NATURAL DEVELOPMENT AND DIETARY REGULATION OF BODY AND INTESTINAL GROWTH IN BROILER CHICKENS

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Corrigenda

In the text and * indicates that the reader should refer to corrigenda listing.

Page 14, L8	"Crypt size and enterocyte migration....40% higher than those in the former two groups." Should read "Crypt size and enterocyte migration rates did not vary significantly between un – selected and relaxed selected chickens but values of these variables in continuously selected birds were 40% greater than those in the former group."
Page 24, L4	"In broiler chick production..." The switch in diet may effect feed intake through problems in palatability, texture of the feed or an inability of the animal to digest the new diet.
Page 29, L21	Manipulation of microbial populations. The precise mechanism of action of oligosaccharides in modifying bacterial populations is still debate. However, the favoured mode of action for mannan – oligosaccharides is one of exclusion through blockade of bacteria adhesion sites on enterocytes.
Page 91 Table 4.1.4	Weight per length of tissue (g/cm), jejunum 1d reads 0.03^{d}. This should read 0.03^{b}.
Page 92 Table 4.1.5	Length (cm). Superscripts for duodenum changed as indicated in text.
Page 98, L1	"had with" should read "hatched with".
Page 103, L16	"massive changes" should read "changes"
Page 104 Table4.2.1	Duodenal villus surface area superscripts for means amend in text.
Page 115 L2	"17 times higher than that sucrase" should read "17times higher than that of sucrase".
Page 168 L2	delete methionine.
Page 172 L17	comment. Reductions in feed utilization with age may reflect the increased maintenance costs of an animal as it ages and grows.
Page 179 L1	"Seven-two one – week –old" should read "Seventy – two 7day old".
Page 206-207	Table 5.2.6 vs 5.27. Faecal digestibility of dry matter between treatments was not significantly different, whereas feed conversion ratio differed significantly. This observation most probably reflects

differing microflora usage of nutrients in birds maintained on diets differing in non –starch polysaccharide composition.

Page 233 L5 "allocated in six to cages" should read "allocated six per cage"

Page 264 L4 "1987" should read "1989".

Page 282 L1 "1991" should read "1992".

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SUMMARY

The pattern of body growth and intestinal development of an Australian strain of broiler chicken, the Steggles x Ross (F_1) in response to different diets was studied. Five experiments were designed to examine the pattern of growth and mechanisms involved. In four other experiments, the mechanisms underlying the gross response of the broiler chicks to dietary ingredients, anti-nutritive factors and growth enhancers were examined.

On the commercial starter diet (0-21 days), absolute feed intake, body weight, feed conversion ratio and the retention of most nutrients increased with age. Between hatch and 21 days, there was a 14-fold increase in body weight. There were reductions in feed intake per unit body weight. Relative to body weight, the weight of the small intestine and its component regions as well as the pancreas peaked by 7 days of age and declined ($P<0.001$) subsequently. Intestinal length in relation to body weight and tissue weight also declined ($P<0.001$) with age. There were significant ($P<0.001$) correlations between body weight on the one hand and the weight of the small intestine; gizzard; liver, and combined weight of these organs.

The intestinal mucosa was well developed at hatch but there were tremendous changes in morphometry at all regions. Duodenal ($P<0.05$) and jejunal ($P<0.01$) crypts increased in depth with age. There was a two-fold increase ($P<0.001$) in duodenal and ileal villus height and a three-fold increase ($P<0.001$) in the jejunum although villus height generally declined distally from the duodenum to the ileum. The apparent surface area of the villus was similarly increased with age in the duodenum and jejunum ($P<0.001$) as well as in the ileum ($P<0.01$). The protein content of the mucosal homogenate

obtained from the duodenum and jejunum but not the ileum varied ($P<0.001$) with age. The DNA content of the homogenates generally declined ($P<0.001$) while there was an increase ($P<0.01$) in the protein:DNA ratio of the homogenate with age in all three regions. While the RNA content of the homogenates increased with age in the duodenum ($P<0.01$) and ileum ($P<0.001$), there was a reduction ($P<0.05$) in jejunal RNA content. The RNA:DNA ratio increased ($P<0.001$) with age in the duodenum and ileum as well as in the jejunum ($P<0.05$). Further examinations of the development in the jejunum revealed peak cell proliferation (labelling index obtained with 5-bromo-2'-deoxyuridine) and migration rate by 7 and 14 days of age respectively, both indices tended to vary ($P<0.001$) with age. Estimated and observed enterocyte life-span increased ($P<0.001$) while there was a reduction ($P<0.05$) in time spent by the enterocytes at the crypt as the chicks became older.

The specific activities of intestinal digestive enzymes, including maltase, sucrase, aminopeptidase N (APN) and alkaline phosphatase (AP) significantly declined ($P<0.001$) with age in all regions of the small intestine. The total activities of these enzymes in all intestinal regions, however, tended to increase ($P\leq0.01$) with age. Enzyme histochemistry also revealed significant increases in total villus enzyme activities in the case of α-glucosidase (AG, $P<0.001$), APN ($P\leq0.01$) and AP ($P<0.05$). There were no significant differences in activities per unit surface area with age in any intestinal region. Enzyme profile maps revealed expression of activities from the crypt to a point close to the villus tip although peak activities occurred at the mid-villus, especially for AG and APN. In the jejunum, enzyme activities were demonstrated in enterocytes that were about 1 hour old and activity was observed in enterocytes that were up to 65 hours old.

The development of amino acid transport in jejunal and ileal brush-border membranes was assessed using L-tryptophan as a model. The uptake rate of 0.04 mM L-tryptophan declined ($P<0.001$) with age in both the jejunum and the ileum, with more uptake occurring in the latter region. Uptake rate per absolute weight of the intestinal regions significantly increased ($P<0.01$) with age and was similar between the jejunum and ileum. L-tryptophan uptake was predominantly ion-independent but responded slightly to the presence of Na^+ and K^+ as well as the anions, NO_3^- and Cl^-. There was a marginal reduction in uptake when valinomycin was included in buffers containing K^+, suggesting some reliance on an inwardly directed membrane potential. L-tryptophan uptake responded to the presence of D-tryptophan, L-lysine, L-methionine, L-alanine and 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH).

The pattern of feed intake, body growth and intestinal development observed on the starter diet was maintained after transfer to a commercial finisher diet between 21 and 35 days of age. The body weight change between hatch and 35 days of age was 35-fold and feed conversion efficiency continued to decline with age. Intestinal mucosal morphometry did not vary during the 14 days of assessment on the finisher diet. The specific activities of the digestive enzymes examined on the finisher diet significantly declined ($P<0.001$) with age. Other differences were of regional origin. Most of the changes observed seemed to be induced by age rather than the effect of dietary change.

The traditional variations in the productivity of broiler chickens on diets based on different cereal grains were observed when diets containing barley, maize, sorghum or wheat, without or with a composite microbial enzyme (Avizyme 1300 with glucanase, arabinoxylase, pectinase activities) were compared. The diets varied widely in non-

starch polysaccharide (NSP) contents; the NSP composition of the barley-based diet was also different from that of the other diets. The weight of the gastrointestinal tract of chicks on the barley-based control diet was significantly higher ($P<0.001$) than that of chicks on the other cereals. On control diets without Avizyme, crypts were deeper ($P<0.01$) and villi longer ($P<0.001$) on the wheat-based diets than on the other diets. There was no effect of Avizyme on these parameters. Chicks on the barley-based diet showed significantly lower jejunal ($P<0.05$) and ileal ($P<0.001$) maltase activities than chicks on the other diets without Avizyme. With Avizyme, the specific activities of jejunal maltase ($P<0.05$) and APN ($P<0.001$) were significantly increased on the barley-based diet. On the barley- and wheat-based diets, there was a general increase in the specific activities of most of the intestinal enzymes while there was a reduction in chicks raised on the maize- and sorghum-based diets in the presence of Avizyme.

Dietary supplementation of the commercial starter diet with the highly viscous NSP, guar gum (GG) and gum xanthan (GX) significantly reduced ($P<0.001$) body weight gain, final body weight and feed conversion efficiency over short and long terms of feeding. The intestinal weight of the chicks on the viscous NSP diets was also increased ($P<0.001$). Over long term exposure to the diets, jejunal ($P<0.01$) and ileal ($P<0.05$) crypt depth was increased while there was an increase ($P<0.001$) in ileal villus length as a result of supplementation with the viscous NSP. Cell size at the jejunum was increased ($P<0.01$) while cell population declined ($P<0.05$) in chicks raised on diets supplemented with GG and GX. Jejunal maltase ($P<0.001$) and sucrase ($P<0.01$) activities were reduced over short term feeding of the viscous diets but enzyme activities were restored to levels similar to or higher than those on the non-viscous diets supplemented with alginic acid or gum arabic after long term exposure to the diets.

There was no adverse effects of the NSP on tryptophan uptake by brush-border membrane vesicles (BBMV).

The supplementation of sorghum-based diets with de-hulled solvent-extracted lupin seed meals increased the gross energy but reduced the apparent metabolizable energy and crude protein contents of diets. Body weight gain and ileal digesta viscosity were increased ($P<0.05$) but there were no significant differences between chicks on extracted meals and whole meals with regards to intestinal mucosal morphometry and activities of intestinal enzymes, except in the case of duodenal AP ($P<0.001$).

Further supplementation of sorghum/lupin-based diets with a commercial mannan-oligosaccharide, Bio-Mos (BM) did not significantly improve productivity. The supplement increased starch digestibility and lactic acid content in the ileum but led to an increase ($P<0.05$) in the viscosity of ileal digesta. Apart from an increase ($P<0.01$) in the length of jejunal villi, the supplement did not significantly alter intestinal mucosal morphometry. Bio-Mos increased the specific activities of jejunal maltase ($P<0.01$), leucine aminopeptidase ($P<0.05$) and alkaline phosphatase ($P<0.001$) and L-tryptophan transport ($P<0.01$) but had no significant impact on these factors at the ileum.

The results of the studies indicated that a rapid development of the small intestine preceded significant overall body growth. Body growth would, however, depend more on the various physiological events such as those related to mucosal growth and renewal, digestive enzyme function, and nutrient transport. Some of the differences observed in productivity of broiler chickens on different diets were traced to events at the intestinal level.

DECLARATION

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 has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Date: 14/8/98

DEDICATION

This thesis is dedicated to my parents, Iji and Priscilla Ijogo for their love of knowledge and foresight.

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PUBLICATIONS ASSOCIATED WITH THIS THESIS

A. Journal

i. **Iji, P.A.** and Tivey, D.R. (1998). Raffinose series oligosaccharides in broiler chicken diets. Wld. Poult. Sci. J. 54 (in press).

B. Full conference papers

i. **Iji, P.A.** and Tivey, D.R. (1997). Intestinal and body growth of broiler chickens on diets supplemented with non-starch polysaccharides. Proc. Nutr. Soc. Aus. 21, 125-128.

ii. **Iji, P.A.** and Tivey, D.R. (1997). Feed utilization and body growth of a new strain of broiler chicken from hatch to 35 days of age. Proc. Aust. Poult. Sci. Sym. 9, 171-174.

C. Abstracts

i. **Iji, P.A.** and Tivey, D.R. (1998). Post-hatch development of intestinal enzyme function in broiler chickens. Aus. Poultry Sci. Sym. 10, 213.

ii. **Iji, P.A.** and Tivey, D.R. (1997). Non-starch polysaccharides and intestinal function in broiler chickens. Workshop on Polysaccharides in Monogastric Nutrition and Health. University of Adelaide, Roseworthy Campus, Roseworthy, Australia, 19-20 September, 1997.

iii. **Iji, P.A.**, Hynd, P.I, Hughes, R. and Tivey, D.R. (1997). Linking intestinal structure and function with growth of broiler chicks on different cereal-based diets and microbial enzyme supplement. International Congress on Nutrition, Montreal, Canada, 27 July - 1 August, 1997, PR 391.

iv. **Iji, P.A.**, Hughes, R. and Tivey, D.R. (1996). Short-term feeding of non-starch polysaccharides to broiler chickens: effects on villus structure and brush border peptidase expression. Proc. Aust. Poult. Sci. Sym. 8, 212.

v. **Iji, P.A.**, Hughes, R. and Tivey, D.R. (1996). The effect of lupins (*L. albus* and *L. angustifolius*) on villus structure and brush border peptidase expression in broiler chickens. Proc. Aust. Poult. Sci. Sym. 8, 213.

D. Research highlight

i. **Iji, P.A.** and Tivey, D.R. (1997). Lupinseed: brighter future for this high protein feedstuff ? Feed International 18, 8-12.

ABBREVIATIONS USED IN THIS THESIS

µl	Microlitre
µm	Micrometre
µM	Micromolar concentration
µmole	Micromole
AA	Alginic acid
Ab.	Absorbance
AG	Alpha-glucosidase
AIB	Aminoisobutyric acid
AME	Apparent metabolizable energy
ANF	Anti-nutritive factor
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APA	Aminopeptidase A
APM	Aminopeptidase M
APN	Aminopeptidase N
ARN	Apparent retention of nutrient(s)
BBM	Brush-border membrane
BBMV	Brush-border membrane vesicles
BCH	2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid
BM	Bio-Mos™
BrDU	5-bromo-2'-deoxyuridine
CBB	Coomassie brilliant blue (Page blue)
cDNA	Complementary DNA

cP	Centipoise
CP	Crude protein
CPM	Counts per minute
CVJ	Crypt:villus junction
DAB	Diaminobenzimidine
DM	Dry matter
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
DPP IV	Dipeptidylpeptidase IV
FAME	Fatty acid methyl esters
FDM	Feed (faecal) dry matter
FOS	Fructo-oligosaccharide(s)
FRP	Final reaction product
GA	Gum arabic
GE	Gross energy
GG	Guar gum
GGT	Gamma-glutamyl transferase
GIT	Gastrointestinal tract
GOD	Glucose oxidase
GX	Gum xanthan
HEPES	*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]
HPR	Hexazonium para-rosaniline
IgA	Immunoglobulin A
IGF	Insulin-like growth factor(s)
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IVDMD	*In vitro* dry matter digestibility
J_{max}	Maximum flux
kBq	Kilobecquerel
kDa	Kilodalton
K_m	Substrate concentration at half maximum flux
kV	Kilovolts
LAP	Leucine aminopeptidase
LSD	Least significant difference
M	Molar concentration
ME	Metabolizable energy
MeAIB	2-methylaminoisobutyric acid
MHA	Methionine hydroxy-analogue
MJ	Megajoule
ml	Millilitre
mM	Micromolar
mm	Millimetre
NBF	Neutral buffered formalin
NHS	Normal horse serum
NRC	National Research Council (USA)
NS	Not significant at stated probability level
NSP	Non-starch polysaccharide
PBS	Phosphate buffered saline
PNP	Paranitrophenol
PPPI	Pig and Poultry Production Institute

PRP	Primary reaction product
RDM	Residual dry matter
RNA	Ribonucleic acid
SCFA	Short-chain fatty acid(s)
SD	Standard deviation
SED	Standard error of difference
SEM	Scanning electron microscopy
TMEn	True metabolizable energy
Tris	Tris[hydroxymethyl]aminomethane
V_{max}	Maximum flux attained at equilibrium substrate concentration
v/v	Volume by volume
VAL	Valinomycin
w/v	Weight by volume

CHAPTER ONE: GENERAL INTRODUCTION

The modern broiler chicken has undergone profound genotypic and phenotypic changes over the years. Over the last few decades there has been a reduction in the production cycle of broiler chickens by about 1 day per year and an increased proportion of neonatal life as part of the production cycle (Nir *et al.*, 1993; Dunnington and Siegel, 1995).

Broiler chickens were developed to ingest large quantities of feeds and attain high body weight at a relatively tender age (Nowland, 1978; Williamson and Payne, 1990). The similarity in amounts of pancreatic enzymes secreted by broiler chicks and pullets may, however, imply a limitation to the ability of the former to economically utilize the large amounts of ingested feeds (Nitsan *et al.*, 1974).

Broiler chickens are increasingly being fed diets high in novel ingredients. The exploitation of cheap legume seeds and cereals has been proffered as a solution to lack of conventional sources and rising costs of poultry diets (Gladstones, 1974; Hill, 1977; Oluyemi and Roberts, 1979; Sonaiya, 1995; Annison *et al.*, 1995). Most often, the substitutes cannot be utilized at high levels in diets due to the presence of anti-nutritive factors (Ruiz *et al.*, 1977; Musquiz *et al.*, 1989). The poor nutritive value of certain replacement legume seeds and non-wheat temperate cereals has been traced to their rather low contents of starch, the predominant carbohydrate being non-starch polysaccharides (NSP), formed by $\beta(1\rightarrow4)$-linkage (Carre *et al.*, 1985; Klopfenstein, 1988; Choct and Annison, 1990; Dahle *et al.*, 1992). Legume seeds may also be rich in raffinose series oligosaccharides and toxic factors such as alkaloids, found in unimproved lupins (Hill, 1977; Muzquiz *et al.*, 1989) in addition to non-starch polysaccharides (Rackis, 1975; Saini, 1989). Animals lack the necessary enzyme

systems to digest NSP and oligosaccharides, which is responsible for the low productivity on diets containing high levels of these factors (Fengler and Marquardt, 1988; Brenes *et al.*, 1989; Centeno *et al.*, 1990; Brenes *et al.*, 1993).

The gross effects of the anti-nutritive factors on poultry performance are known (Bailey *et al.*, 1974; Centeno *et al.*, 1990; Fransesch *et al.*, 1990) but little research has been carried out into the identification of mechanisms of their action. In spite of the high crude protein (CP) contents of lupins, diets based on lupin seed tend to possess a very low protein efficiency ratio as was demonstrated in the rat by Ballester *et al.* (1980). Poultry performance is also reduced when non-wheat temperate cereals constitute a high proportion of the poultry diet (Rotter *et al.*, 1990; Bedford *et al.*, 1991).

Apart from nutritional factors, poultry performance depends on endogenous animal factors, some of which regulate feed processing and utilization at the intestinal level. A large proportion of dietary nutrients, estimated at 20 % in ruminant animals is used for the renewal of the gastrointestinal mucosa (Webster, 1980; Yen, 1989). The exact requirements of the gastrointestinal tract (GIT) in poultry have not been established due to limited studies on intestinal development in the modern strains used in the broiler industry. In the pig, major changes have been observed in gut structure and function with age (Smith, 1988; James *et al.*, 1988). Such changes may influence the overall growth of the body and would need to be examined in poultry, on which so far there are only limited studies (Dibner *et al.*, 1992).

In response to internal and external factors, one of the intestinal functions that may be most affected would be transport of nutrients, including amino acids. The main

functions of the transport systems available for many amino acids as well as the molecular nature of the mechanisms for competitive and non-competitive cross inhibition in poultry have remained largely unidentified.

Most previous studies on poultry nutrition focused on the gross response of the bird to dietary factors and other environmental factors, with little attempt at resolving the mechanisms which regulate the changes observed under different dietary treatments (Ballester *et al.,* 1980; Bogdanov *et al.*, 1988; Al-Masri *et al.*, 1995). In many species, the study of these mechanisms has provided an avenue for the rapid modification of production systems, improvement in animal selection and diagnosis of diseases. The principal aim of this research is therefore to offer physiological explanations for changes that occur naturally with age and those associated with the presence of different factors in broiler chicken diets.

CHAPTER TWO: REVIEW OF RELATED LITERATURE

2.1.0 The modern broiler chicken

Broiler chickens (*Gallus domesticus*) are meat-type birds bred for marketing at an early age. In the development of renowned broiler breeds, the breeding traits of importance that are considered include growth rate, body conformation, rapid feathering and plumage colour (Nowland, 1978). Usually, large numbers of a particular line are hatched and very severe selection pressure is applied at broiler age. In a broiler breeding programme, selection is applied to at least 3 lines of birds: a sire line and 2 dam lines. The idea of using multiple dam lines is to gain additional egg production as well as improve fertility and hatchability from the hybrid vigour. Nowland (1978) reported that the selection pressure could be as severe as utilization of only 5 % of any one line in the first instance. These procedures yield a bird that ranks highly among domestic meat animals in feed intake and growth rate (Isshiki *et al.*, 1992). The impact of selection and development of the broiler chicken is profound. Highly selected broiler chicks weighed over 1000 g at 4 weeks, compared with 700 and 250 g respectively for lines that were relax-selected or un-selected (Smith *et al.*, 1990b).

The original meat-producing breeds of chickens are typified by the Orpington, Cornish Dark and Jersey Black Giant (Williamson and Payne, 1990). Apart from the pure meat breeds, dual-purpose breeds such as the Rhode Island Red, Light Sussex, New Hampshire Red and Australorp were developed in the new worlds. The development of breeds and strains has been very rapid and the broiler chicken is continuously changing. Blair (1975) estimated that 5.1 kg of feed and 74 days were required to produce a 2 kg bird in 1966. Over the next decade, it took 4 kg of feed and 50 days to achieve the same weight target. Such weights are now achievable over 35-40 days and with considerably less feed.

At the intestinal level, the modern broiler chicken develops more rapidly than the egg-type chicken. Yamauchi *et al.* (1992) reported that the duodenal epithelial cells of the newly hatched broiler chick are almost mature and might be able to digest and absorb nutrients more efficiently than would cells present in the egg-type chicken. Relative daily feed intake was observed to peak at 30 % of body weight in the broiler chick at 3 days of age while the peak feed intake of 20 % body weight occurred at 6 days in the egg-type chick (Nir *et al.*, 1993). Nir *et al.* (1993) also observed higher feed intake and growth rate at subsequent ages in meat- than in egg-type chickens. The allometric growth of the small intestine, intestinal contents and liver was greater in broilers than in layers. There were no major differences between layers and broiler chickens in the rate of secretion of pancreatic enzymes (Nir *et al.*, 1993). Broiler chicks hatched with intestinal disaccharidase activities two- to five-fold higher than levels found in layer chicks but this trend was reversed from 7 days of age (Mahagna and Nir, 1996). This could indicate some measure of inefficiency in the utilization of feeds by the modern broiler bird, a limitation completely attributable to the gastrointestinal tract (Nir *et al.*, 1993).

In comparative studies on broiler and egg-type (Leghorn) chicks, Newcombe and Summers (1984) observed longer intestines in broiler chicks between 10 and 21 days of age than Leghorn (layer) chicks but the transit time of feed nutrients, examined with a chromium oxide marker was similar between the two, indicating that broiler chicks were passing feeds faster than the Leghorns (Newcombe and Summers, 1984). The reduction in transit time would reduce the derivation of nutrients, especially if the broiler chicks had lower or similar activities of digestive enzymes as has been previously reported (Nir

et al., 1993; Mahagna and Nir, 1996). It was also noted (Nir *et al.*, 1993) that the early post-hatch period as a proportion of the life of the modern broiler chicken increases with each generation, warranting a continuous review of management standards.

2.1.1. Variations between breeds and strains of broiler chickens

Some of the reasons for the development of strains rather than breeds for the broiler industry are to combine the attributes of known meat breeds and reduce the variability between batches. These targets are largely met but there still appear to be wide differences between strains, including those based on similar breeds. The differences may be due to the pattern and intensity of selection as has been reported by Smith *et al.* (1990b). In F_1 chicks derived from two lines differing in body weight, there were differences between each of the parent lines and the offspring in weights of the proventriculus, small intestine, lungs and length of the oesophagus at common body weight (Katanbaf *et al.*, 1988). The weight of the oesophagus, gizzard, heart, liver, lungs, breast, small intestines also differed at any one common age. The heterosis for most organs was less than 15 % but heterosis for small intestinal weight was greater than 30 %. The results suggest the existence of differences in resource allocations between populations of the same breeds. Similar differences were observed between eight lines of boiler chickens known to differ widely in body weight (Nitsan *et al.*, 1991). There were significant differences in hatching weight, absolute and relative (to body weight) weight of the lungs, liver, pancreas and yolk sac. The activities of trypsin, chymotrypsin and amylase in the pancreas and intestinal digesta were also different. In all lines, the weights of the small intestine, liver and pancreas increased relatively more than the weight of the body during the first week post-hatch, after which the relationship was reversed (Nitsan *et al.*, 1991).

In heavy and light strains of White Plymouth cockerels which had been selected over several generations, the relative weight of most internal organs like the heart, lungs, liver and pancreas generally remained in constant proportion to body weight (Dunnington and Siegel, 1995). The heavy strains had larger GIT as a proportion of body weight until 10 days of age after which GIT weight in light strains was relatively larger. In chicks selected for high body weight, weight gain commenced immediately after hatch whereas chicks selected for a low body weight delayed weight gain to several days after hatch (Turro *et al.*, 1994). Absolute feed intake increased linearly in both lines but at a higher rate in the line selected for high body weight than in those selected for low body weight.

The initial weights of the supply organs, especially that of the small intestine are usually associated with subsequent growth of the demand organs or tissues such as the muscles in avian species (Lilja *et al*, 1985; Sell *et al.*, 1991; O'Sullivan *et al.*, 1992a). Using mathematical models, Vinardell and Marti (1992) established that the relationship between body mass and alimentary tract mass in chickens conformed to a bi-segmented straight line with a change of gradient. The change in gradient was estimated to occur at 90 g body weight, corresponding to 2 weeks of age. It was suggested that functional differentiation of the intestine attains a peak at this age. It is not known if all broiler strains conform to this model. Broiler strains that have been assessed showed peak intestinal growth within the first 7 days of age (Katanbaf *et al.*, 1988; Nitsan *et al.*, 1991; Shanawany, 1994), similar to what has been observed in turkeys (Sell *et al.*, 1991).

In comparative studies on eight lines of broiler chickens, varying in body weight, digestive enzyme levels in the heavy lines were higher than those in the light lines (O'Sullivan *et al.*, 1992b). The foregoing review indicates that a significant amount of the differences in productivity between strains may be traced to events at the intestinal level. This necessitates the current increased interest in studies on the intestinal development of broiler chickens.

2.2.0. The growth of the broiler chicken industry

The broiler chicken industry has grown tremendously in recent years, both in terms of volume and economic importance around the world. Initial developments occurred in the temperate areas of the world, where it took several years, running into centuries in some cases to develop the modern breeds and production standards (Williamson and Payne, 1990). These breeds and production standards were imported by developing countries, so that it took only a few years for some of the latter countries to be almost at par with the original breed developers. Thuraisingham and Wah (1971; cited by Williamson and Payne, 1990) reported that the Malaysian poultry industry progressed by approximately the same degree between 1956 and 1964 as did the Danish industry between 1750 and 1950.

The major advantage possessed by the industry is that poultry generally require less rearing space and are more efficient converters of feed into meat (and eggs) than most domestic animals. While the per capita production of meat from ruminant animals remained static or fell in Latin America, Asia and Africa, the increased production of

both eggs and poultry meat assisted in reducing the gap in supplies of animal protein for the human population in these areas (Jasiorowski, 1973).

The importance and growth of the broiler industry in developed countries are no less than they are in the developing countries. Around the world, there were 12 billion unclassified chickens, providing 48 million tonnes of meat annually (FAO, 1995). Comparatively, 56, 77 and 9.8 million tonnes of meat were obtained from cattle/buffaloes, pig and sheep/goats respectively. Within the same period, there were about 52 million broiler chickens, out of a total chicken population of 70 million in Australia (Castles, 1995; FAO, 1995). In Australia, broiler chicken populations rose from 47 to 52 million between 1988 and 1993 while broiler meat production rose from 400 to 474 tonnes over the same period. The gross value increased from 671 to 833 million dollars. Across Australia, broiler chicken establishments represented 53 % and 0.6 % respectively of all poultry and agricultural establishments as at 1993. The industry's contributions to the gross value of agricultural commodities produced were 13.8 and 3.8 % of the livestock and agricultural sectors respectively. In terms of meat supply, the broiler industry contributes more than do pigs or lamb.

Currently, there is less reliance on pure breeds for meat production. Commercial breeders had used existing stock from which they developed most of the strains or hybrids currently in use (Williamson and Payne, 1990). Commercial strains are developed to cope effectively with prevailing local conditions. The development of hybrids has led to increased feed utilization efficiency and availability of low cost breeder pullets and meat quality in conformity with local prejudices (Williamson and Payne, 1990). Where hybrids are not readily available, crossbreeds or dual-purpose

breeds may be of some value. The rapid growth of the industry in developing countries and many other parts of the world placed a strain on feed supply and led to the near collapse of the industry in some countries (Williamson and Payne, 1990; Sonaiya, 1995). This has necessitated the exploration of alternative, low-cost diets, an area that is further examined in this review (section 2.5.0).

2.3.0. The gastrointestinal tract of poultry

Some of the problems encountered with boiler chicken production may be better managed through an understanding of the intestinal structure and physiology of the species, especially in response to dietary factors in new feed ingredients. The GIT of poultry is similar to that of most non-ruminant mammalian species, except for the presence of the crop and gizzard (Low and Zebrowska, 1986). The small intestine of birds is also comparatively shorter than the mammalian and has a uniform diameter throughout its length. Low and Zebrowska (1986) also reported on the absence of Brunner's glands in the intestinal mucosa of the chicken; the villi are variously described as elipsoidal, leaf-like and plate-like, depending on the region studied (Bayer *et al.*, 1975; Low and Zebrowska, 1986).

It was previously thought that the small intestine of poultry could only be divided into the duodenum and ileum, with no clear demarcations into the three regions observed in mammals (Low and Zebrowska, 1986). Most anatomists have delineated the structure into duodenum, jejunum and ileum (Imondi and Bird, 1966; Bayer *et al.*, 1975; Levin *et al.*, 1983; Wallis and Balnave, 1984). More recently, Branton *et al.* (1988) have observed a constancy in the position of the relic of the yolk stalk (Meckel's diverticulum) with age and suggested that it would be a useful point of demarcation

between the jejunum and ileum. These divisions were recognized and utilized in the course of studies towards this thesis.

Nutrient derivation at the intestinal level has been identified as the major determinant of body growth rate in most avian species and broiler chickens (Lilja *et al.*, 1985; Nir *et al.*, 1993). Changes that occur in enterocyte function during the lifetime of an animal may account for the ability of the small intestine to modify nutrient capture and energy metabolism (James *et al.*, 1988; Tivey and Smith, 1989). Smith *et al.* (1990b) were of the opinion that such changes had not been adequately studied in the chicken.

2.3.1. Structural and functional development of the gastrointestinal tract in chickens

The development of the GIT is regulated along three axes. Some of the changes occur with the age of the animal; other changes are observed on the horizontal axis between different regions of the tract while there could also be variations along the vertical axis, from the crypt to the villus tip. These changes all serve to regulate the efficiency of the gastrointestinal tract through modifications to the surface area available for digestion and absorption.

The changes that occur in the mucosa of the intestine can be profound. Kelly *et al.* (1992) indicated that extensive proliferation of epithelial cells and cyto-differentiation, including changes in the expression of enzymes, receptors and transport systems occurred during ontogenesis in mammals. These changes are regulated through the interaction of endogenous and exogenous factors.

2.3.2. Factors regulating the development of the GIT

The growth of most body organs including the GIT occurs by cellular hypertrophy and/or hyperplasia (Morisset, 1993). Such changes can be assessed by examinations of organ weight or indirectly through measurements of the concentrations of nucleic acids and protein or specific activities of local enzymes (Waterlow *et al.*, 1978). The intestinal tissue, like all tissues made of parenchymal cells grows by cellular hypertrophy and/or hyperplasia but differs from many other tissues in being renewable (Leblond, 1972). The impact of continuous selection of poultry on mucosa development has been highlighted (Smith *et al.*, 1990b). Crypt size and enterocyte migration rates did not vary significantly between un-selected and relax-selected chickens but values of these variables were 40 % higher than those in the former two groups. This suggested that early selection could uncouple the diet-induced changes on crypt hyperplasia from secondary effects on villus structure while later selection may increase appetite (Smith *et al.*, 1990b).

2.3.2.1. Age-related pattern of intestinal development

In breeds and strains of chickens that have been studied, intestinal and other visceral organ weights increased more rapidly than the weight of the body within the first few days post-hatch. Such pattern has been reported for the Anak broilers and the layer breed, New Hampshire x White Leghorn (Dror and Nitsan, 1977; Nir *et al.*, 1993). In turkey poults, the weights of the proventriculus, small intestine and pancreas increased more rapidly than body weight until 6 days post-hatch (Sell *et al.*, 1991). From 6 days of age, the changes in the weight of the pancreas and small intestine appeared to be

parallel to body weight but the weight of the proventriculus continued to increase at a more rapid rate. Gizzard weight peaked by 4 days of age.

Some account of the changes that occur in the intestinal mucosa with age has been provided in section 2.1.0. In mammals, mucosal growth in foetal and neonatal intestine differs from that in adults in four major respects: migration along the crypt/villus axis is slow in early life (Koldvsky *et al.*, 1966); the enterocyte life-span is long (Jarvis *et al.*, 1977); nutrient uptake can occur along the entire crypt/villus axis and not only at the villus apex (Smith, 1981). In studies on the neonatal mouse, Beaulieu and Calvert (1987) reported that mitosis may occur along the entire crypt:villus axis unlike in the adult in which mitosis occurred only in the crypt. It is not known if similar patterns exist in poultry. Bayer *et al.* (1975) also reported that villi from the jejunum and duodenum of one-week old chicks were indistinguishable from one another. Such patterns may have been altered by further selection and breeding; significant differences between different regions in the Arbor Acres and Lohmann breeds at various ages were reported by Uni *et al.* (1995a).

The response of the GIT to feeding may vary with age, as has been shown in the rat (Holt and Kotler, 1987). Intestinal mass was reduced in both young and adults during fasting but this was more severe in the aged than in young rats. Sucrase activity was affected in a similar pattern. During re-feeding of fasted rats, the activities of enzymes, including sucrase and maltase responded more positively in old than in young animals (Holt and Kotler, 1987). Similar results have been reported by Reville *et al.* (1991) in studies on rats. There was a greater reduction in jejunal sucrase activity in old rats than was observed in the young rats, following a 48-hour fasting. Enzyme activities in the

ileum of the aged rats remained unaffected, suggesting that the distribution and levels of enzymes along the GIT may also vary with age. In studies on rats, Holt *et al.* (1985) observed lower specific activities of sucrase, maltase and lactase in the upper intestine of adults than that of the young. This was attributed to an increase in the number of undifferentiated enterocytes in the old animals. In turkey poults, the specific activity of maltase at the jejunum was high at hatch but decreased markedly by 4 days of age. The reduction in specific activity was not compensated for by an increase in total jejunal weight (Sell *et al.*, 1991).

Transport rates are also dependent on age although the changes are not directly related to the fact that transport function is the last in the sequence of functional development by the enterocyte (Smith, 1985). In studies on the pig, Smith (1988) observed that only pre-natally formed enterocytes were capable of absorbing large amounts of protein (amino acids and peptides). The ability of the small intestine to transport lysine, peptides containing lysine and glucose declined markedly with age during the first week of post-natal life. With methionine, there was a net transport at birth and day 4 but this had ceased by 10 days of age (James and Smith, 1976). In the chicken, intestinal transport capacity is established prior to hatch or within a few days of hatch (Pratt and Terner, 1971). Planas *et al.* (1982) demonstrated maximal trans-epithelial transport of leucine within the first 3 days of hatch, followed by a decline with age over the next 4 weeks. In the Arbor Acres, methionine uptake capacity increased in both the duodenum and jejunum between hatch and 7 days of age and remained constant afterwards to 14 days (Noy and Sklan, 1996). A similar trend was observed for glucose while oleic acid uptake in the duodenum did not change between hatch and 14 days of hatch. In the Hubbard-Cross chicks, peak uptake rates in the jejunum coincided with 2 and 5 days

respectively for glutamic acid and β-alanine (Shehata *et al.*, 1984). There was no change in the uptake rate of choline between 2 and 21 days of age.

2.3.2.2. Development and function of different intestinal sites

Although lines of demarcation between intestinal regions are not so clear in poultry, the degree of variation in intestinal structure and function in birds from one site to the other is large, warranting the sub-divisions in line with those found in mammals (Imondi and Bird, 1966). Structural and functional changes may occur at different rates in the different regions of the small intestine. These differences are important in the interpretation of response to dietary or disease factors. In many species that have been studied, the absorptive and digestive potentials of the intestine have been found to vary with the position along the tract (Harrison and Webster, 1971).

Some of the physical differences between intestinal segments have been identified in previous studies on mammals and some strains of poultry (Ferraris *et al*, 1989; Uni *et al.*, 1995a). In pigs and poultry, longer villi have been observed in the proximal intestine than those in the distal regions (Imondi and Bird, 1966; Moon and Skartvedt, 1975; Hall *et al.*, 1983). In earlier strains of chicken, ileal villi were found to be about half the length of duodenal villi and enterocytes migrated more slowly in the former than in the latter (Imondi and Bird, 1966). In studies on the intestine of female chickens, Levin *et al.* (1983) found jejunal villi to be broader than ileal villi. The variation in villus area also showed a similar trend, with the area of ileal villus only 40 % of the area of the jejunal villus.

In rats, 3 days of fasting caused villus and crypt hypoplasia and reduction in migration rates in the jejunum but not the ileum (Holt *et al.*, 1986). There was a reduction in the weight of the small intestine during fasting in rats but the impact was more pronounced in the duodenum than in the jejunum (Holt and Kotler, 1987).

In the avian, there is generally greater intensity in protein digestion and absorption in the duodenum and upper jejunum, compared with the ileum (Low and Zebrowska, 1986). In studies on the chicken, Amat *et al.* (1992) also observed a higher activity of alkaline phosphatase activity in the proximal region of the small intestine while the activity of leucine aminopeptidase was fairly constant throughout the length of the intestine. With carboxypeptidase P, activities were higher in the middle region of the small intestine of rats than in other regions (Erickson *et al.*, 1989). The specific activities as well as profiles of most intestinal enzymes in rats, including sucrase and alkaline phosphatase decreased with age and these changes were more noticeable in the duodenum than in the jejunum (Holt and Kotler, 1987).

With a number of amino and imino acids, Munck and Munck (1992) observed a jejuno-ileal gradient in transport across the brush-border membrane of the rabbit small intestine. For lysine, leucine and β-alanine, the rate of transport at constant concentrations increased from very low levels in the proximal jejunum to maximum values in the most distal 30 cm of the ileum. There was an absence of the β-alanine carrier in the jejunum. There are few reports on the comparative efficiency of the different regions of modern strains of poultry. In one of such studies, Amat *et al.* (1992) found that the jejunum was more permeable to 3-oxy-methyl-D-glucose than the ileum although the compound accumulated to higher concentrations in the ileum than it did in

the jejunum. In the Red Jungle fowl, one of the progenitors of the modern chicken, Jackson and Diamond (1995) also observed higher uptakes of glucose from the jejunum than the duodenum and ileum between hatch and 9 days of age. With proline, the relative importance of the jejunum declined by 7 days of age, when the duodenum was the major site of uptake.

The differences in development and function of the different intestinal regions may be an adaptation towards the management of the effects of adverse dietary factors and disease. One or more regions may be primed to balance the inefficiency of other regions during dietary or disease stress.

2.3.2.3. Development along the crypt:villus axis

The intestinal mucosa is in a constant state of renewal, with cells born in the crypts migrating towards the apex of the villus from where they are extruded into the lumen (Moon and Skartvedt, 1975; Syme and Smith, 1982). The life-span of a cell from 'birth' to extrusion varies between species (Imondi and Bird, 1966; Spielvogel *et al.*, 1972; Smith *et al.*, 1984; Smith *et al.*, 1986). In the rat, enterocyte life-span is about 2 days (Syme and Smith, 1982). In early studies on chicks (Imondi and Bird, 1966; Spielvogel *et al.*, 1972) cell transit times of between 48 and 90 hours from the crypt to the villus apex were observed. A bi-phasic migration rate was exhibited by cells; an initial slow phase lasting for a period of 50 hours followed by a 40-hour rapid phase. It was postulated (Spielvogel *et al.*, 1972) that cells developed digestive and transport functions during the slow phase. This has been shown in later work on the chicken by Smith and Peacock (1989) who reported on the sequential developments in the intestinal

mucosa from the elongation of the microvilli to capacitation of digestive and finally transport functions.

The crypt region comprises approximately 20-25 % of the total depth of mucosa in the chick and was recognized as the zone of cell formation and synthesis of functional molecules as a result of its higher concentrations in RNA and DNA than the apex of the villi (Webster and Harrison, 1969; Spielvogel *et al.*, 1972). Spielvogel *et al.* (1972) observed great differences in intestinal properties between birds and mammals and these have been confirmed by more recent studies. Smith and Peacock (1989) and Smith *et al.* (1990b) observed a gradual increase in the length of the microvilli as enterocytes migrated out of the crypts on to the base of the villi. Chicken microvilli were found to first lengthen outside the crypt, then shorten considerably towards the tip of the villus (Smith and Peacock, 1989). The enterocyte migration rate of chicken was also comparatively slower than that obtained previously for mammalian species, including the hamster, mouse, rat and pig.

In mammals, the physical size of the crypts controls the mode of differentiation of the enterocytes as well as rate of expression of digestive enzymes. The rate of migration by enterocytes influences their functional capacities. In rats infested with nematodes, Wild and Murray (1992) observed significant reductions in cell cycle time and activities of Na^+-K^+-ATPase in the jejunum but not the ileum. The reduction in enzyme activity was attributed to an increased proliferation of cells which migrated out of the crypt too rapidly, although some of it may also be due to damage to the intestinal mucosa. The protein content of enterocytes increases with age, and as the cells migrate up the villus (Morrill *et al.*, 1989). This may influence the pattern of results of experiments in which

specific activities, related to the protein content, rather than total or absolute activities of enzymes or transport rates are reported.

Peak activities of sucrase and maltase were observed close to the crypt in rats while alkaline phosphatase activity was demonstrated on the upper villus than the crypt:villus junction (Holt *et al.*, 1985). These variations may be due to differences in rates of synthesis vis-à-vis cellular migration and extrusion. In the rat, the synthesis of sucrase is more rapid than its degradation at the crypt:villus junction and lower villus (Riby and Kretchmer, 1984). This produces an accumulation of the enzyme towards the crypt. The reduced activities observed towards the villus tip is also achieved through increased degradation in the region, as the rate of synthesis in the crypt declines.

The superior transport efficiency of the upper villus enterocytes has been documented in several species (Kinter and Wilson, 1965; King *et al.*, 1981; Smith, 1985; Debnam *et al.*, 1990). Generally, enterocytes complete microvillus development before they can absorb nutrients (Smith, 1985). In the rabbit jejunum and ileum, a large proportion of alanine (80 %) and lysine uptake took place in the upper third of the villus (Paterson *et al.*, 1980; King *et al.*, 1981). Smith and Peacock (1989) attributed the greater transport efficiency of the upper enterocytes to their close proximity to nutrient-rich digesta. There might also be an induction of transporters by nutrients, a hypothesis which has not been fully investigated in poultry and other farm animals.

The rate of development of transport systems may also vary along the crypt:villus axis. In studies on two systems involved with lysine transport, King *et al.* (1981) observed a greater activity of system Ly_2 than that of another system, Ly_1 in enterocytes in the lower

region of the villi. There was an initial slow phase in the maturation of system Ly_1, followed by a rapid phase which was not observed for system Ly_2.

The response to dietary factors may vary vertically along the crypt:villus axis. The feeding of high-lactose colostra to piglets was observed to stimulate an increase in lactase activity in the lower villus (Tivey *et al.*, 1994). The ingestion of galactose did not produce similar effects.

2.3.2.4. Dietary factors

Dietary nutrients are required for the normal development of the GIT in all animals. In piglets that are weaned late, there is a slow increase in crypt depth and no change in villus height, compared with more complex changes, including a reduction in villus height in individuals that are weaned early (Hampson, 1986). Chicks are hatched directly on to solid feed and would require factors in such feed for both direct and indirect regulation of the growth and development of intestinal mucosa. In species that have been more extensively studied, the gross effects of adverse dietary regimes have been partly attributed to changes in the pattern of intestinal development and function. Dietary protein deficiency is accompanied by a thinning of the intestinal wall (Syme, 1982) but other changes that occur on the mucosal face may be more important to digestive and absorptive functions. Yamauchi *et al.* (1996) reported the denudation of apical surfaces and shrinkage of villi due to fasting over a period of 5 days post-hatch in chicks. Similar findings were reported by Dauncey *et al.* (1983) in trials with piglets.

Adaptation to a low protein diet could occur by a combination of reduced mitotic rate and villus height (Syme and Smith, 1982; King *et al.*, 1983). Changes in villus

structure are always accompanied or preceded by changes in the crypt cell proliferation rate (King *et al.*, 1983; Smith and Peacock, 1989; Smith *et al.*, 1990b). Syme (1982) observed an increase in villus basal width and prolonged gradual change in mucosal structure in rats on low protein diets.

The morphological response to severe protein deficiency is an increased production of crypt cells (Syme, 1982). Syme and Smith (1982) observed that young rats adapted their intestinal physiology to cope with the problems of protein deprivation if energy supply is not restricted. When energy supply becomes limiting over long periods, the animal faces the double risk from infection by enteropathogens and from loss of cellular albumin (Dosseter and Shittle, 1975; Syme and Smith, 1982). High-energy diets, on the other hand have been shown to induce a rapid growth of the intestinal mucosa in pigs (Moore *et al.*, 1989) and chickens (Yamauchi *et al.*, 1996). Nitsan *et al.* (1974) observed increased secretion of digestive enzymes in parallel with increase in food consumption in young chicks although overfeeding did not necessarily increase the specific activity of the pancreatic enzymes (Nir *et al.*, 1974).

In rats, 3 days of fasting caused villus and crypt hypoplasia, through reduction in cell proliferation and migration rates in the jejunum but not the ileum (Holt *et al.*, 1986). Similar results were reported by Reville *et al.* (1991) who also observed a reduction in jejunal sucrase activity in rats but no changes in the ileum, following a 48-hour fasting. The activities of alkaline phosphatase and aminopeptidase N were significantly greater in rats on a high protein diet than those on protein-deficient diets (Zambonino-Infante *et al.*, 1989). Maltase activity also varied with protein content but was less affected than AP and APN. In addition to reductions in the rate of cell proliferation and villus height,

the weaning of gnotobiotic piglets on to a pelleted solid meal reduced the activities of brush-border membrane enzymes (Hall and Byrne, 1989). This was associated with a reduction in weight gain over a 3-week period, after which there was some intestinal repair as a result of increased rate of cell proliferation. In broiler chicken production, chicks commonly encounter a switch in diets from one age to the other. This may influence intestinal function and would require more critical examinations.

Morrill *et al.* (1989) also observed an increase in sucrase-isomaltase and glucoamylase activities all along the villus in rats that were re-fed after 4 days of fasting. Similar reductions in the activities of jejunal disaccharidases were reported in rats, subjected to three days of fasting (Holt *et al.*, 1986). There was an increase in enzyme activities in the ileum during the same period. Normal enzyme levels were restored at both sites following one day of re-feeding.

2.3.2.5. Anti-nutritive factors in feeds

The major setback to broiler chicken production is the reliance of the industry on food sources which are also used directly by humans (Oluyemi and Roberts, 1979; Williamson and Payne, 1990). This has not augured well for the growth of the industry, especially with dwindling supplies of food for human consumption in developing regions of the world. To maintain low costs of production, the industry has increasingly explored feed sources not directly used by humans. Some of the sources contain deleterious factors that tend to reduce productivity but the exact mechanisms at the intestinal level have not been unravelled. The chemical nature and importance of the prominent anti-nutritive factors (ANF) are further reviewed in section 2.5.3.

In broiler chickens, the adaptation to increased dietary fibre levels include increases in the size of the GIT, with pea fibre exerting a stronger impact than wheat or oat brans (Jorgensen *et al.*, 1996). There are, however, variations in the pattern of response observed in diets containing different types of fibres. Some of these differences arise from variation in the chemical composition, the molecular sizes and solubility of the fibre in water (Johnson *et al.*, 1984; Evans *et al.*, 1993; Ward, 1996; Smits and Annison, 1996). In comparisons between guar gum, pectin and oat bran, only guar gum produced any significant increase in mucosal mass and concentrations of RNA and DNA in rats (Jacobs, 1983). Pectin in the diet reduced villus height but increased the crypt depth. The increased proliferation of cells in the crypt was accompanied by a reduction in enterocyte life span and thus, villus height on diets supplemented with pectin and guar gum.

Fibres were thought to maintain cell proliferation through a bulking effect, by mechanical distension or abrasion of surface cells (Cassidy *et al.*, 1981; Sircar *et al.*, 1983) but other studies (Dowling *et al.*, 1967; Stragand and Hagenmann, 1977) suggest the involvement of factors other than physical that may influence cell proliferation. The digestion of NSP and other types of fibre yields short-chain fatty acids, which have been found to induce cell proliferation in various species (Tulung *et al.*, 1987; Mathers and Kennard, 1993; Sakata *et al.*, 1995). NSP present in various fibre fractions may influence cell proliferation and mucosal growth through the regulation of insulin (MacDonald *et al.*, 1991). Maize bran in the diet of rats was observed to increase binding of insulin to receptors in the duodenum and ileum but reduced such binding in the jejunum. Guar gum also decreased binding in the jejunum. These changes will

influence mucosal growth since insulin may be involved in processes pertaining to cell proliferation and growth (Steeb *et al.*, 1997).

Rubio *et al.* (1989) observed extensive erosion, shortening and atrophy of the microvilli in the duodenum, jejunum and ileum of broiler chickens fed raw faba beans as a direct result of the presence of trypsin inhibitors. A similar effect of wheat bran on the intestinal structure of rats has been reported by Jacobs and Schneeman (1981). Wheat bran caused an increase in crypt column and number of cells along the column. It was also shown that the consumption of a diet containing 20 % wheat bran supplement produced mucosal hyperplasia and changes in epithelial cell replication and exfoliation at the colon.

The response of digestive enzymes to ANF is complex and depends on several factors. Viscous NSP and protease inhibitors may form chelates with intestinal enzymes, to reduce their activities (Ikeda and Kusano, 1983; Trevino *et al.*, 1992). The effects of adverse dietary factors may arise indirectly from the influence on tissues and organs outside the gastrointestinal tract (Smith *et al.*, 1990b). Huisman and van der Poel (1989) observed a reduction in the weight of the pancreas, an important organ associated with intestinal development and function when kidney bean was included in the diet. Rubio *et al.* (1989) also reported the degeneration of pancreatic acinar cells in broiler chicks raised on diets containing raw faba beans. On high NSP diets, pancreatic cells undergo hypertrophy and hyperplasia and may increase secretions of digestive enzymes (Ikegami *et al.*, 1990). Most often, enzyme levels cannot be bolstered to normal levels and there is a reduction in the digestion of substrates as a result of the effects of the dietary factors. In instances where pancreatic enzyme secretion is increased, the

enzymes may induce proteolysis of intestinal disaccharidases as has been reported by Tivey and Shulman (1991).

In rats on high fibre diets, Cameron-Smith *et al.* (1994) observed a reduction in post-prandial glycaemia and attributed this to a reduction in digestive function as a result of the increased viscosity in the intestine. Van der Klis *et al* (1993) reported a decrease in the rate of absorption of minerals, including calcium, phosphorus, magnesium and sodium throughout the small intestine of male broiler chicks on diets supplemented with carboxy-methyl-cellulose, a compound with similar structure to NSP in some feed ingredients. There was a tendency for compensatory absorption of most minerals in the lower ileum except for potassium.

Many plant protein sources, including soyabeans contain protease inhibitors, known to reduce the activities of pancreatic and intestinal enzymes (McDonald *et al.*, 1988; Petterson and Mackintosh, 1994). Gossypol, a factor associated with poor growth of pigs and poultry on unimproved cottonseed cakes was shown to reduce the activities of alkaline phosphatase, maltase and sucrase in rats (Baram, *et al.*, 1987).

2.4.0. Functions of the GIT

The GIT performs a variety of functions, chief among which are digestion of dietary components and the absorption of nutrients arising from digestion. The diet influences the GIT to much the same extent as the GIT regulates the overall productivity of the animal as reviewed in the preceding sections. In the avian, the efficiency of the GIT is the major factor regulating body growth since the bone and muscular frameworks are fairly developed at hatch (Lilja, 1983; Anthony *et al.*, 1989). The relationships between

intestinal function, especially at the level of the mucosa has been relatively less studied in poultry than in other farm animals. A greater knowledge of these relationships may further aid the development of new strains as well as formulation of diets to target particular populations and production systems.

2.4.1. Digestion

Nutrient digestion occurs through the action of enzymes derived from organs located outside the gut as well as cellular secretions from the intestine (Bamba *et al.*, 1993; Nir *et al.*, 1993). The lower secretion of pancreatic enzymes by the broiler chicks compared with the layer chicks has been highlighted (Nir *et al.*, 1993; Mahagna and Nir, 1996). This is perceived as a limitation to digestion of the large amounts of feeds ingested by the broiler chicken.

2.4.1.1. Extra-intestinal enzyme secretions

The GIT is linked to important organs from which it receives enzymes, among other factors. The main support organs are the pancreas and gall bladder (Low and Zebrowska, 1986). Pancreatic enzyme functions have been more extensively studied than intestinal enzymes in all animals and especially poultry (Hulan and Bird, 1972; Duke, 1986; Nitsan *et al.*, 1991). Hulan *et al.* (1972) reported that adult White Leghorns secreted 15-20 ml pancreatic juice a day, with secretions generally higher in fed birds than in fasted ones. Pancreatic juice contains enzymes capable of catalysing the hydrolysis of carbohydrates, lipids and proteins and their activities depend on feed composition and feeding regime (Hulan and Bird, 1972; Duke, 1986). The activities of pancreatic enzymes, including trypsin, chymotrypsin and amylase also vary between

heavy and light lines of broiler chickens as has been previously mentioned (Nitsan *et al.*, 1991).

Pancreatic enzymes generally hydrolyse large macromolecules into intermediate compounds, readily digestible by intestinal enzymes (Duke, 1986). With protein, some of the end-products of pancreatic enzymes, the small peptides may be absorbed directly by the chicken.

The principal bile salt in chickens is chenodeoxycholic acid, with small amounts of cholic acid, both conjugated with taurine (Haslewood, 1971). Caple *et al.* (1978) estimated a bile flow rate of 33 µl per minute in adult broiler chickens following surgery, the flow rate reducing to half, four hours later. As in mammals, the principal function of bile salts in poultry appears to be the emulsification of lipids in the digesta to improve digestion by pancreatic and intestinal lipases.

In addition to enzymes secreted by organs associated with the intestine, enzymes of microbial origin are found in the crop and the large intestine (Duke, 1986; Low and Zebrowska, 1986). Lactic acid is the main product of microbial digestion in the crop while acetic acid, with small amounts of lactic, propionic and butyric acids predominate in the large intestine. Microbes found in the GIT of chicken produce diverse metabolic activities with little net benefit to the host. Better growth is achieved through supplementation with antibiotics which reduce the microbial burden (Fuller *et al.*, 1981). In addition to posing a health risk to birds, most microbial digestion occurs post-ileum and little of these nutrients is absorbed (Parsons *et al.*, 1982). More recently, it has become possible to manipulate the relative proportions of microbial species, to

achieve desirable effects through the use of oligosaccharide supplements (Alltech Inc., 1994; Monsan and Paul, 1995). The place and effects of such supplements in broiler nutrition are further examined in section 2.5.3.2 and were assessed in one of the experiments (experiment 5.4) reported in this thesis.

2.4.1.2. Digestion in the small intestine

Most previous research on digestive function had focused on secretions from the pancreas and upper GIT due to difficulties in collecting intestinal secretions that were free of contamination with secretions from outside the intestine (Duke, 1986). Although there were earlier thoughts of possible role for enzymes from outside the pancreas operating within the intestines, it was not until the early 1950s that the role of membrane-bound intestinal enzymes in digestion was realized (Kenny and Turner, 1987). Almost all digestion of dietary components had been ascribed to pancreatic enzymes and it was generally believed that complete digestion of peptides and oligosaccharides released by pancreatic enzymes was completed after transport into the cells rather than on the cell membranes prior to absorption. Rothstein *et al.* (1953; cited by Kenny and Turner, 1987) showed that the digestion of glucose-1-phosphate esters by intestinal loops occurred at the cell surface and was not the result of digestion by enzymes from within the cell (intra-cellular enzymes) or those present in the intestinal contents (pancreatic). From research of those early years, there has been a great advance in research towards further identification of the nature and functions of membrane-bound (ecto-) enzymes (Kenny, 1986; Kenny and Turner, 1987; Kenny *et al.*, 1987). The development of histochemical techniques was a major step in the localization and proper identification of enzymes (Gutschmidt *et al.*, 1980; Gutscmidt and Gossrau,

1981; Lodja, 1981). The major focus of such studies has been enzyme systems found in mammalian species, with few reports on poultry.

Although most intestinal enzymes are integral proteins, bound to the mucosal membrane, a few of such enzymes are only partly anchored and can be released by the action of endogenous peptidases or phospholipases (Kenny and Turner, 1987). There is the additional factor that some membrane-bound enzymes may be structurally similar to cytosolic enzymes. Such enzymes can only be differentiated from one another by close study of their regulating genes (Kenny and Turner, 1987).

Intestinal membrane-bound enzymes are asymmetrically orientated so that the catalytic site is exposed only at the external surface of the cell (Stanley *et al.*, 1982; Kenny and Turner, 1987). Kenny *et al.* (1987) reported the existence of about a dozen peptidases in the intestinal brush-border membrane of most mammalian species although the list of known ecto-enzymes may not be exhaustive, neither are the functions of some ecto-enzymes clearly established (Gutschmidt and Gossrau, 1981).

