of severely catabolic conditions. The evidence that IGF-I peptides can augment growth, and improve food absorption and food conversion efficiency when nutrient absorption is markedly compromised, suggests that IGF-I or, more likely, one of its analogues could be especially valuable in the treatment of gut diseases. More particularly, since IGF-I peptides have such profound effects on gut growth, the therapeutic value of these peptides would appear to be greatest in conditions featuring SGS. When the features of treatment with IGF-I and its analogues are considered in total, its potential as an aid to recovery after extensive gut-resection is clear.

Challenges for the future are to determine the efficacy of IGF-I treatment as a post-resectional therapy in the clinical setting. Specifically, 1) would IGF-I peptides be

effective if administered with a TPN solution? The answer to this is crucial as IGF-I is advanced as a therapeutic agent for patients recovering from massive rather than minor gut resection, since the former is associated with severe catabolism and IGF-I offers the advantage of improving nitrogen retention. Massive resection, in turn, necessitates initial, and often long-term, TPN. However, if IGF-I did prove to be effective in conditions of TPN dependence, supplementation of the TPN solution with IGF peptides would be an obvious means by which IGF-I could be continuously infused; 2) would the efficacy of IGF-I therapy be improved if it were administered in conjunction with other proposed therapeutic agents? Additive anabolic effects of IGF-I and GH have been observed in female hypophysectized rats and dwarf rats (Clark et al., 1992) suggesting that these factors may be optimally administered in combination; 3) would administration of IGF-I peptides increase intestinal length in short gut patients? Although the effects on length were not significant in the gut-resected rats treated with IGF-I in this study, significant effects on length have been observed following IGF infusion to dexamethasone-treated rats (Read et al., 1992). If IGF-I, or one of its analogues, was to have the same effect in gut-resected humans, especially those in which only very short segments of gut remain, its therapeutic potential would be considerably enhanced.

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Appendix 2.1 18% Casein diet recipe:

Choline Chloride	2g/10ml H_2O	Methionine	5g
Mineral Mix	100g	Vitamin Mix	2g
Sucrose	304g	Casein	360g
Starch	1028g	Bran	100g
Peanut oil	100g	Fish oil	1ml

VITAMIN MIX

	50g
Thiamine HCL	3.5g
Riboflavin	1.5g
Niacin, Nicotonic acid	2.5g
Pantothanic acid	7.5g
Pyrodoxal HCL	0.75g
Hydroxocobolamin	0.001g
Inositol	20.0g
p Amino benzoic acid	2.5g
Folic acid	0.5g
Biotin	0.02g
Glucose	11.229g

MINERAL MIX

	1kg
$CaCO_3$	292.9
$CaHPO_4$	3.4
$KH_2(PO_4)$	343.1
NaCl	250.6
$MgSO_4.7H_2O$	99.8
$FeCit.6H_2O$	6.23
$CuSO_4$	1.56
$MnSO_4.H_2O$	1.21
$ZnCl_2$	0.2
KI	0.005
$(NH_4).6(M0_7O_4).4H_2O$	0.025
Na_2SeO_3	0.01

Procedure:

Mix choline chloride with water until it is dissolved. Then, weigh out the mineral mixture, vitamin mixture, methionine and the sucrose. Put these into a mixing bowl, and mix thoroughly using food blender. Add the choline chloride solution and mix well. Weigh this mixture out, and then add starch, casein and the bran, again ensuring to mix well . Next, slowly add the fish and peanut oils while the blender is still running.

Appendix 4.1 Protein Assay protocols

Mucosal+submucosal samples

(1) thaw samples
(2) homogenize for 1 min.
(3) create 2 subsamples (i) 700µl hom. & 700µl 1M NaOH
(ii) 700µl hom. & 700µl 1M NaOH
(4) leave subsamples at 4 C for 48 hrs
(5) vortex digested homogenate
(6) for i & ii for ea. sample, 100µl digested homogenate & 900µl 0.5 M NaOH, **50µl of ea. into 4 wells** (i.e. 2.5µl/well)
(7) Add, 150µl Reagent A every 15s to each well. EXACTLY 10min later add 50µl of 25% Folin (mix well), let stand for 1hr. Read filters 8 & 2

Muscularis samples

(1) thaw and vortex
(2) leave subsamples at 4 C for 48 hrs
(3) homogenize for 1 min.
(4) vortex digested homogenate
(5) for i & ii for ea. sample, 100µl digested homogenate & 100µl 2M HCl & 200µl 1M NaOH & 1600µl 0.5M NaOH , **50µl of ea. into 4 wells** (i.e. 2.5µl/well)
(6) Add, 150µl Reagent A every 15s to each well. EXACTLY 10min later add 50µl of 25% Folin (mix well), let stand for 1hr. Read filters 8 & 2

Standard curve: Stock = 4mg BSA/ml GDW

STOCK	& GDW	& 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	18.75
100	300	400	25
125	275	400	31.25

add **50µl / well**

Reagents: 75ml 2.56% $NaCO_3$ & 750µl 2.56% Na tartrate &750µl 1.28% $CuSO_4.5H_2O$ = REAGENT A
5ml Folin & 15ml GDW = 25% Folin