2.4.1.3. Characteristics and functions of intestinal enzymes

Intestinal enzymes, as have been indicated are responsible for the terminal digestion of extracellularly available substrates, including some disaccharides and peptides. In addition, ecto-enzymes may be involved in the transport of nutrients, as has been demonstrated for alkaline phosphatase in vitamin D-mediated calcium transport (Norman, *et al.*, 1970; Stanley *et al.*, 1982). They may also function in the domain of mechanisms that trigger cell growth and differentiation. The exact role of a few other

enzymes in the intestine is unknown, even when they are present at high concentration (Harrison and Webster, 1971).

Intestinal enzymes appear to be secreted in the crypt but express their activities mainly on the villus (King *et al.*, 1983). The activity of enzymes commences from about the crypt-villus junction (King *et al.*, 1983) although the expression of enzyme activity in the crypt is not uncommon (Silverblatt *et al.*, 1974; Wachsmuth and Tohorst, 1974). In the rat jejunum, microvillus elongation and the appearance of aminopeptidase N (APN) and isomaltase activities were found to attain maximal rates in enterocytes located close to the crypt-villus junction (King *et al.*, 1983). The positional localization of an enzyme depends on its rate of synthesis and half-life, relative to the rate of cell migration and extrusion, as has been reviewed in section 2.3.2.3.

Most of the ecto-enzymes (peptidases) characterized in detail have been shown to be metallo-enzymes, with the apo-enzyme linked to Zn^{2+} (Kenny, 1986; Kenny *et al.*, 1987). Dipeptidylpeptidase IV (DPP IV) is the only known serine peptidase while γ-glutamyl transferase has a completely different catalytic mechanism from the other enzymes. Of the intestinal peptidases studied in mainly mammalian species, Kenny (1986) reported the predominance of aminopeptidases over carboxypeptidases. Exopeptidases cleaving peptide bonds one or two residues from the amino terminus are called aminopeptidases and dipeptidases respectively; those cleaving at the same distance from the carboxyl terminus are called carboxypeptidases and peptidyldipeptidases (Lodja, 1981).

Ecto-enzymes are in general used to mark membrane circulation and some other cellular organelles (Stanley *et al.*, 1982). Aminopeptidase A and M (APA and APM), γ-glutamyl transferase (GGT) and DPP IV have been used as markers of cell transformation due to the effects of toxic factors and malignant lymphoma (Gossrau *et al.*, 1984). Lodja (1981) observed a complete disappearance of DPP IV on stimulation with phytohemagglutinin. In the intestine, these changes may reflect the disruption of cellular maturation as well as digestive and absorptive functions (Webster and Harrison, 1969) and have not been adequately studied in poultry.

2.4.2. Nutrient absorption

The development of nutrient absorptive functions closely follows that of digestion, the former occurring at about 35 hours of age in the rat (Syme and Smith, 1982). Transport function is acquired late in the developmental phase of the enterocyte, with maximal capacity of transport systems occurring in fully differentiated cells towards the tip of the villi (King *et al.*, 1981). Hopfer *et al.* (1973) describes the process as vectorial and predominantly unidirectional from the lumen into the cells and then blood and lymph.

Nutrient transport occurs by both passive and mediated mechanisms. For most nutrients, the active mechanism predominates (King *et al.*, 1981; Black, 1988; Webb, 1990) although the importance of a particular mechanism depends on many factors, notably, substrate concentration. At high substrate concentrations, the contribution of simple diffusion to total transport is enhanced, depending on the permeability of the membrane to a nutrient (Stevens *et al.*, 1984; Black, 1988).

2.4.2.1. Amino acid transport

Amino acids are some of the most important nutrients in poultry nutrition, being largely derived from expensive protein feed sources and synthetic supplements. There are 11 essential or indispensable amino acids in poultry nutrition (McDonald *et al.*, 1988). Among the essential amino acids, lysine, methionine, tryptophan and threonine are regarded as limiting to poultry productivity due to their low concentrations and low availability from most feed ingredients, especially those high in cereal grains as are poultry diets (McDonald *et al.*, 1988; D'Mello, 1993). Although there has been extensive research on the poultry's requirements for these amino acids (NRC, 1994), there are only limited reports on mechanisms regulating their uptake and transport at the intestinal level.

Proteins, unlike carbohydrates are digested into several amino acids and small peptides which can all be absorbed by the intestine (Gray, 1992; Ganapathy *et al.*, 1994). There is therefore a need for the presence of multiple transport systems to handle the absorption of end-products from protein digestion unlike what is required for carbohydrates, yielding one to two terminal end-products and in which only monosaccharides are absorbable (Ganapathy *et al.*, 1994). The efficiency of utilization of dietary and supplemental amino acids depends primarily on the rate of uptake and transport from the GIT into the tissues.

A potential restriction to amino acid transport is the presence of a lipid bi-layer in bio-membranes; amino acids are only soluble in lipids to a limited degree within physiological pH range (Christensen, 1984; Alberts *et al.*, 1994). It is obvious that

external mediation would be required for the transport of amino acids from the intestinal lumen into the tissue. It has long been postulated that the crossing of the lipid bi-layer of the bio-membrane would be the rate-limiting step in the transport of molecules into the cell (Kotyk, 1977). With increasing molecular size and charge, the rate of transport decreases (Nassar, 1989; Alberts *et al.*, 1994).

The fact that amino acid transport is often not limited to a single carrier presents a complex scenario in studies on their transport mechanisms (Christensen *et al.*, 1965). Christensen and Liang (1965) cautioned that the absence of inhibitory action by an analogue on known transport systems does not imply a lack of transport of the analogue nor does the fact that an analogue is not largely transported imply inhibition. Christensen (1982) also observed that the properties of transport systems vary considerably between tissues.

2.4.2.2. Amino acid transport systems

There is a multiplicity of transport systems with overlapping specificities by which amino acids are transported into tissues (Table 2.1). This often creates difficulties in the characterization of system(s) utilized by individual amino acids (Shotwell *et al.*, 1983). Most of the systems have been more extensively studied in eukaryotic cells and mammalian tissues than in poultry (Shotwell *et al.*, 1983; Lerner, 1984; Kushak and Basova, 1988; McGivan and Pastor-Anglada, 1994). The characteristics of the transport systems are not universally applicable due to variation between cell types and species (Lerner and Larimore, 1986; Lerner, 1987).

Table 2.1: Major amino acid transport systems in mammals.

System	Specificity	Tissue distribution	Properties
(i) Neutral amino acids			
A	Small aliphatic amino acids, MeAIB	Widespread	Na^+-dependent
ASC	Small aliphatic amino acids	Widespread	Na^+-dependent
N	Glu, His, Asp	Liver (variant in muscle)	Na^+-dependent
B	Broad specificity; most neutral amino acids	Renal and intestinal brush-border	Na^+-dependent
T	Trp and branched neutral amino acids	In red blood cells, kidney, intestine and placenta	Na^+-dependent; Na^+-independent
L	Mainly branched-chain aromatic amino acids	Widespread	Na^+-independent
(ii) Acidic amino acids			
X_{AG}	Glu, Asp	Renal, intestinal fibroblast	Na^+-dependent
X_c-	Glu, Cys	Hepatocytes, fibroblasts	Electroneutral
(iii) Basic amino acids			
y^+	Arg, Lys, His	Widespread	Na^+-independent
(iv) Neutral and basic amino acids			
B^{o+}	Broad-specificity	Blastocysts, oocytes	Na^+-dependent
b^{o+}	Broad-specificity	Blastocysts, oocytes	Na^+-dependent

Adapted mainly from McGivan and Pastor-Anglada (1994). The convention on nomenclature and classification of properties has not been fully resolved.

Amino acid transport systems are regulated by their individual amino acids and other substrates which may not necessarily be transported (McGivan and Pastor-Anglada, 1994; Ganapathy *et al.*, 1994; McGivan, 1996). Most of the initial research on the identification of amino acid transport systems was led by Christensen and co-workers in studies on mainly whole cells and plasma membranes (Oxender and Christensen, 1963; Christensen, 1964; Christensen *et al.*, 1969; Christensen and Antonioli, 1969).

Although many recent studies are still based on their pioneering work, it has been recognized that transport through the intestinal cell would be different from that observed in many plasma cells due to the polarity of the former (Ganapathy *et al.*, 1994). The overall absorption at the intestine involves transport of the nutrient through the brush-border membrane into the cell and out into the general circulation through the baso-lateral membrane (Figure 2.1). Transport systems operating at the two polarized membranes of the enterocyte appear to differ from one another (Ganapathy *et al.*, 1994).

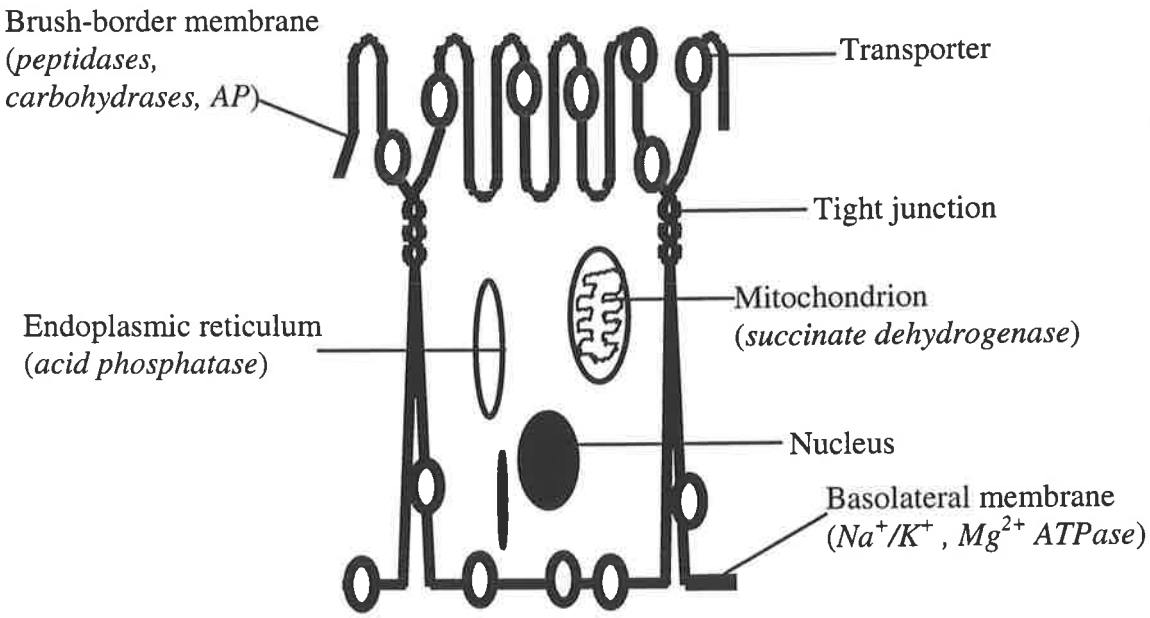


Figure 2.1: An enterocyte showing the two membranes and their respective membrane-bound enzymes.

Apart from the influence of their substrates, transport systems are regulated by a variety of factors, including those of developmental origin; hormones; dietary factors, and ions (Shotwell *et al.*, 1983; Lerner, 1985; McGivan and Pastor-Anglada, 1994). The fact that the GIT assumes responsibility for nutrient derivation immediately after birth or hatch would suggest the pre-natal presence of transport function (Ganapathy *et al.*, 1994). The

rate of transport of most amino acids is maximal at birth or hatch and declines with age in most species (Navab and Winter, 1988; Buddington and Diamond, 1989).

The regulation of amino acid transport by dietary factors involves complex mechanisms. The transport rates of most amino acids and peptides is enhanced by an increase in the concentration of amino acids in the lumen, as occurs immediately post-prandial (Stevens *et al.*, 1984; Ganapathy *et al.*, 1994). There are, however, reports on the inducement of some transport systems, for example A and X_{AG} by substrate starvation (McGivan, 1996).

The hormonal regulation of amino acid transport also varies with the transport system (amino acid) and hormone. Somatostatin has been shown to reduce the transport of lysine and leucine while neurotensin and secretin enhance their transport (Chen *et al.*, 1987).

Amino acid transport systems are frequently classified on the basis of their response to ions, notably sodium (McGivan and Pastor-Anglada, 1994; McGivan, 1996). Other ions that may regulate transport include potassium, lithium, chloride and hydrogen (Aragon *et al.*, 1987; Lerner, 1985; Aragon *et al.*, 1987).

2.4.2.3. The molecular nature of amino acid transporters

The identification of the molecular nature of amino acid transporters is a very recent area of study, even in mammals. King *et al.* (1983) observed that future advances in the understanding of digestive and absorptive functions in the intestine will revolve around studies at the cellular and molecular levels. Ultimately, most of the transporters are

protein in nature but they are not easy to isolate for detailed examination due to a variety of limitations (Karasov *et al.*, 1986; McGivan and Pastor-Anglada, 1994). Most of the information currently available has been obtained from expression cloning of proteins, using principally, oocytes of *Xenopus spp*. Inactivation by radiation has also been used to a limited extent to study the sizes of transporters *in situ* (McGivan and Pastor-Anglada, 1994). McGivan and Pastor-Anglada (1994) postulated that more than one gene product may correspond to a single kinetically defined transport system and tissue-specific isoforms of transport proteins may occur. The cloning of the appropriate genes would be important to studies on the molecular basis for recognition of a wide range of substrates by the transporters.

The first mammalian amino acid transport system to be identified was system y^+ (now called the cationic amino acid transporter 1 or CAT1 (Kim *et al.*, 1991; Wang *et al.*, 1991). A cDNA encoding an electrogenic Na^+- but not Cl^--dependent high affinity glutamate transporter (named EAAC1) was also isolated from the small intestine of rabbit by expression in *Xenopus* oocytes (Kanai and Hediger, 1992). The abnormal glutamate transport associated with certain neuro-degenerative disorders were ascribed to changes in the function of this protein. In addition to this transporter, other transporters, code-named GLT-1 and GLAST, were identified for glutamate and all three showed remarkable similarities to one another and may represent a protein family (McGivan and Pastor-Anglada, 1994). Wells and Hediger (1992) isolated a 2.3 kb cDNA from the rat kidney which was believed to induce the transport of neutral and cationic amino acids in the intestine. The deduced amino acid sequence predicted a 78-kDa protein with a single trans-membrane domain, in contrast to most known membrane transport proteins which possess multiple membrane-spanning regions.

While there may be similarities in the nature of amino acid transporters between mammalian species and poultry, studies on the characterization of transporters in poultry lags behind those of mammalian species. In a review on the comparative aspects of apparent Michaelis constant for neutral amino acid transport in animals, there were wide differences between the chicken and mammalian species in the transport of several amino acids (Lerner and Larimore, 1986). There were also differences between mammalian species in the transport rate of the same amino acids. This may be an indication that transport systems or the nature of transporters may differ from species to species and possibly between strains of chickens used in broiler production.

2.5.0. Feed supply to the broiler chicken industry

Feed manufacture and supply to the broiler chicken industry have become vibrant industries in their own rights. Feed supply has been taken beyond the realm of small farm-level operations and involves extensive research into the identification and impact of various ingredients on poultry productivity. The supply and impact of the two principal groups of feed components, energy and protein are examined in this section. This should not diminish the importance of minor components, most of which, like microbial enzyme production have become big branches of established industries and may command greater economic importance than the supply of the principal feed ingredients.

2.5.1. Energy sources

The traditional energy feedstuff for broiler chickens are the cereals, notably wheat, barley, oats, rye, triticale in temperate areas and maize, sorghum and pearl millet in

subtropical and tropical areas. Although the world output of cereals continues to grow, so also does the direct requirement for cereals by humans, with the result that less and less is available for poultry feeding in many parts of the world (Oluyemi and Roberts, 1979; Sonaiya, 1995).

Novel sources or cheap conventional sources have to be explored. In the temperate regions, more use is made of the less nutritive oats and rye or low quality varieties of wheat and barley (Rogel, 1985; Bedford *et al.*, 1991; Friesen *et al.*, 1991; Miller *et al.*, 1994). Low quality cereals such as unimproved sorghum and millet as well as tubers and brewery by-products are replacing maize in the tropics (Sonaiya, 1995). New diets often reduce poultry productivity but the mechanisms involved have not been fully identified (Campbell *et al.*, 1989; Friesen *et al.*, 1991; Bach Knudsen *et al.*, 1991; Cave and Burrows, 1993).

Most of the mechanisms involved with variation in response, especially at the intestinal level to different cereals have not yet been identified but some attention is currently been paid to the study of cereal carbohydrates, especially NSP and how the nutritive value of low quality cereals could be improved (Annison and Choct, 1991; Bedford *et al.*, 1991; Classen and Bedford, 1991; Dahle, 1992). NSP may become the major determinants of feed quality in future and have been associated with the variability in metabolizable energy of wheat batches (Annison, 1993; Nicol *et al.*, 1993) as well as poor growth of poultry (Campbell *et al.*, 1989; Friesen *et al.*, 1992). The structure and distribution of these compounds are further examined in section 2.5.3.1.

2.5.2. Protein sources

The oilseed cakes are the principal sources of protein for poultry. Around the world, the major oilseeds include soyabeans, groundnuts and rape or canola (McDonald *et al.*, 1988). Less common ingredients of local importance include faba beans, cottonseed, sesame, peas, palm kernels, coconut meal and lupins (Trevino *et al.*, 1992; Petterson and Mackintosh, 1994; Sonaiya, 1995). Animal sources of protein such as fishmeal, bloodmeal, feathermeal and meat/bonemeal have also been used in broiler feeding but are limited in supply. Animal proteins cannot be included at very high levels but may be used to rectify certain amino acid deficiencies in plant protein (McDonald *et al.*, 1988).

As with the major cereals, the price of oilseed meals has been on the increase over the years, coupled with unreliable supply in many areas (Oluyemi and Roberts, 1979; Williamson and Payne, 1990; Sonaiya, 1995). There has been a need to identify cheaper sources of protein, of less direct use to other livestock industries and humans.

Lupins are among the alternative ingredients currently been explored in Australia and other parts of the world (Centeno *et al.*, 1990; Brenes *et al.*, 1993; Petterson and Mackintosh, 1994; Olver and Jonker, 1997). There are over a hundred known species of wild lupins in North America alone but the principal lupins grown for grain in Australia, Europe and North America are the white (*Lupinus albus*) and the narrow-leaf (*L. angustifolius*) lupins (Cheeke and Kelly, 1989; Petterson and Mackintosh, 1994). The protein content of lupins is comparable to that of soyabeans and other traditional oilseed cakes (Ballester *et al.*, 1980; Petterson and Mackintosh, 1994). Petterson and

Mackintosh (1994) have identified *L. albus* as higher in protein content than *L. angustifolius*. The major advantage of lupin seed meal for non-ruminant animals is that unlike soyabean seed meal, it can be used without pre-treatment. In spite of their high protein content and low concentrations of protease inhibitors, animal productivity on lupins and the other legume seed meals is lower than the level observed with oilseed meals such as those of soyabeans (Centeno *et al.*, 1990; Brenes *et al.*, 1993).

Most current research is aimed at identifying the cause of the low productivity, combined with identification of solutions to the problem. Results point to the existence of ANF in many of the alternative feed sources (Watkins *et al.*, 1988; Saini, 1988; Cheeke and Kelly, 1989). The impact of these factors at the intestinal level is an area that has been less studied than the gross effects on productivity.

2.5.3. Anti-nutritive factors in feedstuff

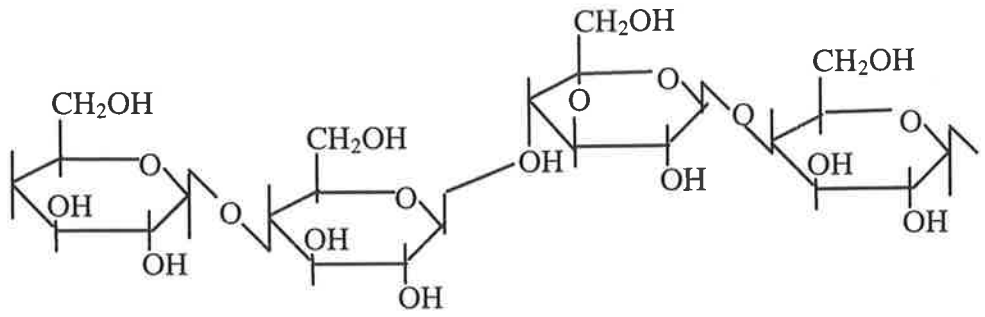
Anti-nutritive factors in foodstuffs fall into two broad classes. Some of the factors are actually potential nutrients which animal enzymes cannot digest (Annison, 1993; Rotter *et al.*, 1990; Campbell *et al.*, 1993). Other factors clearly do not provide any nutrient to the bird and at a high concentration could be toxic (Aslanov *et al.*, 1987; Watkins *et al.*, 1988; Cheeke and Kelly, 1989). The reduction in the productivity of poultry on diets supplemented with poor quality cereals and legume seed meals has been frequently reported (Campbell *et al.*, 1989; Friesen *et al.*, 1991; Friesen *et al.*, 1992; Marquardt *et al.*, 1993) but the operating mechanisms at the intestinal level are poorly understood. The effects of some ANF on the development of intestinal structure and function have been highlighted in section 2.3.2.5. In this section, the structure and distribution as well as impact of ANF on productivity of some of the ANF are presented.

2.5.3.1. Non-starch polysaccharides and toxic anti-nutritive factors

Starch is the predominant carbohydrate in most feed ingredients used to supply energy in poultry nutrition. Some of the carbohydrates in feed ingredients may be in the form of NSP. The common NSP include β-glucans, arabinoxylans and fructans (Classen and Bedford, 1991). Most cereals used in poultry diets contain NSP at different concentrations and there are indications of their presence in some legume species, including lupins (Watkins *et al.*, 1988; Brenes *et al.*, 1989; Saini, 1989; Evans *et al.*, 1993) and cassava (Tovar *et al.*, 1989).

The structure of NSP differs between grains and also between varieties of the same crop (Saastamoinen *et al.*, 1989; Bengtsson *et al.*, 1990; Annison, 1990). The similarities between the different types of NSP, usually is the presence of β(1→4) backbones with or without the presence of β(1→3) side-linkages (Figure 2.2). These linkages generally render NSP resistant to animal enzymes (Dahle and Eikum, 1992). Barley and oats are particularly rich in NSP (Klopfenstein, 1988; Dahle *et al.*, 1992) and there are similarities in structure between rye and wheat pentosans (Choct and Annison, 1990). In oats and barley, the dietary fibre constituents of major importance in the endosperm cell walls are the mixed-linked (1→3), (1→4)- β-D-glucans (mixed-linked β-glucans) while in wheat, rye and triticale, the endosperm cell walls consist mainly of arabinoxylans (Pettersson *et al.*, 1990; Smits and Annison, 1996). The tropical cereals, maize and sorghum are generally low in NSP, and the NSP is made of predominantly arabinoxylans.

a. β-(1→3), (1→4)-D-glucan, the major NSP in barley and oats



b. Arabinoxylan, the major NSP in wheat, maize and sorghum.

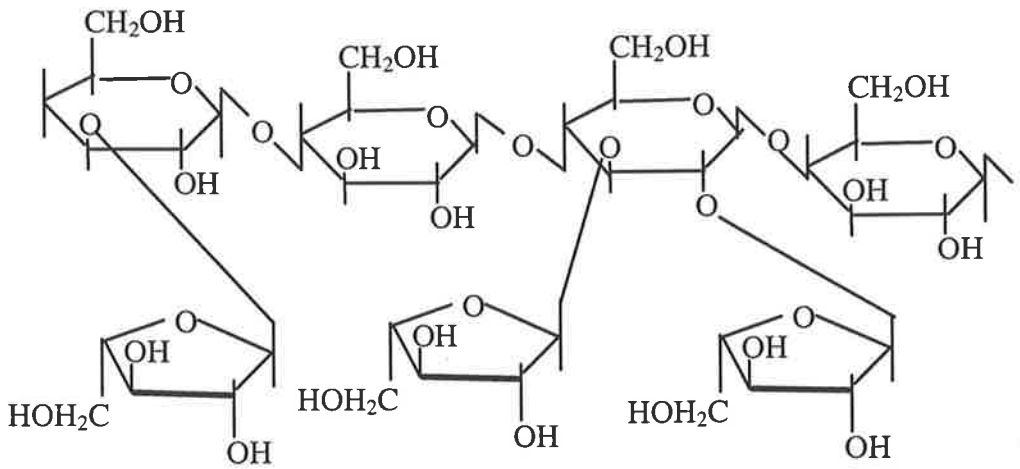


Figure 2.2: The two principal NSP found in cereal grains (Smits and Annison, 1996).

Most digestion of NSP in poultry occurs in the lower gut through the influence of enzymes secreted by resident microbes or microbial enzyme preparations included in the diet (Pawlik *et al.*, 1990; Friesen *et al.*, 1992). Over the entire GIT of pigs, β-glucans are better digested than arabinoxylans but both of these NSP are better digested than cellulose (Bach Knudsen, 1991; Bach Knudsen *et al.*, 1991). Carre *et al.* (1995) reported almost a complete lack of digestion of pea seed NSP in young broiler chicks and only minimal digestion of the material by cockerels, indicating that resident

microbes contribute mostly to the digestion of NSP in non-ruminant animals. Poultry have been generally identified as poorer digesters of NSP than pigs and rats (Huisman and Tolman, 1992; Jorgensen *et al.*, 1996).

The most noticeable effect of NSP in diets is an increase digesta viscosity and this was considered to be the main mechanism by which NSP influence productivity (Salih *et al.*, 1990; Classen and Bedford, 1991; Smits and Annison, 1996). There are still conflicting reports on the effects of NSP on the digestion of other dietary components, including proteins and starch (Green, 1988; Rakovska *et al.*, 1990; Annison, 1990; van der Klis *et al.*, 1993). The variation in research findings may be due to differences in solubility and molecular weight of NSP from different feed sources (Choct and Annison, 1990; Classen and Bedford, 1991; Evans *et al.*, 1993).

Apart from the creation of a viscous environment in the GIT, other effects of NSP have been identified which may regulate poultry productivity in tandem with or more significantly than viscosity. In trials on various soluble commercial NSP supplements in broiler chicken diets, Annison (1989) postulated that the changes in productivity may be linked to alterations in intestinal structure and function, rather than the simple effects of energy dilution. This has been proved in studies on rats fed on diets supplemented with soluble commercial NSP, including guar gum (Johnson and Gee, 1986; Brunsgaard and Eggum, 1995). The NSP induced increased intestinal weight through an increased rate of cell proliferation and there were changes in mucosal structure and function. β-glucans and other NSP may bind to dietary nutrients as well as reduce nutrient mobility and thus impair digestion and absorption (Read, 1987; Klopfenstein, 1988). Ikeda and

Kusano (1983) have also shown that NSP could bind to digestive enzymes to reduce enzyme activities and digestion.

The digestion of NSP by microbial enzymes may provide additional dietary energy as well as short-chain fatty acids and oligosaccharides, all of which are involved in complex mechanisms to induce cell proliferation and mucosal growth or renewal (Mathers and Kennard, 1993; Sakata *et al.*, 1995; Yamauchi *et al.*, 1996). Most of these mechanisms have not been investigated in poultry. A greater knowledge of how NSP operate at the intestinal level will assist in the development of techniques to combat their negative effects.

2.5.3.2. Oligosaccharides

Oligosaccharides are carbohydrate molecules containing between 2 and 10 monosaccharides, which may be pentoses or hexoses. The most common naturally occurring oligosaccharides are of the raffinose series, formed by the combination of α-D-galactose to a sucrose primer and serial elongation of the chain by further additions of the α-D-galactose (Saini and Gladstones, 1986). The common members of these family are shown in Figure 2.3 and their effects on broiler productivity were recently reviewed by Iji and Tivey (1998). The exact impact of the oligosaccharides are still unknown and the conflict may be due to differences in chemical structures and level of supplementation (Waldroup *et al.*, 1993; Oyarzabal and Conner, 1995; Oyarzabal and Conner, 1996; Irish *et al.*, 1995). The conflict has been increased by reports on the positive effects of oligosaccharides on animal productivity and lack of improvements when oligosaccharides are extracted from seed meals (Choi *et al.*, 1994; Okumura *et al.*, 1994; Slominski *et al.*, 1994; Irish *et al.*, 1995). Currently, there is an array of synthetic

commercial oligosaccharides being marketed for use in animal and human nutrition (Monsan and Paul, 1995) although the exact benefits and effects of many of them are yet to be determined.

Like the NSP, more research has been conducted into the gross effects of oligosaccharides and less is known about their impact at the intestinal level. Such information will determine whether or not they are beneficial or detrimental to productivity.

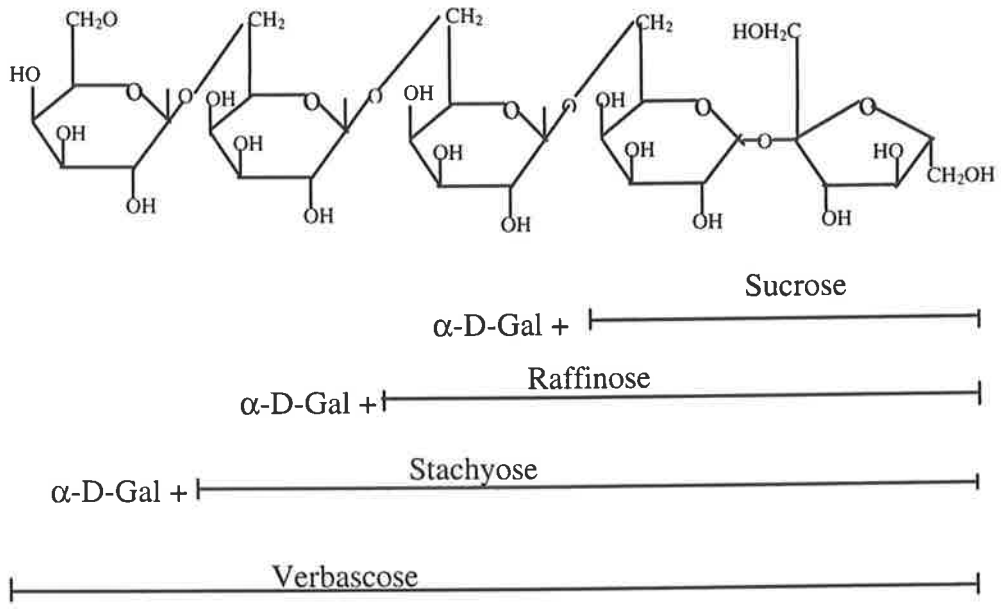


Figure 2.3: The raffinose series oligosaccharides are formed by the step-wise addition of α-D-galactose to a sucrose primer (Saini and Gladstones, 1986).

2.5.3.3. Other anti-nutritive factors in feeds

The growth-depressing effects of certain legume seeds have been traced to the presence of toxic factors, rather than a deficiency in amino acids (Huisman and van der Poel, 1989). In faba beans and cowpeas, the limiting ANF are tannins which complex with starch and proteins to reduce digestion (Thomson and Yoon, 1984; Trevino *et al.*, 1992;

Sonaiya, 1995). Protease (trypsin) inhibitor activities have been demonstrated in a few legume seeds (Petterson and Mackintosh, 1994). In unimproved cotton seed meal, the major ANF is gossypol, whose direct effects on non-ruminant animals are depressed appetite and growth (McDonald *et al.*, 1988). Its effects on enzyme activities have been highlighted in a previous section of this review (Baram *et al.*, 1987). Cheeke and Kelly (1989) have also attributed the low nutritive value of lupins to high levels of manganese, the toxicity of which is characterized by depressed appetite and retarded growth.

2.5.3.4. Combating the effects of anti-nutritive factors

The broiler chicken industry will continue to explore and exploit alternative ingredients since they ensure the lowest costs of production. A lot of research has been undertaken to improve the quality of alternative ingredients for poultry. Further research is also conducted to assess the efficacy of some of the techniques used to improve the nutritive value of the alternative feed ingredients. The direction of research at improving alternative feed ingredients falls into various categories; most of them targeted at the improvement of the feed material and less at the improvement of the animal.

Most of the improvement to dietary ingredients has been achieved through plant breeding. The development of low alkaloid lupins was a huge success although the nutritional value of the low alkaloid (sweet) lupins has not yet been adequately studied (Gladstones, 1974; Hill, 1977; Cheeke and Kelly, 1989; Brenes *et al.*, 1993). The selection of *L. albus* for lower manganese content is also feasible (Oram *et al.*, 1979; cited by Cheeke and Kelly, 1989). Similar efforts have been directed at cereals with the aim of developing less viscous cultivars of barley, oats and rye which would be able to

sustain higher growth rates in broiler chickens than the highly viscous cultivars (Campbell *et al.*, 1989; Campbell *et al.*, 1993; Miller *et al.*, 1994; Boros *et al.*, 1995).

Brenes *et al.* (1993) and Annison *et al.* (1996) reported an improvement in broiler chicken growth through dietary supplementation of lupin-based diets with commercial microbial enzymes. Supplementation with a β-glucanase achieved the same effects of improved animal performance raised on barley and oat diets, more so in hull-less than in hulled barley (Rotter *et al.*, 1990; Campbell *et al.*, 1993; Farrel and Martin, 1993). Depolymerization through the use of a β-xylanase or soaking reduced the deleterious effects of pentosans in diets for broiler chickens (Pawlik *et al.*, 1990; Choct and Annison, 1992). The improvement observed with enzyme supplementation is attributed to improved digestion and absorption of other dietary components (Scholtyssek and Knorr, 1987; Fengler *et al.*, 1988; Pettersson and Aman, 1989). These improvements are achieved possibly through a reduction in digesta viscosity usually noticed in birds fed on rye diets supplemented with enzymes (Bedford *et al.*, 1991). The digestion of NSP may also provide more energy required for intestinal and body growth as has been highlighted above (Moore *et al.*, 1989; Yamauchi *et al.*, 1996).

Pre-treatment of rye by auto-claving and pelleting has been shown to enhance the subsequent response of birds maintained on such diets (Tietge *et al.*, 1991; cited by Bedford *et al.*, 1991). There are conflicting reports as to the benefit of heat treatment of lupins. Petterson and Mackintosh (1994) indicated that heating, flaking and rolling of lupins yielded only marginal improvements in feed quality, stressing that high heat treatment may reduce the utilization of amino acids. Cheeke and Kelly (1989) stated that no remedy to the adverse effects of lupins was achieved through heat treatment

when fed to pigs. The lack of improvement in nutritive value was attributed to the absence of heat-labile deleterious factors in lupin seed.

Research at the animal level to improve their utilization of low quality diets are limited and such research can be developed only with better understanding of the natural pattern of the body and intestinal development, followed by research on responses to the target dietary factors.

2.6.0. Summary

This review has provided information on the variability between different strains of broiler chickens used by the industry and indicated the importance of the GIT to nutrient derivation in mainly mammalian species. The pattern of regulation of the GIT by natural and intestinal factors, especially in mammals was also highlighted. The changes in productivity observed in the presence of alternative ingredients in poultry diets may be traced to events at the intestinal level. The mechanisms have not been adequately studied in poultry and results from such research will aid further genetic development of broiler chickens as well as improve the formulation of diets for the industry.

2.7.0. Potential benefits of research

The benefits of this study will be the development of cost effective diets for broilers in the short term. In the long term, poultry strains could be developed to maintain productivity during adverse dietary challenge. Outside the realm of agriculture, some of the findings will find use in bio-medical research, especially in the study of gastrointestinal diseases in humans.

2.8.0. Objectives of research

The research reported in this thesis had two major objectives:

1. the assessment of the natural pattern of body growth in relation to changes in the structure and function of the GIT in broiler chickens, and

2. the influence of various dietary factors and regimes on gastrointestinal development and body growth.

CHAPTER THREE: RESEARCH PLANS AND METHODOLOGIES

3.1.0. Introduction

Most of the experimental procedures adopted for this research have not been widely employed previously in research on poultry but may have been reported in studies involving other species. Preliminary studies were conducted to establish optimum conditions for the application of these procedures in studies on poultry. This chapter describes the materials and procedures common to more than one of the experiments conducted in the present study. Other materials and methods specific to individual experiments are described under the appropriate experiments. Results of preliminary studies undertaken to set standards for the main experiments are also presented.

3.1.1. Animal management

The main experiments were conducted on the Steggles x Ross (F_1) strain of chicks obtained from the Australian Poultry Pty Ltd. This strain was developed in the mid-1990s from Ross females of Scottish origin and Steggles males originally used in Australia. Some preliminary studies and one of the main experiments were conducted on Ingham IM98 (Inghams Enterprises Pty. Ltd.). For the studies on the natural pattern of development, commercial diets (Milling Industry Stockfeeds, Murray Bridge, South Australia) were used for the entire period. For other studies, the chicks were brooded on the starter commercial diet for 7 days before they were transferred to the treatment diets. For the short term study reported in experiment 5.3, chicks were brooded for 3 weeks on a commercial diet prior to the commencement of a 7-day exposure to the experimental diets.

Preliminary studies revealed differences in body weight, growth rate and feed conversion efficiency between male and female chicks raised on a sorghum/soyabean

meal diet to 28 days of age. No significant differences were observed in mucosal morphometry and activities of α-glucosidase (AG), alkaline phosphatase (AP), aminopeptidase N (APN) and dipeptidylpeptidase IV (DPP IV). For subsequent experiments, the chicks were not sexed and were randomly allocated to treatment diets or sampled in the case of studies on natural development.

Chicks were brooded in battery brooders to ensure adequate control and accurate measurement of performance variables. The brooding temperature was between 32° and 40°C in the first week and was stepped down to the optimum rearing temperature regime, 24-26°C for growing and finishing broiler chickens by 21 days of age. Post-brooded birds were finished in battery cages (63 x 63 x 37 cm). Artificial lighting from normal flourescent tubes was provided for 23¾ hours a day.

All diets were formulated to meet the nutrient requirements specified by the National Research Council (1994) for broiler chickens. Feed and water were supplied *ad libitum.* Birds were weighed at the start of each trial and afterwards at 7-day intervals until the end of the feeding period.

3.1.2. Ethical clearance for animal management

The studies reported in this thesis were authorized by the University of Adelaide Animal Ethics Committee (No. W/054/94). Experimental procedures were designed to minimize stress and pain to animals. Only diets that could be practically used in the broiler chicken industry were formulated and assessed. Prior to the collection of internal organ samples, chicks were euthanatized by intravenous injection with

Lethabarb™ (Virbac Aus. Pty Ltd), a preparation containing sodium pentobarbital, at a dose of 1 ml/kg body weight.

3.2.0. Protocols

To meet the objectives of the present study, experiments were conducted first, to examine both the natural pattern of intestinal development in relation to whole body growth, and secondly, to assess the impact of dietary factors on these parameters.

3.2.1. Natural development of intestinal structure and function.

The structure and properties of the gastrointestinal tract and some accessory organs at different ages were studied. Results obtained provided baseline data for comparisons with subsequent studies on response to dietary challenge. The nutrient composition of the commercial diets that were fed under this protocol is shown in Appendix 1.

3.2.2. Development in response dietary factors

The negative impact of ANF present in many novel as well as certain traditional feed ingredients have been highlighted in Chapter 2. One of the principal aims of this project was to ascertain the link between intestinal function and response to these ANF. The dietary factors considered in this thesis are those that are of contemporary importance to the broiler chicken industry. Studies with this objective were conducted mainly over the long term (14-21 days) and are reported in Chapter 5. Apart from studies on ANF, one of the experiments targeted a pre-biotic commercial product, Bio-Mos™ (Alltech Inc., Nicholasville, KY, USA) currently being included in diets.

The short-term studies were conducted mainly to identify the significance of duration of exposure to dietary factors on intestinal function. Although they may be of limited direct use to the industry, these studies may validate some of the exploratory techniques used for dietary assessment, as commonly undertaken in the classical apparent metabolizable energy (AME) assays.

3.3.0. Dietary analysis

The nutrient composition of some of the diets was calculated from feed composition tables, based on assays conducted on poultry of similar age to the ones used in the studies reported in this thesis. Data for ingredients grown in Australia were used. In addition, the concentrations of some nutrients and ANF were determined according to existing procedures, for experiments reported in chapter 5. Assays were also conducted to determine the *in vitro* digestibility of dry matter (DM) and starch.

3.3.1. Starch content of diets and digestibility of starch

Starch content was determined according to the method of Lever (1972). A 200-mg sample of feed was gelatinized in 9 ml of 0.2 M sodium acetate buffer, pH 4.5 at 100°C in sealed, screw-capped, polypropylene, acid-resistant centrifuge tubes for 4 hours. The tubes were cooled to below 50°C and 1 ml of 0.2 M sodium acetate buffer added. The digesta was incubated with 0.1 mg of a thermo-stable α1: 4α 6-amyloglucosidase/ml at 50°C for 18 hours in a rocking water bath. A sub-sample of the final digesta was incubated with glucose oxidase (EC. 1.1.3.4) at room temperature for 30 minutes after which the absorbance was measured at 610 nm. The enzyme and glucose kit were obtained from Boerhinger-Mannheim (Aus) Pty Ltd.

Based on this assay, the digestibility of starch *in vitro* was derived as the amount of glucose released following digestion. Where the starch contents of the diets and digesta were determined, the digestibility *in vivo* was obtained by difference according to the following equation:

$$St_{diet} - St_{digesta}/St_{diet} \times 100$$

where St_{diet} is starch content of diet and $St_{digesta}$ is starch content of the digesta.

3.3.2. Crude protein content

The crude protein contents of the commercial diets used in experiment 4 and diets fed in experiment 5 were determined with the assistance of personnel at the Nutrition Laboratory of the Pig and Poultry Production Institute (PPPI), Roseworthy, South Australia. The standard Kjeldahl technique was used, in which a known weight of the sample was digested with concentrated sulphuric acid. The digest was distilled on an automatic Kjeltec 1026 distilling unit using boric acid. The distillate was then titrated against 0.1 M HCl to derive the nitrogen content (g/kg) of the sample as:

$$1.4 \times (S_{vol} - B_{vol}) \times Mol_{HCl}/S_{wt}$$

where S_{vol} is the volume of sample distillate titrated to neutrality, B_{vol} is volume of blank titrated to neutrality, Mol_{HCl} is the molar concentration of HCl used and S_{wt} is the weight (g) of the sample digested. The constant, 1.4 represents the amount of nitrogen (0.0014 g) in sample, neutralized by 1 ml of 0.1 M HCl. Crude protein (g/kg) was derived from nitrogen (g/kg) using the factor 6.25.

3.3.3. Non-starch polysaccharides in diet

The total NSP contents of the diets fed in chapter 5 were measured by gas-liquid chromatography according to the procedures of Englyst *et al.* (1992). Finely ground samples, 100-200 mg, in duplicate were solubilized with 2 ml of dimethyl sulphoxide. The samples were incubated for 30 minutes in boiling water after which 8 ml of 0.1 M sodium acetate buffer (pH 5.2; pre-equilibrated at 50°C) were added and incubation continued for 3 minutes at 42±2°C. A fresh solution of porcine pancreatin (1.2 g in 50 ml distilled water) was prepared and centrifuged at 1500 g to obtain a supernatant. 0.5 ml of this supernatant was added to each feed sample followed by 0.1 ml of pullalanase (Boerhinger-Mannheim Aus Pty Ltd) which had been diluted 1:100. The mixture was incubated at 42±2°C in a rocking water bath for 18 hours.

Incubation was terminated with 40 ml absolute ethanol and the incubation tubes were transferred to ice-cold water for 30 minutes. The samples were centrifuged at 1500 g for 10 minutes to obtain clear supernatants. The supernatant was decanted and discarded, without disturbing the residue. Ten millilitres of 85 % ethanol were added to the residue, mixed by inversion and made up to 50 ml with 85 % ethanol. The mixture was centrifuged and the supernatant decanted as described above. The sample was rinsed with 50 ml absolute ethanol, centrifuged and the supernatant discarded. Acetone, 20 ml was added to the residue, mixed, centrifuged at 1500 g for 10 minutes and the supernatant was discarded. The sample was dried in a fume hood until all acetone was removed.

The residue was digested with 5 ml of 12 M H_2SO_4 at 35°C for 1 hour and 10 ml of 1 M H_2SO_4 at 100°C for 2 hours. Internal standards (allose and inositol) were added and the samples were reduced and acetylated, using a modified procedure described by Theander *et al.* (1990). The samples were then analysed on a gas-liquid chromatograph to obtain peaks of individual sugars. Total NSP was calculated from polymerization factors of 0.89, 0.88 and 0.9 for deoxysugars, pentoses and hexoses respectively.

3.3.4. Dry matter digestibility

Dry matter (DM) digestibility, *in vitro* was determined by incubating 1 g of ground sample with porcine pepsin (39°C; 6 hours), followed by digestion with porcine pancreatin (39°C; 18 hours). The slurry was filtered through a glass filter crucible (pore size 1) containing celite as a filter aid. The crucible with the residue was dried overnight at 80°C. *In vitro* dry matter digestibility (% IVDMD) was calculated as:

$$FDM - RDM/FDM \times 100$$

where FDM is the weight of fresh dry matter incubated and RDM is the weight of residual dry matter after digestion and drying.

The digestibility of DM *in vivo* was estimated by difference between diet DM and faecal or digesta DM.

3.3.5. Digesta viscosity

The *in vitro* viscosity of the diets fed in experiment 5.1 was determined as described by Bedford and Classen (1993). A 600-mg sample of finely ground diet was digested in 0.9 ml 0.1 HCl and 2000 U pepsin/ml for 45 minutes at 40°C. The mixture was further incubated with 0.3 ml 1 M $NaHCO_3$ containing 8 mg pancreatin (8 x USP, Sigma

P7545) in sealed tubes for 2 hours with intermittent vortexing. The digesta was centrifuged at 12,700 g to obtain a supernatant on which viscosity was determined. For other experiments, the *in vivo* viscosity of digesta collected at appropriate sites along the gastrointestinal tract was determined. The digesta were frozen (-20°C) immediately after collection. The digesta were then thawed and centrifuged at 10,000 g to obtain a supernatant which was sub-sampled for measurement of viscosity.

Viscosity was measured with a Brookfield DV III rheometer (Brookfield Engineering, Mass., USA) fitted with a CP 40 cone. Measurements were performed at 25°C and shear rate of 0-500s^{-1}. The samples did not exhibit thinning over this range of shear rate.

3.4.0. Animal tissue collection and processing

At the end of each trial, the birds were slaughtered as described in section 3.1.2 and dissected to obtain the visceral organs. The gastrointestinal tract was weighed, full and empty. For the purpose of these studies, most of the focus was on the small intestine and its regions. The duodenum was regarded as the first segment of the small intestine, extending from the gizzard outlet to the ligament of Treitz (Levin *et al.*, 1983). The jejunum extends from this ligament to Meckel's diverticulum while the ileum was regarded as the section between the diverticulum and a point about 40 mm from the ileo-caecal junction (Wallis and Balnave, 1984; Branton *et al.*, 1988).

Experimental samples were collected from the proximal end of each region. The tissue was flushed with ice-cold phosphate buffered saline (PBS, pH 7.4) and slit longitudinally. The mucosa was rinsed of digesta with PBS and subdivided according to

the number of laboratory assessments for which it will be used. Usually, a 5-cm piece was wrapped in aluminium foil and snap-frozen in liquid nitrogen for use in the preparation of brush-border membrane vesicles (BBMV) on which digestive enzyme and transport functions were studied. A 1-cm piece was embedded in liver and snap-frozen for studies on enzyme histochemistry. A third piece was fixed in neutral buffered formalin and used to study mucosal histology. The methods adopted for each of these techniques are described in subsequent sections of this Chapter. The entire procedure from killing to sample storage took between 10 and 12 minutes for each chicken.

Accessory visceral organs such as the liver, pancreas, yolk sac and gizzard were also weighed. The digesta-holding capacities (gut-fill) of the gizzard and the small intestine were measured by difference between full and empty weight of organs. The chickens were not starved prior to sample collection to ensure accuracy of gut-fill.

3.4.1. Histology

Tissues for histology were fixed in neutral buffered formalin, which was identified as the most appropriate fixative in a preliminary study (section 3.4.1.1). The tissues were post-fixed in 70 % ethanol after 24 hours. Tissue slices, each measuring 1-2 mm thick were obtained and enclosed in tissue cassettes (Bayer Diagnostics, Aus. Pty Ltd). The tissues were processed over 16.5 hours in an automatic tissue processor (Shandon, Pittsburgh, USA). Processing consisted of serial dehydration with ethanol, clearing with histolene (Bayer Diagnostics, Aus. Pty Ltd) and impregnation with wax (Appendix 4). The processed tissue was then embedded in paraffin wax.

Sections were cut from the waxed tissue on a Leitz 1512 microtome (Ernst Leitz Westlar GmBH, Austria), cleared of wrinkles by floating on warm water (45-50^{o}C) prior

to mounting on 10 % poly-L-lysine coated slides. The slides were stained by Lilee Meyer's hematoxylin, counter-stained with eosin yellow and mounted in DePeX medium (Bayer Diagnostics, Aus. Pty Ltd) as shown in Appendix 5.

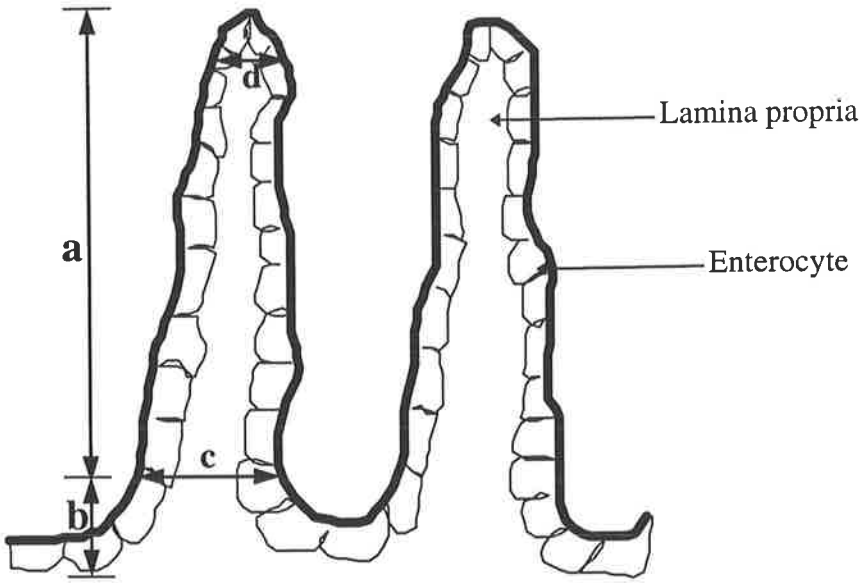


Figure 3.1: Outline of whole villi, showing measurements involved in morphometric assessment of the intestinal mucosa. Morphometric measurements undertaken included villus height (a), crypt depth (b), villus basal width (c) and villus apical width (d). Apparent villus surface area was estimated as (c + d)/2 x a.

Slides were viewed on an Olympus BH-2 microscope and digitized using a video image software, Video Pro (Leading Edge, Bedford Park, South Australia). Images were viewed (optical lens No. 4) to measure the thickness of the external muscle, crypt depth, villus width at the crypt-villus junction, villus height and villus apical width (Syme, 1982; Hamilton *et al.*, 1983). Villus surface area was estimated through trigonometry (Figure 3.1). Fifteen villi were assessed per sample.

3.4.1.1. Selection of fixative for histology samples

Biological tissues for histological assessment require routine fixation. Fixation achieves the multiple purposes of preservation of structural integrity, prevention of decay, hardening for subsequent processing and reduction in loss of cellular constituents.

Table 3.1: Effects of different fixatives and duration of fixation on external muscle thickness and mucosal morphometry (μm).

	Carnoy's fluid			Neutral buffered formalin			Paraformaldehyde			
Duodenum	**2h**	**6h**	**24h**	**2h**	**6h**	**24h**	**2h**	**6h**	**24h**	**SED**
External muscle	226.3	137.5	138.0	186.0	234.9	190.6	199.4	177.7	204.0	35.6
Crypt depth	221.3	234.6	269.5	274.4	287.2	210.8	294.4	236.5	253.1	31.3
Villus height	1411.0^{c}	1496.2^{bc}	1853.6^{ab}	1625.5^{ab}	1665.1^{ab}	1635.0^{ab}	2001.6^{a}	1839.3^{ab}	1889.8^{a}	142.6**
Ileum										
External muscle	234.3^{ab}	163.6^{b}	166.5^{b}	208.4^{ab}	206.3^{ab}	244.2^{ab}	236.3^{ab}	236.1^{ab}	273.4^{a}	24.1***
Crypt depth	157.1	174.4	158.0	151.8	152.6	173.8	162.4	181.8	172.7	13.6
Villus height	680.9	828.6	672.1	699.3	762.0	742.4	735.2	804.4	838.6	61.0

a,b - Mean values in the same row with unlike superscripts are significantly different (**P<0.01; ***P<0.001).

A major limitation encountered in the early phase of the current research was the poor quality of microscopic sections obtained from tissues fixed in Carnoy's fluid. The fixatives tested have been used successfully for tissues from other species (Hall and Byrne, 1989). There are few reports on microscopy of chicken intestinal tissue in current literature. Villi and microvilli from similar intestinal sites are known to be longer in poultry than in mammals (Smith and Peacock, 1989). A preliminary study was therefore conducted to identify the most suitable fixative and optimum duration of fixation for the main studies. Five 4-week-old broiler chicks of the Ingham IM98 strain (Ingham Enterprises Pty Ltd) were used for the tests. Tissues were fixed in Carnoy's fluid, 10 % neutral buffered formalin (NBF) or 4 % paraformaldehyde (compositions in Appendix 3) over 2, 6 or 24 hours. The birds were slaughtered as described in 3.1.3. Slides were obtained and digitized as highlighted in section 3.4.1.

The response of mucosal morphometry to the fixatives is shown in Table 3.1. The major criterion that was used to select the most appropriate fixative was the stability of morphometric values, especially villus height over the longer duration (24 hours) of storage. This was to allow for unimpeded sample collection, prior to transfer into a post-fixative, especially in experiments with a large number of samples. The fixative that yielded the most intermediate values between the two extremes was also assumed to be the one that would least distort natural morphometry. Thirdly and most importantly the subsequent quality of sections obtained from tissues treated in the three fixatives was compared. Overall, 10 % NBF met these criteria the best and was chosen for fixation of samples in the course of these studies, except for the samples that were used to study cell proliferation and migration in experiment 4.2 which were fixed in Bouin's fluid for 2 hours. Regardless of the fixative used, samples were transferred into 70 % ethanol prior to full processing (Appendix 4).

3.5.0. Biochemical estimation of cell size and metabolic activity

A known weight of intestinal tissue was cut into small pieces and vibromixed at high speed in 6 ml of ice-cold Tris buffer (0.05 M Tris[hydroxymethyl]aminomethane, 0.1 M NaCl, 0.01 M disodium EDTA; pH 9). The sample was filtered through a Buchner funnel, 1 mm pore size and the muscular debris discarded. The filtrate was blended at low speed (setting 2) using a tissue PCU-2 Polytron homogenizer (Kinectica GmbH, Luzern, Switzerland). A sub-sample of the homogenate was assayed for protein content according to the method described in section 3.7.1.1.

Nucleic acids (DNA and RNA) were extracted from crude mucosal homogenates of the duodenum, jejunum and ileum and quantified fluorometrically using the method described by Lemmens (1995). 100 µl proteinase K solution (0.2 mg /ml Tris buffer) and 100 µl sodium dodecyl sulphate solution (20 % w/v in Tris buffer) were added to the homogenate in Eppendorf tubes. The mixture was incubated for 15 minutes at room temperature with alternate stirring on a Vortex mixer. This was followed by centrifugation on a bench centrifuge for 15 minutes at 14,000 U/minute on a Micro 12-24 bench centrifuge (Hettich Zentrifugen, Tuttingen, Germany). A 300 µl sub-sample of the supernatant was transferred into Eppendorf tubes in duplicate. To the supernatant, 300 µl of 80 % phenol and 300 µl chloroform/isoamyl alcohol (24:1 v/v) were added and mixed for 10 minutes on a Vortex mixer at room temperature. The mixture was centrifuged and the phenol-chloroform/isoamyl alcohol phase (lower phase) discarded.

Phenol and chloroform/isoamyl alcohol extraction was repeated. Chloroform-isoamyl alcohol (300 µl) was added to the supernatant containing the nucleic acids and mixed for 1 minute on a Vortex mixer at room temperature. The mixture was centrifuged for 5 minutes and the chloroform-isoamyl alcohol phase (lower phase) was removed. The procedure was repeated. The nucleic acids were transferred to new Eppendorf tubes and stored at -20°C prior to spectrofluorometric measurements.

Total nucleic acid content (DNA + RNA) was determined by coupling first to ethidium bromide (excitation = 365 nm, emission = 590 nm), followed by determination of DNA by coupling to bisbenzemidazole (excitation = 352 nm, emission = 448 nm). In each

case, 100 µl of undilute samples were mixed with 3 ml of ice cold ethidium bromide or bisbenzemidazole in 4-clear-sided cuvettes with a 1 cm light path (Kartell, Italy). RNA concentration was obtained by difference between total nucleic acid and DNA concentrations. Measurements were made on a spectofluorometer (American Instrument Company, Silver Spring, MD, USA) using calf-thymus DNA and Bakers' yeast RNA as standards. All chemicals were obtained from Sigma (Aust.) Pty Ltd.

3.6.0. *In situ* localization of enzyme activity in brush-border membranes

The occurrence and expression of activity of intestinal ecto-enzymes has been studied in mammalian tissues for several decades (Wachsmuth, 1976; Gossrau, 1980; 1981). There are very few reports on such studies in the chicken. It was therefore imperative to set appropriate standards for the current research. The research interest of this study was focused on carbohydrases, peptidases and phosphatases. This section presents the standardization of experimental procedures for measuring the *in situ* activities of alkaline phosphatase (AP), aminopeptidase N (APN) and α-glucosidase (AG). In Chapter 2, mention has been made of their general characteristics as observed in mammalian tissues.

Intestinal tissues were obtained from 28-day-old chicks which had been fed a sorghum/soyabean diet. The tissues were flushed with PBS (pH 7.4), slit open and embedded in thin slices of liver prior to snap-freezing in liquid nitrogen. Sections, 8 µm thick were obtained with the aid of a Riechert-Jung cryostat (Cambridge Instruments, GmBH, Germany) at box and object temperature of -25±1° and -15±1°C respectively.

Sections were preliminarily mounted on untreated 1-ounce coverslips at room temperature.

For each of the enzymes tested in subsequent experiments, initial rate activities were determined. The buffers and anisidine salts used were similar to those employed in studies on mammalian tissues (Nachlas *et al.*, 1957; Gutschmidt *et al.*, 1979; Gutschmidt *et al.*, 1980). The incubation temperature was 39°C. The mounting medium was glycerine jelly, prepared locally from 10 g gelatin, 60 ml distilled water, 70 ml glycerol and 0.25 g phenol as preservative.

All assays were conducted in coplin jars, designed to hold 5-7 coverslips. The coverslips, with the tissue sections were then mounted on uncoated slides. The slides were viewed on an Olympus BH-2 microscope and enzyme activity was digitized on computer using the Video Pro software (Leading Edge, Bedford Park, South Australia). At least 5 villi were scored and averaged per tissue (replicate). The optimum duration of incubation was estimated as the time over which half maximal activity occurred or the point of inflection between the rapid and slow phases of activity.

The principle behind enzyme cytochemistry is of the form:

a. Substrate + membrane-bound enzyme $\longrightarrow$ Primary Reaction product (PRP)

b. PRP + anisidine diazonium salt $\longrightarrow$ Final reaction product (FRP)

The FRP is a coloured product deposited thinly on the surface of the villus (brush-border membrane) and can be measured in terms of its absorbance of incident light (Wachsmuth and Donner, 1976). Fixatives included in the incubation mixture confine

the deposition of the PRP and FRP to the original site of enzyme activity. Plate 3.1. illustrates the outcome of procedures designed for the enzymes assessed in this study. Details of the procedures adopted for each enzyme are described in the following sections.

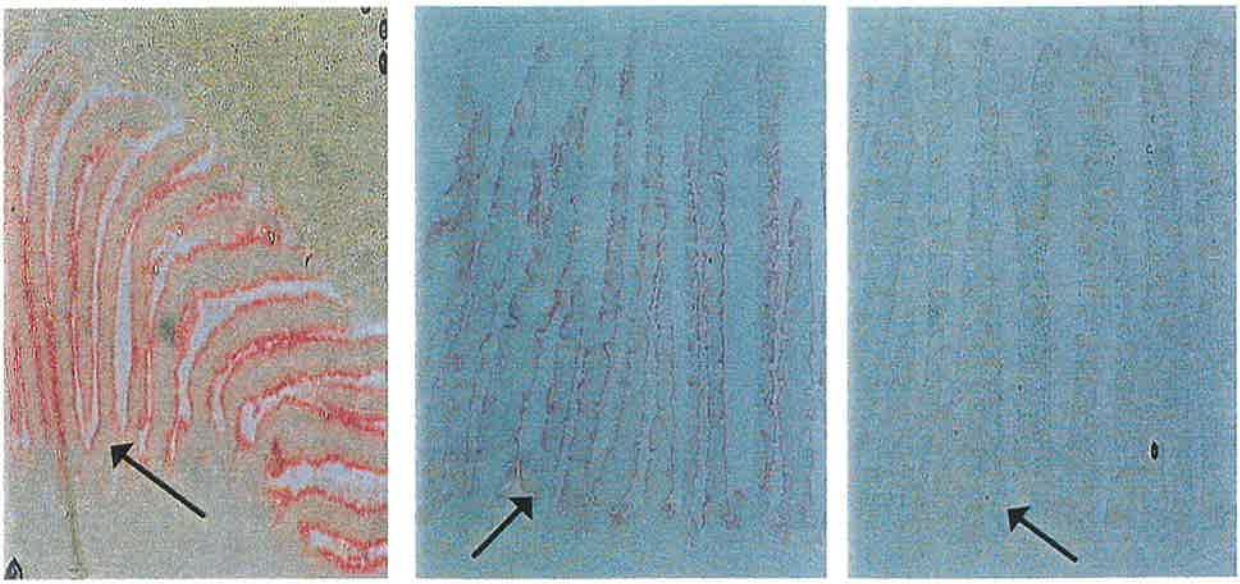


Plate 3.1: The typical localization of intestinal enzymes on jejunal villi of 28-day old chicks, after the procedures described in 3.6.0. Arrows show the crypt region.

3.6.1. Alkaline phosphatase (EC. 3.1.3.1)

The activity of alkaline phosphatase was measured through incubation with naphthol AS-BI phosphate as substrate (Gutschmidt *et al.*, 1980). Tissue sections were prefixed at 4°C in freshly prepared formal calcium (1 g $CaCl_2$ in 91.25 ml distilled water + 8.75 ml formalin) for 0.5, 1, 2, 4, 8 or 10 minutes. The sections were rinsed in 100 - 125 mM Tris-HCl, pH 9.2 at 39°C prior to incubation.

The incubation mixture consisted of 0.63 mM (3.1 mg) naphthol AS-BI phosphate dissolved in 60 µl of dimethylformamide (DMF); 9.94 ml Tris-HCl, pH 9.2 and 10 mg

Fast Red. The reaction was terminated at each time point by rinsing the section in ice-cold 125 mM Tris-HCl, pH 7.5. The sections were post-fixed in neutral buffered formalin for 30 minutes and mounted with glycerine jelly.

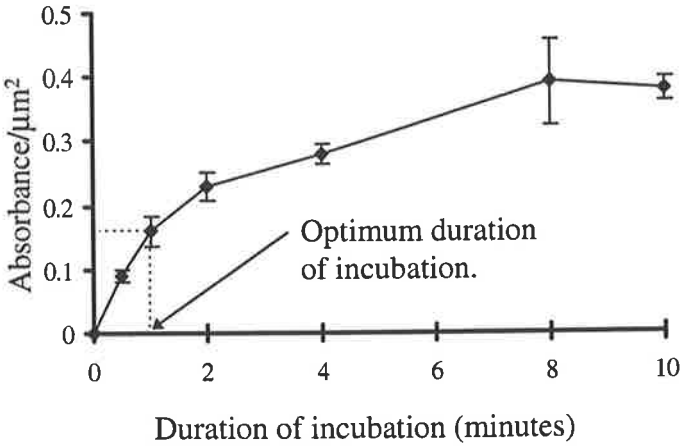


Figure 3.2: *In-situ* activity of alkaline phosphatase (Mean Ab. ± SD) at the ileum with increased duration of incubation.

The activity of AP peaked after 8 minutes of incubation and declined thereafter (Figure 3.1). There was a rapid phase between 0.5 and 1 minutes of incubation, suggesting that optimum rate of activity coincided with 1 minute of incubation in the chicken intestine.

3.6.2. Aminopeptidase N (APN; EC. 3.4.11.2)

The method of Nachlas *et al.* (1957) was used for the histochemical evaluation of APN. L-alanine 4-methoxy-β-naphthylamide was used as substrate while the diazonium salt was Fast Blue BB.

The incubation mixture consisted of 0.7 mM (2 mg) L-alanine 4-methoxy-ß-naphthylamide dissolved in 0.05 ml ethyl alcohol + 0.45 ml H_2O + 5 ml 0.1 M acetate

buffer, pH 6.5 + 4 ml normal saline + 0.5 ml 0.13 % KCN + 5 mg Fast Blue B. The sections were dried at room temperature for 2 hr, followed by pre-fixation in fresh formal calcium at 4°C for 10 minutes. They were rinsed twice in 0.85 % saline at 4°C over 2 minutes and incubated for 1, 2, 3, 4 or 5 minutes. Incubation was terminated by rinsing in normal saline for 2 minutes and post-fixation in 0.1 M $CuSO_4$ and normal saline for 2 minutes each.

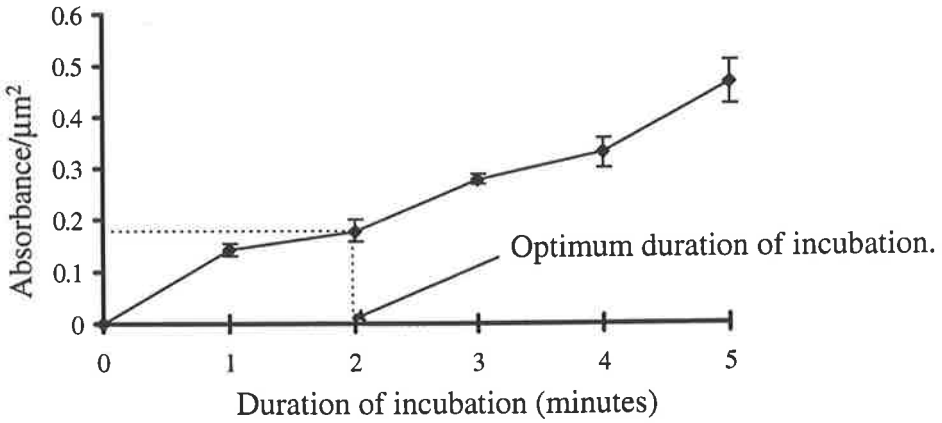


Figure 3.3: *In-situ* activity of aminopeptidase N (Mean Ab. ± SD) at the ileum with increased duration of incubation.

Results are shown in Figure 3.3. There was a linear increase in the intensity of the product over the duration of the incubation tested. The optimum duration of incubation, 2 minutes chosen was the point at which absorbance corresponds to approximately 50 % transmittance and is the duration used with mammalian tissues.

3.6.3. Alpha-glucosidase (AG, EC. 3.2.1.48)

The *in-situ* activity of α-glucosidase (maltase-sucrase complex) was demonstrated through incubation of fresh sections with 6.0 mM β-naphthyl-α-D-gluco-pyranoside as

described by Gutschmidt *et al.* (1979). The diazonium (anisidine) salt was hexazonium-ρ-rosaniline (HPR).

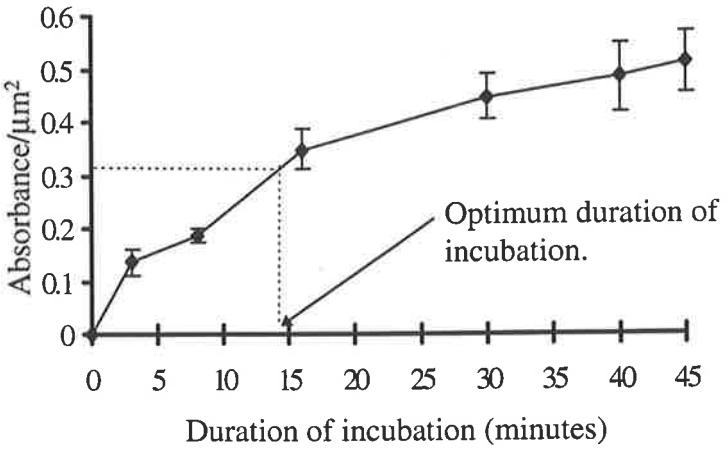


Figure 3.4: *In-situ* activity of α-glucosidase (Mean Ab. ± SD) at the ileum with increased duration of incubation.

The composition of the incubation mixture was 6 mM (18.4 mg) β-naphthyl-α-D-glucose (18.4 mg) dissolved in 0.92 ml DMF, 9.08 ml citrate-phosphate buffer (63.1 ml Na_2HPO_4 (2.84 %) + 36.9 ml 0.1 M citric acid) and 0.6 ml HPR. HPR was made by mixing equal parts of ρ-rosaniline (1 g in 20 ml distilled water + 5 ml conc. HCl) and freshly prepared 4 % sodium nitrite. This mixture was pre-warmed, cooled and filtered through Whatman No 4 paper. The pH was adjusted to 6.0 with NaOH.

Tissue sections were dried at room temperature for 2 hours, prefixed in formal calcium at 4°C for 10 minutes and then washed twice in 0.1 M citrate-phosphate buffer, pH 6.0 for 2-3 minutes twice. Incubation was terminated each time by immersion and washing in ice-cold distilled water for 5 minutes. The samples were post-fixed in 4 % formalin

for 2 hours and serially rinsed in running tap and distilled water prior to mounting with glycerine jelly.

Activity was rapid between 0 and 20 minutes (Figure 3.4) and attained half-maximum value at about 14 minutes, which was adopted as the optimum duration of incubation.

3.7.0 Preparation of brush-border membrane vesicles

The successful extraction of the brush-border membrane of enterocytes (Figure 2.1) was a revolutionary step towards a greater understanding of membrane function, without interference from other cellular structures (Murer and Kinne, 1980). The current techniques rely on the different reactivity of cell membranes and other cellular organelles with the divalent ions, magnesium or calcium. Magnesium is preferred over calcium due to its ability to preserve membrane integrity, although brush-border membranes extracted with the Mg^{2+} are usually more contaminated with basolateral membranes and other cellular debris than those extracted with Ca^{2+} (Aubry *et al.,* 1986).

The brush-border membrane was extracted in the form of vesicles and used to biochemically assess digestive enzyme activities as well as the uptake of amino acids. Vesicles were prepared in line with the method described by Shirazi-Beechey *et al.* (1991).

Fresh frozen samples were cut into small pieces and defrosted in buffer (100 mM mannitol, 2 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES)/Tris, pH 7.1). About 25 ml of buffer were required for 1-2 g of tissue. The mixture was vibromixed for 2 x 30 seconds and filtered through a Buchner funnel, 1

mm pore size. The filtrate was blended in a PCU-2 Polytron homogenizer for 30 seconds at high speed (setting 6). One ml of homogenate was taken for assessment of protein and brush-border membrane marker enzymes. Magnesium chloride from a 2.5 M stock was added to the rest of the homogenate to a final concentration of 10 mM, vigorously mixed and allowed to stand for 20 minutes. The suspension was centrifuged on a JA20 rotor in a J2-21 centrifuge (Beckman Instruments, Palo Alto, CA, U.S.A) at 3000 g for 15 minutes. The supernatant was removed and centrifuged at 30 000 g for 30 minutes. The pellet was re-suspended in a second buffer (100 mM mannitol, 2 mM HEPES/Tris, 0.1 mM $MgSO_4$, pH 7.4) passed through a 25G needle until the mixture was homogenous. The sample was then centrifuged for 45 minutes at 30 000 g and the supernatant discarded. The final pellet was re-suspended in 500 µl of a buffer containing 300 mM mannitol, 20 mM HEPES/Tris, 0.1 mM $MgSO_4$, and 0.02 % sodium azide, pH 7.4. The membranes were made homogenous by sequentially passing through 25 and 27G needles. Membrane vesicles in Eppendorf tubes were then stored in liquid nitrogen prior to use.

3.7.1. Purity of membrane vesicles and specific activities of enzymes

Biochemical assays on brush-border membrane vesicles (BBMV) serve the dual purposes of checking the purity of the membrane extracts as well as estimating the specific activities of enzymes under varying environmental conditions. Ideally, the specific activity of membrane-bound enzymes should be higher in BBMV than in the crude homogenate. The marker enzymes of choice include the disaccharidases, sucrase and maltase; aminopeptidases, and alkaline phosphatase. The activities of Na^+/K^+- and Mg^{2+}-stimulated ATPases could be assessed to determine the degree of contamination of BBMV with the basolateral membrane.

3.7.1.1. Protein

The recovery rate of the membranes was assessed by estimating the protein concentrations in homogenates and vesicles according to the modified techniques of Bradford (1976). The assay utilized the red form of Coomassie Brilliant Blue G-250 (CBB), which turns blue on binding to protein. The reaction was started by adding 1 ml of CBB to 25 µl of dilute vesicles or homogenate, vibromixing and reading at 595 nm after 5 minutes but within 1 hour. Data generated were analysed with the aid of a computer software, Lowry (Elsevier BIOSOFT, Cambridge, UK).

3.7.1.2. Alkaline phosphatase

AP was assayed in line with the modified methods described by Forstner *et al.* (1968) and Holdsworth (1970). The assay system consisted of 50 mM $MgCl_2$, 50 mM Tris (pH 10.1) and the substrate, 10 mM paranitrophenol phosphate (PNP, Sigma 104). The standard was paranitrophenol (Sigma 104-1). The reaction was initiated by incubating 20 µl of dilute vesicles or homogenates with 0.8 ml of Tris buffer, 0.1 ml of $MgCl_2$ and 0.1 ml of PNP at room temperature for 20 minutes. The reaction was terminated by 0.1 ml 40 % trichloroacetic acid. Further colour development was accomplished by adding 2.0 ml of 0.4 M NaOH to 0.1 ml of the primary reaction mixture, which was then vibromixed and read at 410 nm.

3.7.1.3. Aminopeptidase N

The activity of APN was estimated using a modified method of Hino *et al.* (1976). The experimental tube (total volume, 1.05 ml) contained 0.25 ml 0.3 M Tris-maleate (pH

7.0) buffer, 0.5 ml 3 mM alanine ρ-nitroanilide in 2 % Triton X-100, 0.25 ml distilled water and 50 µl vesicles. The standard was 3 mM ρ-nitroaniline. All tubes were incubated at 39°C for 30 minutes and the reaction was terminated with 3.0 ml of 1.0 M acetate buffer, pH 4.2. Absorbance was then read at 380 nm.

3.7.1.4. Sucrase and maltase

The disaccharidases, α-glucosidase (maltase, EC. 3.2.1.20) and β-fructofuranosidase (sucrase, EC. 3.2.1.26) were assayed using the method described by Dahlqvist (1964). The incubation mixture was freshly prepared 100 mM maltose or sucrose respectively in succinate buffer (4 mM sodium succinate, 90 mM sodium chloride, pH 6.0). Homogenates or vesicles, 25 µl were incubated in 475 µl of substrate-buffer for 30 minutes at 39°C. Incubation was terminated by pipetting in 5 folds of 0.2 % Triton X-100 (w/v) in 0.5 M Tris buffer, pH 7.02 at 39°C. Incubation released glucose which was then estimated by the GOD-Perid test kit (glucose oxidase, EC. 1.1.3.4; Boerhinger-Mannheim Pty. Ltd, Australia). The amount of glucose released was measured spectrophotometrically at 610 nm after 30 minutes of colour development at room temperature.

For all enzymes and protein, the samples were read on a UV-120-0 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) in cuvettes with a 1 cm light path. All samples and standards were read in duplicate.

3.8.0. Uptake and transport of amino acids

The study of transport phenomenon was traditionally conducted on intact tissues or whole cells (Kimmich, 1970; Bosin *et al.*, 1975). Both systems are, however, flawed by interference from various cellular systems. Brush-border membrane vesicles, extracted as described in section 3.7.0 offer a useful alternative in which nutrient uptake and transport through the membrane *per* se could be determined. Membrane vesicles possess the advantages that are lacking in intact tissue or whole cells. With vesicles, it is possible to study processes which are specific to a single membrane type without interference from other membranes or intra-cellular structures (Sachs *et al.*, 1980; McGivan, 1992; Proulx, 1996).

In studies described in this thesis, the uptake and transport of tryptophan was assessed. Tryptophan is the third limiting amino acid for poultry, next to lysine and methionine. Its degree of essentiality is the result of low concentrations in cereal grains, especially maize, which are the predominant component of most practical poultry diets (McDonald *et al.*, 1988; D'Mello, 1993). Series of tests were conducted to determine the optimum concentration and conditions to use in the various experiments reported in Chapters 4 and 5. These are described below.

3.8.1. Uptake and transport assay

The uptake and transport of L-tryptophan was assayed on brush-border membrane vesicles according to the procedure described by Wolfram *et al.* (1984). The incubation buffer contained mannitol, HEPES and unlabelled amino acid. The actual concentrations of these varied according to requirements and are indicated under the

various experiments. The buffer was labelled with tritiated L-tryptophan at the level of 74.1 kBq/ml.

Uptake was initiated by incubating 25 µl of undilute vesicles in 25 µl of buffer over a specified time, stated under different experiments. Incubation was terminated by the addition of 1 ml ice-cold stop buffer (176.2 mM NaCl, 15 mM Tris/HCl; 357 mosm/kg; pH 7.4). The mixture was rapidly filtered through nitrocellulose filter (22 µm pore size, Millipore Corporation, Bedford, MA, USA) fitted to a Millipore manifold. The filter was then rinsed twice with 3 ml stop buffer. A blank, consisting of labelled uptake buffer without the vesicle samples was similarly rinsed to account for binding to filter paper. Samples and blanks were run in duplicate. The filter was transferred into scintillation vial, topped with 5 ml of biodegradable counting scintillant (Amersham Aus. Pty Ltd). The radioactivity was determined on a liquid scintillation counter, LS 3801 (Beckman, Irvine, CA, USA) to estimate the uptake and transport of amino acid into vesicular space.

The following preliminary assays were conducted to establish the optimum concentration of amino acid at which other assays were conducted; the optimum duration of incubation, and confirmation of uptake.

3.8.1.1. Time-dependence of L-tryptophan uptake and transport

Jejunal brush-border membrane vesicles from 7-day old Steggles x Ross (F_1) chicks were used to determine the time-dependent uptake of transport of L-tryptophan. Uptake was studied at 0.08 mM and over 3, 5, 10, 20, 40 and 80 seconds. Maximum uptake

occurred at 10 s (Figure 3.5). Subsequent assays were therefore conducted over 10 seconds of incubation.

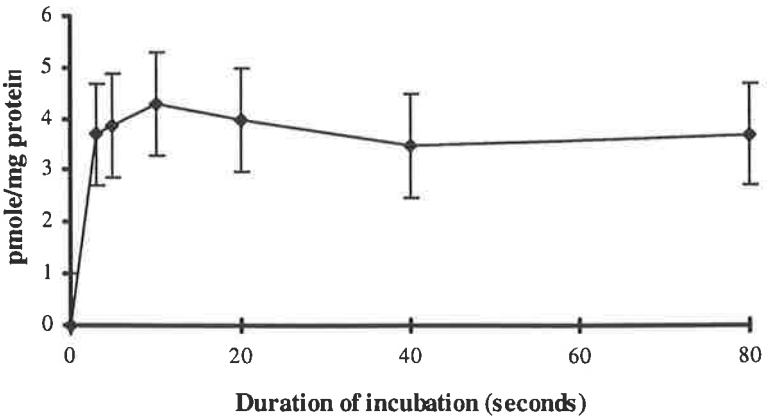


Figure 3.5: Uptake and transport of 0.08 mM L-tryptophan over varying duration of incubation. **Assays were conducted on jejunal BBMV from 3 chicks, each run in duplicate.**

3.8.1.2. Optimum concentration of L-tryptophan for uptake and transport

The uptake and transport of various concentrations of L-tryptophan was assessed. Jejunal BBMV from 7-day old chicks were incubated in buffers containing 0.02 - 0.64 mM L-tryptophan. Maximum uptake occurred at 0.04 mM (Figure 3.6). Previous research on hamsters had shown self-inhibition of L-tryptophan at high concentrations of the amino acid (Spencer and Samiy, 1960).

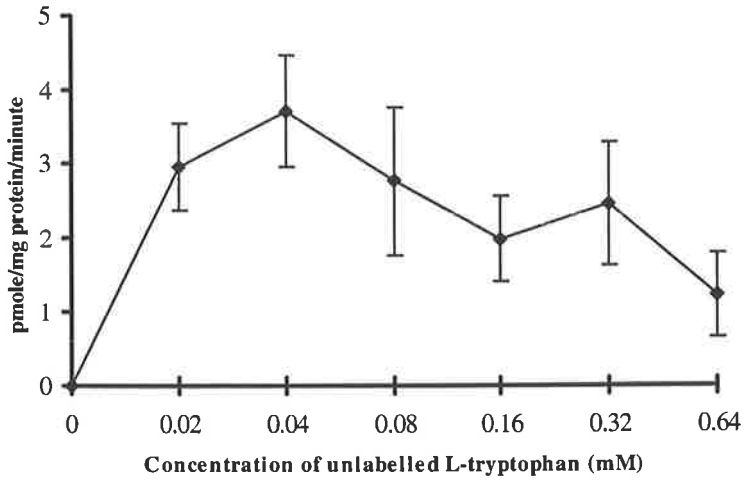


Figure 3.6: Uptake of tryptophan at different concentrations (mM). Incubation was assessed over 10 s for each concentration. Each data point represents the average of 3 birds.

3.8.1.3. Verification of uptake

In studies with membrane vesicles, it is common to over-estimate uptake through inclusion of binding to the membrane. This has been reported to be a common problem with tryptophan over long durations of incubation (Ganapathy *et al.*, 1986). For accuracy, uptake and transport estimates should include only the amino acid taken up and held within the vesicle. This occurs maximally at optimum osmolarity. Vesicles loaded into uptake buffers of low osmolarity lose their turgidity and lyse. Similarly, vesicles loaded into buffers of very high osmolarity shrink in volume and yield low uptake values. The osmolarity of the stop buffer should also be considered since uptake could be lost through transfer of vesicles into a stop buffer of low osmolarity. A stop buffer of very high osmolarity shrinks the vesicles as well, resulting in reduced uptake. Ideally, the storage and uptake buffers should be at similar osmolarities while the stop buffer should be of slightly higher osmolarity (Wolfram *et al.*, 1984). The slight hypertonicity of the stop buffer ensures that the swollen vesicles (following uptake) are

slightly shrunk, to avoid lysis. The osmolarity of all buffers used in this study was assessed on a vapour pressure osmometer, Vapro 5520 (Wescor Inc., Logan, UT, USA).

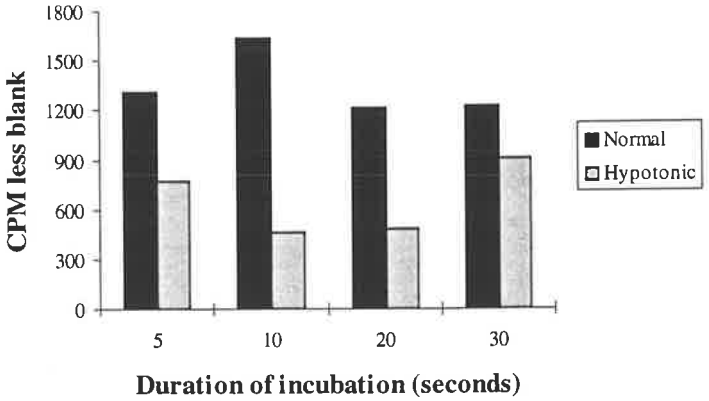


Figure 3.7: Verification of amino acid uptake by brush-border membrane vesicles and degree of binding to vesicles. Vesicles were incubated in regular buffer over the specified durations. Incubation was then terminated with slightly hypertonic stop buffer and rinsed with the same buffer (normal) or with a hypotonic buffer, with half the osmolality of the normal buffer. The counts obtained with blank filters were subtracted from total counts. Tests were conducted with a cocktail of jejunal vesicles from four 7-day-old chicks.

To confirm uptake and determine the rate of binding, uptake was conducted over varying duration of time (Figure 3.7). At each time point, incubation was terminated with the slightly hypertonic ice stop buffer (357 mosm/kg) and the mixture filtered. The filter paper was then rinsed with the stop buffer or a hypotonic stop buffer (178.5 mosm/kg). The results showed that some radioactivity was retained on the membrane even after the capacity of the vesicles for uptake was reduced or lost. Apparent binding varied between 28.4 % (at 10 s) and 74.4 % (at 30 s) when uptake under normal stop buffer condition was compared to uptake with the hypotonic buffer. A considerable amount of uptake was lost when incubation over 10 seconds was terminated and rinsed with the hypotonic buffer (data not shown).

3.9.0. DATA ANALYSIS

The type of experimental design and replication used are described under individual experiments. Most of the data collected were subjected to analysis of variance (ANOVA) as described by Mead and Curnow (1983). Differences between treatments were deemed to be significant only if the F value was significant at a level of probability ≤ 0.05. The differences between mean values were identified by the least significant difference (LSD). Mean values were then presented along with the required standard error of difference (SED) between the means or presented $\pm$ treatment standard deviations (SD). In experiments where other forms of data analyses were performed, they are indicated and described.

CHAPTER 4: NATURAL PATTERN OF BODY GROWTH AND INTESTINAL DEVELOPMENT

4.0.1. GENERAL INTRODUCTION TO CHAPTER

Five experiments were designed to study the natural pattern of whole body and the gastrointestinal tract (GIT) development. For the purpose of these studies, commercial diets (Milling Industries Stockfeed, Murray Bridge, South Australia) were used. Data obtained from experiments conducted in this subsection served to provide a baseline from which subsequent data could be compared. In many ways, too, the results demonstrated the performance of the new strain, Steggles x Ross (F_1) chicks used for most of this project.

Experiment 4.1 contains reports of studies on changes in body and organ weight in relation to feed utilization within the first 21 days when the chicks were maintained on a broiler starter diet. Experiment 4.2 examined the development of the intestinal mucosa and the mechanisms behind the structural changes over the same age period. The natural development of intestinal digestive enzyme function is reported in Experiment 4.3 while the mechanisms involved in neutral amino acid uptake and transport, typified by tryptophan between hatch and 21 days of age are reported in Experiment 4.4. Experiment 4.5 reports the growth, feed utilization and intestinal development of the chicks subsequent to a change from a starter diet to a finisher diet.

4.1.0. EXPERIMENT 4.1: EFFECT OF A COMMERCIAL STARTER DIET ON BODY AND INTESTINAL GROWTH.

4.1.1. INTRODUCTION

The modern broiler chick is hatched with a fairly developed muscular and skeletal framework (Anthony *et al.*, 1989). Subsequent body growth depends largely on nutrition. The GIT, therefore, has an important role to play to ensure optimum body growth. Over the last few decades, the broiler production cycle has been considerably reduced through genetic improvement (Nitsan *et al.*, 1991; Nir *et al.*, 1993; Dunnington and Siegel, 1995). Nitsan *et al.* (1991) estimated a reduction in the production cycle of about 1 day per year over 40 years of selection and genetic improvement. To achieve maximum productivity within the short production cycle, there is a need to maximize nutrient derivation from diet at an early age. This is partly achieved by a large intestinal mass at hatch and rapid development of same in early life (Nir *et al.*, 1993).

The relationships between liveweight and visceral organ weights have been studied in a few poultry species, including some breeds and strains of broiler chickens as was reviewed in chapter two. Dror *et al.* (1977) observed an increase in the weight of many visceral organs with increase in liveweight in early life and then a reduction in later life in both light and heavy strains. Similar relationships have been reported in the Japanese quail (Lilja *et al.*, 1985); turkeys (Sell *et al.*, 1991) and White Plymouth Rock cockerels (Dunnington and Siegel, 1995).

Intestinal growth and function also depend strongly on selection patterns and intensities (Smith *et al.*, 1990). The large differences between lines of broiler chickens, in terms of

weight at hatch and growth rate have been previously highlighted (Nitsan *et al.*, 1991; Turro *et al.*, 1994).

The aim of this experiment was to establish the pattern of natural development of the intestine in relation to body growth in the recently developed Steggles x Ross broiler chicks.

4.1.2. MATERIALS AND METHODS

4.1.2.1. Animals and diets

Eighty mixed sex day-old broiler chicks of the Steggles x Ross (F_1) strain (Australia Poultry Pty. Ltd.) were used for the study. The chicks were brooded together in battery brooders for 14 days. On day 14, the chicks were moved into battery cages.

The chicks were brooded as described in section 3.1.1. A commercial broiler chick starter diet in the form of crumbles (Milling Industry Stockfeeds, Murray Bridge, South Australia) was fed. The nutrient composition of the diet was assessed and found to be adequate in protein, energy, essential amino and fatty acids and the mineral elements - calcium and phosphorus (Appendix 1).

4.1.2.2. Assessment of gross productivity

All chicks were weighed on receipt from the hatchery and subsequently every 7^{th} day. Feed utilization over the period was assessed. Mixed faecal and urinal samples (droppings) were collected from all cages over 24 hours on the last day of each week and bulked. The mixture was dried in an oven at 75°C over 24 hours to a constant weight.

In addition to crude protein (section 3.3.2), the concentrations of dry matter (DM), gross energy (GE), amino acids, lipids, fatty acids and minerals in the feeds and droppings (faeces and urine) were analysed. The GE was measured in an adiabatic bomb calorimeter at the Nutrition Laboratory of the PPPI, Roseworthy, South Australia. Amino acid composition was analysed by the Cereal Improvement Laboratory, Department of Plant Science, University of Adelaide. For all amino acids except tryptophan, initial sample hydrolysis was carried out according to Barkholt and Jensen (1989). The amino acids in the hydrolysates were then measured in an AminoQuant II analyser (Hewlett-Packard, Avondale, PA, USA). Tryptophan analysis was preceded by liquid hydrolysis as described by Hugli and Moore (1972) followed by chromatographic determination according to the method of Sarwar *et al.* (1988). The mineral composition of the diets and droppings was analysed at the Department of Plant Science using the inductively coupled plasma spectroscopy.

Total lipid was extracted and quantified as described by Christie (1989). The fatty acid concentration of the lipid was determined by gas-liquid chromatography of fatty acid methyl esters (FAME). Total lipid was extracted with chloroform:methanol (2:1). The material was dried in a rotary evaporator prior to the preparation of FAME. The FAME were then injected into a Hewlett Packard 5890 II chromatograph fitted with a column, 50 m long and 0.23 mm internal diameter. The carrier gas was hydrogen. Samples were analysed at initial and final temperature of 160° and 220°C respectively over 20 minutes.

The percentage apparent retention of nutrient (% ARN) was calculated as:

$$(Dn \times Fi) - (Fn \times Fo)/Dnc \times 100$$

where Dn is concentration of nutrient in diet, Fi is feed consumed, Fn is concentration of nutrient in faeces, Fo is faecal output and Dnc is the concentration of the nutrient in the feed consumed. All calculations were based on dry matter content of feed and faeces.

4.1.2.3. Sample collection

Sampling for animal tissue commenced on the day the chicks were received and once each week up to 3 weeks of age. Ten to eleven chicks were randomly selected each week and slaughtered as described in section 3.4.0.

4.1.2.4. Data analysis

All data collected were analysed as described in section 3.9.0. Simple and multiple regression analyses were conducted to enable estimation of visceral organ weight from liveweight and to determine the association of the individual organ weights to liveweight.

4.1.3. RESULTS

4.1.3.1. Feed consumption and nutrient retention

Absolute feed intake rose between hatch and day 21 but declined, relative to body weight with age (Table 4.1.1). Feed conversion ratio also increased between hatch and day 21, indicating a decline in the efficiency of feed utilization with age.

The retention of dry matter, gross energy and individual nutrients is summarized in Table 4.1.2. This summary is derived from estimates of total amounts retained in relation to amounts ingested. The amino acid, lysine was retained at higher concentrations than were methionine, tryptophan or threonine.

Table 4.1.1: Feed intake and utilization (mean ± SD), estimated on flock basis.

	Age (days)		
	1-7	**7-14**	**14-21**
Initial weight (g)	50.7 ± 3.68	210.3 ± 19.94	475.3 ± 45.86
Final weight (g)	210.3 ± 19.94	475.3 ± 45.86	856.6 ± 62.12
Growth rate (g/day)	22.8 ± 2.33	37.9 ± 4.12	54.5 ± 3.40
Feed intake (g)	157.1	360.5	556.0
Feed intake (g/100 g body weight)[1]	309.9	171.4	117.0
Feed conversion ratio[2]	1.15	1.55	1.60

1. Feed intake in relation to initial weight for the period. 2. Feed consumed per unit of weight gain.

Calcium and phosphorus retention rates were relatively low, less than 50 % of the amount ingested at all ages. There was an increase in intake and retention with age for all fatty acids and total lipid. Linolenic acid was retained to a greater extent than were oleic and linoleic acids.

4.1.3.2. Body and intestinal growth

The body weight of birds sampled increased ($P<0.001$) with age (Table 4.1.3). There were also significant variations in the weight of visceral organs with age. In relation to body weight, small intestinal weight peaked at 7 days of age and declined ($P<0.001$) subsequently. There was also a reduction in the weights of the gizzard ($P<0.05$) and

liver (P<0.05) with age in relation to body weight. In relation to liveweight, the yolk sac and pancreas were significantly heavier (P<0.001) at hatch than at subsequent ages.

Table 4.1.2: Percentage retention of dietary dry matter and nutrients at various ages.

	Age (days)		
Component	**0-7**	**8-14**	**15-21**
Dry matter	76.9	76.8	78.6
Gross energy	42.5	47.8	56.4
Methionine	71.1	74.2	76.9
Lysine	72.9	89.9	89.1
Tryptophan	47.8	69.3	74.1
Threonine	60.1	65.8	71.1
Alanine	64.8	67.6	73.0
Fat	37.4	50.4	57.9
Oleic acid	42.7	54.9	61.9
Linoleic acid	53.0	62.3	71.6
Linolenic acid	53.8	64.4	79.3
Calcium	18.6	27.0	6.1
Phosphorus	4.6	19.2	9.6

Table 4.1.3: Weight of body and visceral organs (g/100 g body weight) of sampled chicks at different ages.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Liveweight (g)	49.5^{c}	153.1^{c}	399.0^{b}	709.0^{a}	22.17***
Small intestine	4.1^{b}	7.2^{a}	5.0^{b}	3.7^{b}	0.31***
Gizzard	5.6^{a}	4.7^{a}	3.6^{bc}	2.6^{c}	0.43*
Yolk sac	8.1^{a}	0.07^{b}	0.03^{b}	0.01^{b}	0.614***
Liver	4.1^{ab}	4.4^{a}	4.6^{a}	3.2^{b}	0.43*
Pancreas	1.1^{a}	0.6^{b}	0.4^{c}	0.3^{c}	0.04***

a,b,c - Mean values in the same row without common superscripts differ significantly *P<0.05 or ***P<0.001. 10-11 chicks were sampled at each age. Values for the small intestine and gizzard indicate empty weights.

The changes in weight of the small intestine and its component segments are shown in Table 4.1.4. Similar to the pattern observed for the whole intestine, the maximum

weight of the duodenum, jejunum and ileum was reached at day 7, and then declined (P<0.001) towards 21 days of age.

Table 4.1.4: Empty weight of the small intestine and its regions (g/100 g body weight) and weight per unit length of tissue (g/cm).

	Age (days)				
	1	7	14	21	SED
Weight (g/100 g body weight)					
Small Intestine	4.1^{bc}	7.2^{a}	5.0^{bc}	3.7^{c}	0.31
Duodenum	1.0^{c}	1.9^{a}	1.4^{bc}	1.1^{c}	0.14
Jejunum	1.8^{b}	3.2^{a}	2.1^{b}	1.5^{b}	0.16
Ileum	1.3^{b}	2.1^{a}	1.5^{b}	1.1^{b}	0.15
Weight per length of tissue (g/cm)					
Small Intestine	0.04^{a}	0.13^{a}	0.18^{b}	0.21^{b}	0.006
Duodenum	0.06^{c}	0.21^{b}	0.31^{a}	0.38^{a}	0.018
Jejunum	0.03^{d}	0.09^{c}	0.13^{a}	0.15^{a}	0.007
Ileum	0.04^{c}	0.09^{b}	0.12^{ab}	0.15^{a}	0.011

a,b,c - Mean values in the same row with unlike superscripts are significantly different (P<0.001).
Five samples were assessed at each age. Samples used for transport, enzyme and nucleic acid assays in experiments 4.2, 4.3 or 4.4 were not used in assessing intestinal dimensions, to ensure rapid collection and storage.

Intestinal weight per unit tissue length increased significantly (P<0.001) with age. Similar changes were observed for the separate regions - the duodenum, jejunum and ileum. The duodenal tissue was heavier than jejunal and ileal tissues although no statistical comparisons were made between the regions.

Small intestinal length increased (P<0.001) from 46.1 cm at hatch to 130 cm at 21 days of age (Table 4.1.5). Individual regions of the intestine also showed a similar increase (P<0.001) with age. The jejunum and ileum were similar in length and together accounted for 82.2 % and 84.1 % of the length of the small intestine at hatch and 21

days of age, respectively. The jejunum was slightly longer than the ileum between hatch and 7 days of age but this situation was reversed at subsequent ages.

Table 4.1.5: Length of the small intestine and intestinal segments in relation to age and body weight.

	Age (days)				
	1	7	14	21	SED
Length (cm)					
Small Intestine	46.1^{c}	88.0^{b}	117.0^{a}	130.0^{a}	4.20
Duodenum	8.2^{d}	14.9^{c}	19.0^{bc}	20.8^{a}	1.24
Jejunum	20.5^{c}	38.0^{b}	48.1^{a}	54.4^{a}	1.74
Ileum	17.4^{c}	35.1^{b}	49.9^{ab}	54.9^{a}	3.48
Length per unit of body weight (cm/100 g body weight)					
Small Intestine	94.1^{a}	55.7^{b}	27.4^{c}	17.6^{c}	3.68
Duodenum	16.8^{a}	9.4^{b}	4.5^{bc}	2.8^{c}	1.18
Jejunum	41.7^{a}	24.1^{b}	11.3^{c}	7.4^{c}	1.23
Ileum	35.5^{a}	22.3^{b}	11.7^{c}	7.4^{c}	2.26

a,b,c - Mean values in the same row with unlike superscripts are significantly different (P<0.001). For each age group, n=5.

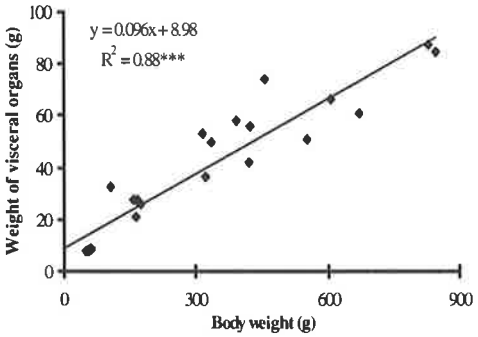
Table 4.1.6: Digesta weight in the small intestine and gizzard.

	Age (days)				
	1	7	14	21	SED
Intestinal contents (g/100 g body wt.)	1.2	1.8	1.5	1.6	0.45
(g/g int. weight)	0.25	0.25	0.30	0.36	0.079
Gizzard contents (g/100 g body wt.	3.7^{a}	2.6^{ab}	0.8^{c}	1.3^{bc}	0.41***
(g/g gizz. Weight)	0.66^{a}	0.58^{a}	0.24^{b}	0.42^{ab}	0.129*

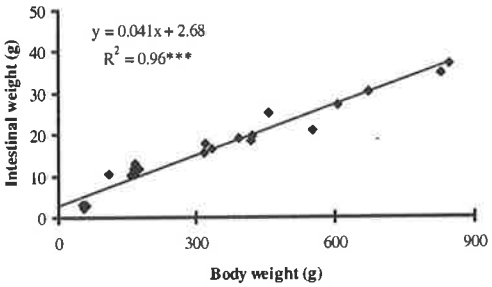
a,b,c - Mean values in the same row without common superscripts differ significantly *P<0.05 or ***P<0.001.

In relation to body weight, small intestinal length declined (P<0.001) with age, varying from 94.1 cm/100 g body weight at hatch to 17.6 cm/100 g body weight. Individual regions varied in a similar pattern.

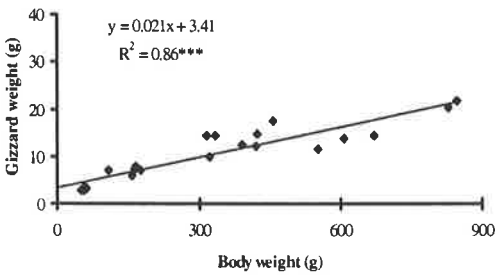
a. Combined weight of visceral organs



b. Small intestine



c. Gizzard



d. Liver

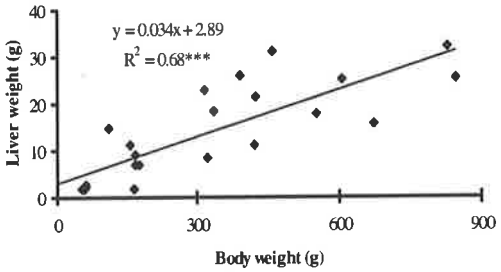


Figure 4.1.1: Relationships between body weight and visceral organ weight. Data from 41 chicks were used for the regression analysis.

There was no significant variation in the weight of intestinal contents (gut-fill) with age (Table 4.1.6). Gizzard contents decreased with age in relation to body weight (P<0.001) and net gizzard weight (P<0.05).

4.1.3.3. Relationships between body weight and visceral organs

There was a highly significant (P<0.001) correlation between the total weight of visceral organs and body weight (Figure 4.1.1). The individual visceral organs also showed separate positive relationships with body weight. The relationships between the

intestines, gizzard and liver on the one hand and liveweight on the other were highly significant ($P<0.001$). The coefficient of determination (R^2) varied between 0.68 and 0.96. The predictive equation for each relationship is shown on the graphs.

4.1.4. DISCUSSION

4.1.4.1. Feed intake and utilization

Dry matter retention was observed to increase with age in this study. In the Red Jungle fowl, one of the progenitors of modern poultry, Jackson and Diamond (1995) observed no strong relationships between age and DM digestibility. As has been reported for other broiler strains (Sarica and Karacay, 1994; Al-Masri, 1995), the overall efficiency of feed utilization declined with age. An extensive search of the literature failed to yield any research reports on the pattern of growth of the Steggles x Ross. The information on the pattern of growth of its parent strains is also scanty. In comparative studies conducted in Australia, Steggles were found to be heavier than Tegels between 12 and 35 days of age (Johnson *et al.*, 1990). Similarly, Arbor Acres chicks were reported to grow more rapidly than Ross chicks up to 7 weeks of age (Holsheimer and Veerkamp, 1992). The growth rate observed in the present strain is similar to that reported by Uni *et al.* (1995a) in research on the Arbor Acres. The Steggles x Ross strain would have been bred to strengthen the advantages possessed by its parent strains. Ross chicks appear to be of higher body weight than Steggles while the latter are the more efficient in feed utilization. This was borne out by results of comparative studies on the Ross x Arbor Acres and Steggles x Arbor Acres broiler chicks (Moran, 1994; Stillborn *et al.*, 1994). The cross obtained with the Ross was heavier but the Steggles-based cross was more efficient at feed utilization although these differences may be of dietary origin.

The low retention of nutrients from the diet in early life is attributed to the supply of nutrients from the yolk sac (Murakami *et al.*, 1988). The initial contribution of the yolk sac ensures adequate preparation of the GIT to solely assume the role of nutrient acquisition in later life. Although nutrient retention increased with age, most of the nutrients would be channelled to the deposition of fat rather than muscle (Hargis and Creger, 1980). This may partly be responsible for the decline in feed conversion efficiency observed in this experiment and in many other on broiler chickens.

The overall increase in retention of amino acids and other nutrients is due to an increased length of the intestine observed with age. Buddington (1994) found that the capacity for nutrient absorption is related to age-dependent absolute increases in dietary inputs. The efficiency of nutrient digestion is known to decline with age as a result of reduced specific activity of enzymes (Thomson and Keelan, 1986; Zelenka *et al.*, 1986; Holt and Kotler, 1987) although absolute nutrient retention may increase with age, as was observed for most nutrients in the present study. The development of digestive enzyme function and amino acid transport is further examined in experiments 4.3 and 4.4.

4.1.4.2. Body growth

The degree of selection imposed on most modern strains has produced individuals which develop rapidly post-hatch as has been observed for the Steggles x Ross in this study. The higher hatching weight and rapid growth of broiler chicks compared with egg-type chicks has been reported in previous studies (Newcombe and Summers, 1984; Mahagna and Nir, 1996).

Turro *et al.* (1994) reported that broiler strains selected for high liveweight initiated weight gain immediately after hatch while lines selected for a low body weight showed delayed weight gain by a few days post-hatch. In the strain assessed in this experiment, body growth did not considerably lag behind intestinal growth. There was a tripling in body weight during the first week; within the same period, intestinal weight, relative to body weight doubled. The Steggles x Ross (F_1) also showed a 14-fold increase in body weight between hatch and 21 days of age.

The rate of post-hatch body growth is partly determined by the distribution of energy devoted to growth between the alimentary tract and the rest of the body (Lilja *et al.*, 1985; Dunnington and Siegel, 1995). In early life, more nutrient is channelled towards intestinal development, and body growth rate is limited until the functional development of the GIT is accomplished. Most of the intestinal growth appeared to have occurred in the first 7 days in the Steggles x Ross strain. There was a reduction in intestinal weight during subsequent age.

4.1.4.3. Growth of visceral organs

The role of the GIT in nutrient processing and acquisition is evident from the growth of the tract observed in the present study. Intestinal development was fastest within the first 7 days, similar to the results reported by Lilja (1983) in studies on the quail and those by Sell *et al.* (1991) in research on turkey poults. In other strains of broiler chicks, the GIT was observed to reach its maximum rate of growth during the first 7 days, post-hatch (Katanbaf *et al.*, 1988; Shanawany, 1994). A similar rapid growth was observed in the strain used in this study. While the weight of the small intestine peaked at 7 days

of age in the Steggles x Ross (F_1), the weights of the other visceral organs relative to body weight were higher at hatch than at subsequent age.

The yolk sac plays an important role during the first few days of life when the massive growth in GIT takes place. The yolk sac is exhausted within 4-14 days post-hatch (Murakami *et al.*, 1988; Nitsan *et al.*, 1991; Ferrer *et al.*, 1995). This pattern of development is observed in the strain studied here; the yolk sac constituted over 8 % of the body weight at hatch but had declined to less than 1 % by 7 days of age.

The present study showed strong positive relationships between liveweight and visceral organ weights. The weakest relationship, though significant was between body weight and liver weight. The liver is essentially not a nutrient deriving organ although its place in nutrient metabolism is significant. Similar positive relationships between body weight and all parts of the GIT in broiler chickens studies between hatch and 56 days of age were reported by Shanawany (1994). For a broiler strain to be thrifty, it is essential that body growth rate exceeds intestinal growth rate, soon after hatch (Nitsan *et al.*, 1991; Dunnington and Siegel, 1995). This ensures increased meat yield in relation to offal weight. The pattern of growth observed in the current study establishes the Steggles x Ross strain as a proficient meat strain.

The importance of the jejunum and ileum to intestinal function could be glimpsed from their relative lengths at all ages. The importance of the duodenum should not, however, be relegated since pancreatic enzymes are secreted into the duodenum. The functional importance of the various regions is further examined in other experiments of this chapter.

4.1.5. CONCLUSION

The Steggles x Ross broiler chick had with a large GIT at hatch and the tract developed rapidly in early life. Such pattern of development is commonly observed in other avian species and strains of broiler chickens. The strain extracted the required nutrients from diets, judging by moderately high levels of retention of assessed nutrients. The mechanisms behind the gross growth of body and GIT are examined in subsequent experiments of this chapter. Experiments in chapter 5 focus on the pattern of intestinal development and body growth of the strain in response to various dietary regimes.

4.2.0. EXPERIMENT 4.2: GROWTH AND DEVELOPMENT OF THE INTESTINAL MUCOSA

4.2.1. INTRODUCTION

The gastrointestinal tract (GIT) constitutes the first barrier to nutrient metabolism in animals (Cant *et al.*, 1996). The metabolic activity of the gastrointestinal mucosa can have tremendous impact on nutrient supply to the whole animal. In cattle, sheep and pigs, over 20 % of the whole-body consumption of oxygen occurs at the level of the GIT (Webster, 1980; Yen, 1989; Reynolds *et al.*, 1991). Most of the energy utilized by the GIT is channelled towards protein synthesis. In chickens, oxygen consumption at various intestinal sites varies between 6.83 and 16.52 nmol/minute/mg protein (Spratt *et al.*, 1990). The efficiency of nutrient utilization could be increased if this apparent nutrient 'loss' at the gastrointestinal tract is reduced. Any measures at effecting changes would depend on an understanding of the role of nutritional and genetic factors in the distribution of nutrients towards various physiological processes.

The intestinal tissue, like other organs composed of parenchyma cells grows by both hyperplasia and cellular hypertrophy (Leblond, 1972). The intestine differs from the liver in that cell proliferation is also accompanied by cell turnover. Mucosal growth is accomplished through an excess of cell production over cell loss (Waterlow *et al.*, 1978). These changes can be monitored through measurements of the concentrations of DNA, protein and RNA in tissues. The rationale for such an assumption is that the DNA content of diploid cells remains relatively constant after formation. Changes in DNA concentration would therefore reflect changes in cell number. Cell size can then be estimated as the ratio of protein to DNA. The relationship between protein and RNA

concentrations are bi-faceted: the RNA/protein ratio is a measure of the potential for protein synthesis while the protein/RNA ratio estimates the extent to which this potential is realized. The RNA:DNA ratio is associated with changes in the amount of protein synthesized per cell or per unit DNA.

The intestine possesses an inherent ability to create and maintain regional differences with regards to mucosal structure, especially villus height (Ferraris *et al.*, 1992). These differences are noticeable in mammals and have been observed in poultry (Uni *et al.* 1995a), even though the delineation of regional sites may not be as precise. Other differences may occur based on age, intestinal region and position along the crypt:villus axis as has previously been reviewed (2.3.2). Poultry have been observed to differ from many mammalian species studied with regards to intestinal development as has already been highlighted (Smith and Peacock, 1989).

Following the changes in absolute mass and length of the intestine observed in experiment 4.1, an experiment was designed to study the mechanisms behind the growth of the intestinal mucosa. The relative importance of the two processes - hyperplasia and hypertrophy was assessed through direct cell counts and utilization of the biochemical indices, DNA, RNA and protein.

4.2.2. MATERIALS AND METHODS

4.2.2.1. Animals and diets

Ninety-six day-old Steggles x Ross (F_1) chicks were used for the study. The chicks were maintained on the same diets and similar rearing conditions as in Experiment 4.1.

4.2.2.2. Sample collection

To facilitate studies on cell proliferation and migration, chicks were injected with 5-bromo-2'-deoxyuridine (BrDU, ICN, Aust. Pty Ltd.) intraperitoneally at a dose of 50 µg/kg body weight on 1, 7, 14 and 21 days of age. At each age, six chicks were killed at intervals of 1, 24, 48 and 96 hours post-administration of BrDU. For general histology, samples were collected from the proximal duodenum, jejunum and ileum of chicks birds killed within one hour of BrDU treatment. The tissues were fixed in 10 % neutral buffered formalin over 24 hours. Only jejunal samples were used in the study of cell proliferation and migration and these were fixed in methacarn (60 % methanol + 30 % chloroform + 10 % glacial acetic acid) for 6 hours. Both sets of samples were transferred into 70 % ethanol at the end of primary fixation.

All tissues were processed as for normal histology (section 3.4.1 and Appendix 4) and sections, 5 µm thick were prepared on microtome. For routine histology, the sections were stained with Lilee Meyer's hematoxylin and counterstained with eosin yellow (Appendix 5).

The sections for routine histology were digitized on computer using the Video Pro image analysis programme as described in section 3.4.1. Five well presented villi were assessed for cellular proliferation and migration. The method used in the preparation of samples for cell proliferation is described in section 4.2.2.3 below.

4.2.2.3. Cellular proliferation and migration

Cell proliferation and migration were traditionally studied by the tritiated thymidine assay but a lot of time is required for the development of incorporated material and autoradiography (Meyer *et al.*, 1989). The development of antibodies to BrDU has increased the efficiency of studying cell proliferation and migration, both as a biomedical aid and for routine research. BrDU is incorporated into S-phase cells and the labelling index closely approximates that of thymidine (Meyer *et al.*, 1989; Thoolen, 1990).

In the present experiment, BrDU-labelled cells were identified by immunohistochemistry, using mouse anti-BrDU antibody (Sigma Aust. Pty Ltd.). Sections were deparaffinized in an oven (80°C) for 45 minutes, followed by clearing in xylene (10 minutes) and absolute ethanol (2 minutes). The sections were incubated in cold absolute methanol + 0.3 % H_2O_2 for 30 minutes; followed by 2 minutes each in 80, 50, 30 % ethanol and distilled water. The sections were immersed in 1 M HCl for 8 minutes at 60°C, then transferred into ice-cold PBS. The sections were then incubated in 10 % normal horse serum (NHS, Sigma) for 20 minutes at room temperature to eliminate non-specific binding.

Sections were incubated in mouse anti-BrDU (1:100) containing 1.32 % NHS for 90 minutes, rinsed in PBS and incubated in biotinylated antimouse IgG (Amersham Aust. Pty Ltd.) diluted in PBS, for 30 minutes. This was followed by incubation in Vectastain (an avidin-biotin complex, coupled with horse radish peroxidase, Dako Aust. Pty Ltd.), rinsing in PBS and incubation in 0.05 % diaminobenzidine (DAB) in PBS and H_2O_2 for

30 seconds. The sections were rinsed in nanopure water and counterstained in dilute haematoxylin (1 part in 8 parts nanopure water) for 2 minutes, rinsed with demineralized water and passed through graded concentrations (30, 50, 80 %) of ethanol and xylene for 2 minutes each. The sections were then mounted in DePeX.