Appendix 4.2 DNA Assay protocols

Mucosal+submucosal samples

(1) thaw samples
(2) homogenize for 1 min.
(3) create 2 subsamples (i) 700µl hom. & 700µl 1M NaOH
(ii) 700µl hom. & 700µl 1M NaOH
(4) leave subsamples at 4 C for 48 hrs
(5) vortex digested homogenate
(6) for i & ii for ea. sample, 500µl digested homogenate & 50µl 5 M HCl & 2.5 ml cold 0.36 M perchloric acid (PCA)
(7) extraction: i) vortex and ice for 30min or longer
ii) centrifuge for 20min at 2500 rpm
iii) remove and discard supernatant; add 1ml cold 0.3M PCA to pellet
iv) vortex and ice for 30min or longer
v) centrifuge (as before) - prepare standard curve
vi) remove and discard supernatant; add 2ml cold 0.5M PCA to pellet
vii) vortex and heat at 70°C for 20min or longer, cool briefly (5-10min)
viii) ice for 30min or longer
ix) centrifuge (as before)
x) supernatnant is sample **100µl/well** (i.e 20µl/well)
(8) Add 200µl diphenylamine reacting solution to each well, mix and leave 17hr at 30°C in covered water bath. Read filter 7

Muscularis samples

(1) thaw and vortex
(2) leave subsamples at 4 C for 48 hrs
(3) homogenize for 1 min.
(4) vortex digested homogenate
(5) create 2 subsamples ea. of 700µl
(6) for i & ii for ea. sample, 500µl digested hom. & 200µl 5 M HCl
(7) take 700µl neutralized hom. & 3.5 ml cold 0.36 M Perchloric acid (PCA)
(8) extraction as for mucosal samples
(9) Add 200µl diphenylamine reacting solution to each well, mix and leave 17hr at 30°C in covered water bath. Read filter 7

Standard curve: Stock = 200µg / ml GDW

STOCK	& GDW	& 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 20min, cool for 5-10min, then ice for 30min
100µl/well

Reagents: 1.5g diphenylamine (DPA) & 100ml acetic acid & 1.5ml conc H_2SO_4 = DPA reagent
200µl acetaldehyde & 9.5ml cold GDW = aqueous aldehyde
100ml DPA & 500µl aqueous aldehyde = DPA reacting solution

Appendix 4.3 Mucosal+submucosal sucrase assay protocol

Samples:

Duodenum: a)50µl homogenate + 3950µl 50mM phosphate (PO4) buffer (triton)
assay 50µl, i.e. 0.625µl/well
b)25µl homogenate + 3975µl 50mM PO4 buffer (triton)
assay 50µl, i.e. 0.313µl/well

Jejunum: as above

Ileum: a)100µl homogenate + 3900µl 50mM PO4 buffer (triton)
assay 50µl, i.e. 1.25µl/well
b)50µl homogenate + 3950µl 50mM PO4 buffer (triton)
assay 50µl, i.e. 0.625µl/well

Blanks: 50µl sample (i.e. a) or b)) + 200µl TGO + 50µl 0.2M sucrose

Standard Curve: 1mM glucose stock (glucose + 50mM PO4 (triton))

nmol gluc./well	gluc. std.(µl)	50mM PO4 (triton)
0	0	500
5	50	450
10	100	400
15	150	350
20	200	300
25	250	250
30	300	200

add 50µl/well

	Sample	Blank	"0" std.	other std's.
homogenate	+	+	-	-
sucrose	+	+	+	+
glucose	-	-	-	+
TGO	+	+	+	+

Methods:

1). add 50µl of: sample to sample and blank wells; standards to standard curve wells
2). plates in water bath at 37°C for 5m. Cover with lids
3). add 50µl 0.2M sucrose to samples and stds.
Mix well and incubate at 37°C for **exactly** 30m.
4). add 200µl TGO to blank wells and immediately add 50µl 0.2M sucrose add 200µl TGO to all remaining wells. Mix well and incubate at 37°C for 30m.
5). read on Eliza reader filters 4 + 8 (492nm + 690nm)

Reagents:

Glucose standard:
1mM stock: make up 10mM stock (i.e. 0.18g D-glucose in 100ml PO4 buffer) then take 1ml upto 10ml with PO4 buffer

TGO:
3.75ml glucose oxidase
15mg horseradish peroxidase
2.5ml O-dianisidine solution (0.1g/ml 95% EtOH)
400ml 0.625M Tris buffer pH 7.0 (i.e. 75.6g/L GDW titrated with HCL to pH 7.0) *only stable for 2 wks., photosensitive so wrap bottle in alfoil

50mM Phosphate (PO4) buffer:
prepare 50mM acidic and 50mM basic phosphate
Acidic NaH_2PO_4 7.8g/L GDW
Basic Na_2HPO_4 (anhydrous) 7.1g/L GDW
titrate acidic with small amount of basic to pH 6.1
+ 0.002% triton (i.e. 200µl/L)

Calculations:

nmol glucose/cm/m = (nmol/well*1000*hom. vol.(ml)) / (incubation time(m)*µl sample assayed*cm. intestine)

Appendix 4.4 Organ weights on day 7 after 80% jejuno-ileal resection and treatment with IGF-I peptides.