Enterocyte proliferation and migration rates were assessed by computer-aided image analysis as described for histology above. Mucosal depth (crypt depth and villus height) and distance migrated by leading cells were measured. For samples collected one-hour post-administration, all labelled cells on the right flank of the crypt were counted and related to the total number of cells in the same region, to yield a labelling index.

4.2.2.4. Biochemical estimation of cellular size and metabolic activity

The relationships between protein, DNA and RNA are useful indicators of cellular size and metabolic activities as highlighted in section 4.2.1. The procedures for determining the concentrations of these biochemical indices have been described in section 3.5.0.

4.2.3. RESULTS

4.2.3.1. Structural development of the intestinal mucosa

Assessments made in the duodenum, jejunum and the ileum revealed ~~massive~~ changes in mucosal structure with age (Table 4.2.1 and Plate 4.2.1). In all three regions, there was a general increase in external muscle thickness with age, although this was significant ($P<0.001$) only in the jejunum.

Table 4.2.1: Intestinal muscle thickness and mucosal morphometry at different sites and ages post-hatch.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
A. External muscle thickness (μm)					
Duodenum	225.9	242.4	208.4	240.5	34.65
Jejunum	180.4^{bc}	175.6^{c}	168.0^{c}	298.7^{ab}	29.00***
Ileum	180.3	172.7	184.6	198.5	27.17
B. Crypt depth (μm)					
Duodenum	127.8^{b}	163.9^{a}	159.3^{a}	174.7^{a}	15.39*
Jejunum	112.0^{bc}	127.7^{bc}	105.0^{c}	152.7^{ab}	12.55**
Ileum	104.9	108.2	105.8	97.9	9.00
C. Villus height (μm)					
Duodenum	908.0^{c}	1298.1^{bc}	1248.5^{bc}	1437.7^{ab}	89.47***
Jejunum	447.6^{c}	693.0^{bc}	1007.5^{ab}	1218.9^{ab}	71.36***
Ileum	316.4^{c}	449.3^{bc}	447.7^{bc}	578.5^{ab}	38.50***
D. Villus surface area (mm^2)					
Duodenum	0.12^{c}	0.18^{bc}	0.19^{bc}	0.23^{abc}	0.025**
Jejunum	0.06^{c}	0.09^{bc}	0.11^{b}	0.17^{a}	0.013***
Ileum	0.03^{c}	0.06^{b}	0.06^{b}	0.07^{a}	0.009**

a,b,c - Mean values in the same row not sharing a superscript differ significantly (*P<0.05; **P<0.01; ***P<0.001).

Crypt depth increased with age in the duodenum (P<0.05) and jejunum (P<0.01) but the change was not significant in the ileum. Villus height significantly (P<0.001) increased with age in all regions although the rate of growth was greater in the jejunum than in the duodenum or ileum. Between hatch and 21 days of age, there was 58.3, 172.3 and 82.8 % increase in villus height in the duodenum, jejunum and ileum respectively. A similar trend was observed for villus surface area in the duodenum and ileum (P<0.01) and the

jejunum (P<0.001). Generally, absolute values of these variables were higher in the duodenum than in the ileum, jejunal values were intermediate.

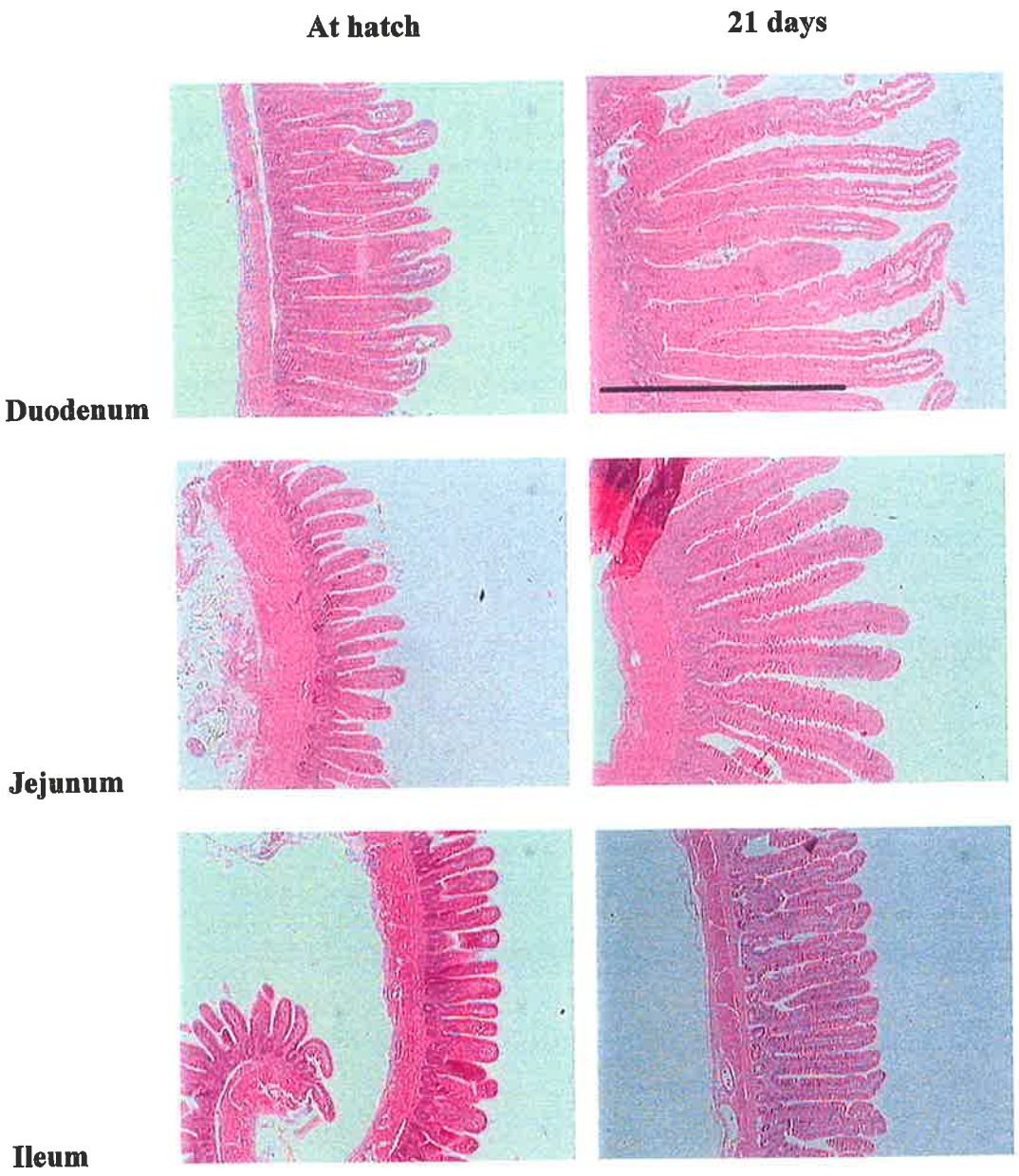


Plate 4.2.1. Mucosal structure at different ages and intestinal sites.
Between hatch and 21 days of age, there were 58.3, 172.3 and 82.8 % increase in villus height at the duodenum, jejunum and ileum respectively. Scale bar = 1000 μm.

4.2.3.2. Protein and nucleic acid contents of intestinal mucosa

The protein content of duodenal mucosa was not significantly affected by age (Table 4.2.2). In the jejunum and ileum, there were significant differences (P<0.001) between

the age groups in terms of mucosal protein content. At all ages except at hatch, protein concentration was higher in the jejunum than in the duodenum or ileum.

Table 4.2.2. Mucosal protein, DNA and RNA contents (mg/g tissue) at different ages and intestinal sites.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Protein					
Duodenum	34.9	37.0	32.3	27.5	3.93
Jejunum	24.3^{b}	79.6^{a}	53.2^{ab}	70.4^{a}	6.86***
Ileum	26.3^{b}	44.1^{a}	33.0^{ab}	26.4^{b}	3.59***
DNA					
Duodenum	3.6^{a}	0.7^{b}	0.6^{b}	0.4^{b}	0.17***
Jejunum	1.7^{a}	1.8^{a}	0.7^{b}	1.0^{b}	0.28***
Ileum	2.9^{a}	1.5^{b}	1.1^{b}	0.9^{b}	0.31***
RNA					
Duodenum	0.6^{c}	0.9^{ab}	1.1^{b}	2.1^{a}	0.2**
Jejunum	3.3^{a}	1.6^{b}	1.7^{b}	1.3^{b}	0.54*
Ileum	0.9^{c}	2.1^{ab}	1.3^{bc}	2.2^{a}	0.30***

a,b - Mean values in the same row not sharing a superscript are significantly different (*P<0.05; **P<0.01; ***P<0.001). Protein was measured in mucosal homogenates extracted with 6 ml of Tris-EDTA buffer, pH 9.0, prior to processing for nucleic acids.

In the three intestinal sites, DNA content declined (P<0.001) with age. These changes were more pronounced at the duodenum and ileum than in the jejunum. At hatch, the duodenum and ileum contained more DNA than the jejunal mucosa. The RNA content of the jejunal mucosa was, however, higher than the concentration in the duodenum and ileum at hatch. There was a significant increase in RNA content between hatch and day 7 in the duodenum (P<0.01) and ileum (P<0.001) while in the jejunum, there was a reduction (P<0.05) in RNA content with age. The RNA content in duodenal and ileal mucosa tended to fluctuate with age.

Table 4.2.3. Relationships between the biochemical indices of mucosal growth.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Protein:DNA ratio					
Duodenum	9.9^{c}	50.4^{b}	53.3^{b}	78.7^{a}	3.23***
Jejunum	15.5^{b}	45.0^{ab}	76.6^{a}	71.4^{a}	8.73***
Ileum	10.7^{b}	30.9^{a}	30.9^{a}	29.4^{a}	2.83***
RNA:protein ratio					
Duodenum	0.02^{c}	0.05^{b}	0.03^{bc}	0.08^{a}	0.006***
Jejunum	0.14^{a}	0.02^{b}	0.03^{b}	0.02^{b}	0.022**
Ileum	0.03^{b}	0.05^{b}	0.04^{b}	0.09^{a}	0.011***
Protein:RNA ratio					
Duodenum	56.6^{a}	19.6^{b}	30.3^{b}	13.9^{b}	6.58***
Jejunum	8.1^{b}	54.7^{a}	32.1^{ab}	55.6^{a}	10.11**
Ileum	32.6^{a}	21.0^{ab}	26.3^{ab}	12.0^{b}	5.23**
RNA:DNA ratio					
Duodenum	0.2^{c}	2.6^{b}	1.8^{b}	5.9^{a}	0.40***
Jejunum	1.9^{ab}	0.9^{b}	2.5^{a}	1.4^{b}	0.48*
Ileum	0.5^{b}	1.5^{ab}	1.2^{b}	2.5^{a}	0.28***

a,b - Mean values in the same row not sharing a superscript are significantly different (*P<0.05; **P<0.01; ***P<0.001).

There was a significant increase (P<0.001) in protein:DNA ratio (a measure of cell size) between hatch and 21 days of age in the duodenum (Table 4.2.3). Similar changes (P<0.001) were observed in the jejunum and ileum but these peaked by the age of 7 days in the ileum and 14 days in the jejunum. The RNA:protein ratio (protein synthesis potential) in the jejunum was highest (P<0.01) at hatch and declined with age. In the duodenum and ileum, this ratio generally increased (P<0.001) between hatch and 21 days of age. While the protein:RNA ratio (actual rate of protein synthesis) declined in

the duodenum (P<0.001) and ileum (P<0.01), there was a general increase in this ratio with age in the jejunum (P<0.01).

In all three intestinal regions, RNA:DNA ratio (protein synthesis per cell) did not show a definite trend although there were significant (P<0.001) variations with age in the duodenum and ileum as well as in the jejunum (P<0.05).

Table 4.2.4: Crypt column count, BrDU-labelled cell number and labelling ratio (mean ± SD) in the jejunum.

	Age (days)			
	1	7	14	21
Total crypt column count	21.6 ± 1.92^{b}	25.9 ± 0.92^{a}	33.6 ± 5.36^{a}	32.4 ± 3.11^{a}
BrDU-labelled cells	10.7 ± 1.36^{b}	13.5 ± 1.32^{a}	13.8 ± 1.35^{a}	12.6 ± 1.17^{ab}
Labelling index (%)[1]	50.0 ± 9.34^{ab}	54.0 ± 3.56^{a}	42.2 ± 3.57^{ab}	38.4 ± 3.32^{b}

a,b - Mean values in the same row not sharing a superscript are significantly different (P<0.001 for total crypt column count; P<0.01 for BrDU-labelled cells and labelling index. 1. Percentage of total cells labelled within one hour of BrDU administration. Cells were counted in 5 crypts of 4 chicks at each age.

4.2.3.3. Enterocyte proliferation and migration

At all ages, enterocyte proliferation at the jejunum was completed and quantifiable within one hour of administration of BrDU (Plate 4.2.2). Crypt column count increased (P<0.001) with age as did the number of BrDU-labelled cells (P<0.01), both values peaked at 14 days of age (Table 4.2.4). The labelling index increased (P<0.01) to a peak by day 7 and declined with subsequent age.

The rate of cell migration increased significantly (P<0.001) with age while there was a decline (P<0.01) in the distance migrated in proportion to mucosal depth (Table 4.2.5). The estimated life-span of enterocytes, based on mucosal depth and rate of migration

varied (P<0.001) between 69.2 hours for chicks treated at hatch and 117.5 hours for chicks treated at 21 days of age. The time spent by enterocytes within the zone of proliferation also declined (P<0.05) with age.

Table 4.2.5: Enterocyte migration rate and estimated life-span in the jejunum at different ages.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Mucosal depth[1] (μm)	579.3^{b}	1051.0^{a}	1381.1^{a}	1292.4^{a}	110.92***
Distance migrated (μm)	206.0^{b}	315.9^{a}	351.2^{a}	305.0^{ab}	27.36***
Rate of migration (μm/h)	8.6^{b}	13.2^{a}	14.6^{a}	12.7^{ab}	1.14***
Migration ratio[2]	0.37^{a}	0.30^{ab}	0.25^{ab}	0.21^{b}	0.040**
Estimated life-span (h)	69.2^{b}	81.4^{b}	95.0^{ab}	117.5^{a}	8.68***
Cell time in crypt (h)[3]	14.9^{a}	12.4^{bc}	10.9^{c}	13.8^{ab}	1.12*

1. Combined crypt depth and villus height. 2. Ratio of migration distance to mucosal depth. 3. Estimated from crypt depth and rate of migration for age group. SED - standard error of difference between mean values. a, b - Mean values in the same row not sharing a superscript differ significantly (**P<0.01; ***P<0.001).

Actual enterocyte life-span increased with age but differed widely from the calculated values. Migration was completed or nearly completed within 96 hours of cell birth in the case of birds treated at hatch and 7 days of age (Plate 4.2.3). In older birds, the leading enterocytes covered only about half to two-thirds of the villus within 96 hours of administering BrDU.

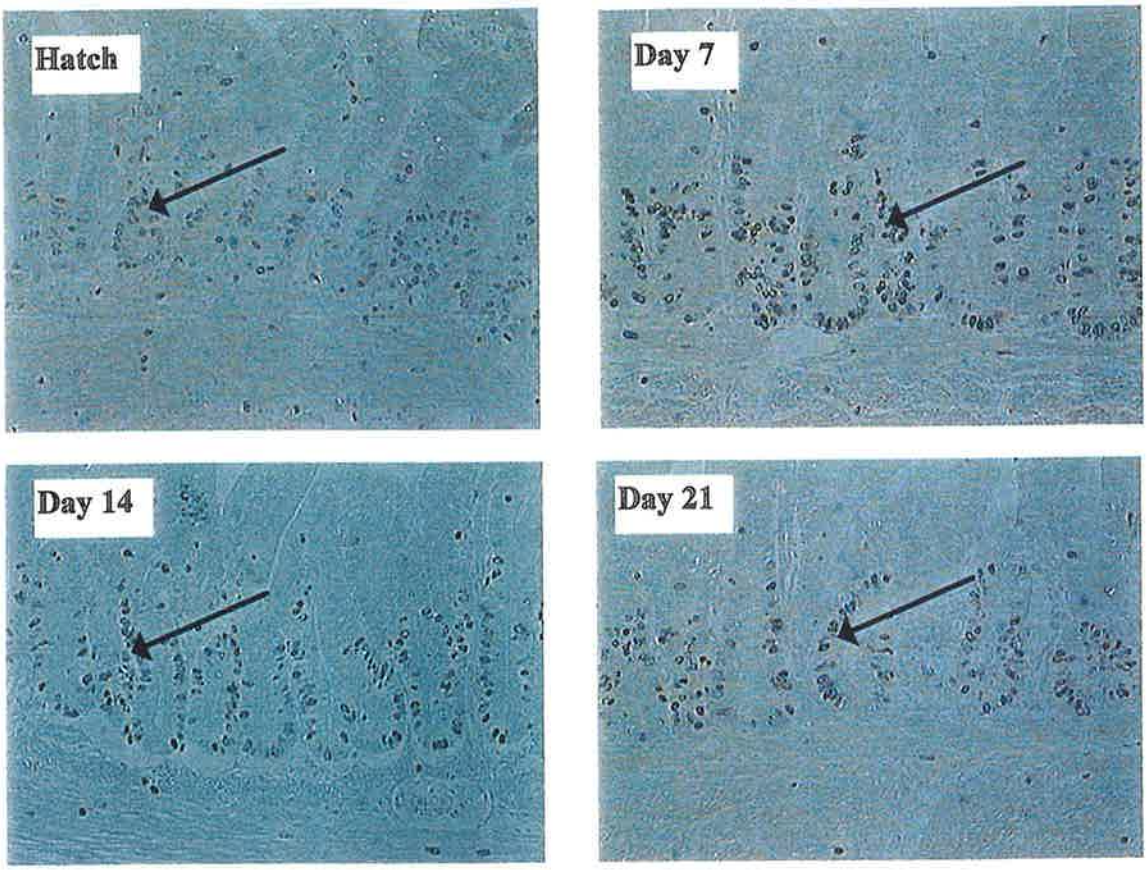


Plate 4.2.2: The rate of cell proliferation estimated 1 hour after administration of BrDU. Cell proliferation was confined to the crypt (*arrowed*) and there was very little migration out of the region within 1 hour of cell formation. Cell proliferation tended to increase with age up to day 7 (Table 4.2.2).

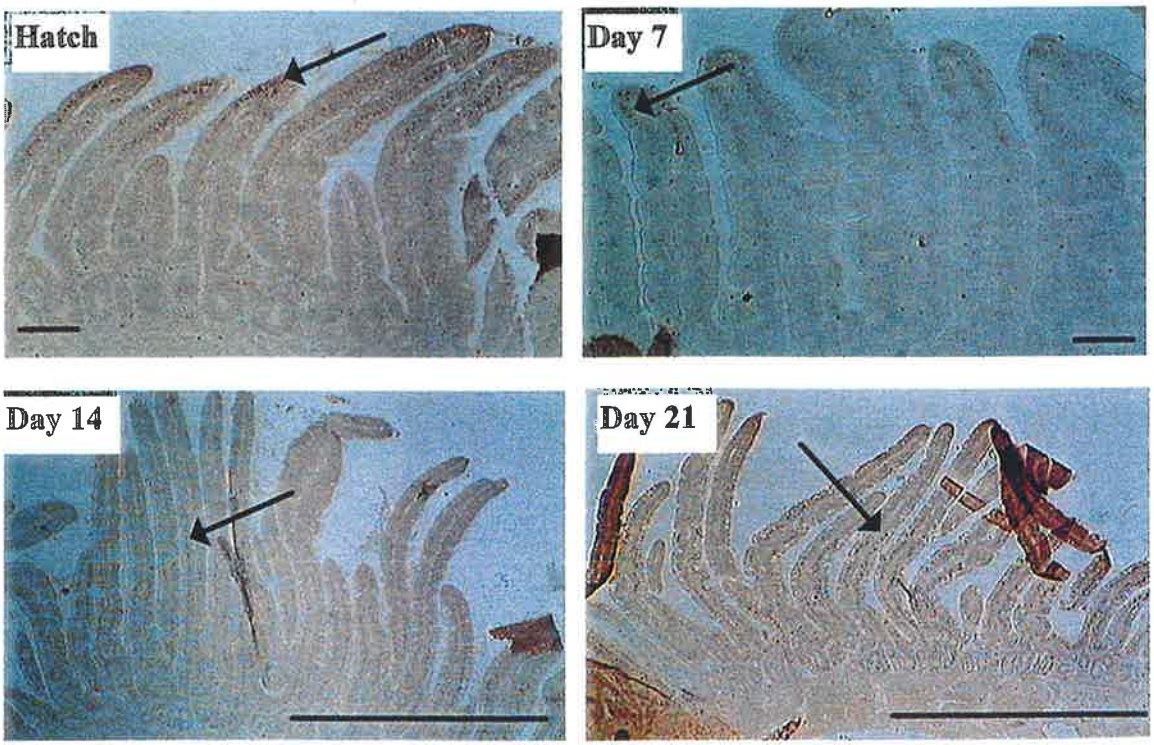


Plate 4.2.3: Extent of cell migration at the jejunum over 96 hours. Arrows show the position of the leading cells at different ages. Scale for hatch/day 7 = 100 μm; for days 14/21 = 1000 μm.

4.2.4. DISCUSSION

4.2.4.1. Mucosal growth

Examination at the level of light microscopy revealed advanced structural development of the mucosa at the three intestinal regions at hatch. Gross changes were, however, noted between hatch and 21 days of age. In most animal species, this increase in mucosal growth is induced by normal exposure to dietary nutrients (Moran, 1985). Baranylova and Holman (1976) reported on the delay and reduction of structural development in the intestine of poultry on restricted feeding post-hatch.

In mammalian species on which extensive studies have been conducted, structural changes in the mucosa are also dependent on nutrient supply (Castillo *et al.*, 1988; Holt *et al.*, 1988; Maxton *et al.*, 1989). These changes become more rapid at birth and most functional molecules are switched on in anticipation of the postnatal roles to be performed by the intestine (Buddington *et al.*, 1991; Grey *et al.*, 1991; Kelly *et al.*, 1992).

Of the three intestinal regions assessed in this study, histological changes were most profound in the jejunum. This is in agreement with results obtained on other strains (Arbor Acres and Lohmann) of broiler chickens (Uni *et al.*, 1995a). It is relatively difficult to compare structural variables between studies due to the differences in methods of tissue processing. If, however, these differences are discounted, both the Arbor Acres (a heavy strain) and Lohmann (a light strain) chicks had considerably shorter villi than the Steggles x Ross at a similar age (7 days). At this age and for the same intestinal region, the villi of the Arbor Acres were only about half the length of the

Steggles x Ross while those of the Lohmann were even shorter. Both studies used 10 % neutral buffered formalin as fixative but may have differed in procedures during further processing. The extent of tissue shrinkage depends on duration of fixation as well as number and duration of each of the steps during dehydration and clearing. There were, however, differences between the Lohmann and Arbor Acres in mucosal growth (Uni *et al.*, 1995a). Both strains are of similar hatching weight to the Steggles x Ross but only the Arbor Acres grows at the same rate as the Steggles x Ross at subsequent age to 14 days. In poultry, intestinal mucosal morphometry is strongly linked to the pattern and degree of selection during the development of the strain although there are still inherent differences between strains as was highlighted in section 2.1.1. Highly selected strains were observed to possess longer villi, larger villus surface area, deeper crypts and more rapid enterocyte migration rate than un-selected or relaxed selected strains. These attributes may increase the energy and protein demands of the GIT in highly selected strains. The mucosal variables were observed to correlate well with the degree of improvement in body growth of the selected strains. The values for mucosal variables, especially villus height are generally higher in poultry than those reported for mammalian species (Smith *et al.*, 1984; Smith and Peacock, 1989).

4.2.4.2. Mechanisms involved in mucosal growth

Intestinal and other organ growth may be due to cellular hyperplasia, hypertrophy or a combination of the two processes (Morisset, 1993). In the strain under study, intestinal growth appeared to be most rapid between hatch and 7 days of age. This is accomplished mainly by an increase in cell size, as indicated by the protein:DNA ratio. There was also an increase in crypt column count with age, which is suggestive of a potential for increased cell proliferation although cell population (DNA concentration)

tended to decline with age at all intestinal sites. The pattern of development in the Steggles x Ross appears to differ from that observed in the Arbor Acres and Lohmann. In the latter two strains, Uni *et al.* (1995b) observed an increase in DNA content of the duodenal mucosa between the 12th day of incubation and 8 days post-hatch. The RNA content was maximal at hatch and 4 days post-hatch in the Lohmann and Arbor Acres respectively. In the strain studied here, the RNA content of the duodenal and ileal mucosal homogenates increased while that of the jejunum declined with age.

The RNA:protein ratio has been proposed by Waterlow *et al.* (1978) as an indicator of ribosomal capacity at translating amino acids into protein. In the present study, the RNA:protein ratio in the duodenum and ileum was low at hatch but high in the jejunum. In the two former sites, RNA:protein ratio increased with age although the change in mucosal protein was minimal. At the jejunum, RNA:protein ratio declined with age, accompanied by a large increase in mucosal protein. The implications of these regional differences to function are not properly understood. The jejunum, being longer than the duodenum and more proximal than the ileum would be an important site for nutrient digestion (Low and Zebrowska, 1986). The absorption of nitrogen, however, is usually higher in the ileum than in the jejunum or duodenum. The relative activities of intestinal enzymes in the various intestinal regions are examined in experiments 4.3 while the transport of an amino acid, tryptophan is reported in experiment 4.4.

4.2.4.3. Cell proliferation and migration

Cell proliferation appeared to be rapid, judging by the large number of cells labelled within one hour of BrDU administration. The cells also migrated rapidly up the villus. These results suggest the possible effects of short periods of exposure to dietary factors.

The increase in labelling index between hatch and 7 days of age may be reflective of an increased rate of cell proliferation to support both crypt and villus growth. The discrepancy between estimated enterocyte life-span and actual values may be due to an unusual reduction in migration rate with age, beyond 14 days of age. The initial increase in cell migration is similar to observations in the rat in which enterocyte migration rate was found to be lower in the neonate than in the adult (Smith *et al.*, 1986). Spielvogel *et al.* (1972) also reported on the bi-phasic rates of cell migration in chickens. Such changes were not assessed in the present study but could reduce the validity of estimates of enterocyte life-span, based on single migration rates. Actual observations, as was undertaken would be more reliable. In mammalian species and earlier strains of broiler chickens, cellular differentiation was observed to occur during migration from the proliferation zone at the crypt to the extrusion zone at the villus tip (Spielvogel *et al.*, 1972; Freeman *et al.*, 1993). In the Steggles x Ross, there appeared to a reduction in time spent by enterocytes at the crypt, the region associated with cell proliferation. Enterocytes that delay their migration from the synthesis zone are usually more mature and functionally competent than cells that migrate rapidly out of the zone (Wild and Murray, 1992).

The distance covered by the cells with time is a function of mucosal depth or more appropriately villus height as well as migration rate. In very young chicks, enterocytes approached the extrusion zone within 96 hours of formation but were only about three-quarters of the distance in chicks older than 14 days. These differences were reflected in the migration ratio, relating distance migrated to mucosal depth. In earlier strains of broiler chicks (2-day old), jejunal enterocyte life-span was about 48 hours (Imondi and Bird, 1966). The villus length reported by Imondi and Bird (1966) at hatch was

comparable to present-day value although improvements to selection techniques have generally enhanced subsequent mucosal growth in modern strains (Smith *et al.*, 1990). The migration rate observed in the present study is considerably higher than values reported for 6-week-old Rhode Island Red chickens by Smith and Peacock (1989); which suggests that the rate of migration may not recover after the reduction observed by 14 days of age. Smith and Peacock (1989) also indicated that the migration rate observed in the Rhode Island Red was lower than values previously reported in research on several mammalian species (Smith *et al.*, 1984). A faster migration rate may be associated with a shorter enterocyte life-span and, possibly, with a greater need for cell replenishment. Both of these processes would increase nutrient demands for the maintenance of the intestinal mucosa.

4.2.5. CONCLUSION

The intestinal mucosa was structurally developed at hatch and grew rapidly with age. This growth is accomplished by initial rapid cell proliferation, cellular hypertrophy and increased rate of migration. The rate of cell proliferation peaked by 7 days while cellular migration increased with age up to 14 days of age. It is not certain why there is a reduction in migration rate subsequent to this age.

The rate and, to some extent, pattern of mucosal growth varies with intestinal site, being greater at the jejunum than at the duodenum or ileum. The growth at the jejunum appears to be modulated by a high concentration of RNA as well as wide RNA:protein ratio at hatch relative to values obtained for the other sites.

4.3.0. EXPERIMENT 4.3: DEVELOPMENT AND LOCALIZATION OF BRUSH-BORDER MEMBRANE ENZYMES IN THE SMALL INTESTINE OF BROILER CHICKENS.

4.3.1. INTRODUCTION

Intestinal enzymes are responsible for the terminal digestion of most dietary macromolecules and play a vital role in regulating the amount of nutrients available for absorption. Apart from digestion, some of the enzymes may be involved in other functions including nutrient transport from the intestine, reception of signals into cells and regulation of cell growth and differentiation (Kenny, 1986).

In mammalian species, enzyme activities are dependent on several factors, some dietary and others of animal origin (James *et al.*, 1988; King *et al.*, 1983; Batt *et al.*, 1995). There is a reduction in intestinal enzyme function within a short period after birth (Holt and Kotler, 1987; Tivey and Smith, 1989; Wild and Murray, 1992). Sell *et al.* (1991) have also reported on the age-dependence of some intestinal enzymes in turkeys. In broiler chickens, variations between lines (Uni *et al.*, 1995a) as well as between chicks on different dietary carbohydrate densities (Biviano *et al.*, 1993) have been established as was reviewed in section 2.1.1. Further studies are required to enable closer assessment of the impact of dietary factors, especially the stress imposed by anti-nutritional factors present in the diet. The dietary regulation of enzyme activity can be fully appreciated if the natural pattern of development is understood.

The overall efficiency of digestion would depend on the contribution of the component sites of the gastrointestinal tract (GIT), especially the small intestine. It is well known

that nutrients are increasingly released as the ingesta progresses through the GIT but there are few reports on the relative activities of similar enzymes at different intestinal sites. There is a paucity of research reports on the regulation of body growth by digestive enzyme function. Such studies would aid the selection of individuals for increased efficiency in feed utilization.

The aim of the present experiment was to examine the natural development of key intestinal enzymes in broiler chicks. The variation in developmental pattern due to age, intestinal regions and the crypt:villus axis were examined. The major focus was on the intestinal carbohydrases, maltase and sucrase; an N-terminal aminopeptidase, APN, and a phosphatase, AP. The exact physiological role of AP is unknown but the enzyme is commonly used as a marker of brush-border membranes. The other enzymes were chosen on the basis of the relative concentrations of their natural substrates, carbohydrates and proteins in normal poultry diets.

4.3.2. MATERIALS AND METHODS

4.3.2.1. Animals and diets

Eighty mixed sex day-old broiler chicks (Steggles x Ross) obtained from Australia Poultry (Australia) Pty. Ltd. were used for the study. The chicks were maintained on a commercial crumble diet, at rearing conditions similar to those described in experiments 4.1 and 4.2.

Samples were collected at hatch and subsequently every seven days until 21 days of age. Seven chicks were randomly selected at each age and euthanatized as described in section 3.4.0. Sub-samples of the small intestine, about 5 cm long were taken from the

proximal ends of the duodenum, jejunum and the ileum and flushed with ice-cold PBS, pH 7.4. The segment was slit longitudinally and further rinsed with PBS. Samples for biochemical assays were wrapped in aluminium foil and snap-frozen in liquid nitrogen. Samples for histochemistry were embedded in strips of liver and snap-frozen in a hexane bath cooled by liquid nitrogen. The samples were stored at -80°C and used to prepare BBMV or frozen sections within one week of collection.

4.3.2.2. Biochemical assays

Previous studies have tended to estimate enzyme activities in the lumenal digesta or mucosal homogenate rather than *in situ* on the intestinal membrane (Siddons, 1969; Palmer and Rolls, 1983). Such results would be biased depending on the degree of contamination with extra-intestinal secretions and the half-life of the enzyme. In the present study, enzyme activities were estimated mainly *in situ*, by histochemical localization or biochemically on purified membranes.

Brush-border membrane vesicles (BBMV) were prepared in line with the method described by Shirazi-Beechey *et al.* (1991), outlined in section 3.7.0. Assays were conducted on fixed substrate concentrations established in studies on mammals. Enzyme activities were assessed in the duodenum, jejunum and ileum. The influence of substrate concentration on enzyme activity was also examined for maltase and sucrase in vesicles obtained from the jejunum and ileum.

4.3.2.3. Enzyme histochemistry

Fresh-frozen tissues were sectioned on a cryostat as described in section 3.6.0. The histochemical assessment of brush-border membrane enzyme activities was conducted

according to modified techniques developed for studies on mammalian tissues as described in sections 3.6.1 and 3.6.3.

4.3.3. Data analysis

Data collected from biochemical assays were analysed as described in section 3.9.0. Data on absorbance per unit surface area were further processed using an Excel macro to obtain activity profiles on the crypt:villus axis. Principally, the macro equilibrated villus length between samples within the same age and intestinal region in relation to absorbance, enabling the construction of profile maps at similar locations of the small intestine. Profile data were also related to cell migration data (experiment 4.2) to determine the approximate age of enterocytes associated with each level of enzyme activity.

Data on specific activities were related to the absolute empty weight of intestinal regions to obtain total activity according to the formula:

$$TA = Sp.\ Ac.\ x\ PY_{muc}\ x\ Int.\ wt.$$

where TA is total enzyme activity; Sp. Ac. is specific enzyme activity per unit membrane protein; PY_{muc} is protein content of mucosa per unit weight of tissue, and Int. wt. is the mean absolute weight of the intestinal region for the age obtained in experiment 4.1.

4.3.4. RESULTS

4.3.4.1. Specific activity of enzymes

The specific activity (expressed as product formed per unit of membrane protein with time) of maltase decreased ($P<0.001$) at all intestinal sites between hatch and day 14,

after which there was an increase towards day 21 (Table 4.3.1). Maltase activity varied significantly between intestinal sites at hatch ($P<0.001$) and was similar at other ages. The specific activity of sucrase at the three intestinal sites was significantly higher ($P<0.001$) at hatch than at the other ages. There were also significant ($P<0.05$) differences between the intestinal sites at hatch and day 7 but not at the other ages.

Table 4.3.1: Specific activity of disaccharidases (μmole glucose/mg protein/minute) in brush border membrane vesicles.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
A. Total maltase					
Duodenum	28.2^{a}	17.6^{ab}	14.7^{b}	17.0^{ab}	3.69
Jejunum	33.9^{a}	19.4^{b}	10.1^{b}	14.5^{b}	2.84
Ileum	49.6^{a}	18.2^{b}	10.0^{b}	15.0^{b}	2.39
B. Sucrase					
Duodenum	4.8^{a}	0.8^{b}	0.5^{b}	0.5^{b}	0.56
Jejunum	6.5^{a}	1.2^{b}	1.0^{b}	0.6^{b}	0.43
Ileum	7.2^{a}	0.8^{b}	0.9^{b}	0.6^{b}	0.31

a,b - Mean values in the same row not sharing a superscript differ ($P<0.001$) significantly.

The specific activity of APN declined ($P<0.001$) between hatch and 21 days of age at all intestinal sites investigated (Table 4.3.2). The differences between the intestinal regions were significant ($P<0.01$) only at day 14. AP activity followed a similar trend and was significantly lower in the ileum than in the duodenum or jejunum at hatch ($P<0.01$), day 7 ($P<0.001$) and days 14 and 21 ($P<0.05$).

Table 4.3.2: Specific activity of aminopeptidase N (μmole *p*-nitroaniline/mg protein/minute) and alkaline phosphatase (μmole *p*-nitrophenol/mg protein/minute) in brush border membrane vesicles.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
A. Aminopeptidase N (APN)					
Duodenum	3.8^{a}	2.8^{ab}	1.1^{b}	1.1^{b}	0.52
Jejunum	3.8^{a}	2.3^{b}	1.7^{bc}	1.0^{c}	0.28
Ileum	4.6^{a}	2.5^{abc}	2.2^{bc}	1.2^{c}	0.50
B. Alkaline phosphatase (AP)					
Duodenum	11.8^{a}	4.6^{b}	2.8^{b}	2.3^{b}	1.35
Jejunum	7.3^{a}	0.9^{b}	3.0^{ab}	1.6^{b}	1.02
Ileum	2.4^{a}	0.9^{b}	0.7^{b}	0.4^{b}	0.28

a,b,c - Mean values in the same row not sharing a superscript differ significantly (P<0.001).

Across all ages and intestinal sites, the activity of maltase was higher than that of the other enzymes examined, being on average, 17 times higher than that sucrase. Maltase activity was also 10 and 15 times higher than the activities of APN and AP respectively across all age groups and intestinal sites.

4.3.4.2. Total activity of enzymes

The total activities of the four enzymes assessed in the three intestinal regions are shown in Table 4.3.3. The total activities of maltase and sucrase in the duodenum significantly increased (P<0.001) with age. The total activities of APN and AP increased (P<0.001) to a peak at 14 days of age and then declined. Between hatch and 21 days of age, there were respectively 12-, 3-, 5- and 4-fold increases in the activities of maltase, sucrase, APN and AP in the duodenum.

Table 4.3.3: Total activity of BBM enzymes (μmole product/minute) at different ages.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Duodenum					
Maltase	12.4^{b}	50.0^{b}	51.1^{b}	153.7^{a}	12.27***
Sucrase	2.1^{b}	2.6^{b}	3.8^{ab}	5.7^{a}	0.56***
APN	1.7^{b}	7.9^{ab}	11.1^{a}	8.9^{a}	1.48***
AP	5.4^{b}	13.4^{b}	29.0^{a}	18.9^{a}	3.14 ***
Jejunum					
Maltase	28.7^{c}	147.7^{b}	110.2^{bc}	339.2^{a}	20.37***
Sucrase	5.5^{b}	9.3^{ab}	10.5^{a}	14.1^{a}	1.62**
APN	3.3^{b}	17.7^{a}	18.9^{a}	22.0^{a}	1.21***
AP	6.2^{b}	6.5^{b}	34.0^{a}	36.4^{a}	5.44***
Ileum					
Maltase	28.7^{b}	64.3^{b}	61.8^{b}	228.8^{a}	28.97***
Sucrase	4.2^{b}	2.9^{b}	5.5^{ab}	8.7^{a}	1.00***
APN	2.6^{b}	8.8^{a}	14.0^{a}	12.6^{a}	1.27***
AP	1.4^{c}	2.9^{bc}	4.8^{ab}	5.8^{a}	0.93**

a,b - Mean values in the same row with unlike superscripts are significantly different (**P<0.01; ***P<0.001).

In the jejunum, the total activities of the four enzymes significantly increased with age within the period tested. The changes in activities between hatch and 21 days of age represented 12-, 3-, 7- and 6-fold increase respectively for maltase, sucrase, APN and AP.

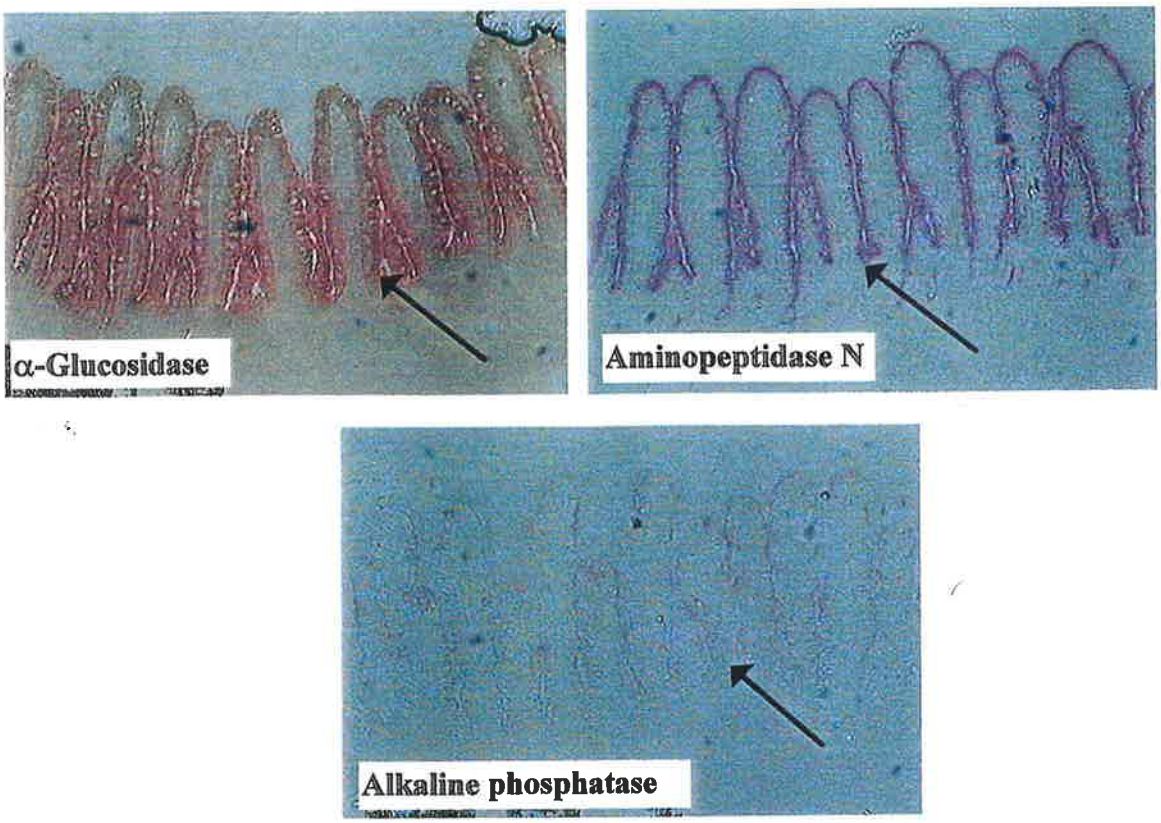


Plate 4.3.1: Localization of brush-border enzymes at the ileum at hatch.
Some enzyme activity was expressed in the crypt region (*arrowed*) although maximal activity occurred on the mid-villus. From top-left (*clock-wise*): α-glucosidase, aminopeptidase N and alkaline phosphatase.

The total activities of maltase, sucrase and APN in the ileum increased ($P<0.001$) with age. Similar changes ($P<0.01$) were observed in the total activity of AP. There was a reduction in the total activity of APN after day 14. Between hatch and day 21, there were 8-, 2-, 5- and 4-fold increases in the total activities of maltase, sucrase, APN and AP respectively.

4.3.4.3. Histochemical localization of intestinal enzymes

The typical localization of AG, APN and AP along the crypt:villus axis is shown in Plate 4.3.1. The enzymes investigated expressed activity throughout the crypt:villus

axis although quantitation was undertaken between the crypt:villus junction (CVJ) and the villus tip.

Table 4.3.4: Total villus activity of α-glucosidase (Ab. ± SD) over the entire villus and per activity per unit area (Ab./μm^2).

	Intestinal region		
Age (days)	**Duodenum**	**Jejunum**	**Ileum**
A. Total activity			
1	12.8 ± 2.24^b	9.9 ± 2.92^b	6.4 ± 0.66^b
21	35.3 ± 5.78^a	28.8 ± 6.55^a	14.6 ± 1.42^a
B. Activity per μm^2			
1	0.37 ± 0.062	0.44 ± 0.010	0.36 ± 0.048
21	0.35 ± 0.038	0.39 ± 0.044	0.39 ± 0.030

a,b - for the same variable, mean values on the same column not sharing a superscript differ significantly ($P<0.001$).

The total villus activity of AG was significantly lower ($P<0.001$) at hatch than at 21 days of age in the different intestinal regions (Table 4.3.4). There were no significant differences in the expression of AG per unit area between the age groups. Activity per unit surface area tended to be higher in the jejunum than in the duodenum or ileum at both ages but these differences were not significant.

The total villus activity of APN significantly increased with age in the three intestinal regions examined (Table 4.3.5). The differences between the ages were highly significant ($P<0.001$) in the jejunum and significant ($P<0.05$) in the duodenum and ileum. There was a slight decline in activity per unit area with age in the duodenum and ileum but not in the jejunum. Activity per unit area tended to be higher in the ileum

than in the duodenum, values for the jejunum being intermediate to the two extreme sites.

Table 4.3.5: Total villus activity of aminopeptidase N (Ab. ± SD) over the entire villus and activity per unit area (Ab./μm^2).

	Intestinal region		
Age (days)	**Duodenum**	**Jejunum**	**Ileum**
A. Total activity			
1	8.8 ± 2.04^b	5.4 ± 0.77^b	6.8 ± 1.02^b
21	21.9 ± 1.72^a	19.7 ± 3.47^a	12.8 ± 2.70^a
B. Activity per μm^2			
1	0.22 ± 0.031	0.23 ± 0.039	0.34 ± 0.040
21	0.17 ± 0.026	0.28 ± 0.053	0.30 ± 0.052

a,b - For the same variable, mean values on the same column not sharing a superscript differ significantly ($P<0.05$ in the duodenum and ileum; $P<0.001$ in the jejunum).

The total villus activity of AP in the duodenum at hatch was significantly lower ($P<0.05$) than at day 21 (Table 4.3.6). There were no significant differences between the two age groups in the jejunum or ileum. In all intestinal regions, there was a slight decline in AP activity per unit surface area with age but this was not significant. At hatch, AP activity per unit area declined distally from the duodenum to the ileum.

The total absorbance for each of the enzymes was highest in the duodenum and lowest in the ileum. Total villus activity in the jejunum was intermediate to those in the duodenum and ileum.

Table 4.3.6: Total villus activity of alkaline phosphatase (Ab. ± SD) over the entire villus and activity per unit area (Ab./μm²).

	Intestinal region		
Age (days)	Duodenum	Jejunum	Ileum
A. Total activity			
1	4.0 ± 1.76^{b}	3.2 ± 1.01	1.9 ± 0.20
21	10.1 ± 1.72^{a}	4.5 ± 0.67	3.8 ± 0.44
B. Activity per μm²			
1	0.15 ± 0.064	0.13 ± 0.052	0.10 ± 0.016
21	0.09 ± 0.014	0.06 ± 0.011	0.09 ± 0.007

a,b - For the same variable, mean values on the same column not sharing a superscript differ ($P<0.05$) significantly.

4.3.4.4. Activity profiles of enzymes on the crypt:villus axis

At hatch, both AP and APN activities were expressed up to 400 μm (44.1 % of villus height) from the CVJ in the duodenum (Figure 4.3.1). For AG, enzyme expression was observed up to 300 μm from the CVJ. Peak activity at hatch occurred at around 100 μm from the CVJ for AG and APN, and 300 μm for AP. At 21 days of age, activity was observed up to 1200 μm (68.7 % of villus) from the CVJ (Figure 4.3.1). Peak activity in the duodenum was observed at about 300 μm for AG and AP, and 500 μm for APN. At both ages, the mean activity per unit area was highest for AG and lowest for AP. The activities of APN and AP on the duodenal villi were more uniform than that observed for AG.

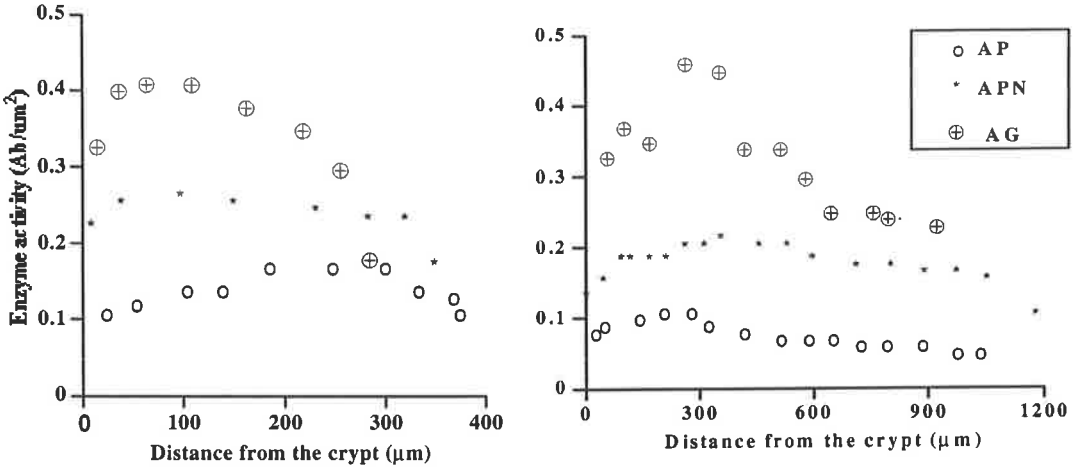


Figure 4.3.1: Enzyme activity profile (Ab./µm^2) in the duodenum at hatch (*left*) and 21 days of age. Each point represents the mean value of 5 birds. Standard errors were below 15 % of mean values.

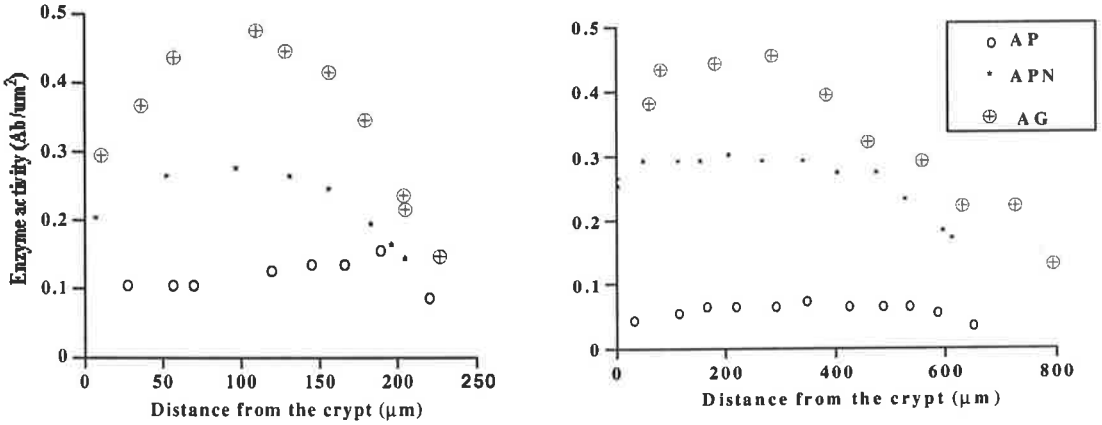


Figure 4.3.2a: Enzyme activity profile (Ab./µm^2) in the jejunum at hatch (*left*) and 21 days of age. Each point represents the mean value of 5 birds. Standard errors were below 15 % of mean values.

In the jejunum, at hatch, enzyme activity spanned the crypt:villus axis up to 250 µm (55.8 % of villus) from the CVJ (Figure 4.3.2a). Similar to the observation in the duodenum, peak AG and APN activities occurred at about 100 µm from the CVJ while AP activity peaked at 200 µm. Peak enzyme activity by day 21 occurred at 300 µm for

all three enzymes, although APN and AP tended to plateau at 200-500 and 200-600 μm from the CVJ respectively.

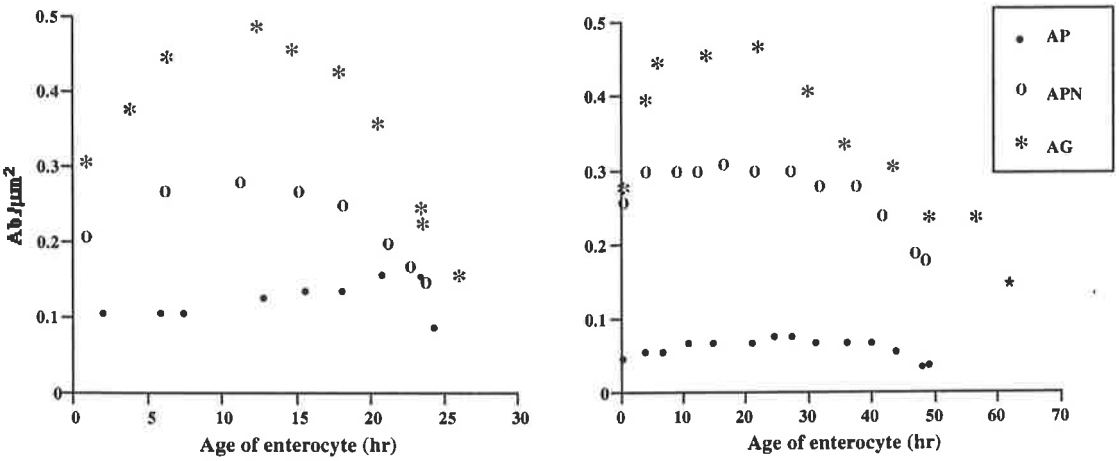


Figure 4.3.2b: Enzyme activity profile (Ab./μm²) at the jejunum relative to the average age of the enterocyte at hatch (*left*) and 21 days of age. Each point represents the mean value of 5 birds. Standard errors were below 15 % of mean values.

The enterocyte migration rates in experiment 4.2 (Table 4.2.5) were related to the enzyme profile data (Figure 4.3.2a) to obtain enzyme profile with age of enterocytes (Figure 4.3.2b). The results showed that most enterocytes were capable of secreting active enzymes within one hour of being formed at the crypt (Figure 4.3.2b). AP activity was not observed in cells less than 2 hours old in day-old chicks. The activity of AP per cell or unit surface area remained relatively uniform for much longer than the activities of AG and APN. Peak activities corresponded to cell locations that were more than 10 hours old in the case of AG and APN and more than 20 hours for AP. With all three enzymes, enterocytes possessed enzyme-secreting capabilities until they were about 25 hours old.

In 21-day-old chicks, cells began secreting active enzymes within less than one hour of formation. Peak enzyme activities for AG and APN were attained when the cells were

22 hours old and for AP at about 28 hours old. Cells continued to produce APN and AP until they were about 50 hours old while active AG secretion continued in cells that were more than 60 hours old. In both age groups (hatch and 21 days), the activity of all enzymes observed on the oldest cells was lower than that observed with the newly formed cells, close to the crypt.

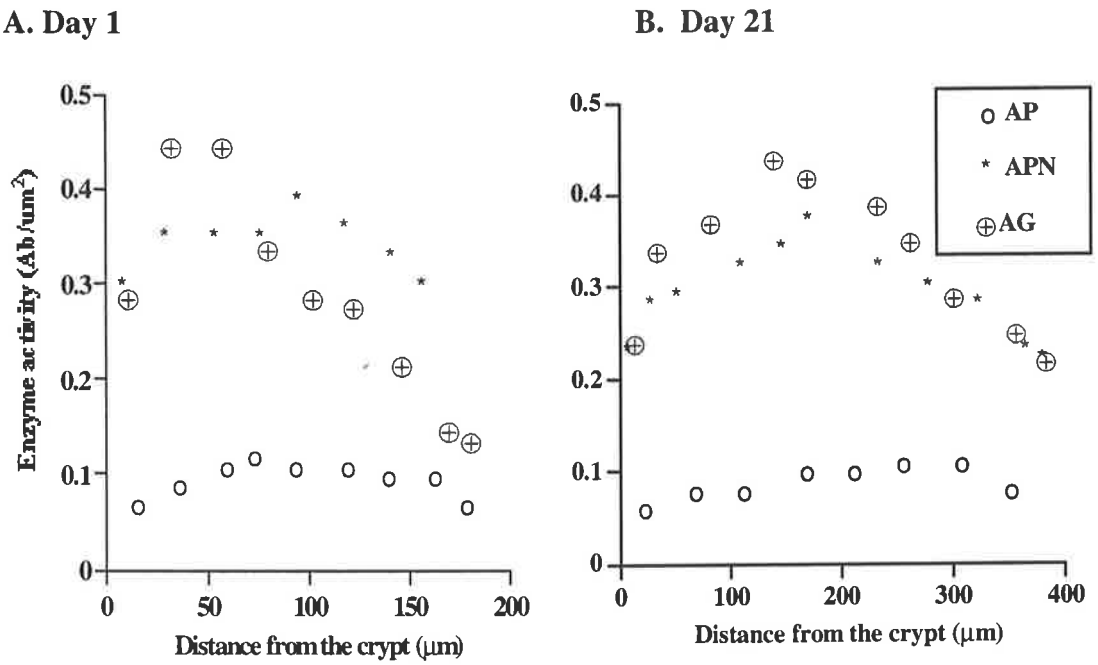


Figure 4.3.3: Enzyme activity profile (Ab./µm^2) in the ileum at hatch (*left*) and 21 days of age. Each point represents the mean value of 5 birds. Standard errors were below 15 % of mean values.

The enzyme activity profiles at the ileum are shown in Figure 4.3.3. The expression of enzyme activity was recorded up to 200 (63.3 % of villus length) and 400 µm (77.2 % of villus) from the CVJ at hatch and 21 days of age respectively. At hatch, peak activity corresponded to 50 µm for AG and about 100 µm from the CVJ for APN and AP. At 21 days of age, peak AG and APN activities occurred at 100-150 µm while peak activity for AP was at 300 µm from the CVJ. In the ileum, especially at hatch, the activities of both AG and APN rose from the CVJ to a peak on the mid-villus and then declined

towards the villus tip. AP activity per unit area was largely uniform between the CVJ and the villus tip.

In the duodenum and jejunum over the two ages examined, AG activity per unit area was the highest while AP activity was the lowest. The expression and distribution of APN at the ileum was similar in pattern to those of AG. The distance at which peak activities were expressed changed with age, as a result of the increase in villus length.

4.3.4.5. Response to substrate concentrations

The response of the two key disaccharidases, maltase and sucrase to variation in substrate concentration was examined in jejunal and ileal brush-border membrane vesicles (Figures 4.3.4 and 4.3.5). The specific activity of maltase in both the jejunum and ileum increased ($P<0.001$) as the enzyme was exposed to increasing concentrations of maltose from 31.25 to 500 mM. At all concentrations, the specific activity of maltase at hatch was higher than the activity at 21 days of age. The pattern of response was similar between the jejunum and ileum.

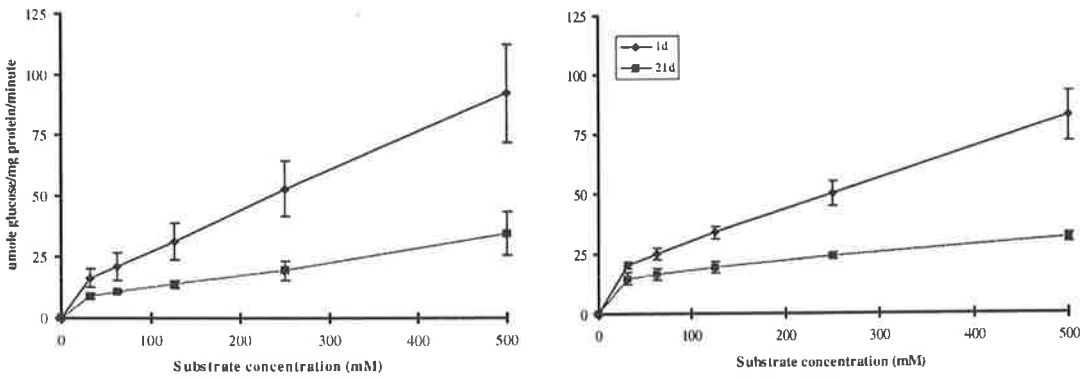


Figure 4.3.4: Response of jejunal (*left*) and ileal maltase to changes in substrate (maltose) concentration. Each point represents the mean value of 4 birds. The specific activity of maltase at the two intestinal sites increased ($P<0.001$) with increase in substrate concentration.

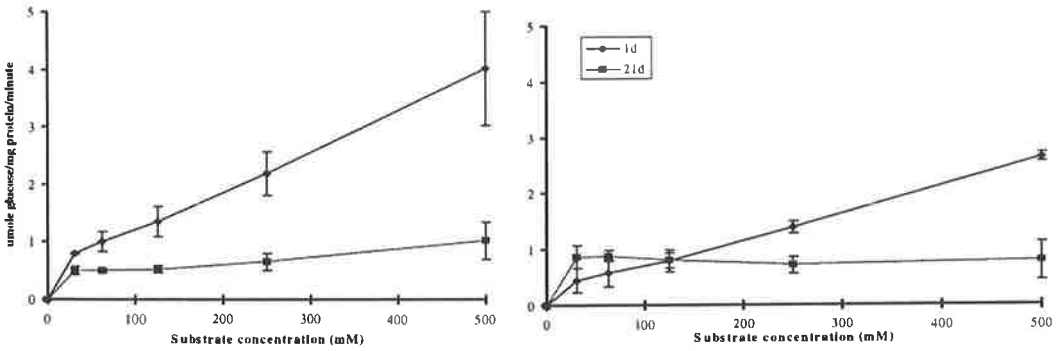


Figure 4.3.5: Response of jejunal (*left*) and ileal sucrase to changes in substrate (sucrose) concentration. Each point represents the mean value of 4 birds. In both intestinal regions, the differences in activity between substrate concentrations were significant at hatch ($P<0.001$) and in the jejunum by day 21 ($P<0.05$); there was no significant variation in ileal sucrase activity by day 21.

The specific activity of sucrase in the jejunum and ileum at hatch also responded more positively ($P<0.001$) to an increase in substrate concentration at hatch than at 21 days of age. In the ileum, at 21 days of age, sucrase activity was higher at 31.25 mM than at the other concentrations tested but this difference was not significant.

4.3.5. DISCUSSION

4.3.5.1. Relative activities of the enzymes

The generally high activity of disaccharidases, especially maltase is a reflection of the nature of the broiler chicken diet, being predominantly carbohydrate. Poultry saliva is deficient in amylase (Low and Zebrowska, 1986), pancreatic and intestinal carbohydrases therefore play a significant role in the digestion of ingested sugars.

Of the three enzymes examined histochemically, AG (maltase/isomaltase-sucrase complex) appeared to be the most highly expressed. Maltose, the main product of hydrolysis of starch by amylases is hydrolysed by sucrase-isomaltase ('sucrase') and one or two maltase-glucoamylases, these being regarded as separate enzyme systems

(Semenza and Auricchio, 1989). In many vertebrate species, the two complexes can be separated by first heat-inactivating the more heat-labile sucrase-isomaltase prior to the assessment of α-glucoamylases. In avian species, the sucrase-isomaltase complex is relatively heat-resistant and any attempt at heat inactivation also results in the loss of α-glucoamylase activity (Hu *et al.*, 1987; Biviano *et al.*, 1993). The two complexes can only be quantified accurately by subjection to specific antisera (Tsuboi *et al.*, 1979; Biviano *et al.*, 1993). In the current study, the specific activities of the two systems were separated on the assumption that maltose hydrolysis depended on maltase while sucrose hydrolysis is more directly dependent on sucrase. The activity of AG is a combination of both enzyme systems.

4.3.5.2. Variation of enzyme activities by intestinal site

The jejunum and ileum appear to be the major sites of disaccharidase activity while APN activity was highest at the ileum. Alkaline phosphatase activity was most pronounced in the duodenum, declining distally towards the ileum. A similar pattern was reported for the rat (Dupuis *et al.*, 1990) and adult White Rock cockerels (Majumdar *et al.*, 1988). In the rat, a reduction in enzyme activity at the jejunum, and probably the duodenum due to senescence may be compensated by enhanced ileal function as has been previously stated (Reville *et al.*, 1991).

Any differences between sites observed in assays on brush-border membranes would be genuine although it is probable that enzymes secreted by the upper segments of the intestine could contribute to digestion at lower sites, depending on their stability. Intestinal enzymes are usually anchored to the intestinal membrane but can be released by factors such as detergents and peptidases (Kenny and Turner, 1987). Some ecto-

enzyme complexes, for example sucrase-isomaltase, are only partly anchored by one of the sub-units although the bonding between the units may be quite strong. Such complexes function both as integral and peripheral proteins.

4.3.5.3. Variation in enzyme activities with age

The general increase in total enzyme expression with age is attributable to the increase in surface area (increased villus height) over which the enzymes are expressed, rather than an increase in the efficiency of individual cells. Total enzyme activity therefore, is highest at the duodenum, with longer villi than at the jejunum and ileum. The increase in total villus enzyme activity with age has been reported in the pig by Tivey and Smith (1989) who observed a 30 % increase in APN activity between birth and 28 days of life. Similar changes have been observed in other strains of broiler chickens and turkeys (section 2.3.2.1)

Age may influence the distribution of enzymes along the longitudinal axis of the gastrointestinal tract. In the present study, this variation was not so obvious except in the case of maltase and sucrase. The wide differences in the specific activities of these two enzymes between intestinal regions at hatch became narrow with age. Alkaline phosphatase was consistently higher in the duodenum than in the other intestinal regions. In rats, Holt *et al.* (1985) reported lower specific activities of sucrase, maltase, lactase and adenosine deaminase in the upper intestine of adult than in young rats. The total activities of the enzymes in the different regions were shown to be dependent on both specific activities per unit membrane protein in the brush-border and on the weight and length of the intestinal region. Although specific activities per unit membrane protein decreased with age, there was generally an increase in the total activities of

digestive enzymes with age in the present study. This change is mainly due to the increase in absolute weight of the intestinal tissues. In a few cases, the increase in intestinal weight or length did not adequately compensate for the drastic reduction in specific activity per unit membrane protein. When specific activities were related to intestinal weight per unit body weight, the results were similar to the pattern observed for specific activity per unit membrane protein (data not shown). These patterns would indicate an ability of the birds to digest large quantities of nutrients ingested with increase in age but digestion may progress at reduced rate, as has been observed in other species (Holt *et al.*, 1985; Sell *et al.*, 1991).

4.3.5.4. Enzyme activity along the crypt:villus axis

Histochemical examination revealed the expression of all enzymes from the crypt to a point close to the villus tip although further processing of the data with the macro tended to obscure this trend. The activity profiles obtained in the present study are similar to those reported in mammals and the sucrase profile under feed restriction in chickens observed by Apine and Sheshukova (1992). The distribution of AP in the strain studied here is similar to that reported by Long *et al.* (1986) who observed a higher activity of the enzyme towards the villus tip than at the crypt or mid-villus in another strain of broiler chickens. Lund and Smith (1987) demonstrated the appearance of sucrase, maltase and trehalase activity first in enterocytes at the base of the villi in 9-day-old rats, with activity increasing and spreading along the entire villus during the following 96 hours. Some enzyme expression at the crypt may commonly occur (Silverblatt *et al.*, 1974; Apine and Sheshukova, 1992). This is often caused by accumulation of enzymes in the crypt when the rate of synthesis surpasses the rate of degradation (Riby and Kretchner, 1984). As the animal ages, there is diminution in

enzyme synthesis rate vis-à-vis increased degradation. This further accentuates the reduction in enzyme activity towards the villus tip. In mammalian species, most activity has been reported on the mid-villus (Lund and Smith, 1987; Freeman *et al.*, 1993).

At both hatch and day 21, enzymes, especially AG and APN tended to accumulate over the first 100 μm of the crypt:villus axis even though the villi varied significantly in length. In poultry, enzyme synthesis by enterocytes closely follows the elongation of microvilli towards the crypt:villus junction (Smith *et al.*, 1990b). The subsequent distribution of enzyme activities on the crypt:villus axis would depend on the rate of enzyme synthesis, cell migration and extrusion as has been demonstrated for sucrase (Riby and Kretchmer, 1984). The rate of cell migration at 21 days of age was higher than that at hatch (see Table 4.2.5). It is therefore possible that enterocytes with similar enzyme potentials could be found within the same region of the villus, especially near the crypt in the young and old chicks.

At the same animal age, enzyme activity is expressed over a larger proportion of the villus in the order of ileum > jejunum > duodenum. The low proportion in the duodenum may, however, be compensated for by the longer villi in the region. These regional differences may be underlined by variation in length of villi over which enterocytes migrate before they become too senescent to secrete digestive enzymes, assuming that cell migration proceeds at the same rate in the duodenum and ileum as in jejunum.

Relating enzyme activities to the age of enterocytes, as was done with data from the jejunum showed an increase in enzyme activities as the enterocytes migrated out of the

region of proliferation and enzyme synthesis. Most enzymes are secreted as inactive precursors at the crypt and acquire activity only as enterocytes progress up the villus, with age (King *et al.*, 1983; Lund and Smith, 1987). Ultimately, peak activity is attained, which in mammalian species, coincides with enterocytes on the mid-villus (Freeman *et al.*, 1993). A similar profile is seen in the poultry, as typified by the Steggles x Ross in the present study, with slight variations for each enzyme. Enterocytes on longer villi from older birds continued to secrete enzymes for much longer than was observed in the shorter villi of young birds. This is due to an increased enterocyte life-span, as was found in experiment 4.2. There is, however, a greater reduction in enzyme activity in the older enterocytes towards the villus tip at day 21 than when chickens were assessed at hatch.

4.3.5.5. Enzyme function and body growth

The natural pattern of enzyme development and function may partly explain the changes in feed conversion efficiency and growth in broiler chickens, with age. Feed conversion efficiency and growth rate usually fall with age, even when feed intake is stable or increased. It may still be possible to improve the efficiency of feed utilization if digestive enzyme function could be increased. Pancreatic enzyme activities are generally similar between egg-type and broiler chicks but intestinal enzyme activity in the latter are usually lower, especially with age (Nir *et al.*, 1993; Mahagna and Nir, 1996). This is regarded as a constraint to feed intake, digestion and growth. The pattern of response of maltase and sucrase to substrate concentration observed in the present study tends to suggest some potential for further enhancement of feed utilization efficiency. It is not certain what the pattern of response would be in the whole animal. In the presence of exogenous enzymes, there is a disproportionately large difference

between the dietary concentration of the enzyme supplement and body growth (Marquardt *et al.*, 1996).

The natural pattern of development in digestive function may also be regulated by factors present in the diet. In the rat, Wiesenfeld *et al.* (1993) observed increases in maltase activity in the presence of sucrose and AP activity in the presence of starch. The influence of some dietary factors are further examined in the next chapter.

4.3.6. CONCLUSION

There is a general reduction in the specific activities of intestinal enzymes with age in broiler chickens. The increase in length of the gastrointestinal tract and surface area of the mucosa with age may, however, compensate for the loss in activity per cell or per unit of intestinal mucosal surface. With age, villi are longer and sustain higher total activities than younger and shorter villi. Enzyme activities are also demonstrated on enterocytes for much longer in older birds as a result of the increase in villus length and enterocyte life-span.

The localization of the intestinal enzymes, both through the gastrointestinal tract and the crypt:villus axis is similar in many respects to patterns observed in mammalian species. Some differences in enzyme activities also exist between intestinal sites and this may influence the pattern of response to anti-nutritive factors when present in the diet.

4.4.0. EXPERIMENT 4.4: DEVELOPMENT OF AMINO ACID TRANSPORT AND CHARACTERISTICS OF THE TRYPTOPHAN TRANSPORT SYSTEM(S).

4.4.1. INTRODUCTION

Absorption is one of the final processes involved in the derivation of nutrients from the diet at the intestinal level. The transport of a nutrient across the enterocyte involves uptake across the brush-border membrane; diffusion through the cytoplasm, and exit across the basolateral membrane into the portal circulation (Stevens *et al.*, 1984). Apart from the cellular membranes, nutrient transport or absorption encounters a water layer and a mucous/glycocalyx layer which together form the unstirred compartment that overlies the enterocyte (Stevens, 1992). Each of these barriers influences absorption to determine the nutrient supply to the animal.

The dissimilar nature of the brush-border and basolateral membranes presents additional complexity to the absorption of nutrients from the intestine (Ganapathy *et al.*, 1994). Nutrient absorption is accomplished by two key mechanisms - diffusion and facilitated transport. Facilitated transport relies on ions and transporters and is facilitated by energy expenditure. Diffusion occurs mainly in response to variation in concentration gradient and may be facilitated by a membrane protein if the membrane is not permeable to the substrate, as is the lipid bi-layer present in many animal membranes (Alberts *et al.*, 1994). The relative importance of the different mechanisms depends on the concentration of the substrate in the intestinal lumen. Immediately after digestion of proteins, for example, the concentration of individual amino acids in the lumen would range from 1 to 25 mM (Stevens *et al.*, 1984). Under such conditions, net intake via

simple diffusion may exceed the rate of mediated transport. As the concentration of the amino acids is reduced, facilitated transport assumes increasing importance until diffusion ceases to be of any significance.

Nutrient transporters are membrane-bound proteins and operate under varying environmental conditions as was previously reviewed in section 2.4.2.2 (Stevens *et al.*, 1982; Stevens *et al.*, 1984; McGivan and Pastor-Anglada, 1994). Many amino acid transporters in the brush-border membrane of the intestine are unique to the intestine, unlike the basolateral membrane transporters which are similar to those found on other plasma membranes (Stevens, 1992). Transporters may be specific to nutrients or may regulate the absorption of several nutrients. The most common transport systems involved in amino acid transport are highlighted in Table 2.1 (section 2.4.2.2). When more than one nutrient is co-transported, the efficiency of transport would depend on the chemical relationships between the different nutrients and between the nutrients and the transporter. Nutrients relate to one another in a variety of ways, including inhibition and stimulation.

Amino acids are some of the most important dietary components and those for which transporters are required. Unlike carbohydrates, proteins are digested into several absorbable amino acids and peptides which vary enormously in chemical structure. Multiple transport systems are therefore required in the transport of proteins from the intestinal lumen (Ganapathy *et al.*, 1994). The degree of importance of the different amino acids has been highlighted in section 2.4.2.1. Several studies have established the requirements for the essential and limiting amino acids and determined the rate of their absorption from the intestinal tract in poultry (Han *et al.*, 1991; NRC, 1994).

Research has also been conducted into the mechanisms involved with the transport of methionine and lysine as well as some non-essential amino acids in chickens (Knight and Dibner, 1984; Brachet *et al.*, 1987; Torras-Llort *et al.*, 1998; Noy and Sklan, 1996; Planas *et al.*, 1997). There is, however, a dearth of research reports on the mechanisms that regulate tryptophan transport in poultry. In other species, L-tryptophan uptake occurs predominantly by the Na^+-independent systems L and T (Lopez-Burillo *et al.*, 1985; Jara *et al.*, 1990; Pankovich and Jimbow, 1991). Tryptophan is required in the synthesis of various proteins and is a major precursor of the vitamin, niacin (nicotinamide) which is involved in hydrogen transfer as a component of the coenzymes, nicotinamide-adenine dinucleotide and nicotinamide-adenine dinucleotide phosphate (Bender and Bender, 1986; McDonald *et al.*, 1988; Sashidhar *et al.*, 1988).

In series of tests, this experiment examined the uptake of L-tryptophan by brush-border membrane vesicles of chicks at various ages. The impact of other substrates and ions was also assessed. The primary objective was to provide initial characterizations for tryptophan transport. Some assays were conducted to identify the preferred routes of transport for the amino acid in broiler chicks. In experiments in the next chapter, the impact of some dietary factors on the uptake of tryptophan by the brush-border membrane was assessed.

4.4.2. MATERIALS AND METHODS

4.4.2.1. Animals and diets

Thirty-two mixed-sex day-old Steggles x Ross (F_1) broiler chicks were used for the study. The chicks weighed 42.4 ± 3.63 g from the hatchery and were reared together in a battery brooder for 14 days. On day 14, the chicks were moved into cages in batches

of 4 per cage. The chicks were maintained on a commercial broiler starter diet and rearing conditions similar to those used for experiment 4.1.

Eight chicks were sampled at hatch and every 7^{th} day until 21 days of age. Chicks were slaughtered and dissected to obtain intestinal tissues as described in section 3.4.0. Intestinal sub-samples, about 5 cm long were taken from the proximal ends of the jejunum and the ileum and flushed with PBS (pH 7.4). The tissues were slit longitudinally, snap-frozen in liquid nitrogen and stored at -80°C until they were used the following day for preparation of brush-border membrane vesicles.

4.4.2.2. Extraction of brush-border membrane vesicles

Brush-border membrane vesicles (BBMV) were prepared in line with the method described by Shirazi-Beechey *et al.* (1991) outlined in section 3.7.0. The specific activities of maltase, alkaline phosphatase and Mg^{2+}-ATPase (EC. 3.6.1.3) were measured in vesicles and related to activities in the homogenates to test the purity of the vesicles. Maltase and AP are preferentially bound to the brush-border membrane while Mg^{2+}-ATPase is localized to the basolateral membrane (Sachs *et al.*, 1980).

The procedures adopted for assessing the activities of maltase and AP are presented in 3.7.1. The activity of Mg^{2+}-ATPase was assessed according to the method of Qin *et al.* (1993). The samples (25 µL) were pre-incubated at 39°C for 5 minutes in 875 µL of a buffer containing 30 mM Tris and 7.5 mM $MgCl_2$, pH 7.6. Incubation was continued for 30 minutes after the addition of 100 µL of 50 mM ATP. The reaction was terminated with 100 µL 40 % ice-cold TCA. The mixture was centrifuged at 2500 g for

5 minutes at 4°C. The colour of the initial reaction product was developed over 20 minutes at room temperature by further incubating 0.5 ml of the supernatant with 2 ml of distilled water, 0.125 ml of Fiske and Subbarrow solution (1 g in 6.3 ml distilled water) and 0.5 ml of acid molybdate solution. The absorbance was read at 660 nm in cuvettes with a 1 cm light path, using a UV-120-0 spectrophotometer. Activity was determined as the amount of inorganic phosphorus released by the membrane-bound enzyme and related to a standard consisting of inorganic phosphorus (20 μg/ml). All chemicals were of analytical grade obtained from Sigma Aus. Pty Ltd.

4.4.2.3. Amino acid uptake into brush-border membrane vesicles

The uptake of L-tryptophan by brush-border membrane vesicles was assessed according to the rapid filtration technique (Wolfram *et al.*, 1984). The details of the procedure for routine assays are provided in section 3.8.1. The procedure was modified to suit trials conducted in this experiment and these are described below and in legends to the individual tables and figures.

Assays were conducted to characterize the system(s) involved in the uptake and transport of L-tryptophan from the small intestine. Some of the assays were conducted simultaneously on BBMV from both the jejunum and ileum although this was not always possible due to limitations in number of samples available, in relation to the number of assays. Each sample was run in duplicate to obtain a mean value for the replicate.

4.4.2.4. Buffers

BBMV were re-suspended in a storage buffer containing 300 mM mannitol, 20 mM HEPES/Tris, 0.1 mM $MgSO_4$ and 0.02 % NaN_3 (pH 7.4) with an osmolality of 341 mosm/kg. The incubation buffers used in assays in this experiment were constituted to be iso-osmotic with the storage buffer. The chemical composition and concentrations of the incubation buffers used in each case are indicated as legend to the results but predominantly consisted of mannitol, HEPES, unlabelled and labelled amino acid. Effectors were added as required and the osmolarity adjusted by varying the content of mannitol. The incubation buffers were labelled with ^{3}H-L-tryptophan at 74.1 kBq/ml of buffer.

A stop buffer consisting of 177.2 mM NaCl and 15 mM Tris/HCl with an osmolality of 357 mosm/kg was used to terminate incubations and rinse the filter. All buffers had a pH of 7.4, similar to the buffer in which the vesicles had been re-suspended.

Prior to use, BBMV were thawed and held on ice. The incubation buffers were stored at room temperature while the stop buffer was maintained on ice at 4°C. All assays were conducted at room temperature over 10 s, determined as the optimum duration of incubation (3.8.2).

4.4.2.5. Uptake and transport of L-tryptophan at different ages and intestinal sites.

The uptake and transport of L-tryptophan was tested at similar concentration, 0.04 mM in vesicles extracted from chicks at hatch and 7, 14 and 21 days of age. At all ages,

uptake was studied in vesicles from both the jejunum and ileum which together constitute 82.2-84.1 % of the small intestine, in terms of length. Uptake was studied in the absence or presence of 50 mM NaCl.

4.4.2.6. Effect of membrane potential and regulation by chloride and nitrate

The impact of various ions on the uptake of L-tryptophan was examined. Principally, sodium-dependent transport was assessed. Transport was also measured when potassium replaced sodium equimolarly in the external medium. The significance of membrane potential was tested by inclusion of valinomycin to facilitate K^+ flux in and out of the vesicles. Two sodium salts, NaCl and $NaNO_3$ at two concentrations were included in the uptake buffer to determine if the anions, rather than Na^+ *per se* were involved in regulating uptake.

4.4.2.7. Interactions with other amino acids and substrates

To test if L-tryptophan uptake was regulated by other amino acids in mixed media as would commonly occur in the digesta, uptake was conducted in the presence of similar or higher concentrations of D-tryptophan, L-lysine, L-methionine or L-alanine. These amino acids are transported by various routes and their impact on L-tryptophan uptake may suggest the existence or absence of shared transport systems. The relative importance of systems L and T by which L-tryptophan has been observed to transit in other species was specifically tested, using their preferred substrates, D-tryptophan for system T and 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH) for system L, both Na^+-independent.

4.4.3. Data analysis

Data were generally expressed as concentration of amino acid taken up by the vesicles in relation to protein content of the incubated vesicles per unit of time. For age studies, the uptake was related to the absolute and relative (to 100 g body weight) weights of intestinal region. The former provides an idea of the capacity of the intestinal region while the latter relates more to the efficiency of the intestinal region. The average weights of the various intestinal regions established in experiment 1 of this chapter (section 4.1.3.2) were the values used to determine uptake capacity and efficiency in this experiment. The results obtained from other assays were expressed simply in relation to mg protein per time. Although assays were conducted over 10 s, the results were extrapolated to one minute.

4.4.4. RESULTS

4.4.4.1. Chemical properties of the brush-border membrane

The protein contents of the homogenates and vesicles are shown in Table 4.4.1. The protein content of the jejunal mucosa decreased ($P<0.01$) with age while the content in the ileum was relatively stable. There were also significant differences with age in protein contents of the BBMV from the jejunum ($P<0.01$) and ileum ($P<0.001$). Protein recovery rate at both sites increased ($P<0.001$) with age and ranged from 2.5 to 5.9 %.

Enzyme activities were measured in both the homogenates and vesicles and were related to the protein concentration per unit weight of tissue. The specific activities of the brush-border membrane-bound enzymes, maltase (Table 4.4.2) and alkaline phosphatase (Table 4.4.3) significantly declined ($P<0.001$) with age. Activities were generally

higher in the vesicles than in the homogenates. For maltase, the maximum enrichment was 6.5-fold while the AP enrichment was up to 8.7-fold.

Table 4.4.1: Protein content of mucosal homogenate and brush-border membrane vesicles.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Homogenate protein (mg/g tissue)					
Jejunum	38.6^{a}	31.2^{ab}	31.3^{ab}	28.2^{b}	2.75**
Ileum	29.4	38.1	36.7	33.6	3.93
Vesicle protein (mg/g tissue)					
Jejunum	1.0^{b}	1.5^{a}	0.9^{b}	1.5^{a}	0.19**
Ileum	0.7^{b}	1.8^{a}	1.8^{a}	2.0^{a}	0.24***
Protein recovery (%)[1]					
Jejunum	2.5^{c}	4.7^{ab}	2.8^{bc}	5.4^{a}	0.55***
Ileum	2.5^{b}	4.7^{a}	4.9^{a}	5.9^{a}	0.48***

1. Protein content of vesicles relative to protein content of homogenates per unit weight of tissue. a,b,c - Mean values on the same row with unlike superscripts are significantly different (**P<0.01; ***P<0.001).

Table 4.4.2: Combined maltase activity (μmole glucose/mg protein/minute) in mucosal homogenate and brush-border membrane vesicles.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Homogenate					
Jejunum	9.5^{a}	5.5^{b}	2.8^{c}	3.3^{bc}	0.75
Ileum	15.5^{a}	7.3^{b}	2.0^{b}	4.5^{b}	1.61
Vesicles					
Jejunum	$36.1\ (3.8)^{a}$	$10.8\ (2.0)^{b}$	$16.1\ (5.8)^{b}$	$9.3\ (2.8)^{b}$	2.56
Ileum	$58.2\ (3.8)^{a}$	$13.6\ (1.9)^{b}$	$13.0\ (6.5)^{b}$	$11.5\ (2.6)^{b}$	0.41

Values in parentheses indicate enrichment; i.e. enzyme activity in the vesicles relative to the activity in homogenate. a,b,c - Mean values on the same row with unlike superscripts are significantly different (P<0.001).

A further purity check on the BBMV extract was conducted by assessing the activity of the baso-lateral membrane-bound Mg^{2+}-ATPase in the vesicles relative to homogenates (Table 4.4.4). At hatch, the activity of Mg^{2+}-ATPase in the BBMV was similar to that in the homogenates but the activity in the homogenates at subsequent ages was higher than activity in vesicles. The maximum de-enrichment obtained was up to 15-fold. In both the homogenates and vesicles, the specific activity of Mg^{2+}-ATPase fell with age. There were significant differences ($P<0.01$) between the age groups with regards to the specific activity of Mg^{2+}-ATPase in jejunal homogenates and ileal vesicles. The specific activity of the enzyme in jejunal vesicles also varied ($P<0.001$) with age.

Table 4.4.3. Alkaline phosphatase (μmole nitrophenol/mg protein/minute) in mucosal homogenate and brush-border membrane vesicles.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Homogenate					
Jejunum	1.3^{a}	0.3^{b}	0.6^{b}	0.6^{b}	0.12***
Ileum	1.6^{a}	0.1^{b}	0.1^{b}	0.1^{b}	0.26***
Vesicles					
Jejunum	4.9 $(3.8)^{a}$	2.6 $(8.7)^{c}$	4.4 $(7.3)^{ab}$	3.1 $(5.2)^{bc}$	0.82*
Ileum	2.2 $(1.4)^{a}$	0.6 $(6.0)^{b}$	0.7 $(7.0)^{b}$	0.5 $(5.0)^{b}$	0.19***

Values in parentheses indicate enrichment; i.e. enzyme activity in the vesicles relative to the activity in homogenate. a,b,c - Mean values on the same row with unlike superscripts are significantly different (*P<0.05; ***P<0.001).

4.4.4.2. Variation in uptake with age and intestinal site

In BBMV from both the jejunum and ileum, there was a significant ($P<0.001$) reduction in the rate of uptake of 0.04 mM L-tryptophan with age (Figure 4.4.1). Uptake was enhanced by the presence of 50 mM NaCl but there was no significant effect of the ions at any age. In the jejunum, the contribution of Na^{+}-dependent uptake to total uptake was

13.0, 27.1, 6.1 and 12.0 % at hatch, and 7, 14 and 21 days of age respectively. In the ileum, Na^+-dependent uptake represented 15.8, 16.7, 13.2 and 11.1 % of the total uptake over the same period. At the same age and irrespective of the presence of Na^+, uptake by ileal BBMV was significantly higher (P<0.001) than uptake by jejunal BBMV.

Table 4.4.4. Mg^{2+}-stimulated ATPase (μmole Pi/mg protein/minute) in mucosal homogenate and brush-border membrane vesicles.

	Age (days)				
	1	**7**	**14**	**21**	**SED**
Homogenate					
Jejunum	50.1^b	91.5^a	42.5^b	60.5^b	8.85**
Ileum	75.5	90.9	91.9	91.0	10.31
Vesicles					
Jejunum	$43.3\ (0.86)^a$	$10.1\ (0.11)^b$	$11.4\ (0.26)^b$	$5.9\ (0.10)^b$	1.80***
Ileum	$69.3\ (0.92)^a$	$12.4\ (0.14)^b$	$9.9\ (0.11)^b$	$5.9\ (0.07)^b$	1.34***

Values in parentheses indicate de-enrichment; i.e. enzyme activity in the vesicles relative to the activity in homogenates. a,b,c - Mean values on the same row with unlike superscripts are significantly different (**P<0.01; ***P<0.001).

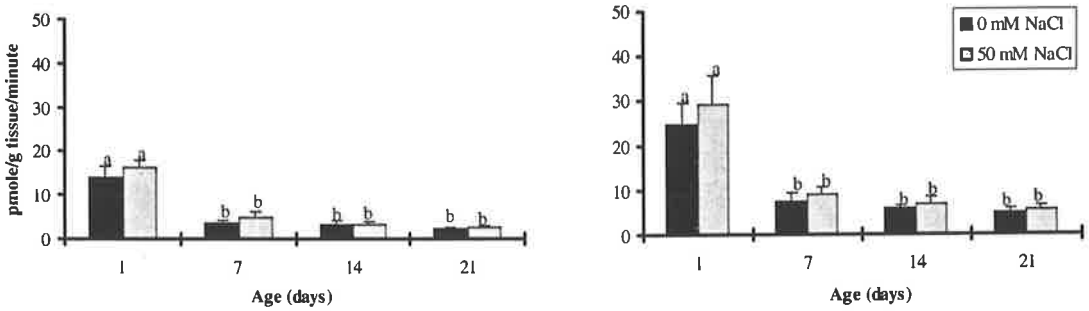


Figure 4.4.1: Effect of age and NaCl on the uptake of L-tryptophan into jejunal (*left*) and ileal BBMV. a,b - Mean values with unlike superscripts are significantly different (P<0.001). The incubation buffers contained 20 mM HEPES, and 0.04 mM unlabelled L-tryptophan, without or with 50 mM NaCl. Mannitol (200.1-300.1 mM) was added to maintain iso-osmolarity. Each bar is the mean value from 4 chicks at each age.

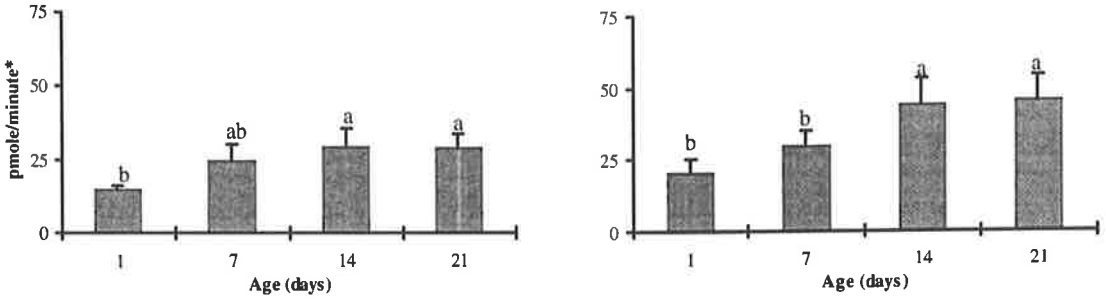


Figure 4.4.2: Capacity of Na^+-dependent and independent uptake of L-tryptophan into jejunal (*left*) and ileal BBMV at different ages. a,b - Mean values with unlike superscripts are significantly different (P<0.01). *Uptake over the entire region; data from Figure 4.4.1 were related to absolute weight of the intestinal region at each age.

The intestinal capacity for uptake was derived by relating the uptake data presented in Figure 4.4.1 to total weight of the two intestinal segments, jejunum and ileum established in experiment 4.1. The data used were those of uptake in the presence of Na^+, to reflect both Na^+-dependent and independent uptake. At both intestinal sites, uptake capacity increased (P<0.01) with age (Figure 4.4.2). The ileum also showed a greater capacity for uptake than the jejunum at all ages considered.

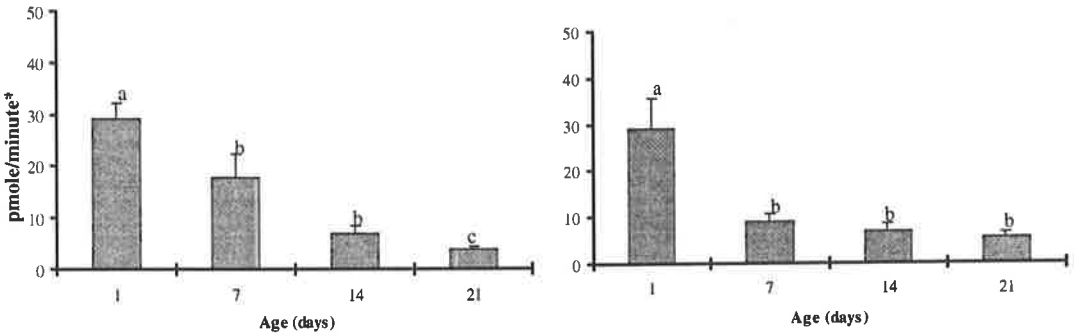


Figure 4.4.3: Efficiency of Na^+-dependent and independent uptake of L-tryptophan into jejunal (*left*) and ileal BBMV at different ages. a,b,c - Mean values with unlike superscripts are significantly different (P<0.001). *Data from the uptake presented in Figure 4.4.1 were related to relative (to body weight) weight of the intestinal region.

Data on total uptake (Na^+-dependent and independent) were further related to the weight of the intestinal segment vis-à-vis body weight (experiment 4.1). This effectively

related uptake to body weight and could appropriately be termed the 'efficiency' of uptake. Intestinal weight per unit body weight varied less with age than did the absolute weight of the intestine or its regions (section 4.1.3.2). Results of this analysis showed a reduction ($P<0.001$) in uptake with age in both the jejunum and ileum (Figure 4.4.3) . The efficiency of uptake at the jejunum was similar to that at the ileum, except at 7 days of age.

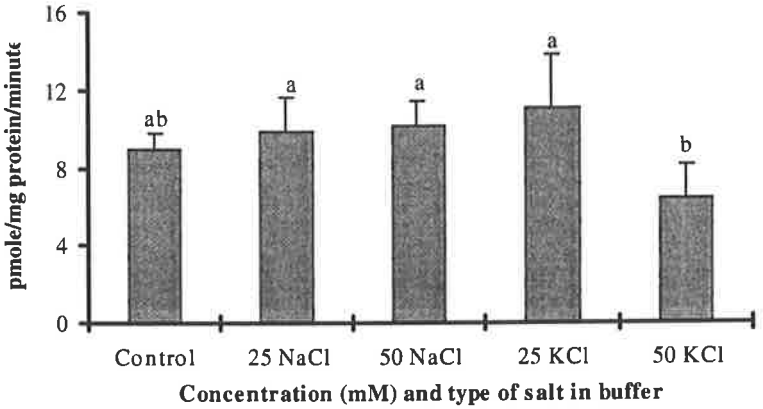


Figure 4.4.4: The regulation of L-tryptophan uptake by sodium and potassium ions in the external medium. a,b - Mean values with unlike superscripts are significantly different ($P<0.05$). Valinomycin was not included in the buffers so as to confine potassium to the external medium. The incubation buffer contained 20 mM HEPES, 0.04 mM unlabelled L-tryptophan and the level of salts indicated. Mannitol was used to adjust the osmolarity. Each mean value is the average of data from the ileum of four 7-day old chicks.

4.4.4.3. The regulation of uptake by various ions

The inclusion of either NaCl or KCl in the external medium influenced the rate of uptake of L-tryptophan when the amino acid was present at a concentration of 0.04 mM in the incubation buffer (Figure 4.4.4). At a concentration of 25 mM, NaCl and KCl increased uptake by 10.0 and 22.2 % ($P<0.05$) respectively. NaCl at a concentration of 50 mM stimulated uptake by 13.3 %. Relative to the control, 50 mM KCl in the external medium reduced uptake by 28.7 % but this was not significant but compared

with the other buffers containing cations, KCl at a concentration of 50 mM significantly reduced ($P<0.05$) the uptake of the amino acid.

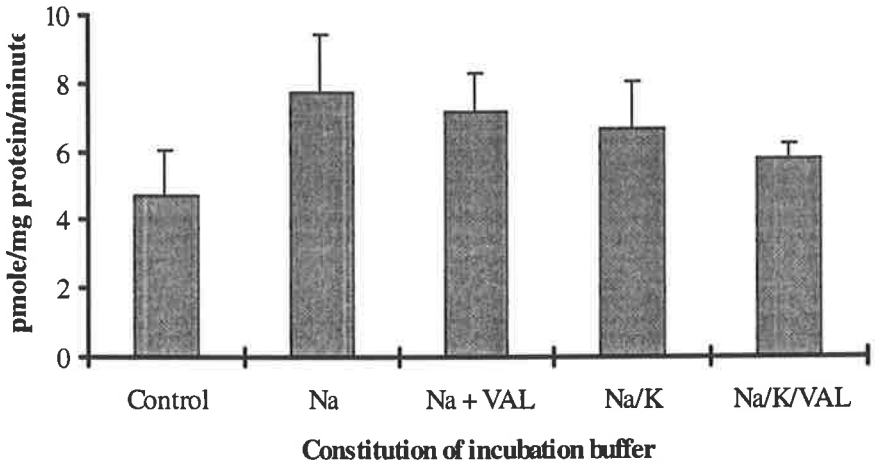


Figure 4.4.5: The regulation of L-tryptophan uptake by sodium and potassium ions in the presence of valinomycin. The incubation conditions were similar to those stated in Figure 4.4.2. Valinomycin (VAL), at a concentration of 2.5 μM was included in the buffer with 25 mM NaCl without or with 25 mM KCl. Jejunal vesicles from four 7-day old chicks were used to obtain the mean value represented by each bar. An equal amount of 95 % ethanol was included in all buffers, including the control without the two salts.

Jejunal vesicles from birds of the same age were used to assess the effect of valinomycin, the potassium-regulating ionophore on uptake in the presence of both Na and K salts (Figure 4.4.5). Valinomycin was re-constituted in 95 % ethanol and included in the incubation buffers at a concentration of 2.5 μM. NaCl at a concentration of 25 mM stimulated uptake by 63.8 and 53.2 % respectively in the absence or presence of valinomycin. In the presence of both NaCl and KCl and in the absence of valinomycin, uptake was increased by 42.6 %. The rate of stimulation by the two salts was only 23.4 % when valinomycin was included in the buffer.

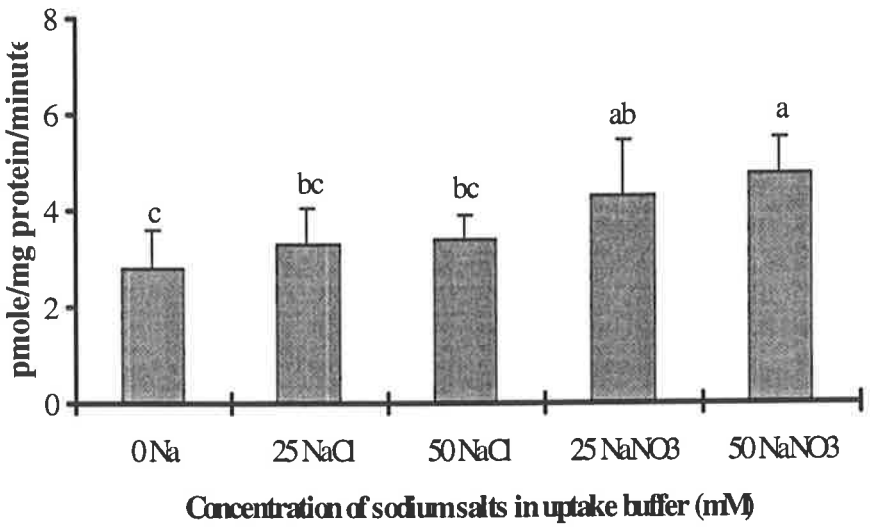


Figure 4.4.6: Uptake of L-tryptophan in the presence of varying concentrations of different sodium salts. a,b - Mean values with uncommon superscripts are significantly different ($P<0.05$). Jejunal vesicles from 4 chicks, 21 days of age were incubated under each condition. The incubation buffers contained 20 mM HEPES in addition to the salts indicated. Mannitol was included to maintain iso-osmolarity.

In 21-day old chicks, $NaNO_3$ significantly ($P<0.05$) stimulated the uptake of 0.04 mM L-tryptophan by jejunal vesicles (Figure 4.4.6). At 25 and 50 mM, $NaNO_3$ increased uptake by 53.6 and 71.4 % respectively. NaCl, at these concentrations also stimulated uptake, by 17.9 and 21.4 % respectively. The ion-dependent component of total uptake with the salts were respectively 15.2, 17.6, 34.9 and 41.7 % for 25 mM NaCl, 50 mM NaCl, 25 mM $NaNO_3$ and 50 mM $NaNO_3$.

4.4.4.4. Interactions with other substrates

The Na^+-independent uptake of L-tryptophan into jejunal vesicles of 21-day chicks was partially inhibited in the presence of 0.391-6.25 mM D-tryptophan, a potent inhibitor of system T in the incubation medium (Figure 4.4.7a). At the highest concentration of D-tryptophan tested, L-tryptophan uptake was reduced by 34.3 %. When both isomers were present in the buffer at similar concentrations between 0.01 and 0.08 mM, however, the D-isomer tended to stimulate the uptake of the L-isomer (Figure 4.4.7b).

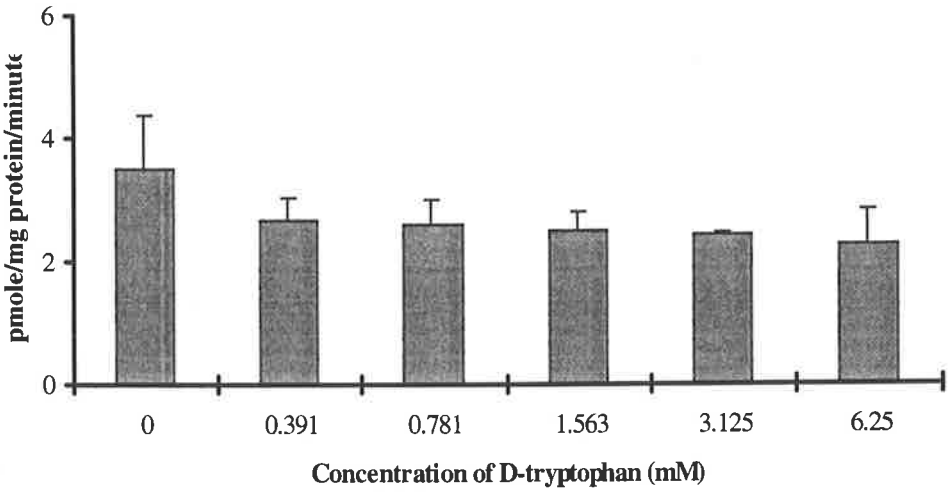


Figure 4.4.7a: Uptake of L-tryptophan in the presence of varying concentrations of D-tryptophan. Assays were conducted on jejunal vesicles from 3 chicks, 21 days of age. The incubation buffers contained 20 mM HEPES, 0.04 mM unlabelled L-tryptophan and D-tryptophan (0.391-6.25 mM). Mannitol was added to maintain iso-osmolarity.

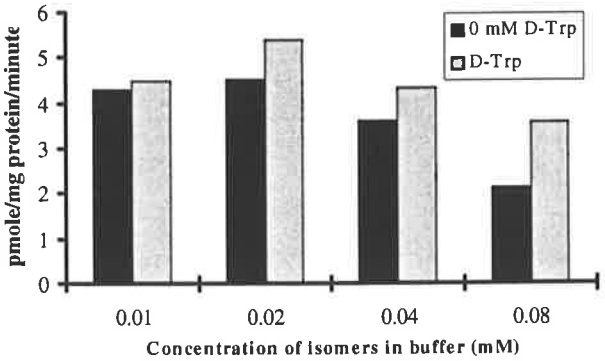


Figure 4.4.7b: Uptake of L-tryptophan at the similar concentrations of D-tryptophan. Assays were conducted on jejunal vesicles from 2 chicks, 21 days of age. The incubation buffers contained 20 mM HEPES, and 0.01-0.08 mM unlabelled L-tryptophan without or with similar levels of D-tryptophan. Mannitol was added to maintain iso-osmolarity.

The inclusion of BCH in the incubation medium significantly ($P<0.05$) enhanced the uptake of 0.04 mM L-tryptophan by jejunal BBMV of 21-day old chicks (Figure 4.4.8). Uptake was maximum at a BCH concentration of 1.563 mM and declined slightly at subsequent concentrations.

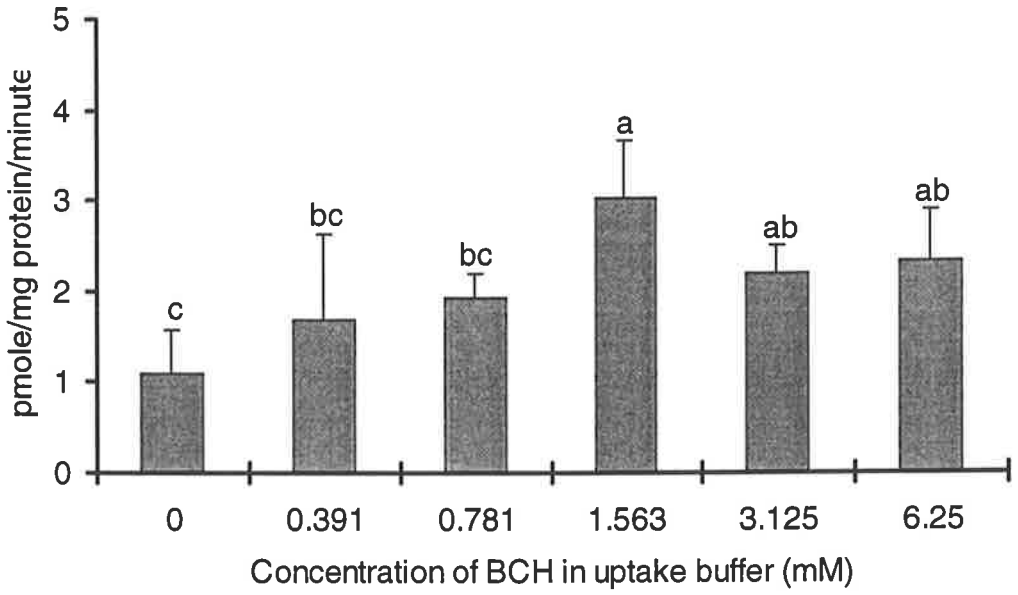


Figure 4.4.8: Uptake of L-tryptophan in the presence of varying concentrations of BCH. a,b,c - Mean values with unlike superscripts are significantly different ($P<0.05$). Assays were conducted on jejunal vesicles from 3 chicks, 21 days of age. The incubation buffers contained 20 mM HEPES, 0.04 mM unlabelled L-tryptophan and mannitol (293.1-300.06 mM).

At similar concentration (0.04 mM) to L-tryptophan, lysine, methionine and alanine in the presence of Na^+ stimulated the uptake of L-tryptophan by 39.3, 37.1 and 16.9 % respectively (Figure 4.4.9). At a higher concentration, 25 mM, these amino acids inhibited L-tryptophan uptake by 15.7, 15.7 and 6.7 % respectively, relative to the control flux. The Na^+-independent uptake of L-tryptophan under the influence of these amino acids was not determined.

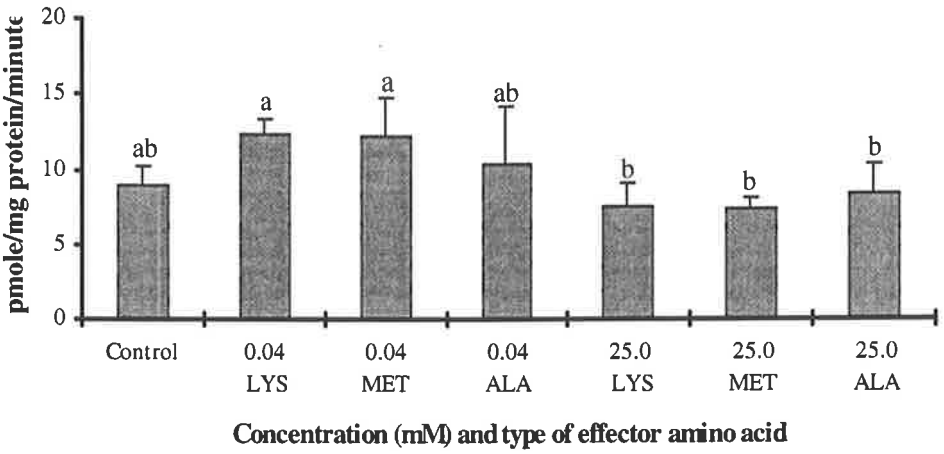


Figure 4.4.9: Uptake of L-tryptophan in the presence of varying concentrations of other amino acids. a,b - Mean values with uncommon superscripts are significantly different ($P<0.05$). Each mean value was derived from data (jejunal BBMV) of 3 chicks, 14 days of age. The incubation buffers contained 20 mM HEPES, and 0.04 mM unlabelled L-tryptophan, the stated concentrations of the effector amino acids and 50 mM NaCl. Mannitol (169.2-220.0 mM) was added to maintain iso-osmolarity.

4.4.5. DISCUSSION

4.4.5.1. Purity of membrane vesicles

The enrichment obtained for the brush-border membrane-bound enzymes is lower than values reported elsewhere for the same procedure used in the extraction of vesicles (Shirazi-Beechey *et al.*, 1991). The differences may, however, be of species origin. The low activity of Mg^{2+}-ATPase in the vesicles would be adequate confirmation of the purity of the vesicles prepared in the present study.

The trend of variation in the specific activities of maltase and AP has been examined in experiment 4.3. Both enzymes are lower in the homogenates at all ages and tend to decline in specific activities with age. The specific activity of Mg^{2+}-ATPase was particularly high in BBMV at hatch and did not indicate the trend in de-enrichment of the enzyme observed at other ages. The procedures adopted for the preparation of BBMV from samples at all ages were identical and unlikely to produce the wide

difference in the trend of Mg^{2+}-ATPase compared to other enzymes. The brush-border membrane is relatively under-developed in both mammalian and avian neonates (Smith *et al.*, 1984; Smith *et al.*, 1990b). Microvilli elongate as the enterocytes migrate out of the crypt and attain maximum length some distance away from the crypt:villus junction. The activities of membrane-bound enzymes increase during the course of microvillus elongation, prior to the acquisition of transport capacity (Smith *et al.*, 1984). It is not known if at any point during these developments there is a similarity between the apical and the basolateral membranes in the distribution of membrane-bound enzymes.

The exact functions of Mg^{2+}-ATPase have not been unravelled. Its close association with Ca^{2+}-ATPase may, however, suggest an involvement in calcium transport (Taffet and Tate, 1992). Since calcium absorption declines with age, the potency of molecules associated with its transport may be high at birth (hatch) and decline with age, as was the case of Mg^{2+}-ATPase in the present study. Prakash *et al.* (1996) observed a large increase in the activity of Ca^{2+}-ATPase in chicken heart muscle within the first one week of hatch. In body muscle, the rate of activity of Ca^{2+}-ATPase was observed to be highest in day-old chicks; intermediate in broiler chickens and lowest in culled layers (Sung and Kwon, 1992).

The method adopted in the preparation of vesicles relied on Mg^{2+} precipitation which is known to yield membranes more highly contaminated with basolateral membranes than those prepared with Ca^{2+} precipitation (Aubry *et al.*, 1986). The current shift to the use of Mg^{2+} has been necessitated by the fact that Ca^{2+} initiates membrane phospholipid breakdown by phospholipase A (Hauser *et al.*, 1980). The loss of membrane phospholipid would adversely affect membrane function.

4.4.5.2. Effect of age on uptake

The results obtained in the present study showed a reduction in uptake with age at both the jejunum and ileum. The efficiency of uptake, when uptake was related to intestinal and body weights also declined with age. The absolute levels of uptake, however, increased with age. There are no reports on the effects of age on uptake or transport rate of tryptophan in the chicken. There is a general reduction in the intestinal uptake of nutrients with age but the trend is more definite for sugars than it is for tryptophan (Rayo *et al.*, 1992). The overall permeability of tryptophan into brain cells was also found to be lower in 12- and 24-month than in 2-month old rats (Tang and Melethil, 1995). This was attributed to a reduction in transporter mobility and number as well as affinity for tryptophan in older animals. A similar reduction in the rate of tryptophan uptake with age into the rat hepatocytes was reported by Saito *et al.* (1991).

In studies on the transport of aromatic amino acids, including phenylalanine, Navab and Winter (1988) associated the reduction in transport rate in older animals with an increase in transport affinity and a decrease in transporter capacity. Other changes which may occur that could influence nutrient transport are changes in number and types of transporters and membrane fluidity (Buddington and Diamond, 1989). Buddington and Diamond (1989) also observed a greater postnatal decline in the uptake of essential amino acids than that of non-essential amino acids in mammals. This would be a constraint to body growth.

Previous studies on other strains of chickens have demonstrated the establishment of intestinal transport capacity prior to hatch or within a few days of hatch (Pratt and

Terner, 1971). The active transport of lysine was observed 4 days before hatch while valine transport commenced 2 days before hatch. In everted intestinal sacs of chicks, Planas *et al.* (1982) demonstrated maximal trans-epithelial transport of leucine within the first 3 days of hatch and noted a decline with age over the next 4 weeks.

There may be some variation in the development of various systems with age. In the rat brain, L-tryptophan uptake has been subdivided into two components, one Na^+-dependent and the other Na^+-independent (Herrero *et al.*, 1985). The Na^+-dependent component appeared 5 days post-natum and increased with age. In the current study, the Na^+-dependent component of uptake attained a peak 7 days post-hatch in both the jejunum (27.1 %) and ileum (16.7 %).

4.4.5.3. Major site of transport

There are conflicting reports on the importance of the various intestinal sites to amino acid absorption. The conflict appears to derive from differences in amino acids, species, strains of the same species and age studied (Lerner, 1984). Lerner (1984) also highlighted the difficulties encountered in making comparisons since the results are often expressed in different ways.

In the current study, the ileum was a more significant site of uptake L-tryptophan through the brush-border membrane than the jejunum. There were no major differences between the ileum and jejunum when total uptake or efficiency of uptake, in relation to body weight was considered. In rats, peptides are absorbed mainly at the proximal small intestine while amino acids are absorbed mostly at the distal portion of the intestine (Matthews *et al.*, 1971; Crampton *et al.*, 1973). Lerner *et al.* (1975) observed a constant

level of methionine absorption from the duodenum to the ileum. Lysine, on the other hand was absorbed principally from the jejunum and ileum and the rate of absorption was similar for both regions in day-old chicks. In 8-week old birds, ileal enterocytes were found to have a greater absorption capacity for glycine and valine than jejunal enterocytes but methionine flux was equal in both regions (Mitchell and Levin, 1981).

4.4.5.4. Regulation of uptake by ions

The results obtained in this study indicated that L-tryptophan uptake relied to some extent on ions. At low concentrations, K^+ stimulated uptake to the same extent as Na^+ but the effect of the former waned as its concentration in the buffer increased. In Highsex broiler chicks, Kushak and Basova (1988) observed a similar stimulation of tryptophan transport by K^+ in the medium and attributed this to a lack of strong specificity of the sodium-binding sites. The competition between sodium and potassium for these binding sites is partially responsible for the reduction in total uptake when potassium is present at high levels in the extracellular medium (Lerner, 1984).