Absolute weight (g)

Treatment group	Final body-weight (g)	Liver	Spleen	Kidneys	Adrenals	Thymus	Heart	Lungs
Vehicle	159.1±3.3	8.08±0.31	0.352±0.020	1.59±0.06	0.029±0.003	0.315±0.046	0.699±0.037	0.864±0.025
IGF-I (0.7 mg/kg bwt/d)	163.6±4.0	7.86±0.31	0.420±0.017	1.71±0.06	0.030±0.003	0.331±0.028	0.650±0.022	0.965±0.043
IGF-I (1.8 mg/kg bwt/d)	165.6±5.0	7.94±0.45	0.429±0.030	1.76±0.05	0.029±0.002	0.343±0.023	0.675±0.021	0.918±0.050
IGF-I (4.5 mg/kg bwt/d)	166.0±4.9	8.04±0.35	0.462±0.018[b]	1.72±0.08	0.030±0.002	0.396±0.013	0.672±0.015	1.076±0.098
des(1-3)IGF-I (0.3 mg/kg bwt/d)	168.3±4.7	8.81±0.46	0.437±0.026[a]	1.76±0.08	0.027±0.003	0.378±0.018	0.745±0.032	0.955±0.032
des(1-3)IGF-I (0.7 mg/kg bwt/d)	160.3±6.0	7.82±0.40	0.389±0.021	1.70±0.09	0.031±0.004	0.381±0.029	0.743±0.026	0.941±0.039
des(1-3)IGF-I (1.8 mg/kg bwt/d)	165.6±4.3	8.41±0.45	0.428±0.021[a]	1.82±0.06	0.028±0.001	0.435±0.031[a]	0.748±0.034	0.974±0.037
LR3IGF-I (0.3 mg/kg bwt/d)	166.0±8.6	7.73±0.36	0.402±0.022	1.64±0.07	0.027±0.002	0.467±0.048[b]	0.704±0.034	1.033±0.090
LR3IGF-I (0.7 mg/kg bwt/d)	167.8±5.0	7.97±0.25	0.423±0.020[a]	1.74±0.07	0.030±0.003	0.470±0.045[b]	0.700±0.014	0.998±0.039
LR3IGF-I (1.8 mg/kg bwt/d)	172.1±4.0	8.78±0.27	0.506±0.045[b]	1.82±0.05	0.027±0.003	0.440±0.034[a]	0.741±0.033	1.103±0.055
			Relative weight (g/kg bw)					
Vehicle	-	50.97±2.51	2.21±0.11	9.95±0.28	0.182±0.019	1.95±0.26	4.39±0.22	5.43±0.10
IGF-I (0.7 mg/kg bwt/d)	-	48.20±2.13	2.57±0.07	10.44±0.30	0.182±0.015	2.03±0.16	3.97±0.08	5.93±0.31
IGF-I (1.8 mg/kg bwt/d)	-	47.93±2.29	2.60±0.18[a]	10.66±0.41	0.176±0.014	2.07±0.12	4.09±0.15	5.53±0.17
IGF-I (4.5 mg/kg bwt/d)	-	48.36±1.21	2.79±0.10[b]	10.35±0.35	0.179±0.011	2.40±0.09	4.06±0.10	6.44±0.45
des(1-3)IGF-I (0.3 mg/kg bwt/d)	-	52.27±1.94	2.60±0.15[a]	10.48±0.44	0.161±0.16	2.25±0.11	4.43±0.14	5.68±0.13
des(1-3)IGF-I (0.7 mg/kg bwt/d)	-	47.67±1.79	2.37±0.12	10.37±0.45	0.186±0.022	2.32±0.15	4.54±0.11	5.73±0.14
des(1-3)IGF-I (1.8 mg/kg bwt/d)	-	49.72±2.61	2.53±0.11	10.78±0.45	0.165±0.006	2.57±0.19[ad]	4.42±0.17	5.76±0.18
LR3IGF-I (0.3 mg/kg bwt/d)	-	46.77±1.60	2.42±0.06	9.98±0.43	0.166±0.012	2.81±0.22[b]	4.26±0.15	6.16±0.22
LR3IGF-I (0.7 mg/kg bwt/d)	-	47.61±1.39	2.52±0.07	10.36±0.19	0.179±0.016	2.80±0.25[be]	4.19±0.10	5.98±0.26
LR3IGF-I (1.8 mg/kg bwt/d)	-	51.08±1.43	2.95±0.27[c]	10.61±0.40	0.157±0.015	2.57±0.21[a]	4.31±0.17	6.45±0.39

Values are means ± SEM for 7-8 rats in each group: [a] $P < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs vehicle-treated rats, [d] $p < 0.05$, [e] $p < 0.001$ vs IGF-I at the same dose.