Certain researchers have suggested that a difference in membrane potential is actually more important than the nature of the ion (Aragon *et al.*, 1987). When lithium replaced sodium in the medium, Aragon *et al.* (1987) observed an increase in the V_{max} of tryptophan transport in vesicles prepared from the rat brain, without a change in K_m. The effect of lithium was attributed to its ability to maintain adequate electrochemical potential across the membrane rather than co-transportation with the amino acid. *In vivo*, the desirable electrochemical gradient is maintained by the actions of Na^+-K^+ATPase (Ganapathy *et al.*, 1994). Using a potential-sensitive dye, Schell *et al.* (1983) showed that amino acid/Na co-transport depolarises the membrane potential and Na^+

affects the carrier affinity but not the maximum flux (J_{max}). At high concentrations of potassium or introduction of valinomycin, a K^+ flux is facilitated, leading to dissipation of membrane potential and a slight reduction in ion-dependence observed in the present study.

The uptake of L-tryptophan was predominantly Na^+ (ion)-independent, similar to the reports by Ganapathy *et al.* (1986) in studies on placental BBMV. However, the relative importance of Na^+-independent uptake may have been over-estimated and that of Na^+-dependent uptake under-estimated in the present studies. Some of the uptake estimated as Na^+-independent could be passive diffusion (Karasov *et al.*, 1986). Diffusion constitutes a significant pathway in membrane vesicles even at physiological concentrations. Stevens *et al.* (1984) estimated a 25 % contribution from diffusion to the transport of phenylalanine at a concentration of 2.5 mM, about equal to the contribution from the Na^+-independent carrier(s).

The differences in uptake in the presence of different sodium salts would suggest some response to the anions. A part of transport stimulation has been attributed to the chloride ion (Lerner, 1985; Aragon *et al.*, 1987). In the present study, nitrate tended to stimulate transport to a greater extent than was observed for chloride. Bio-membranes are permeable to both anions, nitrate being the most permeable of the two (Overholt *et al.*, 1993; Curtis *et al.*, 1993). The entry of the anions into cells or vesicles could create an electrochemical potential, capable of driving L-tryptophan uptake as was observed in this study.

4.4.5.5. Interaction with other substrates

The interactions between L-tryptophan and other amino acids or transport system substrates present two seemingly conflicting facets; one of stimulation at low concentration of the modifier and the other of inhibition. The nature of inhibitive relationship between amino acids is usually competitive, especially if they are transported by the same system (Matthews and Laster, 1965). Both D- and L-tryptophan are transported on the T and L systems while L-methionine and L-tryptophan have been identified as system L substrates in mammalian species (Jara *et al.*, 1990; Pankovich and Jimbow, 1991). A Na^+-independent system speculated by Ganapathy *et al.* (1986) which was responsible for the transport of L-tryptophan in human placenta had very low affinity for D-tryptophan. Similarly, although the uptake of L-tryptophan in human red blood cell by system T is competitively inhibited by D-tryptophan, the latter isomer is not transported by the system (Lopez-Burillo *et al.*, 1985).

The lack of inhibition of L-tryptophan uptake by these amino acids at low concentration may indicate dissimilar routes of transport or higher affinity of shared transporter(s) for L-tryptophan. Enterocyte transport systems which share substrates are also known to interact indirectly, for example through counter-transport (Christensen, 1990). Such interactions can influence the net flux rates and distribution of amino acids across the brush-border and basolateral membranes (Stevens, 1992). The stimulation of uptake by BCH may be due to an enhancement of an inwardly directed membrane potential or counter-transportation, both of which aid L-tryptophan transport. BCH is the preferred substrate for system L (Shotwell *et al.*, 1983; Mertens *et al.*, 1990). These results may, however, imply the lack of significance of system L in the transport of L-tryptophan in the chicken intestine but this would require further verification at BCH levels higher

than 6.25 mM. Some caution is required in the interpretation of inhibition studies when very high concentrations (≥100 mM) of the effector substrate are used (McGivan, 1992). It was postulated (McGivan, 1992) that the inhibition observed under such conditions may be the result of high concentrations of impurities present in the test substrate.

In the red blood cells of pigeons, tryptophan was transported mainly on the L system (Vadgama *et al.*, 1987). The L system was, however, simultaneously present with the Na^+-independent system, asc (a variant of system ASC) by which alanine is also transported (Vadgama and Christensen, 1985). It is probable that some of the Na^+-independent uptake of L-tryptophan may be by the asc system, given the inhibition created by a high concentration of L-alanine.

The existence of a strong sodium-independent system is not in doubt since the contribution of the Na^+-dependent system(s) to total uptake at all the ages studied is less than 40 %. Results from the test involving valinomycin indicated a higher contribution of Na^+- dependent uptake. This may be an aberration caused by the presence of ethanol in the medium although this does not invalidate the results of the effects of valinomycin since an equal amount of ethanol was present in all buffers. The impact of ethanol on uptake and transport of tryptophan from the intestine has not been previously reported. Smith *et al.* (1990a) reported a reduction in alanine transport in studies on liver baso-lateral membrane vesicles obtained from rats which had been on chronic ethanol intake.

L-lysine, L-methionine and L-alanine at high concentrations and in the presence of Na^+ all partially inhibited the uptake of L-tryptophan, as did D-tryptophan. In the rat jejunum, tryptophan competes with lysine for the carrier of basic amino acids across the

brush-border membrane without itself being transported (Munck and Rasmussen, 1975). The overall absorption of tryptophan may be reduced by high concentrations of lysine and methionine if these amino acids pose similar effects at the basolateral membrane. Most feed ingredients used for poultry feeding contain higher levels of lysine and methionine than tryptophan (McDonald *et al.*, 1988). Munck and Rasmussen (1975) also reported that tryptophan may exit the cell across the basolateral membrane by a mechanism shared by lysine after crossing the brush-border membrane by the neutral amino acid transporter(s). The relationship between tryptophan and methionine is more complex. Methionine is a potent *cis* and *trans* inhibitor of tryptophan influx across the brush-border membrane but stimulates the unidirectional transmural flux of tryptophan (Munck and Rasmussen, 1975).

4.4.6. CONCLUSION

It is premature to draw conclusions on the nature of the system(s) responsible for L-tryptophan uptake in the current study. Clearly, two mechanisms, one Na^+-independent and the other Na^+-dependent are involved. The identification of systems by studies on BBMV are limited by the fact that the absence of a given system (through inhibition assays) does not always imply its absence in the intact tissue (Murer and Kinne, 1980). The identification of a system in studies on BBMV is, however, a very strong indication of its presence in the intact tissue. It may be necessary to support these studies in future with those on intact cells or tissues, using a relatively large number of samples.

While uptake rate declined with age, there was an increase in the capacity of the small intestine to transport L-tryptophan, so as to support a larger body mass. The limitation in uptake at the apical membrane may limit overall absorption, especially if transport at

the basolateral membrane is also disrupted. The study of events at both sites would present a clearer picture of the actual capacity of chicks to obtain L-tryptophan.

4.5.0. EXPERIMENT 4.5: THE EFFECT OF FINISHER DIET ON BODY GROWTH, INTESTINAL STRUCTURE AND FUNCTION.

4.5.1. INTRODUCTION

In conventional production systems, broiler chickens are first maintained on a broiler starter diet and then transferred to a finisher diet (Harms *et al.*, 1986; Leeson *et al.*, 1988; Leeson *et al.*, 1992). Certain systems, especially those concerned with breeding still transfer from the starter diet to a grower diet and finally to a finisher (breeder) diet. These diets vary principally in protein content and may also vary in individual amino acid concentrations as well as in mineral elements. Generally, the starter diet is higher in protein as well as individual amino acids than the grower or finisher diet but there may be no change in metabolizable energy (NRC, 1994). The period during which each of these diets is fed varies with producers. Most operations feed the starter for the first 3-4 weeks before transferring to the finisher diet (Leeson *et al.*, 1988; NRC, 1994). Diambra and McCartney (1985) did not observe any differences in growth and body weight between groups of chickens when they were raised on a starter diet for 20, 25, 30 or 35 days before transferring to a finisher diet and reared to 49 days of age.

The changes that occur to intestinal structure and function with age in mammalian species have been more extensively studied than those in poultry (section 2.3.2.1). The principal aim of this experiment was to obtain data on intestinal development in broiler chicks beyond the starter phase period. Although there might be some direct effects of a switch in diets as has been reported for pigs at weaning (Hampson and Kiddler, 1984; Hampson, 1986), the major objective of this experiment was to set baseline data for

some experiments in chapter 5 in which chicks were assessed on varying dietary factors beyond 21 days of age.

4.5.2. MATERIALS AND METHODS

The results presented in experiments 4.1-4.4 were based on trials in which a commercial starter diet was fed between hatch and 21 days of age. At the end of this phase, a commercial finisher diet (Milling Industries Stockfeed, Murray Bridge, South Australia) was introduced. The nutrient composition of the diet is shown in Appendix 1. Eighteen chicks of the same strain as in the preceding four experiments were kept in cages in batches of 3, from which samples were randomly taken on days 28 and 35. Feed and water supplies were *ad libitum*. The rearing temperature was 26±2°C and artificial light was provided for 23¾ hours per day.

4.5.2.1. Production measurement and sample collection

Feed consumption was recorded every 7 days and samples of faeces/urine were bulked by cage and analysed for nutrient retention as described for the first phase (section 4.1.2.2). All chicks were weighed on days 21, 28 and 35. On days 28 and 35, seven chicks chosen at random were euthanatized and dissected for sample collection as described in section 3.4.0. The full weight of the GIT from the outlet of the gizzard to the cloaca was obtained, in addition to weights of the gizzard and liver. Samples were taken from the proximal ends of the duodenum, jejunum and ileum for histology and preparation of brush-border membrane vesicles as described in sections 3.4.1 and 3.7.0 respectively. Vesicles were only used for the assessment of enzyme function as described in 3.7.1, no transport assays were conducted.

4.5.3. RESULTS

4.5.3.1. Feed consumption and nutrient retention

Absolute feed intake per bird was higher (P<0.01) during the last seven days than during the first seven days of the introduction of the finisher diet (Table 4.5.1). Feed intake per unit of body weight, however, declined (P<0.001) with age. Feed conversion ratio and weight gain were also higher during 28-35 days than at 21-28 days of age.

Table 4.5.1: Feed intake and utilization at different ages on the finisher diet.

	Age (days)		
	21-28	**28-35**	**SED**
Feed intake (g/head)	795.5[b]	920.8[a]	39.64**
Feed intake (g/100 g body weight)[1]	92.8[a]	71.2[b]	2.19***
Initial body weight (g)	856.6[b]	1294.1[a]	61.44***
Final body weight (g)	1294.1[b]	1739.4[a]	98.13***
Growth rate (g/day)	62.5	63.6	6.79
Feed conversion ratio[2]	1.82	2.07	0.117

a,b - Mean values in the same row not sharing a superscript differ significantly (**P<0.01, ***P<0.001).
1. Feed intake based on initial body weight. 2. Feed intake (g/head) per unit body weight gain.

Table 4.5.2: Dry matter and nutrient retention (%) at different ages on the finisher diet.

Age (days)	Dry matter	Gross energy	Methionine	Lysine	Tryptophan
21-28	87.0	61.0	74.8	92.4	42.6
28-35	87.9	64.7	74.7	86.6	59.8

The retention of dry matter other nutrients was estimated on flock basis, from bulked samples; no statistical analysis was conducted.

Dry matter and energy retention increased between 21 and 35 days of age (Table 4.5.2). There was a decline in the retention of ~~methionine~~ and lysine within the same period. Tryptophan retention was, however, increased during the second week of transfer to the finisher diet.

Table 4.5.3: Weight of body and visceral organs (g/100 g body weight).

	Age (days)		
	28	**35**	**SED**
Liveweight (g)	1294.1^{b}	1739.4^{a}	82.39***
GIT[1]	6.9	6.5	0.58
Gizzard	2.7^{a}	2.5^{b}	0.38**
Liver	2.7^{a}	2.3^{b}	0.18***

1. Full weight of the GIT from the outlet of the gizzard to the cloaca, including the caeca.
a,b - Mean values in the same row not sharing a superscript differ significantly (**P<0.01, ***P<0.001).

4.5.3.2. Body and visceral organ weights

There was a steady increase (P<0.001) in body weight between 21 and 35 days of age (Table 4.5.3). During the period, there was a 103 % increase in body weight. However, there was a marginal decline in GIT weight, as a proportion of body weight during the period. The weights of the gizzard (P<0.01) and liver (P<0.001), in relation to body weight also declined with age.

4.5.3.3. Morphometry of intestinal mucosa

Results of external muscle thickness and crypt depth are presented in Table 4.5.4 while villus height and apparent surface area are shown in Table 4.5.5. Within any intestinal site, there were no significant differences between the two age groups with regards to

most of the variables assessed. In the jejunum, crypt depth was significantly higher ($P<0.05$) on day 35 than on day 28. There was a general decline in the values of most variables from the duodenum towards the ileum, at any given age.

Table 4.5.4: External muscle thickness and crypt depth at different ages and intestinal site.

	Age (days)		
Segment	**28**	**35**	**SED**
A. External muscle thickness (μm)			
Duodenum	335.6	338.4	88.74
Jejunum	303.3	313.0	59.41
Ileum	276.2	242.1	45.73
B. Crypt depth (μm)			
Duodenum	164.4	176.3	18.87
Jejunum	149.2^{b}	180.4^{a}	15.88
Ileum	124.7	129.7	17.51

a,b - Mean values in the same row not sharing a superscript differ significantly ($P<0.05$).

Table 4.5.5: Villus height and apparent surface area at different ages and intestinal sites.

	Age (days)		
Segment	**28**	**35**	**SE**
A. Villus height (μm)			
Duodenum	1564.0	1728.4	237.06
Jejunum	1330.5	1225.1	161.57
Ileum	694.3	633.4	51.89
B. Villus surface area (mm^2)			
Duodenum	0.31	0.34	0.095
Jejunum	0.19	0.19	0.026
Ileum	0.10	0.09	0.015

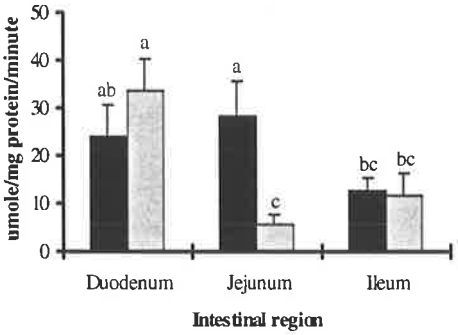
4.5.3.4. Intestinal enzyme activities

The specific activities of four intestinal enzymes on days 28 and 35 are shown in Figure 4.5.1. Maltase activity in the jejunum declined significantly ($P<0.001$) with age; in the duodenum and ileum, its activity was not significantly influenced by age over the study period. On day 28, maltase activity was higher ($P<0.001$) in the jejunum than in the duodenum or ileum but this trend was reversed on day 35 when there was a higher level of expression in the duodenum. Maltase activity was generally higher than the activity of the other enzymes, irrespective of age.

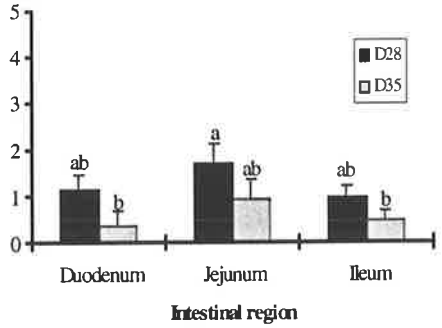
The specific activity of sucrase did not differ significantly with age within any intestinal site, although there were slight reductions with age. At any particular age, there were no significant differences in sucrase activity between the intestinal regions.

The specific activity of APN was significantly higher ($P<0.01$) in the duodenum on day 35 than in the jejunum or ileum but the activity on day 28 was similar between the three intestinal sites. There were slight reductions with age in the jejunum and ileum but there was an increase in the duodenum. The differences between the two age groups within any intestinal site were not significant.

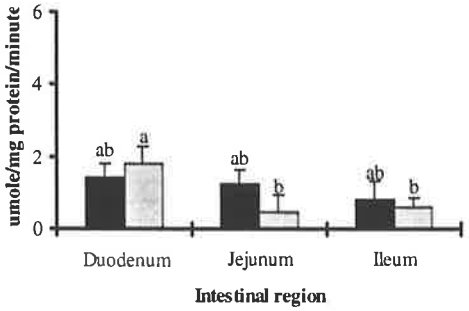
a. Maltase



b. Sucrase



c. APN



d. AP

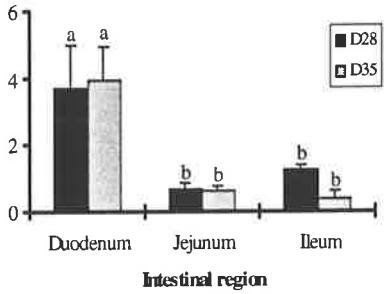


Figure 4.5.1: Specific activities (μmole product/mg protein/minute) of intestinal enzymes at different sites and ages. Mean values not sharing a superscript are significantly different (P<0.01 for APN; P<0.001 for other enzymes).

Within the same intestinal region, there was no significant difference between the age groups in the expression of AP. Its expression was, however, significantly higher (P<0.001) in the duodenum than in the jejunum or ileum in both age groups.

4.5.4. DISCUSSION

4.5.4.1. Feed consumption and body growth

The introduction of finisher diets in the broiler production cycle is somewhat similar to the weaning of most mammalian species on to solid feed, although the impact may not be as drastic. With such change in feed quality, chicks are forced to adjust their feed

consumption appropriately. Young chicks are known to adjust their feed intake in response to changes in the diet much more rapidly than older birds and reductions in dietary energy are matched by increased feed intake (Leeson *et al.*, 1992; Picard *et al.*, 1993). In studies on the effect of nutrient dilution on feed intake, Leeson *et al.* (1992) observed a 70 % increase in feed intake by 35-day-old broiler chickens. It is therefore, possible to further increase broiler productivity or shorten the production cycle considerably by methods which could enhance feed intake at stable nutrient density. In the present study, feed intake per day represented about 13 % of the initial body weight during the 21-28 day period while between 28 and 35 days, the figure was 10.2 %. If calculated on daily basis, these values may be slightly lower but they generally indicate that there is a reduction in feed intake, in relation to body weight with age.

In the present study, absolute feed intake continued to rise while feed intake relative to liveweight declined with age as was observed during the first phase on the starter diet (experiment 4.1). Body growth rate also continued to increase with age. The slight reduction in growth rate in the period 21-28 days may be the result of an adjustment to the new diet. An increase in feed conversion ratio with age was commonly observed in both phases and signified a reduction in the efficiency of feed utilization with age.

4.5.4.2. Visceral organ growth

The weights of the GIT, gizzard and liver relative to body weight fell with age on the finisher diet. In the starter diet phase, the weights of these organs peaked at between 7 and 14 days (section 4.1.3.2). The relative weight of the GIT assessed in this experiment is the full weight and does not account for the digesta weight or the separate weight of the small intestine. It is most probable that the weight of the small intestine

continued to decline after the peak value observed on day 7 in the starter diet phase (experiment 4.1). The reduction in the growth of the GIT and gizzard on the finisher diet and with age was expected. Nutrient deriving organs go through a rapid development in early life and then grow more slowly as priority is shifted to accumulating tissues and organs such as the muscles and bones (Shibata *et al.*, 1983; O'Sullivan*et al.*, 1992a).

4.5.4.3. Intestinal structure and function

There are no reports on changes in intestinal structure and function with age or following transition from starter to finisher diet in poultry. The transition, although similar to weaning may not induce the same level of changes, since poultry are exposed to solid feed immediately post-hatch. The introduction of gnotobiotic piglets to solid feed resulted in damage to the intestinal mucosa, associated with reductions in villus height, crypt cell production and activities of brush-border membrane enzymes (Hall and Byrne, 1989). When normally reared piglets are weaned, there is usually an increase in crypt depth and a reduction in villus height (Hampson, 1986). The trend of changes observed in mucosal structure in the finisher diet phase appears to be similar to those observed on the starter diet and may have been induced by age rather than the new diet. There was a continuous increase in crypt depth at all intestinal sites and a reduction in villus height at the jejunum and ileum. The apparent villus surface area increased slightly at the duodenum but was unchanged at the other intestinal sites.

The specific activities of most of the digestive enzymes assessed declined with age in almost all the intestinal regions, similar to what was observed in the starter diet phase. It is not certain why there were increases in the specific activities of maltase, APN and

AP at the duodenum over the period. The reduction in specific activities of enzymes with age has been commonly observed in studies on mammalian species and other poultry species (Holt and Kotler, 1987; Sell *et al.*, 1991; Wild and Murray, 1992). Although enzyme activity was not assessed by histochemistry, it is most probable that total activities per villus increased with age as was observed in the starter diet phase. This would have been accomplished by an increase in villus height and area with age, in at least the duodenum and jejunum.

4.5.5. CONCLUSION

Broiler chicks maintained an increased consumption in feed with age on the finisher diet as during rearing on the starter diet and feed intake per unit body weight similarly declined with age in both phases. Feed conversion efficiency was lower in the finisher phase than during the starter diet phase but this may be the effect of age rather than the feeding of a new diet. There was no apparent effect of the change in diet on percentage retention of most nutrients. The rate of growth of visceral organs continued to decline with age. There was a steady increase in the development of the intestinal mucosa, associated with a reduction in the specific activities of digestive enzymes with age. The trend in most of the changes observed in the finisher diet phase was similar to that during rearing on the starter diet and these changes were mainly due to age since there was no alternation between diets within any phase.

CHAPTER FIVE: THE EFFECTS OF DIETARY FACTORS ON INTESTINAL DEVELOPMENT AND BODY GROWTH

5.0. INTRODUCTION TO CHAPTER

Poultry productivity varies between diets, especially between the traditional diets and some of the novel low-cost alternative diets. Most of these differences are due to variation in nutrient composition and the efficiency with which nutrients in different nutrients can be utilized. The mechanisms behind these changes are not well understood. Since the intestine is in close proximity with the diet and interacts with dietary factors during digestion and absorption, some of the differences in productivity may be attributable to changes in mechanisms operating at the intestinal level.

The major aim of this chapter was to examine the response of the whole animal to various dietary ingredients and nutrients and to relate this response to changes in intestinal structure and function. Four experiments were conducted to assess both traditional and alternative dietary ingredients as well as supplements. Most of the experiments, as those in chapter 4 were not designed with strong statistical weight to test productivity but mainly to explain the physiological basis of some known phenomena in the poultry nutrition.

5.1.0. EXPERIMENT 5.1: BODY GROWTH AND INTESTINAL FUNCTION ON DIETS BASED ON DIFFERENT CEREAL GRAINS AND A MICROBIAL ENZYME SUPPLEMENT.

5.1.1. INTRODUCTION

Cereal grains constitute the largest proportion of most practical diets for poultry and supply the bulk of energy required by the bird (McDonald *et al.*, 1988). The range of cereals available to the industry and the limitation in their supply as well as exploration of alternative sources have been reviewed in section 2.5.0.

Poultry performance depends to a large extent on the cereal grain included in the diet due to the large proportion of the grain in diets. Starch is the main carbohydrate in cereal grains and is generally composed of two major fractions, amylopectin (75 %) and amylose, both of which may further vary in physical properties such as the nature of crystallization and waxiness (Rogel, 1985; Longstaff and McNab, 1986; McDonald *et al.*, 1988). Amylopectin is more digestible than amylose and cereal starches are generally more digestible than tuber and legume starches (Nitsan and Bartov, 1972; Coates and Rolls, 1981). Apart from starch, cereals contain large amounts of NSP and unlike starch quality, the type of NSP varies widely from one cereal grain to another (Kopinski *et al.*, 1995; Smits and Annison, 1996). Arabinoxylans are the main NSP in wheat, rye, sorghum, maize and triticale but the concentration of the dietary NSP varies with the predominant cereal in the diet (Annison, 1990; Boros *et al.*, 1995; Smits and Annison, 1996). The structures of the two main groups of NSP found in cereals, β-glucans and arabinoxylans are shown in Figure 2.2 (section 2.5.3.1).

Non-starch polysaccharides influence poultry productivity mainly by creating a viscous lumenal environment (Bedford and Classen, 1992; Choct and Annison, 1992). High digesta viscosity depresses the efficiency of digestion by reducing the rate of solute diffusion (Fengler and Marquardt, 1988). Cereal NSP therefore reduce the availability of nutrients from the diet (Annison, 1993; Smits *et al.*, 1997). This effect is responsible for the low apparent metabolizable energy (AME) phenomenon of some Australian wheats (Annison, 1993). There are no known reports on the effects of cereals directly on intestinal structure and function in broiler chickens. The effects of NSP in other species have been reported (Johnson and Gee, 1986; Brunsgaard and Eggum, 1995) and were reviewed in section 2.3.2.5.

The negative effects of cereal NSP can be reduced through chemical extraction or inclusion of microbial enzyme supplements in the diet (Campbell *et al.*, 1989; Bedford, 1995). These measures have been shown to improve the nutritive value of diets and animal growth (section 2.5.3.4). The degree of response to microbial enzyme supplementation depends on many factors, especially the type of cereal as well as concentration and type of NSP (Marquardt *et al.*, 1993).

The present study examined the response of broiler chicks to diets based on different cereals with or without a microbial enzyme supplement. The differences in productivity were related to the mucosal structure of the intestine and function of intestinal enzymes.

5.1.2. MATERIALS AND METHODS

5.1.2.1. Animals and diets

Seven-two one-week-old broiler chicks (Steggles x Ross, F_1; initial weight, 119-138 g), previously brooded under identical conditions were used for the study. The chicks were randomly divided into cage groups of nine birds and assigned to one of 8 diets. There were four main diets based on barley, maize, sorghum or wheat. Each diet contained 650.0 g of one of the cereal grains per kg in addition to (g/kg diet): soyabean meal (250.0), meat meal (60.0), vegetable oil (25.0) and premix (15.0). The premix consisted of (g/kg): lysine (2.5), methionine (3.0), salt (2.5), vitamins/minerals (5.0) and choline chloride (0.8). Each diet was fed without or with an additional supplement of 1.0 g/kg of a commercial microbial enzyme, Avizyme 1300 (*Trichoderma longibrachiatum*; Finnfeeds International, Aus. Pty Ltd.) which has arabinoxylanase-β-glucanase-pectinase activities. The diets were fed for three weeks. Water supply was unrestricted and lighting was for 23¾ hours. The rearing temperature during the first week was 30 ± 1°C; this was reduced to 26 ± 2°C in the last two weeks.

5.1.2.2. Dietary composition and *in vitro* digestibility

In addition to calculated nutrient analysis, the diets were analysed for starch content according to the method of Lever (1972) as described in section 3.3.1. The *in vitro* digestibility of starch was estimated from this assay. *In vitro* digestibility of DM and viscosity of digesta obtained from the diets were assessed as described in 3.3.4 and 3.3.5 respectively.

5.1.2.3. Sample collection

Feed intake and body weight were measured every week and at the end of the feeding period. Five birds per diet, selected at random were slaughtered as described in section 3.4.0. The carcass was weighed prior to removal of visceral organs. The intestine, from the outlet of the gizzard to the cloaca and the liver were weighed. Intestinal tissues were collected from the proximal ends of the jejunum and ileum and used to assess intestinal mucosal morphometry and digestive enzyme function as described below.

5.1.2.4. Histology

Intestinal tissues which had been fixed in 10 % neutral buffered formalin for 24 hours were transferred to 70 % ethanol, sub-sampled and processed as described in section 3.4.1 and Appendix 4. The tissues were sectioned to 5 µm thick and mounted on poly-L-lysine coated slides. The tissues were then stained by the hematoxylin/eosin technique (Appendix 5). Tissue sections were digitized as described in section 3.4.1 to obtain indices of mucosal growth. Crypt depth, villus height and apparent villus surface area at the jejunum and ileum were measured.

5.1.2.4. Biochemical analysis of digestive enzyme activity

Brush-border membrane vesicles (BBMV) were prepared as previously described by Shirazi-Beechey *et al.* (1991), highlighted in section 3.7.0 and used to assess the specific activity of key digestive enzymes. No transport assays were conducted.

5.1.3. RESULTS

5.1.3.1. Feed quality and digestibility

The calculated and laboratory analysed compositions of the diets are shown in Table 5.1.1. The diets were similar in both crude protein and metabolizable energy. Starch content, as determined by chemical analysis was higher in the wheat-based diet than in the other diets.

Table 5.1.1. Nutrient composition (g/kg) of unsupplemented (control) diets.

	Diet (main cereal base)			
	Barley	**Maize**	**Sorghum**	**Wheat**
Dry matter	929.4	916.6	918.0	924.1
Starch	344.4	383.7	388.2	427.7
Crude protein	222.6	216.1	222.6	233.0
Metabolizable energy (ME; MJ/kg)	12.6	13.3	12.8	12.9

Starch content was measured as described in section 3.3.1. Crude protein and ME were calculated from ingredient composition charts.

Table 5.1.2: Total NSP and simple sugar profiles (mg/g) of NSP from different diets.

Diet	Fuc	Rib	Ara	Xyl	Man	Gal	Glu	Total NSP
Barley	1.02	1.12	14.61	21.28	3.06	13.53	30.38	75.8
Maize	1.14	0.57	14.11	12.30	1.48	16.00	14.39	53.4
Sorghum	0.00	0.00	8.85	6.99	1.34	9.60	15.37	37.6
Wheat	0.54	0.40	9.42	11.48	1.24	7.73	11.96	38.1

NSP was measured on microbial enzyme-free samples as described in section 3.3.3. Total NSP was calculated, using polymerization factors of 0.89 for deoxysugars, 0.88 for pentoses and 0.9 for hexoses. Simple sugars represented are fucose (Fuc), ribose (Rib), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glu).

The barley-based diet had the highest content of total NSP while the lowest concentration was observed on the sorghum-based diet (Table 5.1.2). Glucose was

commonly the simple sugar in highest concentration in all samples, except in the maize-based diet. The barley- and wheat-based dietary NSP were composed from predominantly xylose, arabinose and galactose while the major simple sugars in the maize- and sorghum-based diets were galactose, arabinose and xylose, in addition to glucose in each case.

Table 5.1.3. Digestibility of dry matter and starch and viscosity of digesta from diets determined *in vitro*.

	Diet (main cereal base)			
	Barley	**Maize**	**Sorghum**	**Wheat**
Dry matter digestibility (%)	62.9	80.4	79.4	77.0
Starch digestibility (%)	69.3	72.6	76.7	89.3
Digesta viscosity (cP)	3.0	1.8	1.6	2.4

Assays were conducted on samples without enzyme supplement in duplicate for each diet. No statistical comparisons were made.

The *in vitro* DM digestibility of the maize-, sorghum- and wheat-based diets was identical while the digestibility of the barley-based diet was the lowest (Table 5.1.3). The digestibility of starch in the wheat-based diet was higher than that of the other diets; starch in the barley-containing diet was the least digestible, *in vitro*. The *in vitro* digesta viscosity of the unsupplemented barley-based diet was also higher than that of the other diets; the maize- and sorghum-based diets had relatively low viscosity.

Table 5.1.4. The effects of enzyme supplementation on feed intake, feed conversion ratio and body weight.

	Initial weight (g)	Final weight (g)	Weight gain (g)	Feed intake (g/bird)[1]	FCR[2]
Control diets					
Barley	138.0	901.0[b]	763.0[b]	1589.0	2.08
Maize	126.0	1182.0[a]	1056.0[a]	1439.0	1.36
Sorghum	119.0	1178.0[a]	1059.0[a]	1387.0	1.31
Wheat	128.0	1248.0[a]	1120.0[a]	1355.0	1.21
Plus Enzyme					
Barley	132.0	1168.0[a]	1036.0[a]	1638.0	1.55
Maize	126.0	1245.0[a]	1119.0[a]	1458.0	1.26
Sorghum	124.0	1196.0[a]	1072.0[a]	1469.0	1.37
Wheat	126.0	1300.0[a]	1174.0[a]	1440.0	1.23
SED	10.24	80.69	81.37	-	-

a,b - Mean values in the same column with different superscripts differ significantly ($P<0.01$).
1,2 - Feed intake and feed conversion ratio estimates were derived on sub-flock basis and not statistically tested. Other estimates were based on the number of chicks sampled at the end of study.

5.1.3.2. Feed intake and body growth

Feed consumption was highest on the barley-based diets and lowest on the wheat-based diets, irrespective of microbial enzyme supplement (Table 5.1.4). Feed conversion ratio was also lower in chicks that were fed the wheat-based diets than in chicks on the other diets. The highest feed conversion ratio was observed on the barley-based diets. These differences were not statistically tested. Feed intake was increased as a result of supplementation with the microbial enzyme by 3.1, 1.3, 5.9 and 6.3 % respectively on barley, maize, sorghum and wheat. There were 34.2, 7.9 and 4.5 % reductions in feed conversion ratio on the barley, maize and sorghum diets respectively. On the wheat-

based diet, there was a slight increase (1.6 %) in feed conversion ratio as a result of supplementation with the microbial enzyme.

Table 5.1.5. Visceral organ weight (g/100 g liveweight) of birds fed on control and enzyme-supplemented diets.

	Diet (main cereal base)			
	Barley	**Maize**	**Sorghum**	**Wheat**
A. Small and large intestine[1]				
Control	11.3 ± 1.45^{a}	6.9 ± 0.37^{b}	6.6 ± 0.83^{b}	7.2 ± 0.26^{b}
+ Enzyme	7.6 ± 1.44^{b}	6.9 ± 0.58^{b}	6.9 ± 0.61^{b}	6.2 ± 0.29^{b}
B. Liver				
Control	3.1 ± 0.57	3.3 ± 0.36	3.1 ± 0.21	3.8 ± 0.46
+ Enzyme	3.3 ± 0.35	3.2 ± 0.34	3.0 ± 0.36	3.5 ± 0.40

a,b - Mean values in the same row and column not sharing a superscript are significantly different ($P<0.001$). 1. Full weight of the small and large intestines, including the caeca; empty weights were not determined.

Chicks raised on the barley-based diets had significantly lower ($P<0.01$) final body weight and weight gain than chicks on the other diets, regardless of microbial enzyme supplement. There were improvements in final weight and weight gain on all diets but this was significant ($P<0.01$) only in the case of barley. The most improvement in final liveweight (29.6 %) and liveweight gain (35.8 %) as a result of microbial enzyme supplementation was on the barley-based diet. The least improvement was observed on the diet based on sorghum, with 1.5 and 1.2 % increase in final liveweight and weight gain respectively.

Table 5.1.6. The protein content (mg/g tissue) of crude homogenates and brush-border membrane vesicles from chicks on control and enzyme-supplemented diets.

	Diet (main cereal base)			
	Barley	**Maize**	**Sorghum**	**Wheat**
A. Total (mucosal) protein				
Jejunum				
Control	34.5 ± 1.16	28.5 ± 1.52	36.7 ± 8.90	34.9 ± 5.79
+ Enzyme	30.2 ± 3.97	27.3 ± 2.79	31.2 ± 3.51	30.2 ± 2.97
Ileum				
Control	25.6 ± 2.77^{bc}	23.3 ± 3.82^{c}	40.5 ± 5.89^{ab}	39.0 ± 3.71^{ab}
+ Enzyme	26.8 ± 3.12^{bc}	21.7 ± 4.78^{c}	29.1 ± 4.60^{bc}	49.7 ± 8.84^{a}
B. Brush-border membrane protein				
Jejunum				
Control	0.13 ± 0.089^{b}	0.08 ± 0.026^{b}	0.13 ± 0.039^{b}	0.39 ± 0.082^{a}
+ Enzyme	0.12 ± 0.066^{b}	0.11 ± 0.017^{b}	0.15 ± 0.055^{b}	0.34 ± 0.058^{a}
Ileum				
Control	0.14 ± 0.076^{ab}	0.18 ± 0.078^{ab}	0.08 ± 0.025^{b}	0.28 ± 0.047^{a}
+ Enzyme	0.15 ± 0.114^{ab}	0.22 ± 0.085^{ab}	0.08 ± 0.015^{b}	0.28 ± 0.066^{a}

a,b,c - For the same variable, mean values in the same row and column not sharing a superscript are significantly different at the following levels:

	Jejunum	**Ileum**
Total protein	Not significant	P<0.05
BBMV protein	P<0.001	P<0.01

5.1.3.3. Visceral organ weight and mucosal protein content

On the control diet, the intestinal weight of chicks maintained on the barley-based diet was significantly higher (P<0.001) than that of birds on the other diets (Table 5.1.5). The intestinal weight of chicks on the barley-based diet was also reduced (P<0.001) following microbial enzyme supplementation. There was no effect of enzyme

supplementation on the intestinal weight of chicks on the other diets. Neither cereal type nor enzyme supplementation had any significant effects on the weight of the liver.

Table 5.1.7: Morphometry of jejunal mucosa of chickens fed the control and enzyme-supplemented diets.

	Diet (main cereal base)			
	Barley	**Maize**	**Sorghum**	**Wheat**
Crypt depth (μm)				
Control	207.9 ± 40.06^{ab}	164.1 ± 17.77^{b}	170.9 ± 25.70^{b}	230.7 ± 26.72^{a}
+ Enzyme	180.6 ± 10.06^{b}	200.4 ± 6.62^{ab}	187.9 ± 24.00^{b}	179.2 ± 33.73^{a}
Villus height (μm)				
Control	1275.6 ± 151.63^{ab}	1330.5 ± 150.91^{ab}	1114.7 ± 139.41^{b}	1411.9 ± 55.00^{a}
+ Enzyme	1323.4 ± 67.21^{ab}	1325.5 ± 57.82^{ab}	1070.3 ± 130.81^{b}	1313.7 ± 121.68^{ab}
Villus surface area (mm^2)				
Control	0.18 ± 0.038	0.18 ± 0.064	0.13 ± 0.019	0.20 ± 0.052
+ Enzyme	0.15 ± 0.011	0.17 ± 0.015	0.13 ± 0.027	0.17 ± 0.022

a,b - Mean values in the same column or row not sharing a superscript are significantly different ($P<0.01$ for crypt depth and $P<0.001$ for villus height).

There were no significant effects of cereal type or microbial enzyme supplementation on the protein content of jejunal mucosal homogenate (Table 5.1.6). The protein content of the ileal mucosal homogenate from chicks reared on the maize-based diets was significantly lower ($P<0.001$) than that observed in the other chicks. There was no significant effect of enzyme supplementation on the ileal mucosal protein content of chicks on any of the cereal grains tested. Chicks that had been fed the wheat-based diet had a significantly higher ($P<0.001$) protein content in jejunal BBM than chicks maintained on the other diets. There was no effect of microbial enzyme supplementation on BBM protein. In the ileum, the BBM protein content of chicks on diets based on wheat, barley and maize were similar. The protein content of the ileal

BBM of chicks raised on the wheat-based diets was higher ($P<0.001$) than that observed in chicks on the other diets. There was no significant effect of microbial enzyme supplementation of any of the diets with regards to BBM protein content at either intestinal site.

Table 5.1.8: Morphometry of ileal mucosa of chickens fed the control and enzyme-supplemented diets.

	Diet (main cereal base)			
	Barley	**Maize**	**Sorghum**	**Wheat**
Crypt depth (μm)				
Control	146.8 ± 17.50	146.8 ± 22.33	142.2 ± 34.19	169.9 ± 13.11
+ Enzyme	144.1 ± 8.50	161.2 ± 15.83	153.2 ± 25.79	168.3 ± 20.82
Villus height (μm)				
Control	612.2 ± 67.55	700.9 ± 158.24	668.0 ± 59.69	790.0 ± 48.67
+ Enzyme	680.7 ± 86.32	701.9 ± 100.50	672.5 ± 50.51	775.1 ± 109.87
Villus surface area (mm^2)				
Control	0.08 ± 0.008	0.08 ± 0.007	0.10 ± 0.041	0.12 ± 0.030
+ Enzyme	0.09 ± 0.015	0.12 ± 0.028	0.09 ± 0.014	0.10 ± 0.008

5.1.3.4. Morphometry of the intestinal mucosa

Jejunal crypts were significantly deeper ($P<0.01$) in birds that were fed the wheat-based diet than in the other groups (Table 5.1.7). Jejunal villi were also longest ($P<0.001$) in birds that were fed the wheat control diet and shortest on the sorghum-based diet with enzyme supplementation. Chicks on the wheat-based control diet had a larger (not significant) jejunal villus surface area than those on the other cereals. Dietary supplementation with the microbial enzyme did not significantly influence the jejunal mucosal morphometry of chicks on any of the cereal types.

In the ileum, crypt depth, villus height and villus surface area were larger in chicks on the wheat-based diet than on the other cereals but there were no significant effects of cereal type or of microbial enzyme supplement on ileal mucosal morphometry (Table 5.1.8).

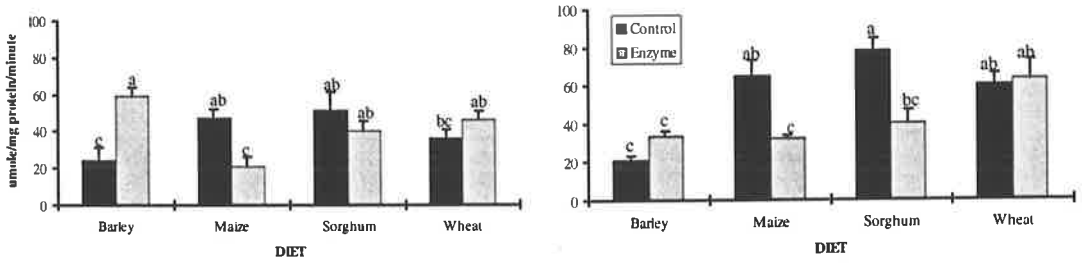


Figure 5.1.1: Specific activity of maltase (μmole glucose/mg protein/minute) in the jejunum (*left*) and ileum of chicks on different diets. a,b - Within the same intestinal region, mean values not sharing a superscript are significantly different (P<0.05 at the jejunum and P<0.001 at the ileum).

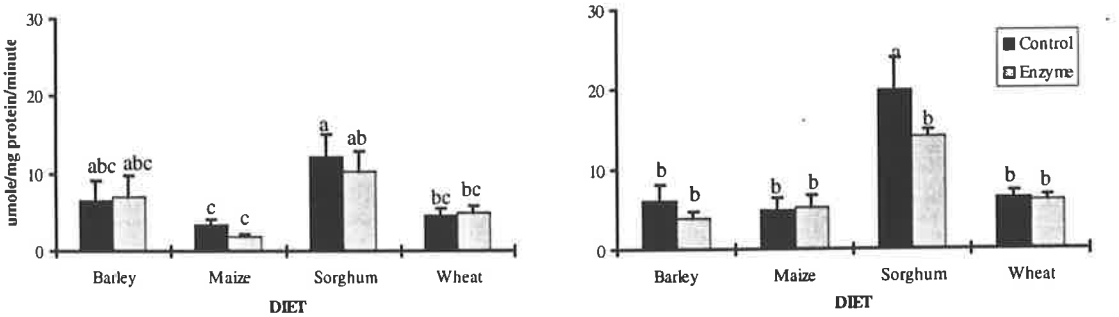


Figure 5.1.2: Specific activity of sucrase (μmole glucose/mg protein/minute) in the jejunum (*left*) and ileum of chicks on different diets. a,b - Within the same intestinal region, mean values not sharing a superscript are significantly different (P<0.001).

5.1.3.5. Activity of intestinal digestive enzymes

On the control diets, the specific activity of maltase in the jejunum was significantly lower (P<0.05) in chicks raised on the barley-based diet than in chicks on the other diets (Figure 5.1.1). Supplementation with the microbial enzyme increased the specific activity of maltase in the jejunum of chicks on the barley- (P<0.05) and wheat-based diets but reduced such activity on the maize- and sorghum-based diets (P<0.05). Chicks on the barley control diet had a significantly lower (P<0.001) specific activity of maltase

in the ileum than the other chicks. Maltase activity was reduced (P<0.001) on the maize- and sorghum-based diets while it was slightly increased on the barley- and wheat-based diets in the presence of the microbial enzyme supplement.

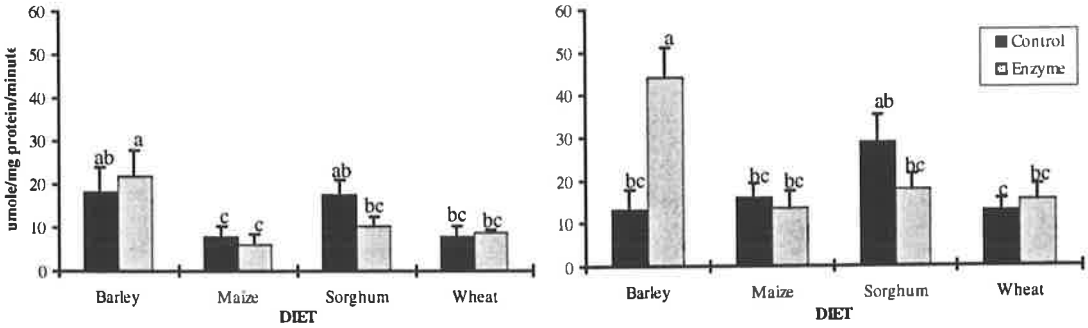


Figure 5.1.3: Specific activity of aminopeptidase N (μmole nitroaniline/mg protein/minute) in the jejunum (*left*) and ileum of chicks on different diets.
a,b - Within the same intestinal site, mean values not sharing a superscript are significantly different (P<0.01 at the jejunum and P<0.001 at the ileum).

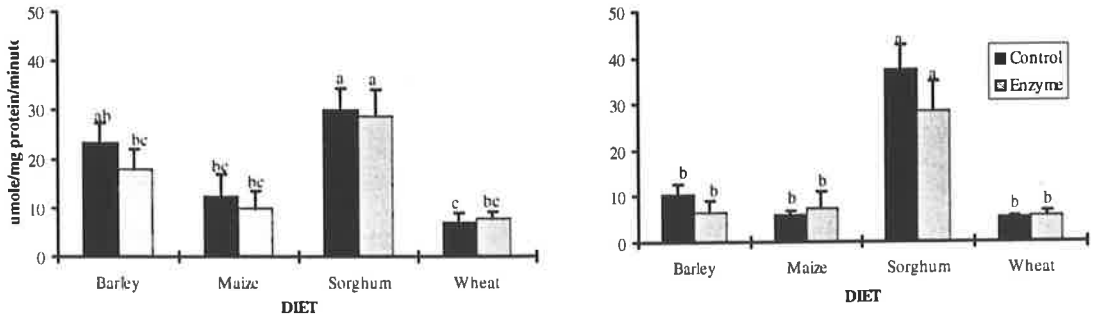


Figure 5.1.4: Specific activity of alkaline phosphatase (μmole nitrophenol/mg protein/minute) in the jejunum (*left*) of chicks on different diets.
a,b - Within the same intestinal site, mean values not sharing a superscript are significantly different (P<0.001).

Sucrase activity in the jejunum was significantly (P<0.001) affected by grain type but not microbial enzyme supplement (Figure 5.1.2). Activity was lower on the maize-based diet than on the other diets. Sucrase activity was significantly higher (P<0.001) in the ileum of chicks on the sorghum-based diets than on the other diets. The sucrase activity of chicks on the sorghum-based diet was significantly reduced (P<0.001) as a result of supplementation with the microbial enzyme.

The specific activity of jejunal APN was lower (P<0.01) in chicks on the maize-based diet than in chicks on the other diets, regardless of microbial enzyme supplementation (Figure 5.1.3). The microbial enzyme supplement had no significant effect on APN activity in chicks on any of the grains. APN activity was increased (P<0.001) in the ileum of chicks on the barley-based diet as a result of supplementation with the microbial enzyme. Chicks that were raised on the sorghum-based control diet showed similar levels of APN as those on the barley- and maize-based diets but these were significantly higher (P<0.001) than the activity observed in chicks on the wheat-based control diet.

There was a higher (P<0.001) specific activity of AP in both the jejunum and ileum of chicks that were fed the sorghum-based diet than chicks on the other diets (Figure 5.1.4). There was no significant effect of microbial enzyme supplementation on the specific activity of AP of chicks on any of the diets.

5.1.4.0. DISCUSSION

5.1.4.1. Feed quality, intake and utilization

Since the predominant carbohydrate in cereal grains is starch, the nutritive value of most cereals would be directly related to the efficiency of utilization of the starch component. This efficiency may, however, be dependent on the concentration and type of NSP in the grain. The poor growth of chicks on the barley-based diets in the present study is similar to the observations made by other researchers exploring low-cost alternatives to maize (Friesen *et al.*, 1992; Marquardt *et al.*, 1993; Almirall *et al.*, 1995). Barley is higher in total NSP than wheat, maize or sorghum (Carre and Melcion, 1995; Kopinski *et al.*, 1995) and this trend was generally observed in this study. Australian wheats have

been reported to vary widely in metabolizable energy content and this was partly attributed to the variable concentration of NSP (Annison, 1990; Choct and Annison, 1990). The wheat sample used in the present study has low NSP and thus supported growth to the same extent as maize or sorghum. Most cultivars of wheat and barley grown in Australia contain more total NSP than sorghum or maize (Choct and Annison, 1990; Kopinski *et al.*, 1995). The NSP of both wheat and sorghum are predominantly pentosan while barley contains about the same amounts of pentosans and β-glucans. The high concentration of glucose in the NSP profile of the barley-based diet would suggest that the sample contained more β-glucans than the other diets. Arabinose and xylose were also higher in the barley-based diet than in the other diets. Kopinski *et al.* (1995) indicated that the pentosan problem in barley may often be masked by the equally high content of β-glucans.

The DM digestibility in this study is a reflection of the level of availability of nutrients in the diets based on the different cereals. The level of *in vitro* digesta viscosity, although low, also reflects the trend *in vivo*, observed by other researchers in studies on the nutritive value of cereal grains for poultry (Carre and Melcion, 1995).

5.1.4.2. Response to microbial enzyme supplementation

Enzyme supplementation generally improved the performance of the chicks in terms of weight gain and feed conversion ratio. This is similar to the results obtained by other workers in research on similar diets and poultry (Bedford *et al.*, 1991; Bedford, 1995). The response obtained with microbial enzyme supplements usually depends on the nature of the NSP present in the diet. The enzyme used in this experiment is a

composite type, targeting pentosans, glucans and pectin (Choct *et al.*, 1996). Most researchers recommend the supplementation of cereal-based diets with composite enzymes which target more than one type of NSP (Annison *et al.*, 1996; Vranjes and Wenk, 1996).

Microbial enzymes improve feed utilization, as was observed in this study by generally enhancing the overall digestion of NSP and a reducing the digesta viscosity (Classen and Bedford, 1991; Almirall *et al.*, 1995). When Choct *et al.* (1996) included wheat soluble NSP extracts in sorghum-based diets, the digesta viscosity of broiler chickens was increased while AME, body growth and feed efficiency were adversely affected. The inclusion of Avizyme 1300, the same enzyme used in the present study significantly reduced the digesta viscosity.

Microbial enzymes may also improve the metabolizable energy of the diet through an increased fermentation of NSP in the hindgut (Rotter *et al.*, 1990; Choct *et al.*, 1996). In trials on rye and wheat and an enzyme supplement, Pettersson and Aman (1989) observed the solubilzation, *in vitro* of pentosans in the presence of a microbial enzyme with pentosanase and β-glucanase activities. *In vivo*, the enzyme supplement increased the digestibility of organic matter, crude protein and starch in the last third of the small intestine. Although the digestion of NSP was not measured in the present study, the improvement in growth and feed conversion efficiency (reduced feed conversion ratio) may have been achieved through an increased digestion of the NSP by the microbial enzyme. The digestion of NSP by microbial enzymes would result in the reduction of viscosity and this has been shown to improve the digestion and availability of other

nutrients including energy and mineral elements (Rotter *et al.*, 1990; van der Klis *et al.*, 1993).

5.1.4.3. Visceral organ weight and mucosal structure

There is a dearth of report on the effects of the major anti-nutritive components of cereals on visceral organ growth and intestinal structure and function in poultry. In the present trial, intestinal weight varied with the type of cereal grain in the diet but a reduction in such weight as a result of microbial enzyme supplementation only occurred on the diet containing barley. In trials on broiler chickens, Smits *et al.* (1997) observed an increase in both the empty and full weights of the small intestine and colon when carboxymethylcellulose was included in the diet. The full weight of the intestines may be increased by an increase in water consumption as well as differences in feed intake in the presence of NSP (Schutte *et al.*, 1993). The change in empty weight of the intestine is a better indication of actual changes in intestinal tissue growth. The results obtained in this experiment would reflect both changes in tissue growth and weight of lumenal contents. On the more viscous barley-based diets, the microbial enzyme supplement reduced the weight of the GIT. This is in contrast to reports by Francesch *et al.* (1994) who did not observe any effects of a crude enzyme preparation from *Trichoderma viride* (predominantly xylanase and β-glucanase) on carcass yield or visceral organ weight of broiler chicks on barley-based diets. The enzyme supplement, however, reduced the DM content of the faeces and incidence of sticky droppings. Some of the contrast in results may be due to the age of the birds and variety of cereal grain fed (Francesch *et al.*, 1994; Jeroch *et al.*, 1995).

There are few reports on the response of intestinal mucosa of chickens to various cereal grains *per se* although the effects of NSP in other animal species as well as chicken have been studied (Jacobs and Schneeman, 1981; Rubio *et al.*, 1989). A high content of rye grain (80 %) in the diet for chicks fed over a similar duration as in the present study resulted in severe damage to intestinal villi and mucous membrane of the small intestine (Rakovska *et al.*, 1990). The integrity of the mucosal structure of chicks in this study was not adversely affected although the grain content of all diets was less than 80 %. Villus height and surface area appeared to vary little with cereal type or enzyme treatment. The involvement of NSP on such changes is further examined in the next experiment.

5.1.4.4. Activity of digestive enzymes

There was a greater variation between the chicks in intestinal enzyme activity than was observed for intestinal weight and mucosal morphometry. NSP, like those present in cereals can physically complex with intestinal enzymes (Ikeda and Kusano, 1983) and cause a reduction in nutrient digestion. This may be responsible for the low activity of maltase in chicks on the barley-based diet without the microbial enzyme supplement.

Apart from maltase activity in the barley-fed chicks, microbial enzyme supplementation did not significantly enhance the activity of intestinal enzymes. Rather, there were reductions in the activity of some of the enzymes as a result of supplementation with the microbial enzyme. The effect of exogenous enzymes on the secretion of endogenous enzymes has not been extensively studied. Almirall *et al.* (1995) observed an increase in the activity of amylase and lipase in broiler chicks on barley-based diets supplemented with a β-glucanase. Endogenous enzyme secretions respond to both

nutrient concentration (Wiesenfeld *et al.*, 1993) and stress from dietary factors (Ikegami *et al.*, 1990). The nature of response can be confounded by the presence of both sets of factors. Dietary NSP have been shown to induce increased activity of pancreatic cells and this could lead to a reduction in the activities of intestinal enzymes (Ikegami *et al.*, 1990; Tivey and Shulman, 1991). Apart from possibly reducing the amount of substrate in the mid and lower intestine, pancreatic enzymes may reduce the activities of intestinal enzymes, especially disaccharidases through proteolysis (Tivey and Shulman, 1991).

The chicks on the sorghum-based diets consistently showed the highest specific activities of the intestinal enzymes assessed while activities were generally low in chicks on the barley- and maize-based diets. The low enzyme activity of chicks on the barley-based diets may be due to actual reductions in the efficiency of cellular synthesis of the intestinal enzymes. It is uncertain why there were reductions in enzyme activities of chicks on the maize- and sorghum-based diets. It is possible that the cellular potential for enzyme secretion was reduced as a result of high pre-jejunal digestion of starch and low concentration of same in the jejunum and ileum.

5.1.5. CONCLUSION

Some of the variation in the productivity of broiler chickens on diets based on different cereal grains could be traced to differences in the concentration and type of NSP both of which negatively impact starch digestibility. There is very little difference in weight of the GIT and mucosal morphometry of the small intestine. Supplementation with a microbial enzyme did not appear to affect intestinal mucosal growth in chickens on the same type of cereal. Enzyme activities varied with cereal type and may affect the

pattern of nutrient digestion. The nature of response to microbial enzyme supplementation appeared to vary with the type of cereal and NSP.

EXPERIMENT 5.2: INTESTINAL DEVELOPMENT AND BODY GROWTH OF BROILER CHICKS ON DIETS SUPPLEMENTED WITH NON-STARCH POLYSACCHARIDES.

5.2.1. INTRODUCTION

Non-starch polysaccharides (NSP) are present in several feed ingredients, ranging from cereal grains to legume seeds (Friesen *et al.*, 1992; Choct and Annison, 1992; Evans *et al.*, 1993; Mohamed and Rayas-Duarte, 1995b). This is a drawback to the exploitation of some of the low-cost alternative ingredients in poultry nutrition. The chemical nature of NSP differs from one ingredient to the other but most NSP generally increase digesta viscosity which interferes with nutrient intake and body growth (section 2.5.3.1). The effects of NSP on intestinal structure and function as well as measures aimed at reducing their impact on animal productivity have been highlighted in chapter two (sections 2.3.2.5 and 2.5.3.4).

In experiment 5.1, the variation in productivity of broiler chickens on various cereals was assessed. While this variation could be due to differences in the chemical nature of starch (Rogel, 1985; Longstaff and McNab, 1986), it could also be the result of differences in the type and concentration of NSP (Kopinski *et al.*, 1995; Smits and Annison, 1996). To define the latter problem, this experiment was conducted to examine the effects of different viscous NSP on the development of intestinal structure and functions. Although the effects of viscous NSP on poultry have been evaluated by previous researchers, the impact of these compounds at the intestinal level have not been widely reported. In the rat, viscous NSP were shown to increase intestinal weight and mucosal cell proliferation rate (Johnson and Gee, 1986; Brunsgaard *et al.*, 1995). Over

a longer duration of feeding as would obtain with a broiler breeder enterprise, the response to NSP may vary with duration of adaptation and response could be transient rather than permanent (Brunsgaard and Eggum, 1995).

The aim of this experiment was to determine the effects of pure viscous NSP on the productivity of broiler chickens and to examine the mechanisms at the intestinal level which regulate the gross response. The effect of duration of exposure was also assessed.

5.2.2. MATERIALS AND METHODS

5.2.2.1. Animals and diets

Seventy-two mixed-sex broiler chicks (Steggles x Ross F_1) which had been identically brooded to seven days of age were used in the study. The chicks were balanced for weight and randomly allocated to cages in lots of 6. The commercial broiler starter diet used in experiments 4.1-4.4 was diluted with alginic acid (AA; a non-viscous NSP), gum arabic (GA, predominantly galacto-araban), guar gum (GG, galacto-mannan) or gum xanthan (GX, gluco-mannan) at 5 % of the diet by weight. Three cage replicates were randomly assigned to each diet for a period of 7 days after which the dietary supplements were reduced to 2.5 % and the diets were fed for a further 14 days. The diets were analysed and found to be isocaloric and isonitrogenous in both phases (Tables 5.2.1 and 5.2.6). Feed consumption and body growth were also monitored.

5.2.2.2. Sample collection

At the end of each phase, three birds per cage (nine birds per treatment) were randomly selected, euthanatized and dissected as described in section 3.4.0. The joint weights of the proventriculus and gizzard as well as weight of small intestine were recorded, full

and empty. The liver was also weighed. Digesta was obtained from the two extreme ends of the small intestine, the duodenum and ileum, using 10 and 20 ml PBS (pH 7.4) respectively and frozen. Digesta were flushed rather than squeezed to avoid damage to the tissues which were subsequently used for histology.

5.2.2.3. Dietary analysis and utilization

Frozen digesta were thawed on ice and centrifuged to obtain the supernatant which was sub-sampled for viscosity measurement as described previously (3.3.5). The viscosity of supplemental NSP and diets was also determined on supernatants obtained from 1 and 10 % solutions respectively.

A sub-sample of ileal digesta was oven-dried at 80°C and analysed for starch digestibility as described by Lever (1972). Faecal samples were also collected over the last 24 hours of the second feeding phase (day 28) and analysed for dry matter content. Ileal and faecal digestibility of dry matter and starch was derived by difference between concentrations in feeds and faeces or digesta. Details of procedures for measuring starch content and digestibility are provided at section 3.3.1.

5.2.2.4. Histology

Intestinal mucosal morphometry was assessed as described in section 3.4.1. Cell size and metabolic activity were estimated through measurements of mucosal protein, DNA and RNA and the ratios between the three factors. The assays for these biochemical indices are described in 3.5.0 and in legends to the results.

5.2.2.5. Enzyme assays

Brush-border membrane vesicles (BBMV) were prepared as previously described by Shirazi-Beechey *et al.* (1991) and highlighted in section 3.7.0. The activities of the intestinal enzymes, maltase, sucrase, APN and AP were assessed as a measure of digestive function. The analytical procedures can be found at sections 3.7.2 - 3.7.4.

5.2.2.6. Amino acid uptake

The uptake of L-tryptophan by BBMV was assessed on samples collected at the end of both feeding phases. Uptake assays were conducted as described in section 3.8.1 at a uniform L-tryptophan concentration of 0.04 mM. The incubation buffer contained 200.06 mM mannitol, 20.0 mM HEPES and 50 mM NaCl, with an osmolality of 340 mosm/kg and pH 7.4. The assays were conducted at room temperature on vesicles from the jejunum and ileum.

5.2.3. RESULTS

The results are being presented separately for the two feeding durations. No comparisons were made between the two phases since the chicks were of different ages.

5.2.3.1. PHASE 1

5.2.3.1.1. Feed characteristics and digestibility

The viscosity of the supplemental NSP varied between 1.38 (AA) and over 2000 cP for GG and GX (Table 5.2.1). Whole diet viscosity ranged from 1.07 cP in the AA-supplemented diet to over 2000 cP in the GG and GX-supplemented diets. There were no significant differences in the viscosity of the duodenal digesta obtained from chicks

on the 4 diets. Ileal digesta viscosity, however, was significantly higher (P<0.001) in chicks that were fed the GX diet than in other chicks. Starch digestibility at the ileum was highest in chicks fed the GG diet and lowest in those on diet GX.

Table 5.2.1: Dietary quality, digesta viscosity and digestibility of diets fed during the first phase.

	DIET			
	AA	**GA**	**GG**	**GX**
Dry matter (g/kg)	905.0	902.0	908.0	907.0
Gross energy (MJ/kg)	18.5	18.6	18.1	18.7
Crude protein (g/kg)	203.0	194.0	205.0	196.0
Starch (g/kg diet)	502.2	517.2	519.7	515.1
Total NSP (g/kg)[1]	101.1	101.1	101.1	101.1
Starch digestibility (%)[2]	92.1	95.1	98.4	85.0
Viscosity (cP)				
Supplemental NSP	1.38	2.27	2000^{+}	2000^{+}
Entire diet	1.07	1.88	2000^{+}	2000^{+}
Duodenal digesta	1.0 ± 0.03	1.1 ± 0.07	1.3 ± 0.24	1.2 ± 0.25
Ileal digesta	1.6 ± 0.27^{b}	1.4 ± 0.17^{b}	2.5 ± 0.75^{ab}	4.2 ± 1.91^{a}

a,b - Mean values in the same row not sharing a superscript are significantly different (P<0.001). 1. The NSP were predominantly gluco-arabinoxylans. The unsupplemented diet had a total NSP content of 98.1 mg/g, a viscosity of 1.15 cP; its glucose, xylose and arabinose contents were 37.8, 35.2 and 28.7 mg/g respectively. 2. Digestibility at the terminal ileum. + - Viscosity was over the range of rheometer used.

5.2.3.1.2. Feed consumption and body growth

Body weight at the end of the first phase of feeding was significantly higher (P<0.001) on the GA diet than on the other diets (Table 5.2.2). Weight gain followed a similar trend. There were no significant differences between the diets with regards to absolute feed intake or feed intake in relation to body weight. Body weight and weight gain were lower on diets with the more viscous NSP, GG and GX. Chicks on the AA- and GA-

supplemented diets also had a significantly better (P<0.001) feed conversion ratio than chicks on diets containing GG and GX.

Table 5.2.2: Feed intake and growth of chicks on different diets during the first phase of feeding.

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
Initial weight (g)	149.0	150.9	147.8	149.8	2.54
Final weight (g)	267.2^{b}	302.6^{a}	195.0^{c}	199.2^{c}	7.49
Weight gain (g)	118.3^{a}	151.7^{a}	47.2^{b}	49.4^{b}	7.83
Feed intake (g/head)	295.8	326.0	280.4	283.4	26.96
Feed intake (g/100 g body weight[1])	198.5	216.0	189.7	189.2	17.13
Feed conversion ratio	2.50^{b}	2.15^{b}	5.94^{a}	5.74^{a}	0.466

1. Feed intake (g/head) in relation to initial weight. a,b - Mean values in the same row which do not share a superscript vary significantly (P<0.001).

5.2.3.1.3. Visceral organ weight and capacity

The combined weight of the proventriculus and gizzard as well as the weight of the liver was similar between treatments (Table 5.2.3). A similar trend was observed in the weight of digesta held by the proventriculus and gizzard. Small intestinal weight was higher (P<0.001) on the GG-supplemented diet than on the other diets. The capacity of the small intestine, in terms of digesta weight (gut-fill) was significantly higher (P<0.01) in chicks on diets containing GG and GX than in those supplemented with GA but were similar to those on diet AA.

Table 5.2.3: Empty weight of visceral organs (g/100 g body weight) and gut-fill of sampled chicks on different diets at the end of the first phase.

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
Proventriculus/gizzard	4.0	4.0	4.5	4.1	0.30
Small intestine	5.2^{bc}	4.8^{c}	7.5^{a}	6.6^{ab}	0.40***
Liver	3.3	3.3	3.5	3.4	0.22
Digesta weight (g/100 g body weight)					
Proventriculus/gizzard	1.8	1.2	2.1	1.5	0.47
Small intestine	2.3^{ab}	1.5^{b}	3.1^{a}	3.3^{a}	0.57**

a,b - Mean values in the same row which do not share a superscript vary significantly (**P<0.01; ***P<0.001).

5.2.3.1.4. Morphometry of the intestinal mucosa

The external muscle thickness and morphometry of the intestinal mucosa were identical between chicks on the four diets at the end of the first phase of feeding (Table 5.2.4). On the same diet, jejunal values were generally higher than ileal values. Ileal villi, for example were only half the length of the jejunal villi.

5.2.3.1.5. Digestive enzyme activity

The specific activity of maltase in the jejunum was highest (P<0.001) in chicks that were fed the AA-supplemented diet and lowest in chicks fed the GX-supplemented diet (Table 5.2.5). Sucrase activity in the jejunal BBMV was also higher (P<0.01) in chicks on the AA diet than in the other chicks. Alkaline phosphatase followed a similar trend, being higher (P<0.05) in chicks on the AA diet than in chicks on the other diets. In the ileum, chicks on the GX-supplemented diet elicited higher (P<0.05) APN activity than

chicks on the other diets. There were no significant differences between the chicks with regards to the activities of the other digestive enzymes measured in the ileum.

Table 5.2.4: External muscle thickness and intestinal mucosal morphometry on different diets during the first phase.

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
A. Jejunum					
External muscle (μm)	257.4	249.3	236.0	226.4	28.42
Crypt depth (μm)	157.9	159.7	176.6	162.2	14.31
Villus height (μm)	1169.9	1131.4	1018.0	981.6	112.83
Villus surface area (mm^2)	0.20	0.21	0.18	0.18	0.030
B. Ileum					
External muscle (μm)	204.0	226.3	223.1	269.7	31.37
Crypt depth (μm)	121.1	127.2	126.7	137.4	9.79
Villus height (μm)	582.1	604.3	564.5	539.0	62.46
Villus surface area (mm^2)	0.10	0.09	0.10	0.08	0.015

5.2.3.1.6. Amino acid uptake

The BBMV used for assessing L-tryptophan uptake were extracted with sucrase and AP enrichments of 2.4-6.1 and 3.5-7.0 folds respectively. There were no significant differences in L-tryptophan uptake between the diets at both intestinal sites (Figure 5.2.1). On the same diet except GA, uptake at the ileum tended to be higher than uptake at the jejunum but this was not statistically tested.

Table 5.2.5: Activity of brush-border membrane enzymes (μmole product/mg protein/minute) in the first phase.

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
A. Jejunum					
Maltase	20.2[a]	19.3[a]	16.9[ab]	11.3[b]	1.89***
Sucrase	1.1[a]	1.2[a]	0.9[ab]	0.6[b]	0.15**
Aminopeptidase N	6.5	6.7	6.2	5.2	1.08
Alkaline phosphatase	8.4[a]	8.3[a]	7.9[a]	3.6[b]	1.68*
B. Ileum					
Maltase	11.1	9.9	12.3	11.6	1.44
Sucrase	0.3	0.4	0.5	0.5	0.10
Aminopeptidase N	5.6[b]	10.0[a]	9.5[a]	11.5[a]	1.70*
Alkaline phosphatase	1.9	1.5	1.5	1.3	0.45

a,b - Mean values in the same row with different superscripts differ significantly (*P<0.05; **P<0.01; ***P<0.001).

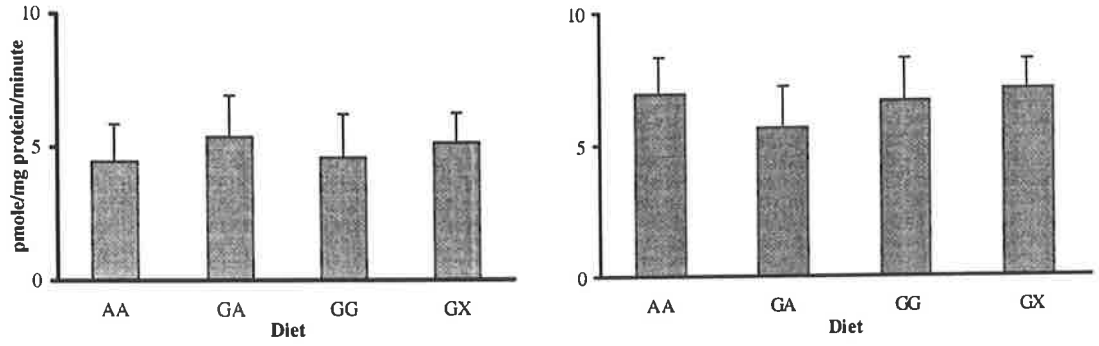


Figure 5.2.1. Uptake of L-tryptophan by jejunal (*left*) and ileal BBBV from chickens on different diets in the first feeding phase.

Samples from five chicks were used to test uptake at each intestinal region. Each sample was assayed in duplicate as described in section 3.8.1. The incubation buffers contained 0.04 mM L-tryptophan, 50 mM NaCl and 20 mM HEPES. Mannitol was included to maintain iso-osmolarity with the vesicle storage buffer.

Table 5.2.6: Dietary quality, digesta viscosity and starch digestibility of different diets fed during the second phase.

	Diet			
	AA	**GA**	**GG**	**GX**
Dry matter (g/kg)	925.0	922.0	926.0	925.0
Gross energy (MJ/kg)	18.0	18.0	18.3	18.1
Crude protein (g/kg)	199.0	200.0	198.0	205.0
Starch (g/kg)	555.1	556.4	511.8	514.9
Supplemental NSP (g/kg)[1]	100.6	100.6	100.6	100.6
DM digestibility (%)[2]	71.4	69.6	77.4	77.9
Starch digestibility (%)[3]	97.5	97.9	76.2	84.9
Faecal moisture (%)	73.5	72.0	79.1	79.6
Viscosity (cP)				
Whole diet	0.94	1.24	1300	2000^{+}
Duodenal digesta	1.0 ± 0.02^{b}	1.0 ± 0.01^{b}	1.3 ± 0.12^{a}	1.3 ± 0.15^{a}
Ileal digesta	1.7 ± 0.06^{b}	1.6 ± 0.10^{b}	3.4 ± 1.22^{b}	21.7 ± 4.56^{a}

1. The NSP profile was similar to that in the 5 % supplemental NSP diets shown in Table 5.2.1. 2. Faecal digestibility. 3. Digestibility at the terminal ileum. a,b - Mean values in the same row not sharing a superscript are significantly different (P<0.001). Statistical analysis were conducted only for viscosity for which samples were collected on bird replicate basis. Other assays were performed in duplicate from bulked samples.

5.2.3.2. PHASE 2

5.2.3.2.1. Feed characteristics and digestibility

The diet supplemented with GX showed the highest viscosity while diet AA had the lowest viscosity (Table 5.2.6). The digesta collected from the duodenum and ileum of birds fed the GX-supplemented diets were also significantly of higher (P<0.001) viscosity than digesta collected from the other chicks. Faecal moisture content followed a similar trend. The ileal digestibility of starch in the birds that were fed the AA- and GA-supplemented diets were comparable and this was reduced in the case of diets GG and GX. Faecal DM digestibility, on the other hand tended to be higher on the diets

containing the more viscous diets than in those with the less viscous diets. Faeces voided by chicks on the more viscous NSP tended to have a higher water content than faeces from chicks on diets AA and GA.

Table 5.2.7: Feed intake and growth of chicks on different diets during the second phase of feeding.

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
Initial weight (g)	293.1^{a}	341.4^{a}	214.3^{b}	215.4^{b}	15.18***
Final weight (g)	842.7^{a}	960.9^{a}	525.2^{b}	476.2^{b}	81.68***
Weight gain (g)	549.6^{a}	619.5^{a}	310.9^{b}	260.8^{b}	30.91***
Feed intake (g/head)	1220.0^{b}	1261.4^{b}	1296.0^{b}	1554.8^{a}	135.47*
Feed intake (g/100 g body weight)[1]	416.2^{bc}	369.5^{c}	589.2^{ab}	721.8^{a}	57.20***
Feed conversion ratio	2.22^{b}	2.04^{b}	4.06^{ab}	5.96^{a}	0.401***

1. Feed intake (g/head) in relation to initial weight. a,b - Mean values in the same row which do not share a superscript vary significantly (*P<0.05; ***P<0.001).

5.2.3.2.2. Feed consumption and body growth

Body weight at the start of the second phase of feeding differed significantly (P<0.001) between diets since chicks were maintained on the same diets fed during the first phase (Table 5.2.7). Final body weight and weight gain at the end of the second phase, as in the first phase were significantly higher (P<0.001) on the GA-supplemented diet than on the other diets. Absolute feed intake per bird (P<0.05) and feed intake per unit of initial body weight (P<0.001) were significantly higher in chicks on the GX-supplemented diet than on the other diets. These differences were reflected on the feed conversion ratio, which was higher (P<0.001) on the GX-supplemented diet than on the other diets.

5.2.3.2.3. Visceral organ weight and capacity

The weight of the proventriculus/gizzard and the liver were identical between chicks on the four diets (Table 5.2.8). Chicks on the GG-supplemented diet had a significantly higher (P<0.001) small intestinal weight than chicks fed the other diets. Small intestinal capacity was highest (P<0.01) in chicks on the GX-supplemented diet. No significant differences were observed between chicks on the diets with regards to digesta weight in the proventriculus/gizzard.

Table 5.2.8: Empty weight of visceral organs (g/100 g body weight) and gut-fill of sampled chicks on different diets during the second phase of feeding.

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
Proventriculus/gizzard	2.8	2.8	3.4	3.6	0.54
Small intestine	3.4^{b}	3.3^{b}	6.7^{a}	5.5^{ab}	0.67***
Liver	3.1	3.2	3.1	3.0	0.50
Ingesta weight (g/100 g body weight)					
Proventriculus/gizzard	1.4	1.3	1.4	2.4	0.71
Small intestine	1.9^{b}	1.7^{b}	3.3^{ab}	5.8^{a}	1.22**

a,b - Mean values in the same row which do not share a superscript vary significantly (**P<0.01; ***P<0.001).

5.2.3.2.4. Morphometry of the intestinal mucosa

The external muscle of the jejunum was significantly thicker (P<0.001) in chickens fed the GX-supplemented diet than in chickens on the other diets (Table 5.2.9). Jejunal crypts were also deeper (P<0.01) in chicks raised on the GX-supplemented diet than in the other chicks. There were no significant differences between the chickens with regards to jejunal villus height and villus surface area. At the ileum, the crypt depth of chicks on the GG-supplemented diet was larger (P<0.05) than that of chicks on the other

diets. Ileal villi were longer (P<0.05) in chicks fed the GA-supplemented diet than in other chickens. Chickens on the GA- and GG-supplemented diets had larger (P<0.05) villus surface area than chicks that were raised on the AA- and GX-supplemented diet.

Table 5.2.9: External muscle thickness and intestinal mucosal morphometry on different diets in the second phase.

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
A. Jejunum					
External muscle (μm)	213.0^{b}	230.7^{b}	216.9^{b}	319.4^{a}	22.33***
Crypt depth (μm)	148.0^{b}	157.5^{b}	170.8^{ab}	195.3^{a}	12.88**
Villus height (μm)	1444.2	1548.3	1354.0	1398.5	133.07
Villus surface area (mm^2)	0.25	0.27	0.27	0.32	0.044
B. Ileum					
External muscle (μm)	200.5	255.5	234.4	273.5	36.35
Crypt depth (μm)	107.9^{b}	122.5^{b}	155.2^{a}	134.6^{b}	13.53*
Villus height (μm)	563.0^{b}	733.1^{a}	677.9^{ab}	620.2^{ab}	56.52*
Villus surface area (mm^2)	0.10^{b}	0.12^{ab}	0.15^{a}	0.10^{b}	0.018*

a, b - Mean values in the same row not sharing a superscript differ significantly (*P<0.05; **P<0.01; ***P<0.001).

5.2.3.2.5. Biochemical indices of intestinal growth and metabolic activity

The protein content of the intestinal mucosa in both intestinal regions was unaffected by the nature of NSP in the diet (Table 5.2.10). At the jejunum, mucosal DNA (P<0.05) and RNA (P<0.001) contents were significantly higher in chicks that were fed the GA-supplemented diet than in those fed the other diets. The ratio between jejunal protein and DNA (cell size) was significantly higher (P<0.01) in chicks on the viscous NSP (GG and GX) than on diets with AA or GA. RNA:protein ratio (potential rate of protein synthesis) was reduced (P<0.01) on diets containing GG and GX but protein:RNA ratio

(actual protein synthesis) was significantly higher on the viscous NSP than on diets supplemented with lowly viscous NSP.

Table 5.2.10: Protein and nucleic acid contents of intestinal mucosa in chickens fed different diets (mg/g wet tissue).

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
A. Jejunum					
Protein	52.6	45.5	47.1	45.5	9.81
DNA	0.8^{a}	0.7^{ab}	0.5^{bc}	0.4^{c}	0.12*
RNA	2.7^{a}	2.6^{ab}	1.0^{bc}	0.6^{c}	0.40***
Protein:DNA ratio	64.4^{b}	66.8^{b}	107.2^{ab}	143.2^{a}	19.95**
RNA:protein ratio	0.06^{a}	0.06^{a}	0.02^{b}	0.02^{b}	0.010**
RNA:DNA ratio	3.4^{a}	3.7^{a}	2.0^{ab}	1.5^{b}	0.51**
Protein:RNA ratio	18.3^{b}	19.0^{b}	49.9^{ab}	81.0^{a}	12.38***
B. Ileum					
Protein	48.0	47.3	36.0	38.7	5.23
DNA	1.2	1.0	0.9	1.3	0.27
RNA	2.6^{a}	2.3^{ab}	1.3^{bc}	0.9^{c}	0.40**
Protein:DNA ratio	43.2	46.6	48.4	31.2	10.18
RNA:protein ratio	0.05	0.05	0.04	0.03	0.010
RNA:DNA ratio	2.3^{a}	2.3^{a}	1.4^{ab}	0.7^{b}	0.30***
Protein:RNA ratio	19.2^{b}	21.7^{b}	37.9^{ab}	47.3^{a}	6.59**

a,b,c - Mean values in the same row not sharing a superscript are significantly different (*P<0.05; **P<0.01; ***P<0.001).

In the ileum, RNA content (P<0.01) and RNA:DNA ratio (P<0.001) were reduced in chicks raised on the more viscous diets. Protein:RNA ratio in the ileum was, however, increased (P<0.01) by the more viscous diets compared to the less viscous diets. No significant differences were observed in the other variables.

5.2.3.2.6. Digestive enzyme activity

There were no significant differences between chicks on the four diets with regards to the specific activities of maltase, sucrase and APN in the jejunum (Table 5.2.11). Alkaline phosphatase activity in the jejunum was significantly higher ($P<0.01$) in chicks on the GX-supplemented diets than in the other chicks. Ileal maltase activity in the ileum was also significantly higher ($P<0.01$) in chicks on the GX diet than in chicks on the other diets. The chicks on all diets responded similarly in terms of the level of expression of the other enzymes in the ileum.

Table 5.2.11: Activity of brush-border membrane enzymes (μmole product/mg protein/minute) in the second phase.

	DIET				
	AA	**GA**	**GG**	**GX**	**SED**
A. Jejunum					
Maltase	15.5	14.2	15.5	17.1	2.87
Sucrase	0.8	0.6	0.9	0.9	0.15
Aminopeptidase N	11.8	9.7	11.3	9.2	2.26
Alkaline phosphatase	4.1^{b}	3.6^{b}	6.3^{ab}	10.2^{a}	1.66
B. Ileum					
Maltase	8.3^{b}	7.6^{b}	9.7^{ab}	11.8^{a}	1.01
Sucrase	0.4	0.3	0.4	0.4	0.04
Aminopeptidase N	3.3	3.3	2.9	3.5	0.34
Alkaline phosphatase	2.2	1.3	1.7	1.9	0.39

a,b - Mean values in the same row with different superscripts differ significantly ($P<0.01$).

5.2.3.2.7. Amino acid uptake

L-Tryptophan uptake by jejunal BBMV was higher (not significantly) in chicks on the diets supplemented with the more viscous NSP than on the diet supplemented with AA

(Figure 5.2.2). The uptake rate of the amino acid by ileal BBMV was significantly lower ($P<0.05$) in chicks on the GA-supplemented diet than in chicks maintained on the other diets.

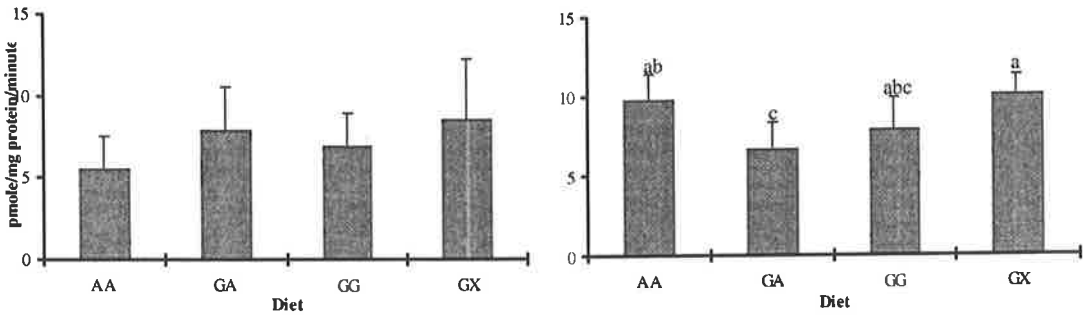


Figure 5.2.2. Uptake of L-tryptophan by jejunal (*left*) and ileal BBBV from chickens on different diets in the second feeding phase.
a,b,c - mean values not sharing a superscript are significantly different ($P<0.05$). Five chicks were used to test uptake at each intestinal region. The incubation buffers contained 0.04 mM L-tryptophan, 50 mM NaCl and 20 mM HEPES. Mannitol was included to maintain iso-osmolarity with the vesicle storage buffer.

5.2.4. DISCUSSION

5.2.4.1. Feed characteristics and utilization

Guar gum (GG) and gum xanthan (GX) were the most viscous of the four NSP tested; there was very little difference in viscosity between the control NSP, alginic acid (AA) and gum arabic (GA). Although the more viscous diets did not affect feed intake over the short duration of exposure, they reduced weight gain and final body weight, suggesting a reduction in nutrient derivation and utilization. This is reflected in the poor feed conversion efficiency (high feed conversion ratio) on the highly viscous diets. There is usually a reduction in digestion and absorption of dietary nutrients in the presence of NSP and increased viscosity (Fengler and Marquardt *et al.*, 1988; Rakovska *et al.*, 1990; Annison, 1993; van der Klis *et al.*, 1993). In the present study, there was little variation in starch digestibility after 7 days on the diets but this was reduced,

especially on the GG- and GX-supplemented diets, following a longer period of exposure to the diets. The specific activities of intestinal membrane-bound carbohydrases were increased following long term feeding of the diets. The enzymes may, however, be unable to access the substrate properly as a result of high digesta viscosity, especially at the ileum. This will tend to reduce the rate of digestion, as was observed for starch.

Compared with the level of productivity observed on the unsupplemented commercial diet in chapter 4 or diets utilized in experiment 5.1, the performance of chicks in this experiment is poor. The fact that productivity on AA and GA was also affected may suggest the involvement of other factors other than viscosity. There may, for example, be an energy dilution involved, for similar amounts of feed ingested. The gross energy content of all diets in this experiment is lower than the value for the unsupplemented finisher diet that was fed in experiment 4.5 (Appendix 1). The AME value (not assessed) may even be lower as a result of the NSP included. The impact of the highly viscous NSP, GG and GX is also obvious from the low body weights and low efficiency of feed utilization. The reduction in NSP level after the first phase was necessitated by the drastic reduction in the growth of the chicks, especially on diets GG and GX.

The negative impact of viscosity on productivity has been confirmed in studies in which wheat pentosans were included in diets for broiler chickens (Choct and Annison, 1992). The extracts increased digesta viscosity and reduced body growth. These effects were largely eliminated through depolymerization of the pentosans with a β-xylanase. The digesta viscosity values shown in this experiment have been under-estimated through dilution with PBS. Higher values would have been obtained if the digesta were

squeezed out rather than flushed but there was a need to reduce damage to the mucosa of tissues that were subsequently used for histology.

Although NSP digestibility was not measured, the results suggest a lack of influence of the NSP on starch digestibility within the first phase. In pigs, the digestibility of cereal NSP varies with the chemical nature of the NSP and is usually in the order of β-glucans>arabinoxylans>cellulose (Bach Knudsen, 1991). The diets did not vary in NSP concentrations although there were minor variations in the chemical nature of the NSP. There was a also very little change in NSP concentrations between the diets supplemented at 5 % and those supplemented at 2.5 %. This may be attributable to the rather high concentration of NSP in the commercial diet. The viscosity of the commercial diet was, however, lower than that of diets that were fed in any of the experiments. Most of the viscosity was derived from the supplemental NSP and this was in excess of the capacity of the rheometer used in the case of GG and GX.

The viscosity of NSP depends on several factors including water solubility of total NSP and its components as well as molecular weight of the NSP (Bedford and Classen, 1992; Choct and Annison, 1992; Ward, 1996). In arabinoxylans, it is the arabinose side chains that impart the water-soluble nature to the NSP; in the absence of arabinose, xylan is insoluble and precipitates without inducing tangible viscosity. The molecular weight of the compounds are also directly related to their viscosity in solution (Bedford and Classen, 1992; Choct and Annison, 1992). NSP of high molecular weights impart considerable viscosity, even when they are present at a low concentration in a mixture (Classen and Bedford, 1992). These factors should be borne in mind when predictions of animal productivity are made from absolute values of NSP.

5.2.4.2. Visceral organ weight and intestinal development

The viscous NSP increased the weight of the proventriculus, gizzard and small intestine but not the weight of the liver. These changes would be due to an actual increase in the rate of cell proliferation, cell size or protein synthesis since empty weights were affected. Johnson *et al.* (1984) observed an increase in intestinal length of rats when fed diets that were supplemented with guar gum and traced this to an increase in mitotic activity within the mucosa.

In the present study, the diets containing the viscous NSP tended to increase crypt depth, especially after a long duration of feeding, although this effect was translated into longer villi only at the ileum. At the jejunum, there was a reduction in DNA concentration (cell population) but this was compensated by the increase in cell size, to maintain similar villus length between chicks on the different diets. The increase in crypt depth in both regions in the presence of highly viscous NSP would suggest a high potential for cell proliferation although this was not supported by an increase in cell population at either site. An increased proliferation of enterocytes could be accompanied by increased migration and extrusion rates but this may be able to enhance villus length, as was observed in the ileum. In rats on diets supplemented with pectin and guar gum, Jacobs (1983) observed an increase in crypt depth but a reduction in villus height which was attributed to an increase in the rate of cell migration and extrusion. Research on other species has shown a greater impact of NSP in the distal than in the proximal GIT (Jacobs and White, 1983; Johnson and Gee, 1986; Brunsgaard and Eggum, 1995). These differences may be due to increased solubilization and viscosity of NSP as the material approaches the distal GIT. There is usually an improvement in the digestion of

NSP towards the lower GIT, through the contribution of a large microbial population. The increased digestion of NSP provides energy-yielding products. In various species, including the rat and chickens, high energy diets have been shown to support increased mucosal growth (Moore *et al.*, 1989; Yamauchi *et al.*, 1996). Mucosal growth may also be enhanced through the indirect effects of hormones such as thyroxine and tri-iodothyronine which are involved in energy metabolism (Yeh *et al.*, 1989; Yeh *et al.*, 1991; Tivey *et al.*, 1993).

The increase in intestinal weight and changes in mucosal structure can be effected by various mechanisms. In the presence of soluble NSP, gastrointestinal and associated cells compensate for the reduction in digestion and absorption by increased cellular hyperplasia and hypertrophy (Ikegami *et al.*, 1990). Dietary fibres may also alter intestinal growth indirectly through interactions with mucosal growth factors. Both intragastric and parenteral administrations of oat β-glucans were shown to increase the levels of immunoglobulin G, G-1 and M in mice (Yun *et al.*, 1997). The influence of different fibres on insulin binding in the rat GIT has been highlighted (MacDonald *et al.*, 1991). Supplementation with guar gum led to a 40 % reduction in the binding of insulin to receptors in the jejunum, compared with the effect of a diet without fibre supplement. Pell *et al.* (1992) reported an increase in enteroglucagon and gastrin when diets containing guar gum were fed to rats. These hormones are involved in regulation of sugar levels in the GIT in addition to changes in the motility of the intestinal tract. They would therefore directly influence substrate digestion as well as absorption and indirectly influence mucosal structure. Another group of agents that may induce intestinal mucosal growth following NSP digestion are short-chain-fatty acids, generally

known to promote cell proliferation (Tulung *et al.*, 1987; Mathers and Kennard, 1993; Sakata *et al.*, 1995).

5.2.4.3. Digestive enzyme activity

In previous research on various pure NSP, including guar and locust bean gums, Annison (1993) was of the opinion that the anti-nutritional effects may not be solely due to energy dilution or viscosity but to specific interaction with some intestinal components. In the present study, there was a reduction in the activity of maltase and sucrase in chicks on the highly viscous diets but these differences had been eliminated or reversed after prolonged exposure to the diets. The initial reduction in enzyme activity is similar to the results obtained by Johnson *et al.* (1984) who observed a reduction in the specific activity of lactase and AP in rats on diets supplemented with guar gum. The change in trend may be due to either an adjustment to the diets or the reduction in the content of supplemental NSP used in the second phase. The changes in the activities of intestinal enzymes may also be brought about by increased secretions of pancreatic enzymes in the presence of NSP (Ikegami *et al.*, 1990). The direct negative effects of some pancreatic enzymes on intestinal enzymes have also been highlighted in experiment 5.1 (Tivey and Shulman, 1991).

There are no reports on the direct inhibition of intestinal enzyme synthesis by NSP but the activities of most enzymes may be reduced through coupling to NSP or physical restriction of enzyme access to substrates (Ikeda and Kusano, 1983; Pettersson and Aman, 1989). The differences in enzyme activities of chicks on the different diets observed in the present study, especially during the first phase may, however, suggest some effect of the NSP on enzyme synthesis. Pancreatic-bile secretions in rats on diets

containing the viscous NSP, sodium alginate, locust bean gum, gum xanthan and guar gum were increased mainly as a result of cellular hypertrophy (Ikegami *et al.*, 1990). In the present study, there was an increase in jejunal enterocyte size (protein:DNA ratio) over long term feeding of the highly viscous NSP. It is not known if such a change led to enhanced synthesis of intestinal enzymes, responsible for the increased specific activities of some of the enzymes observed in that phase.

5.2.4.4. Amino acid uptake

There was no definite trend in the effects of the viscous NSP on amino acid uptake by the brush-border membranes. No previous research on the effect of NSP on amino acid transport in poultry has been reported. In rats, the inclusion of guar gum in the diet has been observed to reduce the transport of glucose into intestinal tissue (Johnson *et al.*, 1984). Reports on depressed absorption of nutrients, including amino acids are more common and may be due to an increase in digesta viscosity and a reduction in movement of nutrients through a dense unstirred water layer rather than changes in the transport capacity of the mucosal membrane (Tulung *et al.*, 1987; Bamba *et al.*, 1993; van der Klis *et al.*, 1993). This effect was demonstrated *in vitro* by Read (1987) who showed a reduction in glucose movement in solutions of xanthan, guar and locust bean gums, individually or when combined.

In pigs, van Barneveld *et al.* (1994) observed a reduction in the ileal digestibility of eight amino acids, including lysine and threonine with an increase in the concentration of GG in sorghum-based diets. Low concentrations of amino acids in the intestinal lumen would limit the rate of absorption from the intestine (Stevens *et al.*, 1984). Over two sampling periods during the day, digestibility was improved at the later collection

(van Barneveld *et al.*, 1994), indicating possibly, an increased adaptation to the NSP or diurnal effects.

5.2.5. CONCLUSION

The results obtained in this experiment tend to suggest strong relationships between NSP and dietary viscosity and the growth of broiler chickens. Although the viscous NSP did not appear to adversely affect DM digestibility, the digestibility of starch was reduced and this may be the case with other key dietary components. Enzyme activities were generally reduced over the short term but tended to recover as the chicks adjusted to the diets. The gross weight of the intestine and distal intestinal morphometry were increased by the highly viscous NSP although the exact mechanism by which this change was effected was unclear. The uptake capacity of the brush-border membrane for L-tryptophan was also not affected by the nature of the NSP, suggesting that changes in absorption on such diets may be regulated more by solute movement through the digesta than through changes in the rate of intestinal transport.

5.3.0. EXPERIMENT 5.3: RESPONSE OF BROILER CHICKS TO SHORT-TERM FEEDING OF WHOLE OR OLIGOSACCHARIDE-FREE LUPIN-SUPPLEMENTED DIETS.

5.3.1. INTRODUCTION

Poultry diets are formulated to minimize costs and optimize productivity. As a result there is currently an increased interest in alternative protein sources (Petterson and Mackintosh, 1994). The low productivity of poultry on alternative diets, including those supplemented with lupins has been reported by previous researchers although the mechanisms involved are not clearly understood (Watkins *et al.*, 1988; Brenes *et al.*, 1989; Saini, 1989). The low nutritive value of legume seeds has been variously attributed to the presence of NSP as well as raffinose series oligosaccharides (Saini, 1989; Brenes *et al.*, 1993; Evans *et al.*, 1993). While the negative impact of NSP in cereals and other ingredients has been established, there is still some controversy as to the effects of oligosaccharides on diet quality and animal growth (Coon, 1990; Leske *et al.*, 1993; Irish *et al.*, 1995). The major link between oligosaccharides and NSP is the resistance of both to endogenous animal enzymes.

Recent research has therefore, focused on establishing the effects of raffinose series oligosaccharides in lupins and other legume seeds on the nutritive quality of diets and poultry productivity (Bryden *et al.*, 1994; Slominski *et al.*, 1994; Irish *et al.*, 1995; Oyarzabal and Conner, 1995). Some of the gross effects of oligosaccharides on diet quality are known but there is less agreement on their effects on animal growth. At the intestinal level, oligosaccharides may alter microbial populations, pH and water balance

but the effects on the intestinal structure and function have been less studied (Choi *et al.*, 1994; Okumura *et al.*, 1994; Oyarzabal and Conner, 1995).

The main objective of the present study was to ascertain the effects of short-term feeding of lupin seed meals from which raffinose series oligosaccharides had been extracted on broiler chick growth. The changes in the structure and digestive function of the small intestine was also assessed and related to diet quality and animal growth.

5.3.2. MATERIALS AND METHODS

5.3.2.1. Animals and diets

Ninety-six mixed-sex Inghams IM98 (Inghams Enterprises Pty Ltd) broiler chicks (initial weight 942.8 g), 21 days old of age were used for this experiment. The experiment was conducted in conjunction with researchers at the Pig and Poultry Production Institute (PPPI), South Australia as part of a major project aimed at determining the nutritive value of Australian-grown lupins. There does not seem to be any major differences in productivity between IM98 and the Steggles x Ross and both have been extensively used to determine the apparent metabolizable energy (AME) of new ingredients and diets at the PPPI (R.J. Hughes, pers. comm). The chicks were randomly assigned in batches of 4 to multi-bird metabolic cages and provided one of four diets. There were 6 replicates (cages) per diet. The experiment was designed primarily for classical AME evaluation.

The four diets were based on sorghum (543 g/kg) and casein (91g/kg). In addition, the diets were supplemented with de-hulled seed meals from two cultivars (Danja and Gungurru) of *L. angustifolius* (300 g/kg). Both cultivars, Gungurru and Danja were

used as whole meals (diets Gung and Danja respectively) or as meals from which most of the oligosaccharides had been removed (diet Gung-OF and Danja-OF respectively) through solvent (ethanol) extraction. Each diet was further supplemented with (g/kg): dicalcium phosphate (26.0), limestone (11.0), celite marker (26.0), vitamin premix (5.0), sodium chloride (3.6) and choline chloride (0.4).

The nutrient compositions of the seed meals and diets were analysed. The diets were cold-pelleted and fed over 7 days, consisting of 3 days of adaptation, followed by 4 days of total faecal collection and measurement of feed consumption. Rearing temperature was 23. 5 ± 1.5°C and artificial light was provided for 23¾ hours per day.

5.3.2.2. Sample collection

At the end of the feeding period, the birds were euthanatized and dissected to enable collection of intestinal samples as described in section 3.4.0. Digesta were collected from the ileum and kept on ice prior to measurement of viscosity on the day of collection (section 3.3.5). Sub-samples of intestinal tissue, about 10 cm long were taken from the proximal end of the duodenum, jejunum and ileum and flushed with ice-cold PBS (pH 7.4). Tissue samples were processed for histology and enzyme histochemistry as described below.

5.3.2.3. Histology

Intestinal mucosal morphometry was assessed as described in section 3.4.1. Crypt depth, villus height and apparent villus surface area were measured on tissues from the duodenum, jejunum and ileum.

5.3.2.4. Enzyme histochemistry

The activities of digestive enzymes on the brush-border membrane were quantified using the histochemical techniques described in section 3.6.0. The activities of AG, APN and AP were measured, in relation to both total villus length and unit area of the villus (per cell).

5.3.3. RESULTS

5.3.3.1. Feed quality, utilization and body growth

The extraction of oligosaccharides from seed meals of both varieties of lupins increased the gross energy (GE) but reduced the crude protein and AME contents of the diets (Table 5.3.1). Feed intake by chicks on the diets containing the extracted meals was significantly lower ($P<0.05$) than that observed on diets with the unextracted seed meals. Body weight gain was significantly higher ($P<0.05$) on the unextracted lupin diets than on the diets supplemented with the extracted meals. Supplementation with the extracted lupin meals led to marginal increases in feed conversion ratio (reduced feed conversion efficiency), compared with diets supplemented with unextracted meals. There were no significant differences between the groups in faecal moisture content but ileal digesta viscosity was increased ($P<0.05$) in chicks maintained on the diets supplemented with the extracted seed meals.

Table 5.3.1: Feed intake, weight gain, feed conversion and apparent metabolizable energy of diets.

	DIET			
	Gung-OF	**Gung**	**Danja-OF**	**Danja**
Initial weight (g)	949 ± 26	946 ± 18	940 ± 43	937 ± 22
Weight gain (g)	376 ± 52^{b}	429 ± 18^{a}	378 ± 43^{b}	420 ± 34^{a}
Gross energy (MJ/kg DM)[1]	17.1	16.4	17.0	16.2
AME (MJ/kg DM)	13.0 ± 0.3	13.8 ± 0.3	12.6 ± 0.5	13.4 ± 0.3
Crude protein (g/kg DM)[2]	234.9	250.5	222.0	239.1
Feed intake (g/day)	111 ± 8^{b}	123 ± 5^{a}	111 ± 10^{b}	122 ± 7^{a}
Feed conversion ratio	2.15 ± 0.25	2.01 ± 0.08	2.13 ± 0.19	2.04 ± 0.06
Faecal moisture (%)	74.0 ± 3.0	73.0 ± 2.0	74.0 ±3.0	74.0 ± 3.0
Ileal digesta viscosity	15.2±10.2^{ab}	6.8 ± 4.0^{c}	17.6 ± 7.0^{a}	8.4 ± 3.0^{bc}

a,b,c - Mean values in the same row not sharing a superscript are significantly different (P<0.05). Gung-OF and Danja-OF are *L. angustifolius* cv. Gungurru and Danja without oligosaccharides respectively. Results are mean ± SD. The gross energy (GE) of the diets was determined on an adiabatic bomb calorimeter. The crude protein (CP) content of individual ingredients was determined and the values used to calculate diet CP; no statistical tests were conducted on GE and CP contents.

5.3.3.2. Morphometry of the intestinal mucosa

No significant differences were observed in the morphometry of the intestinal mucosa of chicks as a result of supplementing the diets with extracted lupin seed meals from both cultivars of lupins (Table 5.3.2). The major differences in mucosal structure were between intestinal regions. Both the crypt depth and villus height decreased (P<0.001) distally from the duodenum to the ileum.

Table 5.3.2: Effects of whole or oligosaccharide-free lupins on intestinal mucosal morphometry (mean ± SD μm).

	DIET			
	Gung-OF	**Gung**	**Danja-OF**	**Danja**
A. Duodenum				
Crypt depth	275.0 ± 40.93	282.0 ± 45.20	280.5 ± 35.19	275.7 ± 18.24
Villus height	1468.1 ± 66.94	1541.4 ± 61.8	1589.4 ± 119.02	1468.6 ± 71.78
Villus surface area	0.23 ± 0.032	0.23 ± 0.017	0.22 ± 0.017	0.20 ± 0.021
B. Jejunum				
Crypt depth	192.7 ± 41.04	191.6 ± 19.38	190.1 ± 12.89	166.6 ± 19.66
Villus height	1023.9 ± 158.83	983.5 ± 108.34	998.9 ± 97.28	1040.8 ± 55.04
Villus surface area	0.15 ± 0.031	0.13 ± 0.013	0.15 ± 0.005	0.13 ± 0.008
C. Ileum				
Crypt depth	154.9 ± 34.84	176.9 ± 38.55	170.1 ± 22.02	157.4 ± 15.36
Villus height	737.1 ± 196.70	810.0 ± 161.04	873.5 ± 83.62	837.9 ± 118.93
Villus surface area	0.12 ± 0.041	0.12 ± 0.032	0.12 ± 0.018	0.11 ± 0.012

Five chicks were assessed per diet; 12-15 villi were measured per sample. Results are mean ± SD.

5.3.3.3. Expression of digestive enzyme activity

When enzyme activities were expressed per unit villus surface area (per cell), no dietary effects were observed on the expression of AG or APN in all three intestinal regions (Table 5.3.3). Chicks raised on Danja-OF expressed significantly lower ($P<0.001$) activity of AP per unit villus surface area than chicks on the other diets but there were no precise effects of oligosaccharide extraction on AP activity per cell. On the Danja-supplemented diet, AP activity was reduced in chicks on the diets supplemented with the extracted meals while there was an increase in the case of cultivar Gungurru. There were also no significant differences between the chicks on the four diets with regards to the activities per cell of any of the digestive enzymes in the jejunum or ileum. Regardless of dietary treatment, AG activity was higher in the jejunum than in the

duodenum or ileum. The activity of APN tended to be higher in the ileum than in the proximal small intestinal regions. The region of highest activity, in the case of AP tended to vary with dietary treatment. The differences between intestinal regions were not statistically tested.

Table 5.3.3: Expression of intestinal enzymes (Ab./μm^2) in chicks on diets with whole or oligosaccharide-free lupins.

	DIET			
	Gung-OF	**Gung**	**Danja-OF**	**Danja**
A. Duodenum				
AG	0.44 ± 0.066	0.42 ± 0.094	0.43 ± 0.032	0.45 ± 0.048
APN	0.34 ± 0.050	0.43 ± 0.133	0.48 ± 0.107	0.43 ± 0.138
AP	0.33 ± 0.018^{a}	0.20 ± 0.019^{ab}	0.14 ± 0.027^{b}	0.24 ± 0.031^{ab}
B. Jejunum				
AG	0.49 ± 0.034	0.54 ± 0.093	0.52 ± 0.099	0.57 ± 0.066
APN	0.41 ± 0.093	0.45 ± 0.189	0.32 ± 0.061	0.42 ± 0.143
AP	0.18 ± 0.057	0.18 ± 0.067	0.17 ± 0.072	0.19 ± 0.086
C. Ileum				
AG	0.50 ± 0.116	0.42 ± 0.021	0.48 ± 0.118	0.49 ± 0.091
APN	0.38 ± 0.083	0.58 ± 0.120	0.47 ± 0.094	0.45 ± 0.133
AP	0.23 ± 0.089	0.27 ± 0.047	0.17 ± 0.023	0.16 ± 0.043

a,b - Mean values in the same row not sharing a superscript are significantly different ($P<0.001$). Results are mean ± SD. Four chicks were assessed per diet and five well presented villi were measured per sample.

The data generated per unit surface area were summed over the entire villus to yield total enzyme activities per villus shown in Table 5.3.4. In the duodenum, the lowest ($P<0.001$) total activity of AP was observed in chicks that were fed on the diet supplemented with extracted Danja meal. There were no differences between the treatment groups with regards to the total activity of AP in the jejunum and ileum or the

activities of the other enzymes in any of the intestinal regions. Irrespective of dietary treatment, the total activities of the three enzymes were generally higher in the duodenum than in the jejunum or ileum, due mainly to regional differences in villus length.

Table 5.3.4: Total activities (Ab./villus) of α-glucosidase (AG), aminopeptidase N (APN) and alkaline phosphatase (AP) in chicks on diets with whole or solvent-extracted seed meals.

	DIET			
	Gung-OF	**Gung**	**Danja-OF**	**Danja**
A. Duodenum				
AG	64.6 ± 12.21	49.6 ± 8.80	55.0 ± 4.06	54.9 ± 7.31
APN	46.8 ± 11.18	56.4 ± 10.53	66.2 ± 10.91	60.0 ± 11.08
AP	41.9 ± 7.11[a]	20.1 ± 5.65[b]	18.5 ± 5.84[b]	30.6 ± 3.03[ab]
B. Jejunum				
AG	36.7 ± 4.51	33.8 ± 7.71	38.7 ± 5.56	42.2 ± 9.88
APN	30.1 ± 6.80	32.8 ± 7.98	24.7 ± 2.40	32.8 ± 5.17
AP	15.2 ± 3.87	15.3 ± 3.38	11.5 ± 3.19	13.4 ± 3.38
C. Ileum				
AG	26.7 ± 6.34	22.7 ± 3.83	27.2 ± 3.45	25.1 ± 4.90
APN	21.9 ± 4.23	34.4 ± 8.37	28.4 ± 7.62	26.0 ± 6.03
AP	15.5 ± 4.22	16.8 ± 3.86	11.2 ± 2.37	11.1 ± 2.51

a,b - Mean values in the same row not sharing a superscript are significantly different ($P<0.001$). Results are mean ± SD. Total villus activity was generated as the summation of enzyme activity (absorbance) per unit area which was measured as described for Table 5.3.3.

5.3.4. DISCUSSION

5.3.4.1. Dietary quality and growth of chicks

The lupins tested in this experiment are two important cultivars of *L. angustifolius* grown in Australia (Evans *et al.*, 1993; Petterson and Mackintosh, 1994). Both cultivars

have been observed to contain similar levels of oligosaccharides (about 8 % of the cotyledonary dry matter), mainly in the form of raffinose and stachyose (Saini and Gladstones, 1986; Evans *et al.*, 1993). Cultivar Danja has slightly higher levels of total NSP but lower soluble NSP than Gungurru.

Most lupins cannot be used beyond 10 % in poultry diets without detrimental effects on productivity (Bogdanov, 1988; Centeno *et al.*, 1990; Petterson and Mackintosh, 1994). It was possible to test the seed meals at the high level (30 %) probably as a result of preliminary de-hulling of the seed. De-hulling has been observed to improve the quality of lupin seed meals since most of the NSP are found in the hulls while the oligosaccharides are more concentrated in the cotyledons (Brenes *et al.*, 1993; Evans *et al.*, 1993; Mohamed and Rayas-Duarte, 1995a).

The extraction of the oligosaccharides led to an improvement in the gross energy of seed meals from both cultivars individually but depressed both the AME and CP (data not shown). This was reflected in the composition of the entire diets and is in contrast to the findings of Leske *et al.* (1993) who observed an increase in the gross energy of soyabean concentrate following the addition of raffinose series oligosaccharides to diets for roosters. Leske *et al.* (1993), however reported a reduction in the true metabolizable energy (TMEn) similar to the reduction in AME observed in the present experiment. Similar changes in ME have been reported by Slominski *et al.* (1994) and Irish *et al.* (1995) in studies on soyabean and canola meals respectively. Slominski *et al.* (1994) also observed an increase in the NSP content of canola seed meal, following extraction with ethanol. An increase in relative NSP content would increase digesta viscosity, as

was observed in chicks on the diets supplemented with the extracted meals in this experiment.

Further contradictions exist with regards to the effects of solvent extraction on other dietary components. In research on soyabean meal, Irish *et al.* (1995) observed an increase in the crude protein content of meals, following extraction with ethanol. Similar changes were reported by Slominski *et al.* (1994) in studies on canola meal and are in contrast to the results obtained in the present study. The increase in protein content of extracted meals may fail to enhance broiler chicken growth as a result of a reduction in the solubility of such protein (Irish *et al.*, 1995). Oligosaccharides are part of the carbohydrate component and may provide some benefit when digested by resident microbes in the lower intestine (Biviano *et al.*, 1993). This was adequately borne out by results obtained in this experiment where AME, crude protein, feed intake and growth rate were depressed as a result of solvent extraction of the meals prior to feeding. The indirect effects of oligosaccharides on animal productivity have been highlighted in section 2.5.3.2 and are further discussed in experiment 5.4. The negative effects of oligosaccharides reported by previous researchers in the presence of lupins and other legume seeds may be due to other factors, including NSP (Centeno *et al.*, 1990; Brenes *et al.*, 1993).

In the present study, chicks on all diets voided equal amounts of water in their droppings. The passage of watery droppings is one of the major symptoms of the presence of NSP in the diet, as was observed with the pure viscous NSP supplements in experiment 5.2. Oligosaccharides may also alter the osmotic potential of the lower GIT and induce the accumulation and passage of water in droppings (Wiggins, 1984). The

similarity in faecal moisture content of chicks on all diets would suggest that oligosaccharides are not particularly responsible for increased water excretion when lupins and other legume seed meals are fed to broiler chicks.

5.3.4.2. Structure and function of the intestinal mucosa

The two cultivars of *L. angustifolius* tested generally had similar effects on intestinal structure and enzyme function of broiler chicks, irrespective of the nature of seed processing. Lupins are higher in NSP than most cereals and these could possibly affect mucosal structure, as was observed over the long term supplementation of diets with pure NSP in experiment 5.2. Over the short-term, pure NSP had no effects on mucosal structure. It is not certain if longer periods of adjustments are required in the case of lupin NSP or oligosaccharides. In experiment 4.2, it was observed that enterocytes of 21-day-old chicks had migrated more than three-quarters of the crypt:villus axis within 96 hours of the incorporation of BrDU at the S phase. The duration of the present experiment was therefore long enough for many enterocytes to have been formed and exposed to the effects of the diets, although longer durations may be required between the first effects of the diet and response by the cells or of the entire mucosa.

5.3.5. CONCLUSION

The extraction of raffinose series oligosaccharides from lupin seed meals improved the gross energy content of the diets but this was associated with a reduction in metabolizable energy and crude protein values and tended to reduce broiler chicken growth. Intestinal structure and enzyme function were not adversely affected by the presence of the oligosaccharides in the diets over the short term. It would be desirable to assess these effects over a longer duration of feeding.

5.4.0. EXPERIMENT 5.4: THE RESPONSE OF BROILER CHICKENS TO SORGHUM-LUPIN DIETS SUPPLEMENTED WITH A MANNAN OLIGOSACCHARIDE.

5.4.1. INTRODUCTION

Oligosaccharides occur naturally in many feed ingredients and were traditionally classified within the indigestible feed fraction collectively known as fibre (Frolich, 1996). The most common natural forms are the raffinose series oligosaccharides (Figure 2.3), described in section 2.5.3.2. As previously indicated, raffinose series oligosaccharides do not succumb to animal enzymes and tend to accumulate towards the posterior end of the GIT where they are digested by microbial enzymes, to release gases that cause flatulence in non-ruminant animals (Fleming, 1981; Saini and Knights, 1984). Previous research has therefore tended to attribute the poor growth of animals on legume seeds to the presence of oligosaccharides. This has been disputed by more recent research (Irish *et al.*, 1995) and the results obtained in experiment 5.3 of this thesis.

More recently, benefits associated with the presence of oligosaccharides in the diet have been identified. In human nutrition, oligosaccharides are regarded as pre-biotics, able to increase or maintain the population of beneficial, non-pathogenic microbial species such as the *Biofidobacterium spp.* (Bailey *et al.,* 1991; Monsan and Paul, 1995). In animal nutrition, oligosaccharides have also been largely shown to reduce disease risk, possibly through an increase in lactic acid concentration and reduction in the proliferation of pathogenic species (Bailey *et al.*, 1991; Choi *et al.*, 1994; Okumura *et al.*, 1994). Beyond the maintenance of health, they have been shown to improve the digestibility of

different dietary fractions (Coon *et al.*, 1990; Leske *et al.*, 1993). These findings have led to the increased manufacture of synthetic oligosaccharides of defined chemical structure for use in animal and human nutrition (Alltech Inc., 1994; Monsan and Paul, 1995) for use in animal feeding.

There is a wide range of commercial oligosaccharides and available types are produced by isomerization of disaccharides, enzymatic hydrolysis of starch and other polysaccharides or direct extraction from the cell wall of microbial species such as yeast (Alltech Inc., 1994; Perry, 1995). Synthetic oligosaccharides are commonly classified according to the major monosaccharide back-bone or method of manufacture. The most common ones used in animal feeding include mannan oligosaccharides, synthesized from yeast cell walls and fructo-oligosaccharides, prepared by transfructosylation of sucrose or hydrolysis of inulin (Alltech Inc., 1994; Monsan and Paul, 1995). The impact of these supplements on the diet and animals varies with the product and probably, with the process of manufacture (Oyarzabal and Conner, 1995). Various trials have demonstrated the benefits of different synthetic oligosaccharides (Bailey *et al.*, 1991; Terada *et al.*, 1994). There are still some inconsistencies in reports on the response of poultry to supplementation with commercial synthetic oligosaccharides, as was highlighted in chapter two (section 2.5.3.2). It is thought that available commercial oligosaccharides and some natural members may improve the health of poultry through an increase in lactic acid concentration and proliferation of beneficial microbes (Choi *et al.*, 1994; Okumura *et al.*, 1994). At the intestinal level, most of the mechanisms responsible for the changes observed in the presence of oligosaccharides in the diet are largely unknown.

The present study was aimed at examining the effects of supplementation with a predominantly mannan-oligosaccharide on broiler chicken productivity. These effects were related to structural and functional changes on the intestinal mucosa.

5.4.2. MATERIALS AND METHODS

5.4.2.1. Animals and diets

Seventy-two one-week old broiler chicks, previously brooded under identical conditions were used in the study. The chicks were randomly allocated in six to cages and fed one of four diets. There were three cage replicates per diet. The composition of the diets is shown in Table 5.4.1. The diets were based on sorghum, whole narrow-leaf lupin (*L. angustifolius*) seed and casein. They were then supplemented with different levels of Bio-Mos™ (BM), a mannan-oligosaccharide derived from the yeast, *Saccharomyces cerevisiae* (Alltech Inc., Nicholasville, KY, USA). Sorghum and lupin seeds were ground to pass through 1 mm screen and mixed with the other ingredients to obtain a mash.

5.4.2.2. Sample collection

The feeding trial was conducted over a period of 21 days during which body weight and feed consumption were monitored on a weekly basis. Three birds per cage (9 birds per diet) were randomly selected and euthanatized by intravenous administration of Lethabarb™ as described in section 3.4.0. The intestinal weight of sampled birds was recorded, full and empty of digesta. The ileal digesta were squeezed out and immediately frozen at -20°C prior to use in the assays described below.

Table 5.4.1: Ingredient composition and analysis (g/kg DM) of diets fed.

	Diet (Bio-Mos level, %)			
Ingredient (g/kg)	**0**	**0.1**	**0.3**	**0.5**
Sorghum	553.4	553.4	553.4	553.4
Whole lupinseed	301.0	300.0	298.0	296.0
Casein	91.0	91.0	91.0	91.0
Vegetable oil	20.0	20.0	20.0	20.0
Limestone	20.0	20.0	20.0	20.0
Rock phosphate	6.0	6.0	6.0	6.0
NaCl	3.6	3.6	3.6	3.6
Vit/mineral premix	5.0	5.0	5.0	5.0
Bio-Mos	0.0	1.0	3.0	5.0
Analysed composition (g/kg)				
Metabolizable energy (MJ/kg)	13.0	13.0	13.0	13.0
Starch[1]	396.9	396.9	396.8	396.8
NSP[1,2]	80.6	80.6	80.6	80.6
Oligosaccharides	44.8	45.7	47.4	49.1
Crude protein[1]	239.0	246.0	246.0	242.0
Methionine	4.01	4.02	4.01	4.01
Lysine	13.15	13.14	13.11	13.08
Calcium	11.34	11.34	11.34	11.33
Phosphorus	4.98	4.98	4.97	4.96

1. Starch, NSP and crude protein were determined by laboratory analysis as described in chapter 3. 2. The NSP consisted principally of galactans and arabinoxylans. Glucose was a major monosaccharide peak observed but may not all be bound in NSP. The metabolizable energy was calculated from data based on assays with broiler chickens. Other components were calculated from Australian ingredient composition tables.

5.4.2.3. Digesta viscosity and pH

A sub-sample of the digesta was centrifuged to obtain a supernatant on which viscosity was measured as described in section 3.3.5. The pH of the supernatant was also

measured using an expandable ion analyser, EA940 (Orion Research, Cambridge, USA).

5.4.2.4. Adherence of microbial cells to digesta

The adherence of microbial cells to the digesta was studied by scanning electron microscopy (SEM). Digesta were fixed in Karnovsky's fixative (4.0 g paraformaldehye and 1.25 g glutaraldehyde in 100 ml PBS containing 4 % (w/v) of sucrose and stored overnight at 4°C. The samples were washed twice by centrifugation and re-suspension in PBS for 30 minutes each wash. The samples were re-suspended in 1:1 (w/v) osmium tetroxide:PBS at room temperature for 2 hours. Samples were dehydrated by washing with increasing ethanol concentrations: 70, 90, 95 %; three washes per concentration, then 100 % ethanol for 30 minutes, followed by another wash for 1 hour. Samples were incubated in 50 % ethanol/50 % acetone (1:1) for 1 hour followed by incubation in 100 % acetone for 1 hour. Samples were then spotted on to SEM stubs and dried at 60°C overnight. The stubs were gold-carbon coated and scanned at 10.0 kV with a Philips XL30G scanning electron microscope.

5.4.2.5. Microbial activity

Microbial activity in the digesta was assessed through the biochemical assessment of lactic acid concentration (Lawrence, 1975). Sub-samples of the digesta from the jejunum and ileum were made homogenous with 15 ml of distilled water. To this mixture were added 2.5 ml of 25 % $CuSO_4.5H_2O$. The mixture was kept at room temperature for 10 minutes after which 2.5 ml of 33 % CaOH were added, vibromixed and stood for another 10 minutes. The solution was made up to 25 ml with distilled water, mixed and centrifuged at 12 000 g for 15 minutes. The pellet was discarded and

the supernatant centrifuged again for 10 minutes. Six ml of concentrated sulphuric acid were added to 1 ml of the supernatant and incubated in boiling water for 5 minutes. The sample was cooled to room temperature in running water and 100 µl of 88.1 mM p-hydroxybiphenyl reagent were added. The sample was vibromixed for 1 minute and absorbance read at 570 nm in cuvettes with a 1-cm light path. Lithium lactate was used as standard. All reagents were obtained from Sigma (Aus.) Pty Ltd.

5.4.2.6. Histology

Intestinal mucosal morphometry was assessed as described in section 3.4.1. The tissues were initially fixed in neutral buffered formalin for 24 hours.

5.4.2.7. Extraction of brush-border membrane vesicles and enzyme assays

Brush-border membrane vesicles (BBMV) were prepared by the method described by Shirazi-Beechey *et al.* (1991) and highlighted in section 3.7.0. The specific activities of membrane-bound digestive enzymes were determined in order to assess the purity of the membranes extracted and to assess the digestive capacity of chicks on the different diets. The activities of maltase, sucrase and AP were measured as described in 3.7.1 while leucine aminopeptidase (LAP; EC. 3.4.11.2) was assayed according to the method of Miura *et al.* (1983). Twenty-five µl of vesicle samples (diluted 1:60) were incubated with 200 µl of distilled water; 100 µl of 200 mM phosphate buffer, pH 7.0, and 100 of µl 8 mM leucine β-naphthylamide at 39°C for 30 minutes. Incubation was terminated with 300 µl of 30 % TCA. The reaction mixture was further incubated at room temperature with 100 µl of 0.3 % sodium nitrite; 100 µl of 1.5 % ammonium sulphamate and 300 µl of 0.1 % (in ethanol) N-1-naphthylethylene diamine-

dihydrochloride, vortexing after the addition of each of these reagents. The absorbance was then read at 560 nm in cuvettes with a 1 cm light path. A standard curve was derived from similarly treating varying concentrations of leucine β-naphthylamide as the samples in the second incubation stage. All reagents were of analytical grade, supplied by Sigma (Aus.) Pty Ltd.

5.4.3. RESULTS

5.4.3.1. Feed consumption and growth

The feed intake and body growth of the chicks are shown in Table 5.4.2. There were no significant differences between birds on the different treatments as a result of supplementation with Bio-Mos (BM) although the supplement tended to increase feed intake and weight gain, especially at the lowest level of supplementation. Feed conversion ratio also tended to decline (not significantly) with increased inclusion level of BM.

Dry matter digestibility was over 70 % generally and appeared to be little affected by supplementation with BM. Starch digestibility was highest on the diet supplemented with 0.5 % BM while digestibility on 0.1 % was lower than the value observed on the control diet. DM and starch digestibility was not statistically tested.

Table 5.4.2: Feed consumption and growth of chickens on different diets.

	Diet (Bio-Mos level, %)				
	0	**0.1**	**0.3**	**0.5**	**SED**
Initial weight (g)	150.9	148.6	150.0	149.8	2.73
Final weight (g)	667.1	729.4	703.3	678.3	33.54
Weight gain (g)	516.2	580.9	553.3	528.5	32.96
Feed intake (g/head)	1150.0	1345.0	1262.0	1154.0	101.50
Feed intake (g/100 g initial weight)	761.8	907.6	841.5	770.6	72.46
Feed conversion ratio	2.24	2.31	2.29	2.19	0.179
DM digestibility[1]	79.9	75.8	78.8	78.0	-
Starch digestibility[2]	65.6	53.8	67.8	76.0	-

1. Determined by comparing feed and faecal values. 2. Determined at the terminal ileum; the starch content of droppings was generally low on all diets. Starch and DM digestibility were determined as described in sections 3.3.1 and 3.3.4.

Table 5.4.3: Empty weight of GIT and pancreas (g/100 g body weight) and weight of digesta.

	Diet (Bio-Mos level, %)				
	0	**0.1**	**0.3**	**0.5**	**SED**
Small intestine	4.4	4.7	4.5	4.7	0.18
Pancreas	0.34	0.40	0.39	0.36	0.031
Proventriculus/gizzard	4.1	4.6	4.4	4.2	0.28
Digesta weight					
Small intestine (g/100 g body wt.)	2.4	2.8	2.8	2.5	0.41
Provent/gizzard (g/100 body wt.)	1.4^{b}	1.7^{a}	1.6^{a}	1.4^{b}	0.12*

a,b - Mean values on the same row not sharing a superscript are significantly different ($P<0.05$).

5.4.3.2. Visceral organ weight and gut-fill

The weight of visceral organs of chicks was similar between treatments (Table 5.4.3). There were also no differences in the gut-fill of the small intestine. Chicks that were fed the diet supplemented with 0.1 % BM had a significantly higher ($P<0.05$) amount of digesta in the proventriculus/gizzard than the amount held by birds on the other diets.

Table 5.4.4: Digesta and faecal characteristics in chickens on the different diets.

	Diet (Bio-Mos level, %)				
	0	**0.1**	**0.3**	**0.5**	**SED**
pH					
Jejunal digesta	5.61	5.82	5.86	5.71	0.207
Ileal digesta	5.95	6.21	5.96	6.09	0.690
Moisture content (%)					
Jejunal digesta	73.8	79.9	77.9	69.9	3.78
Ileal digesta	73.7	77.4	69.2	74.3	6.29
Faeces (droppings)	81.6	77.8	80.5	79.8	-
Lactic acid (mg/g digesta)					
Jejunal digesta			Negligible		
Ileal digesta	4.9	5.5	6.2	6.8	3.38
Viscosity (cP)					
Jejunal digesta	3.0	3.6	4.5	5.0	0.56
Ileal digesta	3.6^{b}	4.4^{b}	7.4^{a}	7.3^{a}	1.41

a,b - Mean values on the same row not sharing a superscript are significantly different ($P<0.05$). Faecal moisture content was determined on bulked samples collected on treatment basis and data were not statistically compared.

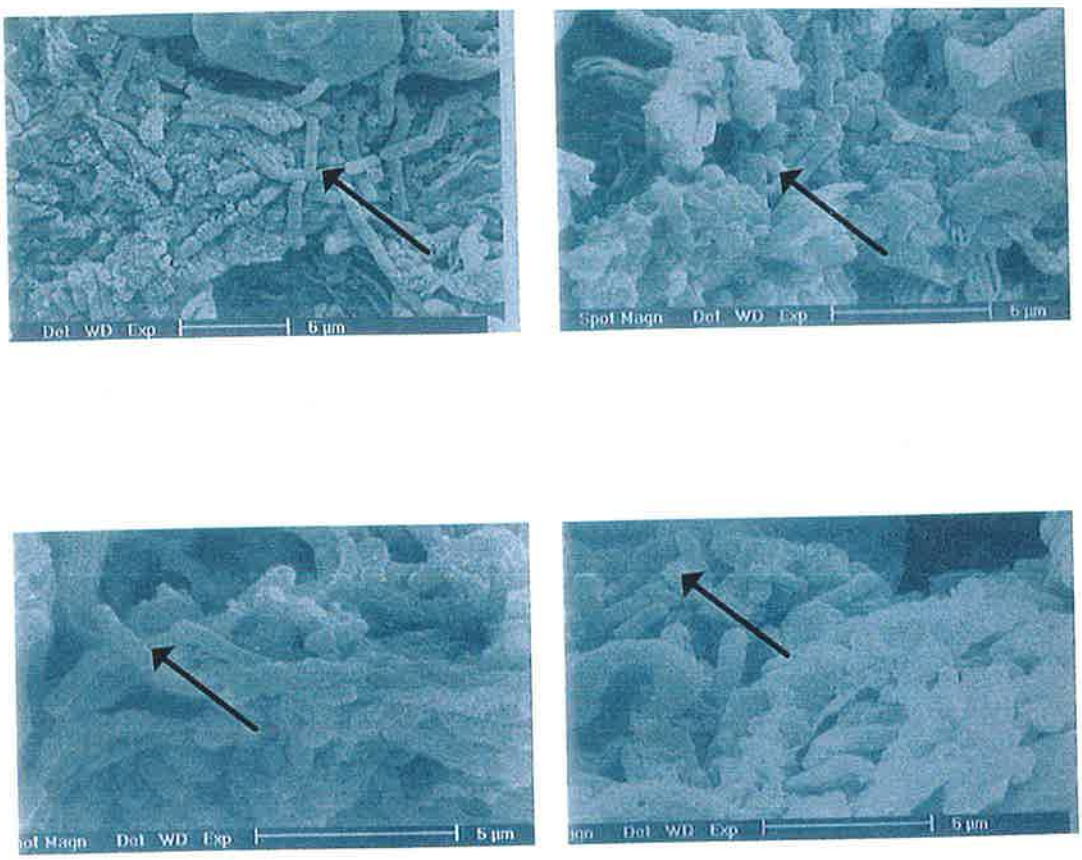


Plate: 5.4.1: Scanning electron microscope photographs showing the incidence of microbial cell (*arrowed*) adherence to the digesta obtained from the terminal ileum in chicks raised on diets with different levels of BM. **From top-left (*clock-wise*) are control, 0.1, 0.3 and 0.5 % BM respectively.**

5.4.3.3. Digesta properties and microbial activity

Digesta pH at the jejunum and ileum was identical between birds on the four diets (Table 5.4.4) although there was a slight increase in birds on the BM-supplemented diets. The moisture content of the digesta at the jejunum and ileum as well as in the faeces (droppings) was also identical in birds on the different diets. Digesta viscosity was generally low at both intestinal sites and was not significantly affected by the supplement in the jejunum. In the ileum, digesta viscosity increased ($P<0.05$) in line with supplemental levels of BM. The lactic acid content of the digesta from the jejunum of chicks on all diets was negligible. In the ileum, lactic acid concentration

tended to increase with the level of BM included in the di*et al*though no significant differences were observed between the diets.

Table 5.4.5: External muscle thickness and intestinal mucosal morphometry on different diets.

	Diet (Bio-Mos level, %)				
	0	**0.1**	**0.3**	**0.5**	**SED**
A. Jejunum					
External muscle (μm)	231.3	241.6	268.7	234.9	30.49
Crypt depth (μm)	206.3	196.1	236.4	197.4	22.45
Villus height (μm)	1245.5^{b}	1252.3^{b}	1277.3^{b}	1553.6^{a}	92.55**
Villus surface area (mm^2)	0.24	0.22	0.23	0.26	0.013
B. Ileum					
External muscle (μm)	223.3	241.6	276.7	188.7	43.96
Crypt depth (μm)	152.2	149.9	164.5	153.7	16.61
Villus height (μm)	799.6	724.9	769.9	797.2	69.77
Villus surface area (mm^2)	0.13	0.11	0.14	0.14	0.021

a,b - Mean values on the same row not sharing a superscript are significantly different ($P<0.01$).

Scanning electron microscopy did not reveal any obvious differences in the incidence of microbial cell adherence to the ileal digesta obtained from chicks on the different diets (Plate 5.4.1). The bacteria observed were predominantly bacilli. The species were not identified but the increase in lactic acid concentration in chicks on the BM-supplemented diet would suggest domination of the population by lactophilic species.

5.4.3.4. Morphometry of intestinal mucosa and biochemical indices of mucosal growth

The jejunal villi of chicks on the 0.5 % BM-supplemented diets were significantly longer ($P<0.01$) than villi of chicks raised on the other diets (Table 5.4.5). There were no significant differences between the birds with regards to other morphometric measurements made in the jejunum or ileum.

There were also no significant differences between birds on the four diets with regards to the concentration of protein in the mucosal homogenate of the jejunum or ileum (Table 5.4.6). DNA content followed a similar trend. The RNA content of the ileal mucosal homogenate was significantly increased ($P<0.05$) in chicks raised on diets supplemented with 0.3 and 0.5 % BM.

The protein:DNA ratio of the jejunal mucosal homogenate was higher ($P<0.05$) on the 0.1 % BM-supplemented diet than in chicks on the other diets. In the ileum, this was unaffected by BM supplementation. There were also no differences between the chicks with regards to RNA:protein ratio, protein:RNA ratio or RNA:DNA ratio of the jejunal mucosal homogenate. In the ileum, RNA:protein ratio and RNA:DNA ratio were higher ($P<0.01$) on the 0.3 and 0.5 % BM-supplemented diets than on the low BM and control diets. The protein:RNA ratio of the ileal mucosal homogenate was, however, highest ($P<0.01$) on the 0.1 % BM diet and declined with increasing levels of BM in the diet.

Table 5.4.6: Concentrations (mg/g wet tissue) of protein and nucleic acids in the mucosa of chickens on different diets.

	Diet (Bio-Mos level, %)				
	0	**0.1**	**0.3**	**0.5**	**SED**
A. Jejunum					
Protein	35.2	35.3	30.3	35.4	4.06
DNA	0.9	0.8	1.1	1.0	0.13
RNA	1.9	2.1	1.9	1.8	0.22
Protein:DNA ratio	39.1^{ab}	44.1^{a}	27.6^{b}	35.4^{ab}	5.50*
RNA:protein ratio	0.05	0.06	0.06	0.05	0.009
Protein:RNA ratio	18.5	16.8	15.9	19.7	2.76
RNA:DNA ratio	2.1	2.6	1.7	1.8	0.46
B. Ileum					
Protein	32.1	31.9	26.3	30.8	3.68
DNA	1.1	1.3	0.9	1.1	0.19
RNA	1.4^{b}	1.1^{b}	2.0^{ab}	2.8^{a}	0.50*
Protein:DNA ratio	29.2	24.5	29.2	28.0	4.73
RNA:protein ratio	0.05^{ab}	0.03^{b}	0.08^{ab}	0.09^{a}	0.016**
Protein:RNA ratio	22.9^{ab}	29.0^{a}	13.2^{b}	11.0^{b}	4.17**
RNA:DNA ratio	1.3^{bc}	0.9^{c}	2.2^{ab}	2.6^{a}	0.42**

a,b - Mean values on the same row not sharing a superscript are significantly different (*P<0.05; **P<0.01).

5.4.3.5. Specific activities of brush-border membrane enzymes

The specific activities of brush-border membrane enzymes are shown in Table 5.4.7. The protein content of jejunal brush-border membrane declined (P<0.01) with increasing level of BM supplementation. There were also significant increases in the specific activities of maltase (P<0.01); LAP (P<0.05) and AP (P<0.001) in the jejunum as a result of supplementation with BM.

In the ileum, the protein content of brush-border membrane extracted from birds on the control diet was significantly higher (P<0.05) than that observed in birds on the other diets. There were no significant differences between the groups in the specific activities of the four membrane-bound enzymes assessed.

Table 5.4.7: Brush-border membrane protein (mg/g wet tissue) and specific activities of membrane-bound digestive enzymes (μmole product/mg protein/minute) in chickens on Bio-Mos supplemented diets.

	Diet (Bio-Mos level, %)				
	0	**0.1**	**0.3**	**0.5**	**SED**
A. Jejunum					
Protein	1.9^{a}	1.9^{a}	1.3^{ab}	1.1^{b}	0.26**
Maltase	9.0^{b}	9.4^{b}	17.0^{a}	15.4^{ab}	2.32**
Sucrase	0.6	0.7	0.8	0.7	0.14
Leucine aminopeptidase	72.8^{c}	85.1^{bc}	118.3^{a}	100.4^{ab}	26.6*
Alkaline phosphatase	5.1^{b}	5.2^{b}	8.4^{a}	9.3^{a}	1.06***
B. Ileum					
Protein	2.0^{a}	1.8^{a}	1.2^{b}	1.6^{ab}	0.22*
Maltase	14.8	14.1	15.3	15.1	1.51
Sucrase	0.6	0.6	0.7	0.7	0.08
Leucine aminopeptidase	25.0	24.1	25.8	25.9	3.22
Alkaline phosphatase	5.1	4.9	5.7	5.2	0.63

a,b - Mean values on the same row not sharing a superscript are significantly different (*P<0.05; **P<0.01; ***P<0.001).

5.4.3.6. Uptake of L-tryptophan by brush-border membrane vesicles

The uptake of 0.04 mM L-tryptophan by jejunal brush-border membrane vesicles was significantly higher (P<0.01) in chicks raised on the 0.5 % BM than chicks on the other diets (Figure 5.4.1). Tryptophan uptake by jejunal BBMV tended to increase in line

with the level of BM in the diet. A similar trend was observed in the ileum although there were no significant differences between the groups.

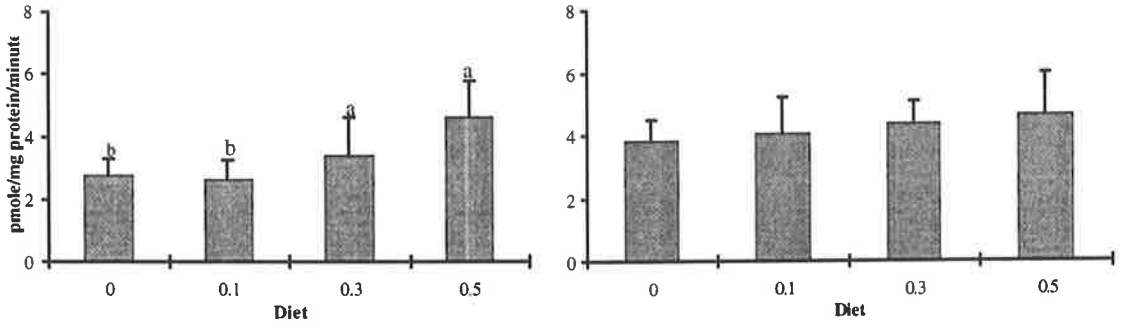


Figure 5.4.1: Uptake of L-tryptophan by jejunal (*left*) and ileal BBBV from chickens on diets supplemented with different levels of BM. a,b - mean values not sharing a superscript are significantly different ($P<0.01$). Five chicks were used to test uptake at each intestinal region. The incubation buffers contained 0.04 mM L-tryptophan, 50 mM NaCl and 20 mM HEPES. Mannitol was included to maintain iso-osmolarity with the vesicle storage buffer.

5.4.4.0. DISCUSSION

5.4.4.1. Feed quality and utilization

The body weight and feed conversion ratio observed in this experiment are poorer than those obtained in experiment 5.1 on the sorghum/soyabean meal diet or the commercial finisher diet used in chapter 4. Although birds were maintained for a shorter period of time in experiment 5.3, the feed conversion ratio appeared to be better on the de-hulled lupin seed meal than on seed meals made from the whole seed used in the present experiment. The differences in the nutritive value of lupins and oil-seed cakes have been reported by several workers (Watkins *et al.*, 1988; Olver and Jonker, 1997). In comparison with de-hulled lupin seeds, whole seeds contain a high level of NSP, which are more concentrated in the hulls than in the rest of the seed (Evans *et al.*, 1993; Mohamed and Duarte-Rayas, 1995b). NSP interfere with the digestion of nutrients, as evident from the low starch digestibility relative to what was observed in experiment

5.1. The digestibility of starch *in vivo* was lower in the present experiment than it was in experiment 5.2, even though the digesta viscosity on diets in experiment 5.2 were higher than that observed in the present experiment. The diets in this experiment contained large amounts of NSP but did not produce digesta of high viscosity. This may be due to the low solubility of lupin NSP (Evans *et al.*, 1993; Mohamed and Rayas-Duarte, 1995b).

The oligosaccharide supplement appeared to have very little impact on the feed conversion ratio, even at levels higher than the inclusion rate, 0.2 % recommended by the supplier. There was, however, an improvement in weight gain and final liveweight, especially on the lowest level of supplementation. While these were not statistically significant, commercially, they would amount to considerable benefit, if the level of supplementation is kept to the minimum. The highest level of supplementation marginally improved feed conversion ratio and starch digestibility but weight gain and final body weight were lower than those on diets with lower BM levels and control diet. In spite of the fact that animals lack endogenous enzymes capable of hydrolysing oligosaccharides, oligosaccharides may be digested to considerable extent by enzyme systems of resident microbes in the GIT. In trials on broiler chicks, Carre *et al.* (1995) observed a significant digestion of α-oligosaccharides, including raffinose, stachyose and verbascose present in a high-pea diet compared with complete lack of digestion in the case of NSP in young chicks. The digestion of oligosaccharides and NSP was not assessed in the present study but substantial amounts of the former would be prone to digestion, especially under the influence of the commercial pre-biotic supplement, which like most oligosaccharides is known to increase the population of non-pathogenic

microbes (Alltech Inc., 1994). Such microbes may provide the necessary enzymes for the digestion of dietary oligosaccharides.

5.4.4.2. Visceral organ weight and characteristics of the digesta

The weight of the intestines as well as that of the proventriculus/gizzard in chicks on all diets in the present experiment appears to be high, probably as a result of the high content of whole lupin seed. In poultry, various regions of the GIT respond differently to high dietary fibre levels. Summers and Leeson (1986) observed an increase in the weight of the gizzard in broiler chicks raised on a high fibre diet but there was a reduction in intestinal weight. In contrast to the trend in poultry, high levels of fibre in the diet increased intestinal weight in pigs and rats (Pond *et al.*, 1989). There were no clear effects of BM on visceral organ weight or intestinal gut-fill. Although there was an increase in feed intake in chicks on the BM-supplemented diets, this did not increase organ weights but tended to increase the volume of the proventriculus and gizzard. In broiler chickens raised on methanol-extracted pea diets to which oligosaccharides had been added, Trevino *et al.* (1990) observed an increase in the length of the small intestine and the caeca. Broiler chicks that were fed on a diet supplemented with fructo-oligosaccharides (FOS) also had heavier full caeca than chicks on a control diet (Oyarzabal and Conner, 1996). This was attributed to a longer retention of the supplement in the caeca for microbial digestion and the accumulation of fluid caused by a difference in osmotic potential. It is unlikely that these effects could occur in the proximal GIT where microbial populations and digestion of oligosaccharides are low (Coon *et al.*, 1990).

There were no apparent effects of the oligosaccharide supplement on digesta pH or moisture content of both the digesta and droppings. Faecal moisture content was relatively high, compared to that of the intestinal digesta. The retention of oligosaccharides in the lower GIT during microbial digestion tends to create an osmotic potential, leading to the accumulation of fluids in the region (Wiggins, 1984). This may result in passage of watery droppings and in some instances, diarrhoea. Different oligosaccharides may be digested to different degrees and this would be related to the transit time and possible effects on osmotic potential. Although the BM supplement tended to increase the viscosity of the ileal digesta, this may not be responsible for any of the differences in weight gain observed between chicks on the different diets. The viscosity of the digesta in both intestinal regions was low, compared to values obtained, even after dilution of the digesta, in experiment 5.2. This may be due to the low concentration of NSP in sorghum, the basal cereal ingredient as well as the low solubility of the lupin NSP, which has been previously highlighted (Choct and Annison, 1990; Evans *et al.*, 1993; Kopinski *et al.*, 1995).

5.4.4.3. Microbial activity

The BM supplement tended to increase the concentration of lactic acid in the digesta in the ileum where the acid was in measurable quantity. This is one of the recognized mechanisms by which oligosaccharides influence animal productivity (Choi *et al.*, 1994; Okumura *et al.*, 1994). Microbial digestion of oligosaccharides yields lactic acid, in addition to other short-chain fatty acids (SCFA) and this is believed to suppress the proliferation of pathogenic microbes. The increase in population of Lactobacilli is also directly detrimental to the growth of Salmonella serotypes (Oyarzabal and Conner,

1995). Schoeni and Wong (1994) reported on the inhibition of Campylobacter colonization when broiler chicks were raised on diets supplemented with FOS.

Direct changes in pH may also be as important as the type of SCFA accumulated in chicks on oligosaccharide-supplemented diets. In broiler chicks that were fed diets supplemented with lactosucrose, Terada *et al.* (1994) observed an increase in the concentrations of acetic and butyric acids through prolonged feeding and this led to a reduction in the frequency of occurrence of some clostridia and staphylococci. Pathogenic bacteria translocate the wall of the GIT to infect internal systems by first attaching to oligosaccharides on the intestinal mucosa (Wells, 1990; Spaeth *et al.*, 1990). Monsan and Paul (1995) speculated that dietary oligosaccharides may reduce infection by creating an alternative site for the attachment of pathogenic bacteria. In addition to creating an alternative site for microbial attachment, BM and a synthetic fructo-oligosaccharide, FOS are particularly not good substrates for pathogenic bacteria (Alltech Inc., 1994; Choi *et al.*, 1994). There was no clear evidence of the effect of BM on microbial adherence to the digesta in this experiment but the increase in lactic acid concentration would indicate changes in the relative proportion of microbial (bacterial) types.

5.4.4.4. Development of the intestinal mucosa

There is a lack of report on the effect of lupins generally or of oligosaccharides on the growth of the intestinal mucosa. At the same age (28d), crypt depth at the two intestinal sites was higher in chicks in the present experiment than was observed in chicks on the commercial finisher diet (experiment 4.5). Jejunal villi were of a similar length except on the diet supplemented with the highest level of BM. Ileal villi were longer in chicks

on all diets in the present experiment than was observed in experiment 4.5. There were no obvious effects of BM except in the case of jejunal villi, which were longer on the highest BM diet than on the other diets. Apparent villus surface area was unafffected and this may be due to the trend in basal and apical widths of the villus, on which the value of surface area was partly based. Oligosaccharides promote a build-up of lactic acid in the GIT which is low under conventional diets (Barnes *et al.*, 1979). While lactic acid is less effective at inducing mucosal cell proliferation than butyric acid (Sakata *et al.*, 1995), fructo-oligosaccharides have been observed to alleviate salmonella-induced necrosis of the caecal mucosa and enhance the micro-villus length of broiler chicks (Choi *et al.*, 1994). It is not known if such changes would involve the entire villus.

In the jejunum where there was an increase in villus length, there were only marginal increases in the DNA content of the mucosa in birds on the BM-supplemented diets and no definite trend in the protein:DNA ratio. This may indicate that villus growth on the two high BM diets occurred mainly by an increase in cellular hyperplasia rather than hypertrophy. In the ileum, there were significant changes in RNA concentration as well as RNA:protein, protein:RNA and RNA:DNA ratios. These changes were, however, not translated into mucosal growth, except for slight changes in the villus surface area. The changes in these biochemical indices may underlie variations in other aspects of mucosal function such as digestive enzyme activity and nutrient transport.

5.4.4.5. Digestive enzyme function

Although some attention has been devoted to studying the pattern of oligosaccharide digestion in animals, there are no previous reports on the effects of natural or synthetic

oligosaccharides on animal enzyme activities. There were no significant changes, on the basis of solvent-extraction of meals on intestinal enzyme activities reported in experiment 5.3. It is not certain what the outcome would have been over a longer duration of feeding. The increase in specific activities of most of the enzymes, especially in the jejunum with further supplementation of an oligosaccharide, BM in this experiment may suggest a positive effect of the materials on enzyme activities. These effects were not noticeable in the ileum, where most microbial digestion of oligosaccharides would have occurred (Coon *et al.*, 1990). The response of mucosal function may not, however, be entirely dependent on the digestion of the supplement at any particular site. The effects of oligosaccharides on microbial translocation have been highlighted in this discussion (Wells, 1990; Spaeth *et al.*, 1990; Monsan and Paul, 1995). The protection offered to intestinal cells by dietary oligosaccharides would enable the cells to develop normally, both structurally and functionally as well as reduce disease risk (Monsan and Paul, 1995). In humans, perfusion with oligosaccharides was shown to increase the concentrations of various immunoglobulins (IgG, monomeric IgA and IgM) as well as albumin (Colombel *et al.*, 1992). These factors may be responsible for the increased growth and functional activity in the jejunum rather than the site of major digestion of oligosaccharides, the ileum.

Oligosaccharides are digested mainly by microbial enzymes and such digestion occurs mainly towards the distal GIT, which limits the availability of released nutrients to the animal since absorption is low (Hellendoorn, 1979; El-Faki *et al.*, 1983; Coon *et al.*, 1990). Oligosaccharide digestion in the distal GIT leads to accumulation of gases such as hydrogen, carbon dioxide and methane which are responsible for the flatulence observed in non-ruminant animals on high legume diets (Fleming, 1981; Saini and

Knights, 1984). Monsan and Paul (1995) have proposed that dietary oligosaccharides may stimulate microbial enzyme synthesis and activity as well as endogenous glycolytic enzyme secretion. These assertions were based on the large increase in body weight gain observed in animals when relatively small amounts of oligosaccharides are included in the diet. In the present experiment, the specific activities of all the tested enzymes in the jejunum were higher on the BM-supplemented diets than they were on the control diet. It is not known if this is related to the changes in the intestinal mucosa, especially with the fact that jejunal villus height and surface area were increased by the supplement.

Compared with the study on natural development (experiment 4.5), the specific activities of maltase and sucrase in the jejunum were lower in the present experiment but were similar in the ileum. The specific activity of AP was higher in this study than it was in the study on natural development. These differences may influence animal growth rates. LAP showed a higher activity than APN tested in the other studies and was considerably higher in the jejunum than in the ileum. These differences may, however, originate from variation in basal ingredients as well as the particle consistency of diets (Nir *et al.*, 1994).

5.4.4.6. Amino acid transport

As with digestive enzymes, the differences observed in L-tryptophan uptake occurred mainly in the jejunum. There were also slight increases in the uptake of the amino acid by ileal BBMV of chicks as a result of supplementation with BM. These changes may be due to the healthy nature of membranes in the presence of oligosaccharides (Choi *et al.*, 1994). Apart from this, there are no reports on the effects of oligosaccharides on

nutrient transport. It is significant to note that the specific activity of LAP was considerably higher in chicks on the BM-supplemented diets than it was in those on the control diet. LAP is one of the enzymes responsible for the terminal digestion of peptides and was assessed in this experiment as a substitute for APN studied in the other experiments. If the activities of other peptidases were similarly increased as that of LAP, it would be plausible to assume that the amino acid pool in the digesta would be higher in chicks on the BM-supplemented diets than it would be on the control diet. Under conditions of high nutrient concentration, there is a greater contribution of passive transport to total transport (Stevens *et al.*, 1984), leading to an increase in the overall absorption of the nutrient. It is likely that the digestion of proteins and other dietary components was increased in much the same way as that of starch under the influence of the oligosaccharide supplement.

5.4.5. CONCLUSION

The high rate of inclusion of whole lupin seed in this experiment tended to adversely affect chick growth and final body weight. The impact of supplementation with the mannan oligosaccharide was a marginal improvement in growth which at the farm level would be of considerable economic significance. The supplement tended to increase feed intake and would reduce feed conversion efficiency at high rates of inclusion. Most of its effects in the GIT were confined to the jejunum where there were changes in villus height, enzyme activities and amino acid transport. The supplement may be ideal to high risk disease environments where it may be able to suppress the proliferation of pathogenic microbes through an increase in lactic acid concentration.

CHAPTER SIX: GENERAL DISCUSSION AND FUTURE RESEARCH

6.0.1. Feed consumption and utilization

The strain studied demonstrated the gross characteristics of most of the useful broiler strains reared around the world. On the tested commercial diets, its body growth and feed conversion efficiency were impressive, compared with most of the important strains used around the world.

An increase in nutrient retention with age is associated with a large increase in body weight. Most of the retained materials may not, however, be directed to the deposition of muscle but accumulation of fat, which may be responsible for the reduction in feed conversion efficiency as the birds grew older. The reduction in digestive efficiency with age also entails wastage of nutrients from an increased intake of feed. Such changes have been observed in other species and strains of poultry (Hargis and Creger, 1980; Thomson and Keelan, 1986; Holt and Kotler, 1987; Sell *et al.*, 1991).

The various dietary interventions showed their unique impact on feed consumption and utilization. Feed intake and feed conversion efficiency varied between chickens on a barley-based diet and diets based on maize, sorghum and wheat. These differences have been reported by other researchers (Marquardt *et al.*, 1993; Almirall *et al.*, 1995) but the mechanisms involved have not been completely understood. The response of the broiler chicks to a microbial enzyme supplement also differed between cereal types. Most of the differences could be attributed to variation in concentration and type of NSP, rather than variations in content, type and digestibility of starch (Classen and Bedford, 1991; Carre and Melcion, 1995; Kopinski *et al.*, 1995).

The impact of pure NSP on feed consumption and body growth was in direct proportion to the viscosity of the material. Chicks on diets containing highly viscous NSP tended to increase feed intake, perhaps in an attempt to derive sufficient nutrients. This led to significant losses in feed conversion efficiency. Digesta viscosity would also regulate nutrient absorption through changes in digesta mobility (Tulung *et al.*, 1987; van der Klis *et al.*, 1993) although there are also reports that some NSP may complex with digestive enzymes (Ikeda and Kusano, 1983) to reduce the efficiency of digestion and thus, of the material available for absorption.

Lupins would be useful replacement for some of the more costly protein sources, provided the seed is de-hulled prior to feeding. Further solvent extraction of lupin seed meals, however, tended to reduce the growth of broiler chicks through mainly an increase in digesta viscosity. Whole lupin seed meals, without de-hulling was of low quality and also reduced productivity, compared with what was obtained on the commercial diet and diets based on soyabeans. Oligosaccharides may not be responsible for the reduction in poultry productivity in the presence of lupin seeds. Similar results have been obtained by Slominski *et al.* (1994) and Irish *et al.* (1995) although there are still dissenting reports as to the neutral or positive effects of oligosaccharides in broiler chicken diets. The differences observed between various research reports may be due to variation in types of oligosaccharides tested (Alltech Inc., 1994). Additional supplementation with a commercial oligosaccharide (a pre-biotic) improved body growth but this was associated with high feed intake and low feed conversion efficiency.

6.0.2. Growth of visceral organs

On the commercial diet, body growth was preceded by a relatively large GIT which grew to a peak within the first week of life. This is the pattern of development reported for most avian species (Lilja, 1983; Katanbaf *et al.*, 1988; Sell *et al.*, 1991; O'Sullivan *et al.*, 1992a; Shanawany, 1994). A different growth pattern would be a constraint to the derivation of nutrients in later life and may reduce dressing percentage (meat yield in relation to offal components) (Nitsan *et al.*, 1991; Dunnington and Siegel, 1995).

The growth of the GIT was regulated by the diets tested in subsequent studies. Chicks raised on barley-based diets generally had heavier GIT than those on the other cereals. This may be as a result of higher levels of NSP in barley-based diets as shown by results obtained with the viscous NSP in experiment 5.2. It is not known if these changes were exclusively induced at the mucosal level or were associated with an increased length of the GIT, as was observed by Johnson *et al.* (1984). The results indicate the loss in productivity that could be incurred by raising poultry on high-NSP diets. The large GIT would be an economic waste due to its impact on nutrient partitioning between mucosal renewal and whole body growth. The chicks on diets with viscous NSP also appeared to undergo a second wave of increased intestinal growth which would be at the expense of body growth.

The supplementation of diets with whole lupin seed meal tended to increase intestinal weight. Oligosaccharides are known to increase the full weight of the GIT through prolonged retention of the material in the lower GIT for microbial digestion as well as accumulation of fluids caused by changes in osmotic potential (Wiggins, 1984;

Oyarzabal and Conner, 1996). The retention of feed materials for long periods of time may lead to intestinal distension and increased empty weight of the affected region. The digesta viscosity of chicks on un-dehulled lupin-supplemented diets was relatively low, even at high levels of inclusion. This may be due to the fact that a large proportion of lupin NSP are insoluble in water (Evans *et al.*, 1993; Mohamed and Rayas-Duarte, 1995b).

There was generally very little direct effect of dietary treatments on liver weight in experiments in which this was assessed. This may be the pattern for organs not in direct contact with feed material. The pancreas, being an important source of enzymes for digestion often responds to dietary substrates, and may be stimulated to increase or decrease enzyme synthesis and secretion depending on the conditions present in the proximal small intestine (Centeno *et al.*, 1990; Ikegami *et al.*, 1990).

6.0.3. Development of the intestinal mucosa

The intestinal mucosa was well developed at hatch but there were significant changes with age. Similar changes have been reported for other poultry and mammals when feed supply is unrestricted (Maxton *et al.*, 1989; Smith *et al.*, 1990b). The most rapid growth occurred in the jejunum, the median region of the small intestine, similar to reports on the Arbor Acres and Lohmann strains of broiler chickens (Uni *et al.*, 1995a). This may aid feed digestion and nutrient absorption. The jejunum and ileum together constitute a large proportion of the small intestine and may be the areas of greatest intestinal function. Digestive function at the duodenum would be mainly supported by enzymes secreted from the pancreas.

An examination of the mechanisms responsible for mucosal growth suggested that growth occurred mainly by cellular hypertrophy and less by hyperplasia. Cell proliferation would remain vital to the renewal of the intestinal mucosa. At the jejunum, cell proliferation was rapid, with several cells labelled by BrDU within a short period of administration. The rate of cell migration from the region of proliferation varied with age. These differences would be important to the functional capacity of cells (Wild and Murray, 1992) and the age by which they become senescent. Enterocyte proliferation was rapid at all ages and this was accompanied by a rapid rate of migration. These changes were significant for the fact that short term exposure to dietary components may have an impact on mucosal structure and functions.

On diets containing viscous NSP, there was a tendency for greater changes in the distal GIT (ileum) than in proximal regions, similar to reports by other workers (Jacobs and White, 1983; Johnson and Gee, 1986; Brunsgaard and Eggum, 1995). These changes may be brought about by an increase in the digestion of NSP as the material transits into a region of high microbial population.

Compared with chickens raised on the commercial diet supplemented with a non-viscous NSP (alginic acid), chickens on the diets supplemented with whole lupin seed had reduced intestinal RNA content (potential rate of protein synthesis) and enterocyte size (protein:DNA ratio). It is not known how and if these differences contributed to the low body weight of the chickens on the whole lupin diets. Cell populations (DNA content) were similar between both sets of chickens. The differences in basal ingredients and particle consistency of diets may also influence the response of chicks to

the diets used in the two studies. In diets supplemented with lupins, significant differences would also occur between meals prepared from de-hulled seeds and those made from whole seeds (Brenes *et al.*, 1993).

6.0.4. Digestion and nutrient transport

The specific activities of all digestive enzymes declined with age. This is the pattern observed with mammalian species and may partly be responsible for the reduction in feed conversion efficiency with age (Tivey and Smith, 1989; Wild and Murray, 1992; Sell *et al.*, 1991). The high specific activities in early life were mainly due to low membrane protein content in the intestinal mucosa, in relation to enzyme proteins. Absolute enzyme levels increased with age and this was accomplished by an increase in intestinal length and villus height. Histochemical techniques also revealed little change in enzyme activities per unit villus area (cell) with age. The limiting factor to digestive function may therefore, be the length of the intestine, and possibly villus length.

Although specific activities of enzymes were distributed along the GIT in much the same way as has been reported in other species (Majumdar *et al.*, 1988; Dupuis *et al.*, 1990) total villus enzyme activities declined distally from the duodenum to the ileum. A considerable amount of the diet would therefore be digested in the duodenum, although this region is much shorter than the jejunum or ileum.

The distribution of enzyme activities along the crypt:villus axis was similar to what has been reported in mammalian species (Lund and Smith, 1987; Freeman *et al.*, 1993). Some enzyme expression was observed at the crypt, the major zone of synthesis; peak activities were, however, observed on the mid-villus. The actual distribution of enzyme

activity on the villus varied with the type of enzyme but the pattern was less affected by age. The total villus surface area over which enzyme activity was expressed also increased with age. This would aid the digestion of increased absolute amounts of feeds ingested. The estimated age at which jejunal enterocytes began to secrete active enzymes did not significantly vary with age but enterocytes retained this capacity for much longer in older animals. This again was accomplished by an increased length of the villi and slight reduction in the migration rate of enterocytes.

Most of the differences between the productivity of broiler chickens on various cereal grains may be attributed to differences in the specific activities of digestive enzymes. There was no clear pattern in relation to the concentration and type of NSP present in the tested cereals. Commonly, nutrient digestibility of diets based on barley and other highly viscous cereals such as rye and triticale is reduced, compared with values observed for maize, sorghum or wheat (Fengler *et al.*, 1988; Friesen *et al.*, 1992; Marquardt *et al.*, 1993). The present research showed that cereal type had an effect on the specific activities of digestive enzymes. The major effects of NSP on digestive function may be mainly associated with the creation of a viscous digesta environment and a reduction in nutrient-enzyme interaction (Ikeda and Kusano, 1983; Johnson *et al.*, 1984). The results obtained in studies on cereal type and pure NSP suggest that dietary factors could as well alter membrane function. The trial on pure NSP reported in experiment 5.2 showed a reduction in the specific activities of enzymes on the highly viscous NSP over a short duration of feeding. Interestingly, however, enzyme activities were increased after long term exposure. Such an adaptation to NSP has not been previously reported but Brunsgaard and Eggum (1995) have observed permanent

changes in the mucosal structure of rats on diets supplemented with cellulose, guar gum, pectin and retrograded starch.

The short term feeding of solvent-extracted de-hulled lupin seed meals did not alter digestive enzyme function, compared with values in chicks that were fed un-extracted seed meals (experiment 5.3). The specific activities of both maltase and sucrase in the jejunum of chickens on the whole lupin diet (experiment 5.4) were reduced compared with values observed on the commercial finisher diet (experiment 4.5). This may be partly responsible for the low body weight of the chicks in experiment 5.4.

The pattern of tryptophan transport observed in the present research may provide a clue to the transport of other neutral amino acids. The rates and efficiency of transport both declined with age. Total transport over the length of the GIT was increased with age, to cater for increased level of material available for absorption (Stevens *et al.*, 1984). Tryptophan uptake was predominantly Na^+-independent but did not appear to be largely undertaken through the L or T systems in broiler chickens. This is in contrast to the research findings on other species (Vadgama *et al.*, 1987; Pankovich and Jimbow, 1991). The ileum was the major site of uptake and this agrees with established findings that amino acids are mainly absorbed in the distal intestine while peptides are absorbed in the proximal region (Matthews *et al.*, 1971; Crampton *et al.*, 1973).

There are no previous research reports on the effect of NSP on amino acid transport in poultry. Johnson *et al.* (1984) had observed a reduction in the transport of glucose by mice on diets supplemented with guar gum. In the present study, there was no definite pattern of the effect of viscous NSP in the diet on tryptophan transport. *In vivo* and in

the presence of a viscous digesta, nutrient absorption may be reduced (Bamba *et al.*, 1993; van der Klis *et al.*, 1993) but this does not seem to be the result of a reduction in the function of the brush-border membrane. The negative effects of NSP on nutrient absorption may be due to a reduction in nutrient mobility (Read, 1987) and low concentration of available nutrients for transport.

The uptake of tryptophan in chickens raised on the whole lupin seed diets (experiment 5.4) was lower than that observed on the commercial diet which was supplemented with the non-viscous NSP (diets AA and GA in experiment 5.2) and fed to chicks of a similar age. The improvement in uptake with increased level of BM supplementation (total oligosaccharide) would suggest that oligosaccharides were not responsible for the reduction in transport. Starch digestibility on the former diets was also lower than that observed in other experiments. It is possible that the digestion of protein and other components would have been similarly affected. This would lead to a reduction in the concentration of material for absorption. It is not known how such events would eventually influence the ability of the BBM to transport nutrients but digestive function is known to respond to nutrient concentration (Wiesenfeld *et al.*, 1993). The studies on NSP showed that membrane transport function was not affected by exposure to the viscous materials. The limiting factor to absorption in the presence of NSP may be a restriction to nutrient mobility (Read, 1987; Tulung *et al.*, 1987; van der Klis *et al.*, 1993; Tang and Melethil, 1995).

6.0.5. Management of broiler chicken diets

The present research examined some of the practices commonly adopted in the industry towards solving the problem of feed scarcity; the presence of anti-nutritive factors in

feed ingredients, and routine attempts at enhancing productivity. The results obtained demonstrated improvements in body growth and feed conversion efficiency when a microbial enzyme supplement was included in cereal-based diets, as has been previously reported (Petterson and Aman, 1987; Classen and Bedford, 1991; Almirral *et al.*, 1995. Microbial enzymes target NSP in diets which are ordinarily not susceptible to animal enzymes (Classen and Bedford, 1991). The supplement tested also tended to induce the activity of some endogenous enzymes on some cereals but reduced the activities of these enzymes in chickens on other cereals. This should be considered when enzyme supplements are selected for use in diets.

The choice of lupin seed as an alternative to oilseed cake would need to be more carefully assessed. Its oligosaccharide component may not be deleterious as has been previously assumed. De-hulling appeared to be an adequate measure at improving the quality of lupin seed, as has been previously reported (Brenes *et al.*, 1993). Whole lupin (un-dehulled) seed appears to be low in quality and depressed productivity considerably when included in the diet at very high levels as borne out by the present research. However, ethanol extraction of the meal to eliminate oligosaccharides did not yield positive results. It may be necessary to include an NSP-targeting microbial enzyme in diets with high levels of de-hulled lupins. Such measures have been shown to further improve broiler growth (Annison *et al.*, 1996). De-hulled un-extracted lupin meals did not adversely affect digestive enzyme function but the feeding of whole seeds reduced enzyme activities as well as uptake of tryptophan by BBM. The inclusion of a commercial mannan-oligosaccharide, in addition to the raffinose series oligosaccharides tended to improve the growth of chicks but this was associated with high feed intake and reduced feed conversion efficiency. The commercial pre-biotic enhanced the

specific activities of some digestive enzymes as well as tryptophan uptake, especially in the proximal GIT.

6.1.0. Direction of future studies

The present research is one of the first to comprehensively study the Steggles x Ross (F_1) strain under varying rearing conditions. The research also proceeded in the line of the current trend at understanding some of the physiochemical phenomena associated with the nutrition of broiler chickens. This is a nascent area of research and no results would be regarded as conclusive since some of the basic areas of nutrition have not been understood even after several decades of research.

It is hoped that the results presented in the current research would open avenues for discussion and more concerted investigations into each of the areas covered. More specifically, the following areas would need to be further investigated:

1. There are large individual variations between chicks of the same strain and this variation was observed in growth parameters as well as physiochemical variables. In the poultry industry, individual variations may be remedied through supplementation with growth-enhancers, notably microbial enzymes. While some of the variation may be due to the instability of an F_1 generation, there may be genuine inherent genetic variability within strains. It may be necessary to establish if there are any relationships between physiochemical and genetic factors within strains and whether production could be improved using physiological factors as an aid to selection.

2. There is very little information on the molecular nature of digestive enzymes and nutrient transporters in poultry. An understanding of the nature of these factors would

again be useful in further breeding. The identification of enzyme and transporter genes is also still a new area, especially farm animals. The short life cycle of poultry confers an advantage to the rapid conclusion of such studies, compared with the difficulties that would be confronted in other farm animal species.

3. Some of the assays utilized in the present research may find wider application in the poultry industry in the near future. An attempt has been made to diagnose various intestinal diseases and thriftiness in broiler chicks using intestinal enzyme profiles (Konrad *et al.*, 1991). Irish *et al.* (1997) have also developed an assay system based on AP to assess the effects of lectins on poultry productivity. A major breakthrough would be achieved if rapid non-invasive techniques could be developed, based on the protocols described in this thesis.

4. Lupins and other pulses are increasingly being exploited as alternative protein sources, especially in Australia. The availability of low-alkaloid varieties was a break-through pioneered in Australia. Other factors which appear to be constraints to the realization of the full potential of lupins need to be investigated through nutritional research. The impact of oligosaccharides on digestive enzyme function and nutrient transport (absorption) deserves particular attention.

APPENDICES

Appendix 1: Composition of commercial diets fed

	Diet[1]	
	Starter	**Finisher**
Dry matter (g/kg)	911.0 (900.0)	911.0 (900.0)
Gross energy (MJ/kg)	17.5	19.06
Total NSP (g/kg)	98.1	ND
Crude protein (g/kg)	225.0	185.5
Lysine (g/kg)	11.6 (11.0)	9.9 (10.0)
Methionine (g/kg)	3.7 (5.0)	3.5 (3.8)
Threonine (g/kg)	8.2 (8.0)	7.8 (7.4)
Lipid (g/kg)	48.5	49.5
Linoleic acid (g/kg)	174.0 (100.0)	158.0 (100.0)
Calcium (g/kg)	123.0 (100.0)	114.0 (90.0)
Phosphorus (g/kg)	8.0 (45.0)	77.0 (35.0)

1. The starter diet was fed from 1 to 21 days weeks and finisher diet from 21 to 35 days. Both diets contained 12000 IU and 3500 IU/kg of vitamins A and D3 respectively. Vitamins E, K, B2, B12, pantothenic acid and niacin amounted to a total of 80.02 mg/kg. NRC recommendations are in parentheses. ND - not determined.

APPENDIX 2: Composition of buffers used.

The following buffers were used for assays described in this thesis, in addition to others whose compositions were revealed under the appropriate sections. The compositions shown are for the regular constituents only, the required pH was obtained by adjusting with NaOH or HCl.

1. ***Formal calcium***

1 g $CaCl_2$

91.25 ml distilled water

8.75 ml formalin.

2. ***0.1 M phosphate buffer***

8 ml 0.2 M $NaHPO_4$

42 ml 0.2 M Na_2HPO_4

50 ml distilled water

3. ***0.1 M acetate buffer***

197 ml 0.1 M sodium acetate

0.1 M acetic acid

4. ***0.1 M Citrate-phosphate buffer***

63.1 ml Na_2HPO4 (2.84 % w/v)

36.9 ml 0.1 M citric acid

5. *Hexazonium para-rosaniline*

Equal parts of solution A (1 g pararosaniline (Sigma P7632) in 20 ml ROH) and 5 ml conc. HCl) and solution B (freshly prepared 4 % sodium nitrite).

6. *0.3 M Tris-maleate*

9.08 g Tris

8.7 g maleic acid

250 ml distilled water.

APPENDIX 3: Fixatives

1. *10 % Neutral buffered formalin*

10 ml formaldehyde 40 %

90 ml distilled water

0.35 g sodium dihydrogen phosphate (anhydrous)

0.65 g dihydrogen phosphate (anhydrous).

2. *Bouin's fluid*

75 ml picric acid (saturated aqueous)

25 ml formaldehyde 40 %

5 ml glacial acetic acid.

3. *Carnoy's fluid*

60 ml absolute ethanol

30 ml chloroform

10 ml glacial acetic acid.

4. *Paraformaldehyde*

40 g paraformaldehyde in 100 ml distilled water.

(This was heated and 10 N NaOH added dropwise to clear the cloudiness; then cooled to 4^0C, filtered and stored at 4^oC).

5. ***Methacarn***

60 ml absolute methanol

30 ml chloroform

10 ml glacial acetic acid

APPENDIX 4: Automatic tissue processing schedule (Programme A)

Step	Chemical	Duration (Hr)
1	70 % ethanol	Pass
2	70 % ethanol	1.0
3	80 % ethanol	1.0
4	95 % ethanol	0.5
5	95 % ethanol	1.0
6	Absolute (97.5-100 %) ethanol	2.0
7	Absolute (97.5-100 %) ethanol	1.0
8	50 % histolene* + 50 % ethanol	2.0
9	Histolene	2.0
10	Histolene	2.0
11	Wax	2.0
12	Wax	2.0

*Histoclear or any other safe clearing agent could be used.

APPENDIX 5: Hematoxylin/eosin fixation schedule

Chemical	Duration
1. Histoclear or histolene*	3 minutes
2. Histoclear or histolene	3 minutes
3. Absolute ethanol	2 minutes
4. 80 % ethanol	2 minutes
5. 30 % ethanol	2 minutes
6. Distilled water	20 seconds
7. Hematoxylin (Lilee-Mayer)	5 minutes
8. Tap water	5 seconds
9. 1 % HCl in 70 % ethanol	1 second
10. Tap water (running)	10 minutes
11. Eosin	1 minute
12. Tap water	Rinse twice
13. 80 % ethanol	5 seconds
14. Absolute ethanol	2 minutes
15. Absolute ethanol	2 minutes
16. Histoclear (histolene)	5 minutes prior to mounting in DePeX.

*Safer replacements for xylene.

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